Molecular Testing of Thyroid Nodules

A Review of Current Available Tests for Fine-Needle Aspiration Specimens

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• ***Context.***—**Fine-needle aspiration of thyroid nodules is a reliable diagnostic method to determine the nature of thyroid nodules. Nonetheless, indeterminate cytology diagnoses remain a diagnostic challenge. The development of multiplex molecular techniques and the identification of genetic alterations associated with different follicular cell–derived cancers in the thyroid have led to the introduction of several commercially available tests.**

Objective.—**To summarize the most common commercially available molecular testing in thyroid cancer, focusing on the technical features and test performance validation.**

Data Sources.—**Peer-reviewed original articles, review articles, and published conference abstracts were reviewed to analyze the advantages and limitations of the most common tests used in the evaluation of thyroid needle aspirations.**

Conclusions.—**The most common tests available include the Afirma Gene Expression Classifier, ThyGenX, and ThyroSeq. The excellent negative predictive value (NPV) of the Afirma test allows it to be used as a "rule out" test. ThyGenX analyzes a panel of DNA mutations and RNA translocation fusion markers to assess the risk of malignancy with good NPV and positive predictive value. ThyroSeq is a next-generation sequencing–based gene mutation and fusion test that has been reported to have the best NPV and positive predictive value combined, suggesting that it can be used as a "rule in" and "rule out" test. Molecular testing of cytology specimens from thyroid nodules has the potential to play a major role in the evaluation of indeterminate thyroid lesions.**

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Thyroid nodules are common lesions encountered in the general population, with an estimate of 10 to 18 million individuals in the United States having a thyroid nodule.[1] Fine-needle aspiration (FNA) of thyroid nodules is an integral part of the workup of these patients and is included in the recommended guidelines by the American Thyroid Association (ATA) for the assessment of thyroid nodules owing to its high sensitivity and specificity.[2] Although most thyroid lesions are classified as category II (benign) according to the Bethesda System for Reporting Thyroid Cytopathology (TBSRTC),[3] some cases of FNAs of the thyroid are more challenging and cannot be classified as benign or malignant by the morphologic findings, leading to an indeterminate diagnosis. These indeterminate diagnoses include the categories of atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS, Bethesda category III) (Figure 1) and follicular neoplasm/suspicious for follicular neoplasm (FN/SFN, Bethesda category IV) (Figure 2), based on the TBSRTC.[3] The implied risk of malignancy according to the TBSRTC should vary from 5% to 15% in category III (AUS/FLUS) and 15% to 30% in category IV (FN/SFN).[4]

The indeterminate diagnosis category represents a challenge to clinicians, as most nodules with AUS/FLUS or FN/SFN cytology are found to be benign in the surgically resected specimens. However, the probability of malignancy is considered too high for clinical follow-up.[3,4] For instance, FNAs of lesions classified as FN/SFN carry a 15% to 30% risk of malignancy. The wide interobserver variability in the indeterminate category of thyroid FNAs further complicates the issue.[5,6] In an attempt to improve accuracy in the indeterminate FNA category, several studies have evaluated different markers in thyroid FNAs. Early immunohistochemical markers such as galectin-3,[7] HBME-1,[8] fibronectin-1, CITED-1, and cytokeratin 19[9] have yielded less than optimal results. The relative low specificity and lack of reproducibility among different laboratories make immunohistochemical studies difficult to use as a daily diagnostic tool to differentiate benign from malignant thyroid lesions.

Advancements in molecular studies have allowed the identification of genetic alterations associated with different follicular cell–derived cancers in thyroid.[10,11] Based on these findings, molecular tests have been developed to assist in determining if a lesion is benign or malignant. The goal of this review is to provide an updated overview of the currently available molecular tests for thyroid FNA specimens. The technical features and limitations of these tests in the clinical setting will also be discussed.

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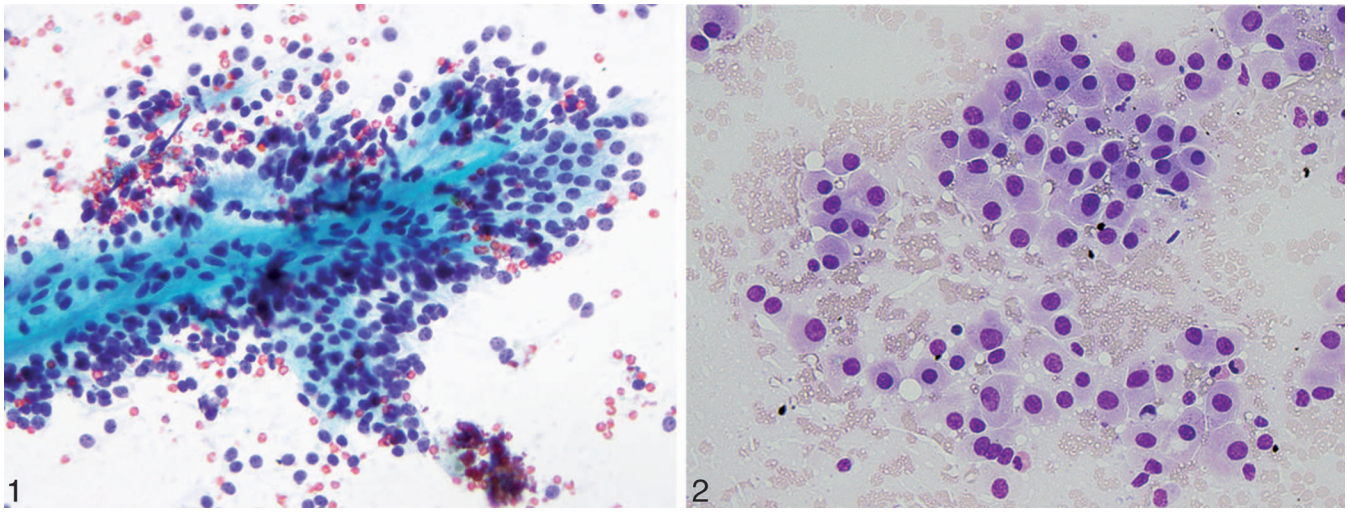

Figure 1. *Bethesda category III: atypical follicular lesion of undetermined significance. The specimen shows a cluster of cells with a papillary architecture but lacks the nuclear changes of papillary carcinoma (Diff-Quick; original magnification ×400).*

Figure 2. *Bethesda category IV: a lesion composed predominantly of Hürthle cells (Papanicolaou; original magnification ×400).*

AFIRMA GENE EXPRESSION CLASSIFIER

The Afirma Gene Expression Classifier (GEC) (Veracyte Inc, South San Francisco, California) is a test based on microarray technology used to analyze the mRNA expression of 167 different genes. These 167 genes include 2 sets of genes, one with 25 genes representing less common entities seen in the thyroid and another with 142 genes comprising the most common entities seen in thyroid cancer. The selected gene profile is based on the gene expression identified from FNAs of surgically proven benign and malignant thyroid nodules.[12,13] Only FNA lesions designated as FLUS/AUS and (suspicious for) Hürthle/follicular neoplasm are accepted for analysis. Two dedicated FNA passes are collected from each thyroid nodule and immediately stored in a proprietary nucleic acid preservative solution. The test is offered through a Clinical Laboratory Improvement Amendments of 1988 (CLIA)–certified reference laboratory (Veracyte) and generates 2 possible results, benign and suspicious.

The Afirma GEC test was validated in a blinded prospective multicenter trial involving 265 nodules with indeterminate cytology and histologic follow-up.[13] The original cytology diagnosis in this validation set included 129 AUS/FLUS, 81 FN/SFN, and 55 "suspicious for malignancy" nodules. The results of the study showed that the Afirma GEC test had a high negative predictive value (NPV) of 95% and 94% for nodules in the AUS/FLUS and FN/SFN categories (Bethesda categories III and IV), respectively. Therefore, the risk of malignancy in these categories when the Afirma GEC test result was benign ranged from 5% to 6% and closely approached the NPV in thyroid FNAs morphologically diagnosed as benign. However, in the "suspicious for malignancy" category (Bethesda category V), the NPV was only 85%, leading to a 15% risk of malignancy. These results supported the indication to use the Afirma GEC test only in lesions with diagnosis included in the Bethesda III and IV categories. Despite the promising NPV values seen if the Afirma GEC test results had a diagnosis of benign, a suspicious diagnosis was less helpful. The positive predictive value (PPV) in this test was much lower than expected (38% for AUS/FLUS and 37% for FN/SFN).[13] Therefore, these findings indicate that the test could be useful as a "rule out" test. If the diagnosis was benign in the indeterminate category, the patient could be followed up clinically with no need for surgery. However, if the diagnosis was suspicious, the diagnosis remained indeterminate and a surgical consultation was recommended, but not necessarily a surgical procedure.

Several subsequent studies have evaluated the effectiveness and value of the Afirma GEC test. A large comprehensive prospective study reported by Alexander et al[13] evaluated 4812 thyroid FNAs from 3789 patients. Among these samples, 5.52% samples were selected for GEC analysis. The overall sensitivity of the Afirma GEC test was 92% with an NPV of 93% (95% for AUS/FLUS, 94% for a follicular neoplasm, and 85% for a lesion suspicious for malignancy).[13] Two recent retrospective multicenter studies[14,15] demonstrated a 100% NPV with a benign Afirma GEC test result. However, a study performed in a community hospital–based thyroid surgery practice showed a lower NPV (89.6%) than other studies in the literature.[16] The Afirma GEC test is expected to provide the most useful information in a practice setting with a prevalence of malignancy in indeterminate thyroid lesions of 15% to 21%. In this environment, the performance characteristics would be predicted to approximate those reported in the validation study.[13] The test may still provide some useful information in settings where the prevalence of malignancy is at 12% to 25%. Outside this range, however, the test seems unlikely to provide information that would alter management. In populations with a pretest probability of less than 12%, the PPV would be predicted to be less than 20%, and therefore the risk of cancer remains low regardless of the GEC result. In populations with pretest probability greater than 25%, the NPV is predicted to be less than 94%.[14]

The role of a suspicious result by Afirma GEC is less well defined. The reported PPVs range from 14% to 57% in various studies, which largely limits its clinical utility to predict the risk of malignancy.[14–17] As pointed out by Marti et al,[14] the risk of malignancy really depends on each individual institution's prevalence of malignancy. Hürthle

cell–rich lesions appear to represent another limitation to the Afirma GEC test. There is a tendency for the Afirma GEC test to report a high percentage of benign Hürthle cell nodules as suspicious.[13,15,16,18,19] These studies indicate that the risk of malignancy for a suspicious Afirma result is lower for aspirates with Hürthle cell cytology (19%–23%) than for those without a prominent population of Hürthle cells.

In 2014, Veracyte introduced the Afirma Malignancy Classifiers (AMCs) to further enhance the Afirma GEC test as a comprehensive diagnostic tool to assess the risk of malignancy, including medullary carcinoma. The AMC tests are performed only on FNA samples carrying a suspicious and malignant cytomorphologic diagnosis or a suspicious Afirma GEC result. The AMCs include an isolated mRNA profile for medullary thyroid carcinoma and/or *BRAF* V600E gene mutation to further assist a clinical decision. The expression of 5 genes that are differentially identified in medullary carcinoma tested in the AMCs include calcitonin-related polypeptide α (*CALCA*), carcinoembryonic antigen–related cell adhesion molecule 5 (*CEACAM5*), secretogranin III (*SCG3*), sodium channel voltage-gated type IX α subunit (*SCN9A*), and synaptotagmin IV (*SYT4*). As a new test, the AMC validation has not been widely verified but a high PPV and NPV were achieved in a study by Veracyte presented at the 23rd American Association of Clinical Endocrinologists Annual Scientific and Clinical Congress.[20] The validation of Afirma *BRAF* test was compared to a sensitive quantitative polymerase chain reaction (qPCR)–based test for *BRAF* V600E mutations. A high positive percentage agreement of 90.4% and negative percentage agreement of 99.0% with qPCR on the test set were observed. The sensitivity for malignancy of the *BRAF* test was 43.8% (consistent with published prevalence of *BRAF* V600E in papillary thyroid carcinoma), while the specificity was 100%, identical to qPCR results obtained on the same samples.[21] In summary, the Afirma GEC has had a significant clinical impact on clinical decision and patient management. A negative Afirma GEC result led to a dramatic decrease of histologic thyroid surgical resection rate from 74% to 7.6% on cytologically indeterminate nodules in a multicenter cross-sectional cohort study.[22] On the other hand, 82% of patients with Afirma GEC suspicious result underwent surgery, compared with only 6% of those with Afirma GEC benign result.[17]

ThyGenX TEST

The identification of mutations in different types of thyroid carcinomas and the advances in the multiplexing analysis of cytology material allowed the development of genomic mutation–based tests. Gene mutations or translocation fusions associated with well-differentiated thyroid cancer have been extensively described previously.[23] Among the different mutations, *BRAF* mutation is the most common and specific mutation seen in papillary thyroid carcinoma (PTC) with a reported incidence of 40% to 45%, while rat sarcoma viral oncogene homolog (*RAS*) and (Ret proto-oncogene) *RET/PTC* gene mutations are identified in 10% to 20% of PTCs. It is believed that approximately 70% of PTCs carry genetic alterations, either point mutations in the *BRAF* and *RAS* genes or present with *RET/PTC* or *TRK* rearrangements.[10,24,25] All of these genetic alterations are able to activate the mitogen-activated protein kinase (MAPK) pathway. Follicular carcinoma is the second most common thyroid cancer, among which *RAS* is the most common genetic alteration and is seen in 40% to 50% of follicular neoplasms followed by *PAX8/PPARγ* (30% to 35%).[10] These mutations are present in 70% to 75% of the follicular neoplasms and are mutually exclusive.[26] Other molecular mutations, including *TP53* and *CTNNB1* genes, may also occur in thyroid cancers although they are more often associated with poorly differentiated and anaplastic carcinomas.[27–29] Medullary thyroid carcinomas frequently harbor *RET* gene point mutations, which are seen in both familiar and sporadic forms.[30]

The high specificity of these genomic alterations has led to the development of molecular tests that include a panel of genetic alterations commonly seen in PTC and other thyroid neoplasms. Of note, one of these tests was originally marketed by Asuragen (Austin, Texas) as miRInform. The test consisted of an 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*). Although highly specific, the test lacked sensitivity, as not all thyroid neoplasms contained the specific genetic alterations evaluated. The test is based on the findings reported by Ferraz et al[11] and internal company findings.

Since its original introduction, the test has been modified and is currently offered by Interpace Diagnostics, a subsidiary of PDI Inc (Parsippany, New Jersey) as ThyGenX thyroid oncogene panel. ThyGenX uses a next-generation-sequencing (NGS) platform to identify more than 100 genetic alterations across 8 genes associated with thyroid malignancy. It includes the original genetic alterations analyzed in the miRInform test as well as testing for *PIK3CA* mutation, which is involved in the formation and progression of follicular carcinoma and anaplastic carcinoma.[31] The ThyGenX test requires only 1 dedicated FNA pass (at least 50 ng of cellular material to be collected into the provided RNA preservative vial RNA*retain*), and only cases diagnosed as AUS/FLUS or FN/SFN are accepted for ThyGenX analysis.

More recently, Interpace Diagnostics introduced a new molecular test, ThyraMIR. This test is based on the analysis of 10 different microRNAs (miRNAs) including miR-29b-1-5p, miR-31-5p, miR-138-1-3p, miR-139-5p, miR-146b-5p, miR-155, miR-204-5p, miR-222-3p, miR-375, and miR-551b-3p. This test is meant to be used in conjunction with ThyGenX when the ThyGenX result is negative. Several studies[32–34] have shown that these miRNA molecules are involved in the cell-cycle progression, differentiation, and proliferation in thyroid pathology, which indicates the potential diagnostic value of miRNA in cytologically indeterminate thyroid nodules. Interpace advocates the use of a combination of both ThyGenX and ThyraMIR on the basis of findings from a histologically blinded, multicenter, cross-sectional cohort study in nodules of indeterminate cytology (Bethesda categories III and IV). It demonstrated a sensitivity and specificity of 89% and 85%, respectively, while the NPV and PPV were reported as 94% and 74%, respectively. When both test results were negative, the residual risk of cancer was very low (6%) in an environment where the underlying malignancy rate was 32%.[35] The reported NPV was 94%, similar to that of the Afirma GEC validation study; however, the PPV of 74% was higher than the one achieved by the Afirma GEC.

ThyroSeq TEST

ThyroSeq is a NGS-based gene mutation and fusion panel initially designed to target 12 cancer genes with 284 mutational hot spots.[36] The performance of this genetic panel was initially evaluated by using thyroid tumors cells and cell lines with known genetic alterations. The ThyroSeq test had a 100% analytic accuracy, as it correctly detected all pathogenic mutations in previously positive thyroid tumor samples and cell lines. In addition, all mutations detected by ThyroSeq were confirmed by other molecular testing methods, such as Sanger sequencing, real-time PCR, or coamplification at lower denaturation–PCR, revealing 100% correlation among the different techniques. The initial validation of ThyroSeq panel by Nikiforova et al[36] showed the presence of the tested gene mutations or translocations in various thyroid malignancies, including 19 of 27 classic PTCs (70%), 25 of 30 follicular variant PTCs (83%), 14 of 18 conventional carcinomas (78%), 7 of 18 oncocytic follicular carcinomas (39%), 3 of 10 poorly differentiated carcinomas (30%), 20 of 27 anaplastic thyroid carcinomas (ATCs) (74%), and 11 of 15 medullary thyroid carcinomas (73%). In contrast, only 5 of 83 benign nodules (6%) were positive for mutations. A subsequent prospective single-institution study of 513 surgically resected thyroid nodules with indeterminate cytologic diagnosis showed that if any of the above gene mutations or fusions were identified, the PPV was 88% and 87% for AUS/FLUS and FN/SFN, respectively.[37] The high PPV of the gene mutation test is similar to the values obtained in other similar studies[38,39] and indicates that the ThyroSeq test could potentially be used as a "rule in" test.

In 2014, an enhanced version of the test was introduced as ThyroSeq v2. The newly expanded version of the test included a more extensive panel of DNA alterations (14 genes, including >1000 mutations) and RNA alterations (42 fusions, 16 genes for expression). These added molecular alterations had been previously associated with cancer diagnosis, prognostication, and optimal targeted therapy selection. A study using ThyroSeq v2 panel showed increased accuracy with a reported sensitivity and specificity of 90% and 93%, respectively, a PPV of 83%, an NPV of 96%, and accuracy of 92%.[40] These results suggested that ThyroSeq v2 may potentially function as both "rule out" and "rule in" test for nodules with indeterminate cytology. Similar to other molecular tests, the predictive values of ThyroSeq correlate with the prevalence of malignancy within a particular practice setting. The above study by Nikiforov et al indicated that with a pretest probability of malignancy in the range of 5% to 15% for AUS/FLUS cytologic diagnosis, ThyroSeq v2 would be expected to have an NPV of 98% to 99% and a PPV of 40% to 69% as based on Bayesian modeling. Therefore, in the situations of low pretest probability of malignancy, although ThyroSeq v2 would remain as an effective "rule out" test (good NPV), a relatively low PPV (due to a low end of PPV value) makes it an unsatisfactory "rule in" test. Furthermore, as a general rule, the increased chance of detecting "false-positive" molecular abnormalities might be associated with the expanded NGS-based mutational profile. Finally, owing to the limited studies and data from literature, the value of ThyroSeq v2 needs further investigation.

In addition to ThyroSeq, other formats of NGS-based molecular tests for thyroid nodules have been investigated. Le Mercier and colleagues[41] from Université Libre de Bruxelles, Brussels, Belgium, carried on a pilot NGS-based study by using a commercially available 50-gene panel kit (Ion AmpliSeq Cancer Hotspot Panel version 2; Thermo Fisher Scientific, Carlsbad, California) to evaluate indeterminate FNA cytology of thyroid nodules. The Ion AmpliSeq Cancer Hotspot Panel v2 is an extensively expanded panel designed to amplify 207 amplicons covering approximately 2800 COSMIC mutations from 50 oncogenes and tumor suppressor genes. Similar to ThyroSeq, the test only needs a minimal amount of tissue DNA and has a low turnaround time (it is possible to complete the entire process in about 3.5 hours). Materials were extracted from either cell block (preferred specimen) or smear slides (if cell block is insufficient) in all indeterminate FNA cases. The results were categorized into 2 classes: "molecular test negative" (including patients carrying germline polymorphisms, mutations of unknown clinical significance, or no mutation) or "molecular test positive" for patients carrying pathogenic mutations. Taking histologic diagnosis as the gold standard, the sensitivity and specificity of the molecular test for the diagnosis of malignancy were 71% and 89%, respectively. The PPV and NPV were 63% and 92%, respectively, with an accuracy of 85%. Nonetheless, more studies with this panel are indicated, as the initial studies were performed with a limited number of cases.

OTHER MOLECULAR TESTS FOR THYROID CANCER

Although not designed to use thyroid cytology material, thyrotropin receptor (TSHR) mRNA test also helps to classify the cytologic indeterminate thyroid nodules. TSHR mRNA was developed by Cleveland Clinic Laboratories (Cleveland, Ohio) to use a quantitative reverse transcription-PCR–based assay to detect TSHR mRNA levels in peripheral blood. Functional TSHRs are constantly produced by thyroid cancer cells, and circulating TSHR mRNA was not detected in normal thyroid tissue or benign thyroid diseases.[42] On the other hand, elevation of TSHR mRNA is strongly associated with thyroglobulin antibody–positive patients in the follow-up of thyroid carcinoma. Based on the above findings, a preoperative test for serum TSHR levels suggests a potential role in characterizing thyroid nodules. Technically, 7 mL of whole blood is collected in 2 EDTA tubes and transported on ice. Total RNA is extracted, reverse transcribed, and then subjected to PCR testing. Absolute quantity is measured against a reference value (prepared from thyroid cancer RNA levels). The validation of TSHR mRNA was tested in one study including 18 indeterminate FNA cytology cases. The results indicated TSHR mRNA correctly classified 14 cases with a sensitivity of 75% sensitivity and a specificity of 78%. The combined diagnostic performance characteristics for TSHR mRNA and FNA cytology were as follows: sensitivity 95%, specificity 83%, PPV 84%, and NPV 95%. All the values are significantly higher than the results obtained from FNA cytologic diagnosis alone.[43] However, owing to the labile nature of RNA in a blood test, TSHR mRNA measurement should be always used and interpreted in view of the clinical context, radiographic findings, and FNA cytologic diagnosis. In addition to the above common somatic mutations and gene arrangement in thyroid cancer, certain miRNA expression levels have been reported to be altered in benign and malignant thyroid lesions.[44–46] These data may indicate a potential diagnostic value of using miRNAs in preoperative molecular testing.

Table 1. Summary of 2015 American Thyroid Association Management Guideline Recommendations for Thyroid Molecular Testings According to the Bethesda Categories

Recommendation No.	Summary	Bethesda Categories			Recommendation Level	Level of Quality Evidence
		ATP/FLUS	FN/SFN	SUSP		
13	Patient counseling of the molecular tests	X	X	X	Strong	Low
14	Tests should be performed in CLIA/CAP-certified laboratories	X	X	X	Strong	Low
15 (A)	Repeated FNA or molecular tests	X			Weak	Moderate
15 (B)	Surveillance or diagnostic surgical excision for inconclusive molecular tests	X			Strong	Low
16 (A)	Diagnostic surgical excision or molecular test		X		Weak	Moderate
16 (B)	Diagnostic surgical excision if molecular test is inconclusive		X		Strong	Low
17 (A)	Surgery, possible gene mutation panel			X	Strong	Low
17 (B)	Possible molecular testing for *BRAF* or gene mutation panel if such data would be expected to alter surgical decision making			X	Weak	Moderate

Abbreviations: ATP/FLUS, atypia of undetermined significance or follicular lesion of undetermined significance; CLIA/CAP, Clinical Laboratory Improvement Amendments of 1988/College of American Pathologists; FNA, fine-needle aspiration; FN/SFN, follicular neoplasm or suspicious for follicular neoplasm; SUSP, suspicious for malignancy.

2015 ATA MANAGEMENT GUIDELINE RECOMMENDATIONS FOR THYROID MOLECULAR TESTINGS

The new ATA guidelines include several recommendations for the use of molecular testing in cases of indeterminate thyroid FNAs. The proposed use of molecular testing in indeterminate thyroid FNA specimens is to assist diagnosis and management plans.[2] The new guidelines recommend patient counseling regarding the potential benefits and limitations of testing. The molecular testing should be performed in a CLIA/College of American Pathologists–certified molecular laboratory, or international equivalent, as reported quality assurance practices may be superior than in other settings. The revised guidelines suggests that molecular studies can be used in cases diagnosed as AUS/FLUS with worrisome clinical and sonographic features to supplement malignancy risk assessment in lieu of proceeding directly to either clinical surveillance or diagnostic surgery. Lesions included in the Bethesda category IV (FN/SFN) show a 15% to 30% estimated risk of malignancy and surgical lobectomy is the suggested management plan. However, if molecular testing is either not performed or inconclusive, surgical excision may be considered for removal and definitive diagnosis of an FN/SFN thyroid nodule. Finally, if the cytology is reported as suspicious for papillary carcinoma, management should be similar to that of malignant cytology. However, mutational testing for *BRAF* or a mutation marker panel may be considered if such data would alter surgical decision-making.

Interestingly, the ATA recommendation guidelines state that since long-term outcome data on companion use of molecular testing to guide therapeutic decision making is still insufficient, it is still debatable whether molecular testing should be used in routine clinical practice for patients with cytologically indeterminate thyroid nodules. A summary of the ATA recommendations for the use of molecular studies can be seen in Table 1.

OTHER ISSUES RELATED TO THYROID MOLECULAR TESTS

Yip et al[47] reported cost savings if molecular studies were performed and the costs were less than $870. They concluded that molecular testing of cytologically indeterminate FNA can allow cost savings and improve patient care by providing an indication for optimal initial surgical management with total thyroidectomy when molecular testing is positive. Li et al[48] used a different approach with a Markov decision model based on a hypothetical cohort of adult patients and found that the treatment costs of patients undergoing molecular testing was $10 719 compared with $12 171 when no molecular testing was used (at a price of $3200 per test). The cost savings were primarily proportional to the number of diagnostic surgeries avoided. Nonetheless, the commercially available molecular tests are expensive tests. The costs for insured patients for either Afirma GEC or ThyroSeq v2 are capped at $300; however, uninsured patients face significant costs. The list prices for these tests are the following: $4875 for Afirma GEC and MTC (medullary thyroid carcinoma), $1675 for ThyGenX, $3300 for ThyraMIR, and $3200 for ThyroSeq.[49] The real impact of thyroid molecular tests in the country's health care costs still needs to be determined in view of the increased incidence of thyroid nodules diagnosed in recent years.

It is important to remember that the preanalytic factors, such as specimen sampling and preservation, are critical to ensure an accurate molecular testing result. The dedicated FNA passes to be used for molecular testing should be obtained from the same location as the one in which the on-site adequacy assessment was performed. Furthermore, the samples should be preserved and shipped strictly according to the individual testing instructions.

The recent reclassification of noninvasive encapsulated follicular variant of papillary thyroid carcinoma as noninvasive follicular tumor with papillary-like nuclear features (NIFT-P) will alter the risk of malignancy in Bethesda categories II, IV, and V. Since NIFT-P shares

Table 2. Comparison of Currently Available Molecular Tests for Indeterminate Thyroid Cytopathology Fine-Needle Aspiration Specimens

	Afirma[a]	ThyGenX[b]	ThyroMIR[b]	ThyroSeq[c]
Methodology	mRNA gene expression	Multiplex PCR by sequence-specific probes	MicroRNA expression	Next-generation sequencing
Test report	Benign/suspicious	Specific gene mutation/ translocation	Negative/positive	Specific gene mutation/ translocation
Specimen collection	2 dedicated FNA passes	1 dedicated FNA pass with at least 50 ng of cellular material	Same as ThyraMIR	1–2 drops from first pass if adequate cellularity on smear slide
Strength	High NPV	High PPV	Good NPV and PPV when combined with ThyGenX	High NPV and PPV
Limitation	Low PPV	Low NPV	Limited validation data	Limited validation data
Cost[d]	$4875 for Afirma GEC and MTC $975 for Afirma MTC alone $475 for Afirma BRAF alone	$1675 for ThyGenX alone	$3300 for ThyraMIR (reflex test)	$3200

Abbreviations: FNA, fine-needle aspiration; GEC, gene expression classifier; MTC, medullary thyroid carcinoma; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value.

[a] Veracyte Inc, South San Francisco, California.
[b] Interpace Diagnostics, a subsidiary of PDI Inc, Parsippany, New Jersey.
[c] ThyroSeq v2 is commercially offered by CBL PATH, Rye Brook, New York, while the test is performed and interpreted in the Division of Molecular and Genomic Pathology at the University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania.
[d] Data derived from Nishino.[49]

similar molecular profile as follicular carcinoma,[50] the reclassification will affect the PPVs of the different molecular tests. The PPV is expected to decrease for all currently available molecular tests, as NIFT-P was considered a malignant neoplasm in the validation studies and subsequent studies. For instance, in a recent study, Medici et al[51] found that 17 of the 362 thyroid nodules were *RAS*-positive by miRInform Thyroid molecular test. The results showed that 7 of 10 *RAS*-positive resected nodules represented NIFT-Ps.

CONCLUSIONS

In summary, the ultimate goal of thyroid molecular testing is to accurately assess the nature of thyroid nodules and reduce the diagnostic uncertainty of cytologically indeterminate thyroid nodules before surgery. Long-term outcome data on companion use of molecular testing to guide therapeutic decision making are still insufficient, with different tests demonstrating different characteristics and strengths (Table 2). The use of molecular testing in thyroid FNA has led to a major change in practice, particularly in lesions classified in Bethesda categories III, IV, and V. A negative result in the Afirma test has resulted in a major decrease in the number of surgeries performed in lesions classified as categories III and IV. However, a suspicious Afirma result has not demonstrated an optimal PPV. On the other hand, the risk of malignancy from an abnormal ThyroSeq or ThyGenX test result appears to be superior to that of the Afirma test. Additional studies from a larger cohort of studies with longer follow-up are recommended before any particular test is considered superior to the other. The identification of the new biomarkers in thyroid will most likely lead to enhanced versions of the current tests or development of new tests. It is still under debate whether molecular testing should be used in routine clinical practice. Therefore, molecular testing must be always performed and interpreted within the context of the clinical, radiographic, and cytologic findings.

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