Review

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Review

"Current State of Molecular Cytology in Thyroid Nodules: Platforms and their Diagnostic and Theranostic Utility"

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Abstract: The high prevalence of thyroid nodules and increased availability of neck ultrasound have led to an increased incidence of diagnostic thyroid fine needle aspirations, with approximately 20% yielding indeterminate results. The recent availability of molecular tests has helped guide the clinical management of these cases. This paper aims to review and compare three main commercially available molecular cytology platforms in the U.S. – Afirma GSC, Thyroseq GC, and ThyGeNEXT+ThyraMIR. Sequential improvements of the Afirma GSC and Thyroseq GC tests have led to higher positive and negative predictive values, sensitivity, and specificity. Comparative studies revealed similar diagnostic performance between these tests, with considerations for factors such as cost and processing time. Thyroseq GC provides detailed genomic information and specific management recommendations. ThyGeNEXT+ThyraMIR, though less studied, presents promising results, particularly in miRNA analysis for weak driver mutations. Challenges in interpreting results include variations in reporting and the evolving nature of testing platforms. Questions persist regarding cost-effectiveness and the utility of ultrasound characteristics in selecting candidates for molecular testing. While molecular testing has primarily served diagnostic purposes, advancements in understanding genetic alterations now offer therapeutic implications. FDA-approved options target specific genetic alterations, signaling a promising future for tailored treatments.

Keywords: thyroid cancer; fine needle aspiration; thyroid nodule; molecular cytology; genetic analysis; mutation

Introduction

Thyroid nodules are highly prevalent in the general population and easily detectable by neck ultrasound which is a noninvasive diagnostic imaging modality that has become more readily accessible in the last 20 years. The rise in the detection of thyroid nodules has caused an increase in the number of diagnostic thyroid fine needle aspirations (FNA) performed. About 20% of the cytology results from thyroid FNA come back as an indeterminate result which includes atypia or follicular lesion of indeterminate significance (Bethesda III) (AUS/FLUS) and follicular neoplasm/suspicious for follicular or oncocytic (formerly Hurthle) neoplasm (Bethesda IV) [1]. 10 to 30% of these nodules will ultimately yield a malignant pathology[1]. For many years, there has been a significant need for accurate tools that can help identify which of these nodules carry a malignant pathology from those with indolent behavior without the need for invasive lobectomy or thyroidectomy procedures [2]. In recent years different molecular cytology platforms have become available to help guide clinical management in these patients. In this paper, we aim to review and compare the performance of the 3 main platforms currently commercially available in the U.S.

Background

Before the recent emergence of molecular cytology applied to thyroid FNA, former American Thyroid Association (ATA) guidelines indicated that when a thyroid FNA resulted in an indeterminate diagnosis, the clinician could either repeat the FNA, indicate a thyroid lobectomy or continue surveillance with a follow-up ultrasound [3]. In approximately 60-70% of such instances, repeating the FNA will reclassify the nodule to a different Bethesda category, and in approximately 50%, it will result in a benign diagnosis, which has a greater NPV than molecular tests [4]. Further strategies available to help guide management in such indeterminate cases included seeking a second cytopathology opinion and correlating the cytology findings with the sonographic characteristics of the nodule. [5]

With the advent of various molecular cytology platforms becoming more readily available, the 2015 ATA management guidelines indicate that "for nodules with AUS/FLUS cytology, after consideration of worrisome clinical and sonographic features, investigations such as repeat FNA or molecular testing may be used to supplement malignancy risk assessment in lieu of proceeding directly with a strategy of either surveillance or diagnostic surgery. Informed patient preference and feasibility should be considered in clinical decision-making. (Weak recommendation, Moderate-quality evidence). If repeat FNA cytology and/or molecular testing are not performed or are inconclusive, either surveillance or diagnostic surgical excision may be performed for an AUS/FLUS thyroid nodule, depending on clinical risk factors, sonographic pattern, and patient preference. (Strong recommendation, Low-quality evidence)" [3].

These guidelines leave much room for clinical judgment, where many different factors need to be considered. Some information we know regarding ultrasound or molecular profile for all thyroid nodules might not necessarily apply to the subgroup of nodules with indeterminate cytology. There are certain nuances that apply specifically to this group that need careful consideration. [6]For instance, more than 70% of ATA high suspicion nodules on sonography end up being malignant, with most of those yielding a Bethesda category VI result on FNA; however, when we look at the nodules that have yielded a Bethesda category III result or indeterminate cytology, the predictive value of ultrasound decreases significantly. Similarly, we know that the *BRAF* V600E mutation is harbored in about 66% of papillary thyroid cancers, [7] Formatting...however, when looking at the cancers with Bethesda category III and IV cytology, we find a lower prevalence of BRAF mutations and a higher presence of other mutations such as RAS mutations. [8]

When considering the use of molecular testing to risk stratify a thyroid nodule with indeterminate cytology, we should aim for a test with a high negative predictive value (NPV) and high positive predictive value (PPV) to help better triage patients for thyroid surgery. Ideally, performance would be similar to that of a Bethesda II (benign) and Bethesda VI (malignant) cytology, where there are only 3% false positive and false negative rates. [9]

When evaluating the currently available molecular testing options, several important factors should be considered. These include the number of studies conducted to evaluate the performance of each test, the duration of follow-up of patients, the percent of patients that underwent the gold standard diagnostic test, which is histopathological surgical confirmation, whether they included Bethesda category III, IV, and/or V as indeterminate cytology, as well as the type of study: prospective vs retrospective, single vs multicenter, blinded vs unblinded. A notion of the prevalence of malignancy in the tested population vs. the rate of malignancy in an institution is also very important, as this can affect the negative and positive predictive value of the test. In contrast, the sensitivity and specificity are inherent and unchangeable characteristics of a test that would not be modified by changes in the prevalence of the disease.

Of note, there has been a significant evolution in the information provided by the different molecular tests since they first became commercially available. Initial reports were binary as either positive or negative with a clear negative and positive predictive value. With a better understanding of the different mutations follicular thyroid cells can acquire and the different risks for malignancy each of these can convey, current reports usually provide either a benign or negative result vs. a positive prognostic value with different types of positive results depending on the specific mutation

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identified. This level of granularity in the report makes it more challenging to calculate a general positive predictive value for each test. An additional point of consideration in the evaluation of these tests is the variations present in the classification and reporting as a positive or negative molecular test result of pre-malignant neoplasms such as noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) and the performance of the tests in oncocytic neoplasms.

Currently commercially available molecular tests for indeterminate thyroid nodule cytology

In the U.S., currently, there are 3 commercially available molecular tests in use for diagnosis of indeterminate thyroid nodule cytology: *Afirma GSC,* which is an RNA-based genomic sequencing classifier (GSC) using gene expression to infer the malignant potential of a nodule; *ThyGeNext+ThyraMIR*, which is a multiplatform test that involves an assessment of genetic alterations of DNA, mRNA and gene expression regulators known as micro RNA; and *Thyroseq GC,* a multigene genomic classifier that started as a purely DNA test but in recent years has also added mRNA testing for a gene expression classifier.

Afirma GSC

When a sample is sent for *Afirma* testing, it is first run through initial classifiers to identify rare neoplasms and lesions with >95% risk of malignancy (strong driver mutations), such as parathyroid, medullary thyroid cancer, *BRAF*, and *RET:PTC* fusion. Then a follicular content index is run, which identifies samples with sufficient molecular content. If there is not enough material, the results will be reported as insufficient. Following this, the ensemble classifier leverages multiple machine learning algorithms to derive a final benign vs. suspicious result, and lastly, a Hürthle Classifier analysis has been added to better classify Hürthle/oncocytic cell lesions as benign or malignant. [10]

The *Afirma GSC* classifier identifies about one-third of indeterminate cytology samples as suspicious, a result that carries an overall 50% risk of malignancy (ROM). Of those, 44% have an identifiable alteration (variant/fusion) that can be detected with the most recently developed *Afirma Expression Atlas*, significantly increasing their ROM and providing more granular data that can guide clinical management.

Reviewing the literature available to assess the performance of the *Afirma* test, one can conclude that about two-thirds of the *Afirma GSC* tests yield a negative result. The sensitivity and NPV vary between 90 and 100%, while some studies have suggested a lower NPV. [11.] The PPV is around 60% which is significantly improved compared to the 40% seen with the older version of the *Afirma GEC* test.[12] When looking at the clinical utility of the test, results show that 33 to 40% of tested patients underwent surgery, and about 60% or more of the tested patients will be able to avoid unnecessary surgery by using this test. [13 14 15 16 17 18 19 20 21 22 23 24]

It is important to highlight that when discussing nodules with premalignant histopathology that are classified as benign, such as follicular adenomas and NIFTP, these benign lesions and their malignant counterparts, such as follicular carcinoma and follicular variants of PTC, often have the same molecular test result profiles. It is likely that in many cases, it might just be a matter of time before a "benign" premalignant nodule acquires additional genomic alterations that lead to invasion and thus fulfill criteria for malignancy. It is worth mentioning that *Afirma* suspicious nodules that are benign on final histopathology are more often clonal neoplasms such as NIFTP or follicular adenoma (FA), in contrast, *Afirma* benign nodules are more often hyperplastic nodules without premalignant potential. If we consider that neoplasms such as FAs could be considered premalignant and surgical removal is the appropriate treatment, the "false positive" rate of the *Afirma* test would be much lower. [25]

Thyroseq

Molecular testing of DNA from indeterminate cytology thyroid nodules for cancer mutations has had multiple modifications through the years, with progressive improvement in its sensitivity. Usually, with improvements in sensitivity, there tends to be a loss of specificity, though fortunately,

this hasn't been the case with this test. [26] The procedure of *Thyroseq* testing in its most recent version involves several steps. First, there is an assessment of DNA and RNA adequacy for testing; this is followed by a cellular composition determination for example, medullary thyroid cancer or parathyroid tissue. Then next generation sequencing (NGS) analysis is conducted for four classes of genetic alterations in 112 genes: (i) Mutations (>12,000 variants), (ii) Gene fusions (>150 types), (iii) Copy number alterations, (iv) Gene expression alterations. The results are processed by a proprietary Genomic Classifier, and finally, the test result interpretation is based on a knowledge database of >3,000 cases with known surgical outcomes allowing to provide an assessment of cancer probability and risk of cancer recurrence. [27]

Ultimately, reports of *Thyroseq* test provide a negative or positive result, and the positive results provide a probability of malignancy or NIFTP (if applicable) as well as very detailed genomic information and clinical management guidance recommendations. A negative result carries a ROM of 3 to 4%, and simple observation is recommended. A positive result is sub-classified into different categories: cases with mutations in genes that carry a ROM of <10% are classified as *currently negative*. This group comprises most commonly NIFTP or neoplastic hyperfunctioning nodules with indolent behavior for which active surveillance is recommended. Nodules with RAS-like mutations or gene expression alterations (GEA) that carry a ROM of 30 to 80% are classified as *positive RAS- like* and recommended to undergo thyroid lobectomy. Nodules with oncocytic morphology that harbor copy number alterations (CNA) with a ROM of 40 to 80%, are classified as *positive Hürthle cell type* with an intermediate ROM and are recommended to undergo either lobectomy or total thyroidectomy. For nodules classified as *positive intermediate-risk*, based on the presence of BRAF-like mutations or GEA, total thyroidectomy or lobectomy is recommended based on a ROM ranging from 95 to 100%. Lastly, nodules classified as *positive high-risk*, based on mutations associated with a 98 to 100% ROM, total thyroidectomy with or without cervical lymph node dissection is recommended.

The *Thyroseq* test performance has been validated in many studies. In one large prospective, double-blind study, the sensitivity and specificity were relatively high, with a negative predictive value of 97% and a positive predictive value of 66% [27]. Some other studies have shown a lower positive predictive value depending on how they group the mutations. In terms of clinical utility, the use of this test seems to help avoid about 61% of unnecessary surgeries. [28] [29] [19]

A study looking at the Thyroseq test's performance by specific histopathology type showed it was able to accurately predict 11/11 NIFTP, 24/27 papillary thyroid cancer (PTC), 21/22 follicular variant (FV), 10/10 Hürthle cell carcinomas, 3/4 follicular carcinomas, 1/1 medullary thyroid cancer, and 1/1 metastatic carcinoma. [27]

ThyGeNEXT/ThyraMIR

ThyGeNext/ThyraMIR is a two-step test, the first step is the ThyGeNEXT panel that tests for 10 gene mutations and 38 gene fusions, responsible for the most commonly occurring malignancies. Step 2 is the ThyraMIR which tests the expression of 10 miRNA genes. The miRNA analysis may be valuable in predicting the behavior of nodules with weak driver mutations such as RAS, but further studies should be conducted to prove this concept. [30] A retrospective study evaluating the impact of pairwise miRNA expression analysis on risk stratification of 178 indeterminate thyroid nodules found significant improvement in the diagnostic accuracy of the test. [31]

ThyGeNext/ThyraMIR has a three-tier reporting system as either negative, moderate, or positive. A study analyzing this test's performance showed that in the negative result group, only 4 out of 81 samples turned out to be malignant vs. 35 out of 47 in the positive result group. The moderate group has a risk of malignancy that is similar to the pre-test risk of malignancy, and in the validation cohort, a moderate result occurred in 28% of patients. The test had a benign call rate of 46%, and positive results were found in 26%. Since this is a non-binary test, the interpretation of the moderate risk category in calculating PPV and NPV has a major effect on these predictive parameters. The test can be sent on 1 dedicated pass, slides, or cell blocks and does not require refrigeration. [30]

Comparison of Molecular tests

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Most of the available studies conducted to compare the performance of different molecular tests provide data comparing the performance of *Afirma* vs. *Thyroseq* tests with limited comparison data for the *ThyGeNEXT/ThyraMIR* test against the others.

An independent, head-to-head test comparison study conducted at UCLA performed a prospective parallel randomized trial of 372 Bethesda III-IV nodules that were randomized to *Afirma GSC* (n=201) or *ThyroSeq v3* (n=171) test. The results showed that the diagnostic performance of both tests was high with no statistical difference; there was a lower percentage of inadequate samples for *Thyroseq* 4% vs 9% with Afirma and a higher benign call rate for *Thyroseq* 60% vs 53% with *Afrima.* [19] A meta-analysis that included 12 validation and real-world experience studies that reported the performance of Thyroseq (530 nodules) and Afirma (471 nodules) also showed comparable results. [26] Other studies comparing both tests have also found very similar performances between the two tests in terms of NPV, PPV, benign call rate, and clinical utility. [10] [27] [32] [33]

Of note, the detailed report that *Thyroseq* provides, describing the specific molecular alterations found including the variant allele frequency for mutations, can add valuable information to help clinical management decisions. Ultimately, as highlighted in one of the aforementioned studies' conclusions, the choice of molecular test to be used may hinge on factors other than the diagnostic performance, such as cost, processing time, sample inadequacy rate, and information regarding specific mutations that can guide future treatment. [19] For instance, the TERT promoter mutation is a well-known high-risk mutation that can be detected by the *Thyroseq* and the *ThyGeNEXT/ThyraMIR* tests but not by the *Afirma* test. A summary of the key features of the 3 main commercially available molecular tests can be found in Table 1.

Table 1. Comparative overview of commercially available molecular cytology tests in the diagnosis of thyroid nodules.

Characteristic	ThyroSeq GC	Afirma GSC	ThyGeNEXT/ThyraMIR **fewer number of studies available*
Methodology	RNA sequencing DNA sequencing	RNA sequencing	RNA sequencing DNA sequencing microRNA classification
NPV	~97%	>90%	~97
PPV	~66%	~60%	~75
Test result categories	- Negative - Positive (subdivided in subcategories)	- Negative - Suspicious	- Negative - Moderate - Positive
Can detect specific targetable mutations: BRAFV600E, TERT, RET/PTC, ALK	BRAF V600E TERT RET/PTC ALK	BRAF V600E RET	BRAF V600E TERT RET/PTC ALK
Collection process	1 dedicated pass or diagnostic cytology slides or cell blocks	1 to 2 dedicated passes	1 dedicated pass or diagnostic cytology slides or cell blocks

Many questions remain regarding the cost-effectiveness of these highly valuable tests and whether the ultrasound characteristics of thyroid nodules can be used to select which nodules with indeterminate cytology are better candidates for molecular testing. A study conducted at the University of Miami aiming to investigate this question compared the performance of older versions of the *Afirma and Thyroseq* tests *(Afirma GEC* and *Thyroseq v2).* The results showed that the sonographic risk of thyroid nodules, classified by ATA-US and TIRADS guidelines, alone was not an adequate predictor of malignancy. There was a modest correlation of the sonographic risk category with molecular test results, and the NPV of both molecular tests was not altered by the sonographic risk category. While 75% of high sonographic risk nodules were *Thyroseq v2* positive, the NPV remained high in this category. The PPV of *Afirma GEC* was higher in higher sonographic risk categories, while the PPV of *Thyroseq* was similar in nodules regardless of the sonographic risk category.[11]Another multicenter *Thyroseq* and ultrasound study found that neither the ATA nor TI-RADS scoring systems further informed the risk of cancer/NIFTP beyond that predicted by *Thyroseq* . [34]

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Therapeutic implications for the future

Molecular testing in thyroid nodule FNA has been primarily used for diagnostic purposes. Because of its high negative predictive value, many surgeries can be avoided in patients with indeterminate cytology and negative molecular testing. Recent advances in our understanding of the association between genetic alterations, cancer phenotype, and risk of aggressive behavior have allowed us to use the information provided by molecular tests to further guide management. Molecular markers may assist in therapeutic decisions beyond radioactive iodine treatment, although this practice has yet to be widely adopted. [6] The Food and Drug Administration has approved therapeutic options for the treatment of advanced BRAFV600E-mutated thyroid carcinomas, NTRK fusions, RET-mutated medullary thyroid carcinoma, and RET-fusion papillary thyroid cancer. [27] As advances in the field continue to evolve, it is predicted that further treatments targeting specific genetic alterations will continue to become available.

Conclusion

Molecular tests are valuable tools in the management of thyroid nodules with indeterminate cytology. Testing algorithms have evolved and improved rapidly in the last few years to improve specificity and PPV while maintaining high sensitivity and NPV. Currently, there are more robust studies to support the clinical validity and utility of *Afirma GSC* and *Thyroseq* GC when compared to ThyGeNEXT+ThyraMIR.

There is considerable heterogeneity in the methodology and results of various studies of molecular tests. Caution must be applied when interpreting the results of past studies as they often involve older versions of the platforms, and there has been a rapid rate of enhancements of the testing platforms in recent years. It appears that molecular test performance is not meaningfully altered by sonographic risk and that test performance is relatively similar in nodules of various sonographic risk categories. Long-term follow-up studies of outcomes in patients undergoing molecular testing are needed, especially for non-operated cases with negative test results.

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