

## INVITED COMMENTARY

# Indeterminate thyroid nodules in the era of molecular genomics

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**Abstract**

**Background:** Indeterminate thyroid nodules are diagnosed in up to 30% of fine-needle aspirations and the risk of malignancy in these cases are highly variable. Consequently, managing these nodules has been a challenge. While a diagnostic thyroidectomy would help clarify the pathology, there is the risk of developing surgical-related complications for a procedure that may not have been necessary and associated high costs. Genomic testing of indeterminate thyroid nodules may help better guide management.

**Methods:** We present an unbiased comprehensive review of available molecular testing for classifying indeterminate thyroid nodules, as well as their strengths and limitations, with the objective to allow practitioners to choose the best testing modality for their patients.

**Results:** Molecular testing of these nodules provided a platform to help distinguish benign versus malignant nodules, providing more confidence to rule in or rule out the likelihood of thyroid cancer in indeterminate nodules.

**Conclusion:** Genomic testing has evolved to more comprehensive panels to better stratify indeterminate nodules, including Hürthle cell neoplasms and noninvasive follicular neoplasm with papillary-like nuclear features. Understanding the methodology of each available test improves patient care and reduces unnecessary costs.

**KEYWORDS**

indeterminate thyroid nodules, thyroid cancer, Thyroseq

## 1 | INTRODUCTION

Thyroid nodules are a common finding, especially in women and individuals older than age 60 years. While they can be discovered through palpation in up to about 5% of cases (Brander, Viikinkoski, Nickels, & Kivisaari, 1991), nodules are detected through ultrasound imaging in up to 68% of the time. (Guth, Theune, Aberle, Galach, & Bamberger, 2009). Excluding thyroid cancer remains the most clinically significant task when assessing a thyroid nodule, as only 5%–15% of evaluated thyroid nodules are found to be malignant (Frates et al., 2006). A

nodule is selected for fine-needle aspiration (FNA), the most sensitive means to distinguish benign from malignant nodules, based on symptoms and sonographic features. The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) reports one of six cytologic categories, reliably establishing whether the nodule is benign (Bethesda II) or malignant (Bethesda VI) in about 70%–80% of cases (Bongiovanni, Spitale, Faquin, Mazzucchelli, & Baloch, 2012; Cibas & Ali, 2017). Bethesda I characterizes a lesion as nondiagnostic due to inadequate cellular yield. For the remaining cases, the FNA diagnosis falls into one of three indeterminate categories

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due to lack of definitive malignancy characteristics: atypia of undetermined significance or follicular lesion of undetermined significance (AUS, FLUS, Bethesda III), suspicious for neoplasm or suspicious follicular neoplasm (Bethesda IV), and suspicious for malignancy (Bethesda V). The risk of malignancy in these groups range from 10% to 75% based on the 2017 TBSRTC update (Cibas & Ali, 2017). The uncertainty of cancer in these indeterminate nodules complicates management, as the risk of malignancy is less certain. Patients with indeterminate cytology may undergo repeat FNA for cytology or diagnostic surgery (either total thyroidectomy or thyroid lobectomy). In about 75% of the cases, the final outcome is the finding of a benign thyroid nodule (Bongiovanni et al., 2012). Diagnostic surgeries not only pose a risk of operative complications, but also unnecessary costs to the patient and medical system as a whole. Furthermore, a hemi-thyroidectomy may be inadequate for some patients with cancer who would actually benefit from a total thyroidectomy based on extent of the malignancy. Alternatively, subsequent completion thyroidectomy could well have been avoided if the team of surgeons and clinicians had the availability of additional information to better characterize cancer risk of the thyroid nodule prior to the original surgery.

Over the past 30 years, the incidence of thyroid cancer continues to rise, at least partially due to incidental findings discovered on imaging. The increase consists predominantly of small papillary thyroid cancers or other early stage type thyroid cancers. As a result, there has been a three to four-fold increase in annual thyroidectomies (Jegerlehner et al., 2017) and overall surgical and surveillance costs exceeding \$1.6 billion (Lubitz et al., 2014). Meanwhile, there has been only a small change in the rate of mortality (Lim, Devesa, Sosa, Check, & Kitahara, 2017; Pellegriti, Frasca, Regalbuto, Squatrito, & Vigneri, 2013; National Cancer Institute 2019), suggesting that we are overdiagnosing and overtreating many small, indolent thyroid cancers. In addition to cost, overtreatment of low risk thyroid cancers poses exposure to potential surgical and medical complications for procedures and treatments that are not expected to impact overall patient mortality. Recognizing the consequences, a number of recent developments are changing the approach towards these indolent tumors. In the revised 2015 guidelines, the American Thyroid Association advocates a “less is more” approach for select low risk thyroid cancers and nodules (Haugen et al., 2016) based on outcomes data. As an example, a formerly considered malignant tumor known as encapsulated follicular variant of papillary thyroid cancer, has now been de-escalated to being a pre-malignant tumor referred to as non-invasive follicular tumor with papillary-like nuclei (NIFTP) (Haugen et al., 2017; Nikiforov et al., 2016).

Molecular testing has emerged as a companion tool to stratify cytologically indeterminate nodules, guiding clinicians into the appropriate clinical decision. Therefore, these

tests may help avoid unnecessary surgery for benign nodules while distinguishing more high-risk cancers that may benefit from a total thyroidectomy up front.

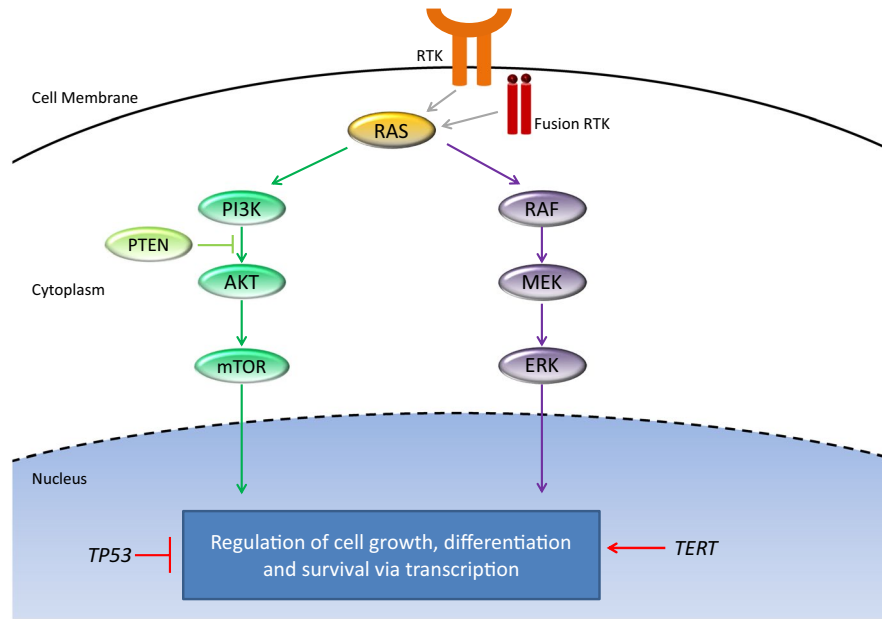
## 2 | GENETICS OF THYROID CANCER: BASIS OF MOLECULAR TESTING

A thyroid nodule grows when it escapes normal feedback mechanisms that regulate cell proliferation, leading to a clinically evident tumor mass. Unregulated cell division can result from mutations in both oncogenes and tumor suppressor genes. Understanding the mutations present in a tumor may better explain the clinical characteristics of benign versus malignant thyroid nodules, provide diagnostic information and assist in directing therapy.

It is known that thyroid cancers frequently harbor genetic alterations in the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways (Figure 1). The most common mutations in papillary thyroid cancer (PTC), comprising about 80% of all thyroid cancers, are point mutations in *BRAF* and *RAS* genes (Cohen et al., 2003; Kimura et al., 2003; Lemoine et al., 1988; Suarez et al., 1988) followed by fusions involving *RET* (Grieco et al., 1990), *NTRK* (Pierotti et al., 1995), or *ALK* (Chou et al., 2015). These mutations are almost always mutually exclusive (Soares et al., 2003), suggesting similar or redundant downstream effects. The Cancer Genome Atlas (TCGA) project described the genomic landscape of 496 cases of PTC, extending beyond known driver mutations, identifying novel mutations such as *EIF1AX* or new mutations of known drivers (*BRAF*, *RET*, or *ALK*). Through the use of various platforms, this comprehensive evaluation reduced the genomic obscurity of PTCs from 25% down to about 3% (Agrawal et al., 2014) and provided information on specific mutations inducing various signaling pathways, gene expression, or histopathologic tumor characteristics.

In follicular thyroid cancer (FTC), point mutations of *RAS* and rearrangements of *PPAR $\gamma$ /PAX8* genes are most commonly found. Additionally, mutations in components of the PI3K pathway, such as in *PTEN* or *PIK3CA*, have been reported, though are less common (Xing, 2013).

Poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC) are characterized by a higher number of mutations including those listed previously, but also more often associated with *TP53* and *TERT* promoter mutations associated with a more aggressive phenotype (Landa et al., 2016). *TP53* and *TERT* promoter mutations may also be seen in more differentiated thyroid cancer, either alone or in combination with other mutations. Medullary thyroid cancer (MTC), different from the previously discussed follicular cell-based thyroid cancer, is a cancer of the parafollicular cells or c-cells. The most



**FIGURE 1** Two common pathways implicated in thyroid cancer. The MAPK and the PI3K pathways are activated by the receptor tyrosine kinase (RTK) or by a fusion RTK. RTKs may include VEGFR, RET, FGFR, while fusion RTKs include RET/PTC, NTRK, and ALK. Increased activity of either of these RTKs (orange or red bars) by growth factors or increased expression by these sites, as well as mutations along the pathway, results in oncogenic activation of these pathways. Either pathway activation leads to tumor progression, differentiation, and decreased cell death. The MAPK pathway may be activated by mutations within RAS (*H-,N-,K-*) or RAF, most specifically *BRAFV600E*. The PI3K pathway is negatively regulated by PTEN and may be activated by mutations in RAS, PIK3CA, PTEN, AKT, and mTOR. *TP53* is a tumor suppressor gene and plays a role in regulating cell death, hence mutations within this gene lead to increased cell proliferation. *TERT* is a gene encoding the enzyme telomerase, and a mutation within the promoter region allows cells to overcome senescence, therefore increasing tumorigenesis. Patients with *TERT* promoter mutations that coexist with BRAF or RAS mutations have a worse prognosis

common genetic alteration in MTC is the *RET* point mutation, present in about 95% of hereditary forms and 45% of sporadic cases (Romei et al., 2016), though *RAS* mutations may also be found in some sporadic MTCs (Ciampi et al., 2013).

Enhanced activity of the MAPK and PI3K signal transduction cascade, *TERT* promoter, or inhibition of the tumor suppressor gene, *TP53*, can lead to uncontrolled cell growth, and potentially lead to the evolution or aggressive nature of a thyroid cancer. Hence being able to capture this data early on in the management course may be very valuable for surgical planning, especially in the setting of indeterminate nodules.

### 3 | EVOLUTION OF GENOMIC TESTING FOR INDETERMINATE NODULES

The introduction of TBSRTC has improved the consistency in characterizing the cytology assessment of thyroid nodules, however the rates of reporting indeterminate cytology varies from 5% to 40% (Cibas & Ali, 2009) primarily related to the experience of the interpreting pathologist. Molecular testing has emerged as a tool to improve clinical decision making for indeterminate nodules. While this form of testing originated as a means to assess for the presence of specific mutations,

such as *BRAFV600E* or *RAS*, it is known that the absence of a single mutation does not exclude thyroid cancer. Single mutation analysis slowly expanded to multi-gene assays such as 7-gene (*BRAF*, *NRAS*, *HRAS*, *KRAS*, *RET/PTC1*, *RET/PTC3*, and *PAX8-PPAR $\gamma$* ) analysis in the early 2000s which was able to identify 70% of thyroid cancers (Nikiforov et al., 2011). While this expansion demonstrated an improved specificity and positive predictive value (PPV), the sensitivity and negative predictive value (NPV) were insufficient for clinical use, failing to diagnose about 30% of cancers (Cantara et al., 2010; Nikiforov et al., 2009, 2011). Hence, a more effective method to “rule out” thyroid cancer was developed primarily based on gene expression. Improvements of “rule in” tests have been seen with next generational sequencing (NGS) through clonal amplification, DNA synthesis, and parallel sequencing.

### 4 | AVAILABLE MOLECULAR TESTS

#### 4.1 | Veracyte™: Afirma® thyroid FNA analysis

The Afirma® Gene Expression Classifier (GEC) utilizes two additional samples acquired by FNA. RNA is extracted from

the cells in the sample and, through microarray technology, the sample's gene expression is compared to the profile of a "control group." This is a two-step process: first screening the expression profile to a specific control group of 25 gene expressions of MTC, metastatic tumors, and parathyroid tissue; if there is no match, then the test proceeds to the second step where the sample is further analyzed by the main 142 gene profiles (Alexander et al., 2012). The crux of the test relies on having adequate representation of the control group to compare the sample against.

A prospective, double-blinded multi-center validation study assessed 265 indeterminate nodules and reported a sensitivity of 92% and a specificity of 52%. The NPV for TBRTC III, IV, and V diagnoses were 95%, 94%, and 85%, respectively (Alexander et al., 2012). Based on these findings, the recommended use was for the TBSRTC III and IV. With its high sensitivity and NPV, Afirma<sup>®</sup> GEC was identified as a "rule out" test. The PPV was quite low at 37%–38% (Alexander et al., 2012) on the initial version of the test. Other groups have shown the rate of confirmed malignancy in GEC suspicious to vary, between 17% and 47% (Alexander et al., 2014; McIver et al., 2014). However, corollary tests assess the gene profile for MTC or *BRAFV600E* and have improved the test's specificity.

While the intention of the GEC was to minimize unnecessary surgeries, the rate of surgery did not necessarily change at all institutions. A retrospective evaluation of one center's experience, after the introduction of the Afirma<sup>®</sup> GEC, interestingly revealed the rate of Bethesda III and IV classification actually increased and surgical rate was statistically unchanged, though an upward trend was noted (Sacks et al., 2016). Several studies showed cytology more often predicted malignancy than the "suspicious by Afirma<sup>®</sup>" GEC, particularly for patients with TBSRTC category IV (McIver et al., 2014) and showed no difference for those with TBSRTC category III (Roychoudhury et al., 2017), suggesting there was not added benefit in performing the test. Furthermore, Hürthle cell changes seen in cytology demonstrated more variability in NPV and higher rates of GEC-suspicious for those with histologically benign lesions (Brauner et al., 2015; Harrell & Bimston, 2014; McIver et al., 2014), questioning the value of performing GEC testing in these nodules.

These limitations and advances in genomic characterization, through enhanced measurements of RNA transcriptome expression and sequencing, lead to the development of Afirma<sup>®</sup> Genomic Sequencing Classifier (GSC), which sought to improve the specificity while maintaining the sensitivity and NPV. The GSC uses RNA sequencing to better assess indeterminate nodules, especially distinguishing Hürthle cell neoplasms from benign Hürthle cell changes and classifiers that may identify medullary thyroid carcinoma, parathyroid lesions, and certain mutations, such as *BRAF V600E* and *RET/PTC*. The validation study utilized the same population as the original version GEC. The results found an increased

specificity and PPV with a higher rate of benign designation (Patel et al., 2018) (Table 1). This conclusion was further shown on two separate studies, where GSC demonstrated a higher benign call rate, especially for TBSRTC III and IV and for Hürthle cell lesions (Angell et al., 2019; Harrell, Eyerly-Webb, Golding, Edwards, & Bimston, 2019).

Afirma<sup>®</sup> has also introduced an Xpression Atlas as an add-on test for GSC suspicious results or in TBSRTC category V and VI nodules. This panel assesses 761 DNA variants and 130 RNA fusions in 500 genes. Although a validation study has not yet been published, preliminary analysis of this test applied to the original GEC and GSC population demonstrates low sensitivity and specificity in TBSRTC category III and IV nodules. In the TBSRTC category V and VI groups, the variant-only sensitivity increased to 79.2% (CI: 57.8–2/9%) and specificity 40% (CI: 5.27%–85.3%); data on gene fusions was not available, due to having a low prevalence in all nodules (Babiarz et al., 2018).

## 4.2 | Thyroseq<sup>®</sup>

Thyroseq<sup>®</sup> originated from the polymerase chain reaction (PCR)-based 7-gene panels of the late 2000s (Nikiforov et al., 2011). However, given the limited number of mutations assessed, this test had a higher false negative rate. In 2013, the test expanded to include 15 mutations, and through the use of next generational sequencing (NGS) of RNA and DNA, there was an improved ability to detect multiple genetic alterations using fewer cells, especially beneficial when the sample is from a fine needle aspirate. ThyroSeq<sup>®</sup> v2 expanded the panel to include approximately 90% of the mutations encountered in PTCs based on the TCGA findings as well as in other cancers (Agrawal et al., 2014). This assay includes testing for point mutations and small insertions/deletions in 14 genes (including *AKT*, *BRAF*, *CTNNB1*, *GNAS*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PTEN*, *RET*, *TP53*, *TSHR*, and *TERT*; *EIF1AX* was included in v2.1), 42 gene fusions that occur in thyroid cancer (including *RET/PTC*, *PPAR $\gamma$* , *NTRK1*, *NTRK3*, *BRAF*, and *ALK*) as well as 16 gene expressions. A positive result is given based on the allelic frequency depending on the type of mutation present, ranging from  $\geq 5\%$  to 10%, and is included in the final report. The presence of certain mutations, such as *BRAFV600E* or *RET/PTC* will more strongly confirm malignancy, whereas other mutations such as *RAS* may suggest either a low-risk cancer or a NIFTP (Nikiforov et al., 2016).

In a single institution study to assess test performance, 96 nodules with TBSRTC category III and 143 nodules as TBSRTC category IV were assessed under this panel, and sensitivity for malignancy was found to be 91% (CI: 80%–99%) and 90% (CI: 80%–99%), specificity 92% (CI: 86%–98%) and 93% (CI: 88%–98%), NPV 77% (CI: 61%–93%) and 96% (CI: 92%–95%) and a PPV of 77% (CI: 61%–93%)



**TABLE 1** Test performance results from validation studies

| Test Name  | Methodology              | Bethesda category | Patients (N) | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|--|--------------------------|-------------------|--------------|-----------------|-----------------|---------|---------|
| Afirma <sup>®</sup> GEC (Alexander et al., 2012)                     | Micro-array              | III               | 129          | 90              | 53              | 38      | 95      |
|  |                          | IV                | 81           | 90              | 49              | 37      | 94      |
| Afirma <sup>®</sup> GSC (Patel et al., 2018)                         | RNA sequencing           | III               | 114          | 93              | 71              | 51      | 97      |
|  |                          | IV                | 76           | 88              | 64              | 42      | 95      |
| Thyroseq <sup>®</sup> v0 (Nikiforov et al., 2011)                    | PCR                      | III               | 247          | 63              | 99              | 88      | 94      |
|  |                          | IV                | 214          | 57              | 97              | 87      | 86      |
| Thyroseq <sup>®</sup> v2.1 (Nikiforov et al., 2015)                  | DNA and RNA NGS          | III               | 96           | 91              | 92              | 77      | 97      |
| Thyroseq <sup>®</sup> v2 (Nikiforov et al., 2014)                    | NGS                      | IV                | 143          | 90              | 93              | 83      | 96      |
| Thyroseq <sup>®</sup> v3 (gene classifier) (Nikiforova et al., 2018) | NGS                      | III               | 84           | 98              | 82              | N/A     | N/A     |
|  |                          | IV                | 74           |                 |                 |         |         |
|  |                          | V                 | 17           |                 |                 |         |         |
| Thyroseq <sup>®</sup> v3 (gene classifier) (Steward et al., 2019)    | NGS                      | III               | 154          | 91              | 85              | 97      | 64      |
|  |                          | IV                | 93           | 97              | 75              | 98      | 68      |
| ThyGenX <sup>®</sup> /ThyraMIR <sup>®</sup> (Labourier et al., 2015) | NGS and miRNA expression | III               | 58           | 94              | 80              | 68      | 97      |
|  |                          | IV                | 51           | 82              | 91              | 82      | 91      |
| RosettaGx Reveal <sup>™</sup> (Lithwick-Yanai et al., 2017)          | miRNA                    | III and IV        | 189          | 74              | 74              | 43      | 92      |

Abbreviations: GEC, genomic expression classifier; GSC, genomic sequencing classifier; miRNA, microRNA; NGS, next generational sequencing; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value.

and 83% (CI: 72%–95%), respectively (Nikiforov et al., 2014, 2015). Filtering out germline gene variants or variants of no known clinical importance allows for the relatively higher PPV. With these statistics, Thyroseq<sup>®</sup> v2 was being marketed as both a “rule in” and “rule out” test.

Test performance of v2 was assessed in several single-institution and inter-institutional studies (Marcadis et al., 2019; Taye et al., 2018; Valderrabano et al., 2017) and noticeably deviated in statistical accuracy compared to the original single-institution validation study and among themselves. This could be attributed to differences in pre-test probability of risk of malignancy at each institution as well as the reclassification NIFTP into the benign category (Nikiforov et al., 2016) (the reclassification occurred in 2016 after the original studies), and inevitably lowered the PPV. Of note, one study looked at cost benefits of implementing Thyroseq<sup>®</sup> v2 versus diagnostic surgery for indeterminate TBSRTC category III and IV nodules, and found that Thyroseq<sup>®</sup> v2 was found to be cost effective in comparison to diagnostic surgery particularly for nodules with TBSRTC IV cytology (Rivas et al., 2018).

Version 3 of ThyroSeq<sup>®</sup> was developed to improve the test's overall accuracy, and to expand the existing v2 panel to include 112 genes with newly discovered genetic alterations related to thyroid nodules and cancer, including point mutations, insertions/deletions, gene fusions, copy number, and gene expression alterations. In the validation study specifically evaluating the

genomic classifier (GC), sensitivity increased from v2 to 98%, though specificity went down to 82% (Nikiforova et al., 2018). However, the test was further assessed in a multi-center, blinded, prospective study at 10 sites. In this population of 247 TBSRTC category III and IV patients found an overall cancer prevalence of 28%, sensitivity 94% (CI: 86%–98%), specificity 82% (CI: 75%–87%), NPV 97% (CI: 93%–99%), and PPV 66% (CI: 56%–75%) (Steward et al., 2019). Specifically applied to Hürthle cell nodules in this series, the GC negative call rate was 53% and all 10 carcinomas were identified.

### 4.3 | ThyGenX<sup>®</sup>/ThyraMIR<sup>®</sup>

ThyGenX<sup>®</sup> is a targeted NGS mutational panel for the detection of five genes (*BRAF*, *KRAS*, *HRAS*, *NRAS* and *PIK3CA*) and three gene fusions (*RET/PTC1*, *RET/PTC3*, *PAX8-PPAR $\gamma$* ) associated with thyroid papillary carcinoma and follicular carcinoma. Alone, this panel has a low sensitivity given the number of missed mutations. ThyraMIR<sup>®</sup> is a micro RNA (miRNA) gene expression classifier that is based on the evaluation and expression of 10 miRNAs by PCR. ThyraMIR<sup>®</sup> is usually ordered as a reflex to a negative ThyGenX<sup>®</sup> test or positive *RAS* mutation. The tests are marketed to be used in combination, strengthening the PPV. The validation study was a multi-center retrospective sampling of 109 patients with TBSRTC category

III and IV nodules with known surgical outcomes. With a malignancy prevalence of 32% (35/109), mutations were detected in 69% nodules with thyroid malignancy. Of the mutation-negative specimens, miRNA testing identified 64% of malignant cases and 98% of benign cases. Overall sensitivity was 89% (CI: 73%–97%), specificity 85% (CI: 75%–92%), PPV 74% (CI: 58%–86%), and NPV of 94% (CI: 85%–98%) (Labourier et al., 2015). They further broke down the statistics based on Bethesda stage (Table 1).

In the multi-center retrospective clinical experience study that assessed risk of malignancy and outcomes such as probability of surgery and overall survival, 180 patients were followed over a 2-year period; only 14% had malignancy. A negative ThyGenX<sup>®</sup>/ThyraMIR<sup>®</sup> result in nodules with TBSRTC category III or IV cytology correlated with a high probability of non-surgical treatment, (only 11% underwent surgery) and a high probability of survival without malignancy (92%) for up to 2 years follow up. A positive result equated to a 57% probability of malignancy and increased chance of surgery. For nodules with weak driver mutations (such as *RAS*, *PAX8/PPAR $\gamma$* , *PIK3CA*), a positive miRNA test supported the likelihood of cancer while negative results downgraded that risk. This study illustrated real-world decisions on surgical treatment as well as risk of malignancy based on test outcomes (Sistrunk et al., 2019). In terms of its strength to diagnose NIFTP and avoid more extensive surgery, the GC portion of the test did not significantly differ between NIFTP and invasive encapsulated follicular variant of PTC, however there were individual pair-wise differences among the panel of 10 miRNAs to potentially make this distinction.

A newer version of the NGS, ThyGeNEXT<sup>®</sup> expanded the mutation panel by 5 additional DNA markers (including *TERT*, *RET*, and *PTEN*) and 32 RNA fusions (such as *NTRK*, *ALK*, and *RET*), totalling 18 and 38, respectively. A recent study correlating the expanded ThyGeNEXT<sup>®</sup> combination with the existing ThyraMIR<sup>®</sup> showed an improvement in detecting strong drivers of thyroid cancer by 8%, where *BRAFV600E* and *TERT* promoters are the most common. In addition, this panel also increased detecting coexisting drivers by 4%, where *TERT* was the most common often paired with *RAS* (Jackson et al., 2020).

#### 4.4 | RosettaGX Reveal<sup>™</sup>

The RosettaGX Reveal<sup>™</sup> is a diagnostic assay, similar to ThyraMIR<sup>®</sup>, and designed to classify indeterminate thyroid nodules as benign or suspicious for malignancy by miRNA profiling and using a single FNA stained smear (Benjamin et al., 2016). The assay measures 24 miRNAs, up from 10 of ThyraMIR<sup>®</sup>. There is also a miRNA profile specific to medullary thyroid carcinoma.

In the clinical validation analysis, this retrospective multi-center study assessed 189 indeterminate samples with pathologic follow up. The results, while not as strong as the other tests, were quite promising, especially given the fact that the test could be performed from a routinely stored FNA smear, avoiding unnecessarily FNA passes or repeat biopsies. However, this cohort did not take into account NIFTP, since this classification came after the study, or any oncocytic (Hürthle cell) carcinomas.

In another retrospective analysis comparing the performance of RosettaGX Reveal<sup>™</sup> to Afirma GEC, Reveal<sup>™</sup> outperformed GEC in a cohort of 81 cytologically indeterminate samples, where final pathology resulted in 63 as benign/NIFTP and 18 malignant. Reveal<sup>™</sup> also demonstrated a higher specificity of 64% compared to 28.4% in the GEC. Among the 7 NIFTP patients, specificity and PPV were higher in Reveal<sup>™</sup> and more accurately confirmed benign Hürthle cell lesions compared to GEC. However, among the 18 malignant patients, GEC more correctly classified the nodules as “suspicious” 94% versus 78% in Reveal<sup>™</sup>.

## 5 | CONCLUSION

Over the last couple of decades, our knowledge has expanded to better understand the genetic expression of a malignant thyroid nodule. This knowledge has been applied to the pre-surgical setting, especially when stratifying malignancy risk in an indeterminate nodule. Molecular testing has evolved from single mutational assessments to more broad genetic panels to help better characterize indeterminate nodules that may have otherwise been subjected to unnecessary and costly lobectomy. With the reclassification of NIFTP as a pre-malignant entity, assessing the existing tests or developing better methods to predict this pathology in the pre-surgical setting is necessary. Knowing which mutation is present, especially those associated with more high risk cancers, such as *BRAF* and/or *TERT*, may also be beneficial to recommending a more complete surgical resection up front as well as guiding more targeted systemic therapies if indicated in the future. It should be noted that in all of these studies, the number of malignant nodules included were relatively low, and test performance is very much dependent on the institutional prevalence of malignancy in their population and variation in pathology interpretation. While there have been some institutional test comparisons among the same cohort, larger more multi-center assessments are needed.

## CONFLICT OF INTEREST

Sarika N. Rao, none; Victor J. Bernet, none.

## AUTHOR CONTRIBUTION

Both Drs. Rao and Bernet equally contributed to the concept and design of the review paper and as well as drafting and finalizing the manuscript.

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**How to cite this article:** Rao SN, Bernet V. Indeterminate thyroid nodules in the era of molecular genomics. *Mol Genet Genomic Med*. 2020;8:e1288. <https://doi.org/10.1002/mgg3.1288>