Preoperative Molecular Markers in Thyroid Nodules

Zeyad T. Sahli1, Philip W. Smith2, Christopher B. Umbricht1 and Martha A. Zeiger2*

1Department of Surgery, The Johns Hopkins University School of Medicine, Baltimore, MD, United States,
2Department of Surgery, University of Virginia, Charlottesville, VA, United States

The need for distinguishing benign from malignant thyroid nodules has led to the pursuit of differentiating molecular markers. The most common molecular tests in clinical use are Afirma® Gene Expression Classifier (GEC) and Thyroseq® V2. Despite the rapidly developing field of molecular markers, several limitations exist. These challenges include the recent introduction of the histopathological diagnosis “Non-Invasive Follicular Thyroid neoplasm with Papillary-like nuclear features”, the correlation of genetic mutations within both benign and malignant pathologic diagnoses, the lack of follow-up of molecular marker negative nodules, and the cost-effectiveness of molecular markers. In this manuscript, we review the current published literature surrounding the diagnostic value of Afirma® GEC and Thyroseq® V2. Among Afirma® GEC studies, sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV) ranged from 75 to 100%, 5 to 53%, 13 to 100%, and 20 to 100%, respectively. Among Thyroseq® V2 studies, Se, Sp, PPV, and NPV ranged from 40 to 100%, 56 to 93%, 13 to 90%, and 48 to 97%, respectively. We also discuss current challenges to Afirma® GEC and Thyroseq® V2 utility and clinical application, and preview the future directions of these rapidly developing technologies.

Keywords: thyroid cancer, non-invasive follicular thyroid neoplasm with papillary-like nuclear features, molecular test, Afirma, Thyroseq

INTRODUCTION

Thyroid nodules are common among adults over the age of 60 years, with a prevalence of 50–70% (1, 2). Moreover, the incidence of thyroid cancer in the United States has increased by 211% between 1975 and 2013 (3), due to both an improved detection of small (<2 cm) thyroid nodules by thyroid ultrasonography and a true increase in thyroid cancer incidence (4). Nevertheless, the vast majority (85–95%) of thyroid nodules are benign (5). For this reason, the ability to distinguish between benign and malignant nodules is important in order to spare patients unnecessary diagnostic surgery.

Fine needle aspiration (FNA) to facilitate this distinction first became widely practiced in the early 1980s (6), and is widely recognized as the gold standard initial diagnostic procedure in the differential diagnosis of thyroid nodules, being accurate, safe, and cost effective (7, 8). The sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), false-negative (FN) rate, and false-positive (FP) ranges of an FNA are 88.2–97.0%, 47.0–98.2%, 52.0–98.0%, 89.0–96.3%, 0.5–10.0%, and 1.0–7.0%, respectively (9).

In 2009, the Bethesda classification system for FNA reporting was introduced by the National Cancer Institute, and recently, revised, included six categories based upon cytopathological features, with an associated malignancy rate and standardized management recommendation for each...
category (Table 1) (10). FNA reliably establishes the diagnosis of a benign or malignant nodule in 70–80% of all cases (11) and has decreased the proportion of benign nodules unnecessarily resected from 86 to 50% (12). However, 20–30% of FNA cases have indeterminate or suspicious cytological results that include Bethesda III, IV, and V categories (12) and, of these, 6–75% are malignant on final surgical pathology (13, 14). Due to the uncertainty of malignancy in these patients, their management has been challenging, usually including a repeat FNA or a diagnostic lobectomy. For this reason, the need for distinguishing benign from malignant lesions in this subset of thyroid nodules has led to the pursuit of differentiating molecular markers.

Interest in achieving this distinction increased in 2002 with the recognition of the oncogenic role of the BRAF V600E mutation in approximately 58–69% of papillary thyroid cancers (PTC) (15, 16). However, genetic testing for BRAF V600E alone for the detection of PTCs is inadequate for clinical decision making due to its low sensitivity of 60% for PTC (17). Indeed, our group first published its use in indeterminate and suspicious thyroid lesions and found it to add minimal clinical value (18). In addition to studying the diagnostic utility of BRAF V600E, numerous studies have investigated the association of BRAF V600E and patient prognosis. However, the correlation between BRAF V600E and clinical features of PTCs has yielded inconsistent results. Some studies report that BRAF V600E is associated with a more advanced phenotype including an increased risk of lymph node metastasis, cancer recurrence, and patient mortality (19–21), while others report no such associations (22). Moreover, thyroid cancer with BRAF V600E and TERT promoter mutations has been associated with worse clinico-pathological outcomes (23, 24).

BRAF testing can also be useful in deciding treatment in the setting of known metastatic thyroid cancer. Direct tyrosine kinase inhibitors, such as vemurafenib (25), dabrafenib (26), and sorafenib (27), have been shown to be effective in BRAF V600E metastatic thyroid cancers.

Since mutational analysis of single genes has not proven adequate in guiding management decisions in indeterminate or suspicious thyroid nodules, attention turned to using panels of molecular markers. Currently, the most common molecular tests in clinical use are Afirma® Gene Expression Classifier (GEC) and Thyroseq® V2 (28). Introduced in 2011 by Veracyte, the Afirma® GEC has been considered a “rule-out” malignancy test. It includes a 42-gene expression molecular assay and uses microarray technology to measure the mRNA expression profiles to determine whether a thyroid nodule is “suspicious” or “benign.” The test’s primary aim is to spare patients with cytologically indeterminate FNA samples unnecessary diagnostic surgery (29). Among indeterminate/suspicious nodules (Bethesda Type III–V), the test has both a high Se (92%) and high NPV (93%) (30) (Table 2). In contrast, it has a low Sp (52%) and PPV (47%), and cannot accurately identify malignant lesions alone.

Thyroseq® v2, introduced in 2014 by CBL Path, is designed to identify malignant thyroid nodules by next generation sequencing (NGS), detecting 14 thyroid cancer-related genetic mutations, including RAS and BRAF mutations, 42 types of gene fusions associated with thyroid cancer, including PAX8/PPARY and RET/PTC rearrangements, and mRNA expression levels for 16 genes; it is therefore considered a “rule-in” malignancy test (29). Among Bethesda Type III – IV nodules, the test is marketed as having a high Se, Sp, PPV, and NPV of 90–91%, 92–93%, 77–83%, and 96–97%, respectively, as well as having the ability to stratify risk based on the mutation detected (52, 53) (Table 3).

In this manuscript, we review the current published literature surrounding Afirma® GEC and Thyroseq® V2, discuss current challenges to their utility; and clinical application, and preview the future directions of these rapidly developing technologies.

### CLINICAL MANAGEMENT OF INDETERMINATE THYROID NODULES

The 2015 American Thyroid Association (ATA) (60) and the 2016 American Association of Clinical Endocrinologists (AACE) (9) clinical guidelines recommend “considering” molecular testing for indeterminate nodules. If molecular testing is being considered, ATA recommends that patients “should be counseled regarding the potential benefits and limitations of testing and about the possible uncertainties in the therapeutic and long-term clinical implications of results” (strong recommendation, low-quality evidence) (60). However, long-term outcome data on the use of molecular markers for therapeutic decision-making is currently unavailable. A recent report estimated that standard application of the GEC for all indeterminate thyroid nodules would result in only a 7.2% decrease in thyroidectomy volume (61). Similarly, two studies by our group showed that molecular markers did not significantly affect the surgical decision-making process, where only 7.9–8.4% of patients had altered clinical management as a result of molecular testing (39, 62).

---

**TABLE 1** The Bethesda system for reporting thyroid cytopathology (10).

| Bethesda group | Diagnostic category | Abbreviation | Before NIFTP reclassification (NIFTP malignant) | After NIFTP reclassification (NIFTP benign) |
|----------------|---------------------|--------------|-----------------------------------------------|---------------------------------------------|
| I              | Non-diagnostic/unsatisfactory | –            | 5–10%                                        | No change                                   |
| II             | Benign               | B            | 0–3%                                         | No change                                   |
| III            | Atypia of undetermined significance/follicular lesion of undetermined significance | AUS/FLUS | 10–30%                                       | 6–18%                                       |
| IV             | Follicular neoplasm/suspicious for follicular neoplasm | FN/SFN       | 25–40%                                       | 10–40%                                      |
| V              | Suspicious for malignancy | SM          | 50–75%                                       | 45–60%                                      |
| VI             | Malignant            | M            | 97–99%                                       | 94–96%                                      |
| Author | Year | Study type | Follow-up period | Bethesda category | Sample size | GEC suspicious | Total surgery | Malignant nodules | NIFTP | Diagnostic value |
|--------|------|------------|------------------|-------------------|-------------|---------------|--------------|-----------------|-------|------------------|
| Afirma® GEC (30) | 2012 | Prospective, blinded multicenter | 301 days (Median) | Total 265 | 165 | 265 | 85 | – | 92 | 52 | 47 | 93 |
| | | | | III 129 | 74 | 129 | 31 | – | 90 | 53 | 38 | 95 |
| | | | | IV 81 | 49 | 81 | 20 | – | 90 | 49 | 37 | 94 |
| | | | | V 55 | 42 | 55 | 34 | – | 94 | 52 | 76 | 85 |
| Hang et al. (31) | 2017 | Retrospective, single institution | – | Total 133 | 112 | 133 | 27 | 21 | 100 | 24 | 42 | 100 |
| | | | | III 42 | 39 | 42 | 6 | 4 | 90 | 6 | 23 | 67 |
| Harrison et al. (32) | 2017 | Retrospective, single institution | – | Total 100 | – | 37 | 13 | – | – | – | 35 | – |
| | | | | IV 10 | – | 4 | 1 | – | – | – | 25 | – |
| Kay-Rivest et al. (33) | 2017 | Retrospective, multicenter | – | Total 172 | 83 | 77 | 38 | – | – | – | 49 | – |
| Samulski et al. (34) | 2016 | Retrospective, single institution | – | Total 294 | 133 | 128 | 33 | 11 | 93 | 17 | 39 | 81 |
| | | | | III 166 | 60 | 60 | 15 | 5 | 96 | 19 | 40 | 88 |
| | | | | IV 122 | 73 | 68 | 18 | 6 | 92 | 15 | 39 | 76 |
| Wu et al. (35) | 2016 | Prospective, single institution | – | Total 245 | 132 | 128 | 63 | – | 94 | 31 | 57 | 83 |
| | | | | III 217 | 115 | 107 | 55 | 32 | 93 | 29 | 58 | 79 |
| | | | | IV 28 | 17 | 21 | 8 | – | 100 | 38 | 50 | 100 |
| Yang et al. (36) | 2016 | Retrospective, single institution | – | Total 189 | 94 | 67 | 32 | – | 100 | 15 | 51 | 100 |
| | | | | III 165 | 81 | 55 | 26 | 8 | 100 | 7 | 49 | 100 |
| | | | | IV 24 | 13 | 12 | 6 | – | 100 | 50 | 67 | 100 |
| Chaudhary et al. (37) | 2016 | Retrospective, single institution | – | Total 158 | 85 | 73 | 28 | – | 100 | 15 | 100 | 20 |
| | | | | III 16 | 41 | 45 | 8 | 100 | 50 | 67 | 100 |
| | | | | IV 14 | 22 | 22 | 7 | 100 | 50 | 67 | 100 |
| Abeykoon et al. (38) | 2016 | Retrospective, single institution | – | Total 24 | 17 | 16 | 12 | – | – | – | 49 | – |
| | | | | III 11 | 6 | 4 | 2 | 100 | 67 | Null | – |
| | | | | IV 13 | 11 | 12 | 10 | 100 | 67 | Null | – |
| Marti et al. (45) | 2015 | Retrospective, multicenter | – | Total 103 | 104 | 70 | 27 | – | 100 | 16 | 43 | 100 |
| | | | | III 62 | 45 | 47 | 6 | – | 100 | 8 | 14 | 100 |

(Continued)
### Table 2 | Continued

| Author                        | Year  | Study type                  | Follow-up period | Bethesda category | Sample size | GEC suspicious | Total surgery | Malignant nodules | NIFT-P | Diagnostic value |   |   |   |
|-------------------------------|-------|-----------------------------|------------------|-------------------|-------------|----------------|---------------|------------------|--------|------------------|---|---|---|
| McIver et al. (47)           | 2014  | Prospective, single institution | 9.5 months       | III               | 72          | 44             | 36            | 6                | –      | 83               | 10| 16| 75|
|                               |       |                             |                  | IV                | 63          |                | 31            | 5                | –      | 100              | 0 | 20| Null|
| Lastra et al. (48)           | 2014  | Retrospective, single institution | –               | Total             | 132         | 62             | 50            | 22               | –      | 100              | 7 | 54| 100|
|                               |       |                             |                  | III               | 68          | 23             | 18            | 11               | –      | 100              | 0 | 61| Null|
|                               |       |                             |                  | IV                | 64          | 39             | 32            | 11               | –      | 100              | 10| 37| 100|
| Aragon Han et al. (49)       | 2014  | Retrospective, single institution | –               | III, IV           | 37          | 36             | 37            | 16               | –      | 100              | 5 | 44| 100|
| Alexander et al. (50)        | 2013  | Retrospective, multicenter   | 8.5 months       | Total             | 339         | 148            | 132           | 54*              | –      | 98               | 13| 44| 91|
|                               |       |                             |                  | III               | 165         | 66             | 48            | 23               | –      | –                | – | – | – |
|                               |       |                             |                  | IV                | 79          | 73             | 65            | 24               | –      | –                | – | – | – |
|                               |       |                             |                  | V                 | 13          | 9              | 8             | 6                | –      | –                | – | – | – |
| Harrell et al. (51)          | 2013  | Retrospective, single institution | –               | Total             | 58          | 36             | 35            | 18               | –      | 94               | 24| 57| 80|
|                               |       |                             |                  | III               | 22          |                | –             | –                | –      | 100              | 33| 64| 100|
|                               |       |                             |                  | IV                | 8           |                | –             | –                | –      | 75               | 0 | 38| 0 |

### Table 3 | ThyroSeq V2 literature review.

| Author                        | Year  | Study type                  | Follow-up period | Bethesda category | Sample size | Thyroseq suspicious | Total surgery | Malignant nodules | NIFT-P | Diagnostic value |   |   |   |
|-------------------------------|-------|-----------------------------|------------------|-------------------|-------------|---------------------|---------------|------------------|--------|------------------|---|---|---|
| ThyroSeq® v2 (53)             | 2015  | Retrospective, Single Institution | –               | III               | 465         | 31                  | 95            | 22               | –      | 91               | 92| 77| 97|
| ThyroSeq® v2 (52)             | 2014  | Single Institution          | –                | IV                | 143         | 42                  | 143           | 39               | –      | 90               | 93| 83| 96|
| Taye et al. (54)              | 2017  | Prospective, Multicenter    | –                | III, IV           | 156         | 51                  | 63            | 10               | 3      | 91               | 45| 27| 96|
| Valderrabano et al. (55)      | 2017  | Retrospective, Single Institution | –               | Total             | 190         | 45                  | 102           | 15               | 5      | 70               | 77| 42| 91|
|                               |       |                             |                  | III               | 104         | 22                  | 52            | 5                | 2      | 43               | 71| 19| 89|
|                               |       |                             |                  | IV                | 86          | 23                  | 50            | 10               | 3      | 85               | 84| 65| 94|
| Shrestha et al. (56)          | 2016  | Retrospective, Single Institution | –               | Total             | 39          | 23                  | 39            | 14               | –      | 93               | 60| 57| 94|
|                               |       |                             |                  | III               | 27          | 15                  | 27            | 9                | –      | 89               | 61| 53| 92|
|                               |       |                             |                  | IV                | 12          | 8                   | 12            | 5                | –      | 100              | 57| 63| 100|
| Khatami et al. (57)           | 2016  | Retrospective, Single Institution | 3–6 months       | III, IV, V        | 42          | 7                   | 7             | 4                | –      | –                | – | 57| – |
| Tolaldo et al. (58)           | 2016  | Prospective, Single Institution | –               | III, IV           | 148         | 51                  | 45            | 20               | –      | 95               | 60| 66| 94|
| Shrestha et al. (59)          | 2016  | Retrospective, Single Institution | –               | III               | 41          | –                   | 41            | –                | –      | 83               | 62| 90| 48|
|                               |       |                             |                  | IV                | 14          | –                   | 14            | –                | –      | 80               | 56| 50| 90|
For these reasons, patient benefit from molecular marker use in routine clinical practice is likely marginal. Moreover, the AACE 2016 guidelines recommend molecular testing to complement cytologic evaluation in indeterminate nodules (Grade A recommendation), but only when the “results are expected to influence clinical management” (Grade A recommendation) (9). Testing for detection of \textit{BRAF}, \textit{RET/PTC}, \textit{PAX8/PPRG}, and \textit{RAS} mutations can be considered (Grade B recommendation). Furthermore, with the exception of \textit{BRAF} \textit{V600E}, there is insufficient evidence “to recommend in favor of or against the use of mutation testing as a guide to determine the extent of surgery” (Grade A recommendation) (9).

Importantly, molecular testing is not recommended in patients with an indeterminate thyroid nodule if other indications for surgery are present such as a nodule greater than 4 cm, compressive symptoms, or personal preference (63). The utility of molecular testing in Bethesda Type V nodules at institutions with a high prevalence of malignancy is low, and provides little additional benefit from a “positive” test result due to the similar PPV as that of a Bethesda Type V FNA result. Moreover, a diagnostic lobectomy would still be recommended in the case of a “negative” result. Finally, a limitation of the current molecular markers is their insufficient data to recommend use among pediatric patients (≤18 years) (64). Until these tests can be validated using this patient population, they cannot be routinely used to complement the indeterminate FNA cytology results.

**CURRENT PUBLISHED LITERATURE**

Current published literature regarding Afirma® and Thyroseq® V2 validation studies are summarized in Tables 2 and 3. The data were summarized from the results of a PubMed search for English language studies that reported diagnostic accuracy in observational clinical settings published for GEC and Thyroseq® V2 up to November 30, 2017. References from the retrieved articles were also searched for additional studies. Inclusion criteria included reporting molecular marker diagnostic accuracy or enough information to calculate sensitivity, specificity, NPV, and PPV among Bethesda Type III or IV lesions. All calculations were made using the available published information. To adhere to current clinical guidelines, non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) and malignant pathologies were categorized as malignant or “requiring resection.” Of the published literature, only two studies included pediatric patients in their cohort (31, 32).

Among Afirma® GEC studies, Se, Sp, PPV, and NPV ranged from 75 to 100%, 5 to 53%, 13 to 100%, and 20 to 100%, respectively. Among Thyroseq® V2 studies, Se, Sp, PPV, and NPV ranged from 40 to 100%, 56 to 93%, 13 to 90%, and 48 to 97%, respectively. Valderrabano et al. report that the wide variation among reported diagnostic values can be explained by different defining characteristics of the study populations such as institutional prevalence of malignancy sample size, Bethesda Type included or combination thereof used, the proportions of each Bethesda Type, the definition of “benign” used in the study, and Hürthle cell (HC) predominance (65). Furthermore, among post-validation studies, the molecular test outcome itself influenced the clinical management.

Supporting this, numerous studies have reported a lower specificity or higher false positive rate in GEC tests among indeterminate nodules with HC predominance. Brauner et al. reported 86% (37/43) patients with a GEC suspicious result had unnecessary surgery (46). The authors include a grouped cohort analysis of 122 HC predominant nodules between 2012 and 2014, showing 85 of 95 (89.5%) benign pathologies identified as GEC suspicious (46). Another study by Parajuli et al. reported on GEC’s increase in false positive rate among HC predominant nodules, but did not observe the same increase in Thyroseq® V2 (66). Additional studies assessing Thyroseq®’s performance in HC predominant nodules are lacking.

One component of GEC, the Medullary Thyroid Cancer (MTC) Classifier, has been far less studied (Table 4). Among the few existing studies, Se, Sp, PPV, and NPV ranged from 91 to 100%, 100%, 98 to 100%, and 99 to 100%, respectively. Further evaluation of the MTC Classifier is needed to establish its clinical efficacy.

**CURRENT CHALLENGES OF MOLECULAR MARKER ASSAYS**

Current challenges to the application of molecular markers are fourfold: (A) the recent introduction of the histopathological diagnosis NIFTP, (B) the correlation of genetic mutations within both benign and malignant pathologic diagnoses, (C) the lack of follow-up of molecular marker negative nodules, and (D) the cost-effectiveness of molecular markers.

**Non-Invasive Follicular Thyroid Neoplasm With Papillary-Like Nuclear Features**

In March 2015, Nikiforov et al. introduced the new histopathological term NIFTP, previously known as encapsulated follicular

| TABLE 4 | Medullary thyroid cancer (MTC) classifier and diagnostic value. |
|---------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Author  | Year | Study type | Follow-up period | Bethesda category | Sample size | MTC suspicious | Surgery MTC prev. | Diagnostic value |
|---------|------|------------|------------------|-------------------|-------------|----------------|------------------|-----------------|
| Pankratz et al. (67) | 2016 | Retrospective, single institution | – | – | 27 | 26 | 27 | 27 | 96 | – | – | – |
| Kloos (68) | 2016 | Prospective, blinded multicenter | – | III–VI | 10,488 | 43 | 43 | 42 | – | – | 98 | – |
| Alexander et al. (30) | 2012 | Prospective, blinded multicenter | 301 days (median) | III–VI | 441 | 4 | 441 | 4 | 100 | 100 | 100 | 100 |
| Training set (69) | 2010 | Prospective, blinded multicenter | – | – | 220 | 22 | 220 | 20 | 91 | 100 | 100 | 99 |
| Chudova et al. (69) | 2010 | Prospective, blinded multicenter | – | I–VI | 48 | 0 | 48 | 0 | – | 100 | – | 99 |
variant of papillary thyroid cancer, representing an indolent entity with very low risk of recurrence (70). Major diagnostic characteristics of NIFTP include features of FVPTC, such as a follicular growth pattern and nuclear features of PTC (enlargement, crowding, elongation, irregular contours, grooves, pseudoinclusions, and chromatin clearing), but a lack of vascular or capsular invasion, key features differentiating NIFTP from FVPTC.

This new diagnosis represents a dramatic shift in thyroid pathology where an estimated 61% of lesions previously classified as FVPTCs will now be classified as NIFTP, thus decreasing the percentage of “malignancies” on final pathology compared with FNA. On pre-operative cytology, NIFTP is associated with FNA Bethesda Category III, IV, or VI in 15, 56, 27, and 2% of tumor samples, respectively (71). As a consequence, this has created a shift in the malignancy rate associated with each Bethesda category. Strickland et al. evaluated a cohort of 655 FNAs with subsequent resection specimens (72). When taking into account the new NIFTP diagnosis, indeterminate, and suspicious FNA samples of Bethesda III, IV, and V had an absolute decrease in rate of malignancy by 17.6, 8.0, and 41.5%, respectively (72). Similarly, Faquin et al. reported an absolute decrease in rate of malignancy in Bethesda III, IV, and V by 13.6, 15.1, and 23.4%, respectively (73).

Despite NIFTP’s extremely low-recurrence rate of 0.6% (two cases), there remains disagreement regarding NIFTP’s true malignant potential (74–80). Despite its likely benign and, at worst, indolent nature, current ATA guidelines recommend lobectomy as definitive therapy for NIFTP. More importantly, however, and, apropos of this review, Afirma® GEC and Thyroseq® V2 validation studies occurred before the establishment of NIFTP as a distinct entity. Because of this one needs to be circumspect about the real utility of these marker panels. And, as a consequence, these molecular diagnostic panels require recalibration to appropriately account for the newly introduced entity, NIFTP; a lesion that should likely not be considered malignant (70, 81, 82).

Correlating Mutations to Pathology

The correlation between presence of mutations and malignancy is imprecise. Among 967 Bethesda Type III, IV, and V nodules, the detection of any mutation conferred the risk of histologic malignancy of 88, 87, and 95%, respectively (83). However, even in nodules with no detected mutations, the malignancy rates were 6, 14, and 28%, respectively. A systematic review by our group included 8,162 patients, of whom 42.5% had benign lesions (84). Among the benign lesions, RAS mutations, RET/PTC rearrangements, and PAX8/PPARGAMMA rearrangements were present up to 48, 68, 55% of the time, respectively. Thus, benign nodules frequently harbor mutations, while some malignant lesions harbor no detected mutations. The combination of the variable and potentially high level of mutations among benign nodules may explain the low specificity and PPV seen in Afirma. Furthermore, their prominence in benign lesions may also challenge the reported PPV of Thyroseq V2.

This issue is further complicated when an indolent tumor, such as NIFTP, should be resected according to current ATA guidelines. NIFTP is commonly associated with RAS mutations (8/27, 29.6%) and its diagnosis is incompatible with the presence of BRAF V600E mutations (70, 79). Moreover, Nikiforov et al. describe that 22% (6/27) of NIFTP samples harbor no detectable mutations. To conform to the recommendation that this indolent lesion be resected, new validation studies must show the reliable identification of NIFTP by molecular markers, an unlikely occurrence given the fact that benign lesions also harbor them.

Molecular Marker Negative Nodule Follow-Up

Despite a number of studies exploring the diagnostic value of GEC and Thyroseq® V2, the current published literature includes discrepancies in the follow-up of molecular marker negative nodules and their consideration as a benign pathology (85, 86). Consequently, this may lead to inaccuracies in diagnostic value calculation. A systematic review by Duh et al. (85) highlighted these issues. They included 12 studies and discussed the exclusion of cytologically indeterminate, GEC benign nodules from diagnostic performance calculations (malignant versus benign), leading to an erroneous decrease in Sp and NPV. This is due to the lack of surgical pathology specimens to establish a definitive diagnosis, as well as a lack of follow-up of GEC benign nodules to establish a reference diagnosis. To establish a diagnostic “reference standard” in these nodules that have not undergone surgery and include them in calculations, the authors argue that they should be considered as “true negative” only if no suspicious changes are noted on scheduled interval ultrasound examinations. However, even the natural history of benign thyroid nodules has been described to involve size changes. Indeed, a 5-year prospective study involving 1,567 sonographically or cytologically benign thyroid nodules showed nodule growth in 11.1% (87). However, thyroid cancer was diagnosed in five original nodules (0.3%), of which, only two had an increase in size. Furthermore, a retrospective study ranging from 1 month to 5 years, reported that 39% of the 268 benign thyroid nodules showed at least a 15% change in nodule volume (88). Only one of the 74 repeat-FNAs was malignant. The authors conclude that an increase in nodule volume alone is not a reliable predictor of malignancy.

Two studies have described their experience with follow-up of GEC benign nodules on ultrasound. A study by Angel et al. including 56 patients with cytologically indeterminate, GEC benign nodules followed for a median of 13 months exhibited similar growth (≥20% in two dimensions or ≥50% in volume) to cytologically benign nodules (86). Furthermore, in a grouped cohort analysis by Kloos et al. of 443 GEC benign patients in six studies with a reported follow-up time of 7–26 months, 380 patients (85.8%) were spared unnecessary surgery (89). Clearly, the currently available follow-up periods are inadequate for a definitive assessment, and larger, prospective studies are needed to further evaluate the behavior of cytologically indeterminate,
molecular marker “negative” thyroid nodules to help guide recommendations for management.

Cost-Effectiveness

The cost-effectiveness of GEC and Thyroseq® has also been an intense area of research. The cost for GEC and MTC is $4,875 while the cost for Afirma® MTC alone is $975 and that of Afirma® BRAF is $475 (Table 5). The cost of Thyroseq® V2 is $3,200 (29). Despite these high costs, insured patient costs are capped at $300 for either GEC or Thyroseq® V2. Numerous studies have reported on the cost-effectiveness of both GEC (90, 91) and Thyroseq® V2 (92).

A 5-year cost effectiveness study of routine use of GEC reported 74% fewer operations for benign nodules with no increase in untreated cancers. Compared with standard clinical management based only on indeterminate FNA results, GEC may lower overall costs (standard cost $12,172 versus GEC cost $10,719) and improve quality of life for patients (91). Another study reported that to be cost effective, GEC’s specificity would have to be greater than 68% and decrease the number of unnecessary surgeries performed on benign nodules by more 50% (90).

However, a study by Yip et al. compared the average cost per patient with Bethesda IV nodules larger than 1 cm extending 10 years from follicular neoplasm diagnosis in three groups: standard of care, GEC, and Thyroseq®. The authors reported a 13% increase in average cost per patient when using GEC at $13,027 (range $12,373–$13,666) when compared with the standard of care $11,505 (range $10,676–$12,347), but a 30% reduction in those using Thyroseq® cost $7,683 (range $7,174–$8,333) (92).

Despite the conflicting results of GEC cost-effectiveness studies and the paucity of analyses on Thyroseq®, these studies highlight the need to closely examine cost-effectiveness with the use of genetic studies. However, the true impact of thyroid molecular tests on a population’s health care costs can only be determined taking cancer prevalence into account. Furthermore, cost-effectiveness also depends on proper education to ensure that these tests are only used in indicated clinical settings.

REFERENCES

1. Gharib H. Changing trends in thyroid practice: understanding nodular thyroid disease. Endocr Pract (2004) 10(1):31–9. doi:10.4158/EP.10.1.31

CONCLUSION AND FUTURE DIRECTION

Molecular markers are a rapidly developing field despite the current limitations. Veracyte and CBL Path have begun to address the challenges discussed here. In 2017, Veracyte launched the Afirma® Genomic Sequencing Classifier (GSC), its newest product which tests a total of 10,196 genes. The Afirma® GSC test system is composed of a series of classifiers including parathyroid (mRNA expression), MTC (mRNA expression + variants), and RET/PTC fusion (fusion transcripts). GSC also includes a follicular content index (mRNA expression), HC index (mRNA expression and mitochondrial transcripts), and Hürthle neoplasm index (mRNA expression and chromosomal level loss of heterozygosity). The reliance on mRNA could prevent detection of mutations undetectable in transcriptome-based assays, such as telomerase reverse transcriptase (hTERT) promoter mutations. Veracyte states that GSC addresses the weaknesses of GEC by significantly increasing molecular test specificity, using a validation cohort with 15 NIFTP samples, and improving the diagnostic performance among HC lesions. Among Bethesda Type III and IV nodules, Veracyte quotes a Se, Sp, NPV, and PPV of 91, 68, 96, and 50%, respectively, when compared with the GEC parameters of 89 50, 93, and 46%, respectively. Among HC lesions, Sp has increased from 11.8 to 58.8%.

Similarly, in 2017 CBL Path sought to address similar issues with the release of Thyroseq® V3 (93). It has an expanded its assay from 56 to 112 genes, detecting mutations, gene fusions, gene expression alterations, and copy number variations. In a prospective double-blind multicenter study using 257 nodules with Bethesda Types III–V (including 11 NIFTP samples, 10 HC carcinomas, 34 HC adenomas, and 5 hyperplastic nodules with HC predominance), Thyroseq® V3 is reported to have an increased diagnostic value, including a Se of 98% (from 96.9%) and specificity of 81.8% (from 74.0%). Moreover, HC samples had an Se and Sp of 100.0 and 66.7%, respectively.

As experience accumulates with these next generation tests, we will gain a better understanding of how well they mitigate the limitations and challenges addressed herein. As our understanding of genetic drivers of malignant cancers and our understanding of NIFTP’s malignant potential becomes clearer, molecular markers will continue to more accurately identify malignant nodules as well as to spare patients from unnecessary surgery.

AUTHOR CONTRIBUTIONS

ZS conducted the literature review. ZS, PS, and CU drafted and revised the manuscript. MZ drafted and revised the manuscript and, as the senior and corresponding author, takes full responsibility.

2. Guth S, Theune U, Aberle J, Galach A, Bamberger C. Very high prevalence of thyroid nodules detected by high frequency (13 MHz) ultrasound examination. Eur J Clin Invest (2009) 39(8):699–706. doi:10.1111/j.1365-2362.2009.02162.x
42. Wong KS, Angell TE, Strickland KC, Alexander EK, Cibas ES, Krane JF, et al. Noninvasive follicular variant of papillary thyroid carcinoma and the Afirma gene-expression classifier. Thyroid (2016) 26(7):911–5. doi:10.1089/thy.2015.0644
43. Zhu Q-L, Faquin WC, Samir AE. Relationship between sonographic characteristics and Afirma gene expression classifier results in thyroid nodules with indeterminate fine-needle aspiration cytology. AJR Am J Roentgenol (2015) 205(4):861–5. doi:10.2214/AJR.14.13984
44. Celik B, Whetsell CR, Nassar A. Afirma GEC and thyroid lesions: an institutional experience. Diagn Cytopathol (2015) 43(12):966–70. doi:10.1002/dc.23378
45. Marti JL, Avadhani V, Donatelli LA, Niyogi S, Wang B, Wong RJ, et al. An independent study of a gene expression classifier (Afirma) in the evaluation of cytologically indeterminate thyroid nodules. J Clin Endocrinol Metab (2014) 99(1):4069–77. doi:10.1210/jc.2013-3584
46. Brauner E, Holmes BJ, Krane JF, Nishino M, Zurakowski D, Hennessey JV, et al. Performance of the Afirma gene expression classifier in Hurthle cell thyroid nodules differs from other indeterminate thyroid nodules. Thyroid (2015) 25(7):789–96. doi:10.1080/10432560.2015.100049
47. McIver B, Castro MR, Morris JC, Bernet V, Smallridge R, Henry M, et al. Performance of a next-generation sequencing assay (ThyroSeq v2) in the evaluation of indeterminate thyroid nodules with indeterminate fine-needle aspiration cytology: an institutional experience. Cancer Cytopathol (2014) 122(10):737–44. doi:10.1002/cncy.21455
48. Han PA, Olson MT, Fazeli R, Prescott JD, Pai SI, Schneider EB, et al. The impact of molecular testing on the surgical management of patients with thyroid nodules. Ann Surg Oncol (2014) 21(6):1862–9. doi:10.1007/s10434-014-3508-x
49. Alexander EK, Schorr M, Klopfer J, Kim C, Sipos J, Nahban F, et al. Multicenter clinical experience with the Afirma gene expression classifier. J Clin Endocrinol Metab (2013) 99(1):119–25. doi:10.1210/jc.2013-2482
50. Harrell R, Biston D. Surgical utility of Afirma: effects of high cancer prevalence and oncocytic cell types in patients with indeterminate thyroid cytology. Endocr Pract (2013) 20(4):364–9. doi:10.4158/EP13330.OR
51. Nikiforov YE, Carty SE, Chiosea SI, Coyne C, Durvuu U, Ferris RL, et al. Highly accurate diagnosis of cancer in thyroid nodules with follicular neoplasm/suspicious for a follicular neoplasm cytology by ThyroSeq v2 next-generation sequencing assay. Cancer (2014) 120(3):3627–34. doi:10.1002/cncr.29038
52. Nikiforov YE, Carty SE, Chiosea SI, Coyne C, Durvuu U, Ferris RL, et al. Impact of the multi-gene ThyroSeq next-generation sequencing assay on cancer diagnosis in thyroid nodules with atypia of undetermined significance/follicular lesion of undetermined significance. Thyroid (2015) 25(11):1217–23. doi:10.1080/10432560.2015.03005
53. Taye A, Gurciullo D, Miles BA, Gupta A, Owen RP, Inabnet WB, et al. Clinical performance of a next-generation sequencing assay (ThyroSeq v2) in the evaluation of indeterminate thyroid nodules. Surgery (2018) 163(1):97–103. doi:10.1016/j.surg.2017.07.032
54. Valderrabano P, Khazai L, Leon ME, Thompson ZJ, Ma Z, Chung CH, et al. Evaluation of ThyroSeq v2 performance in thyroid nodules with indeterminate cytology. Endocr Relat Cancer (2017) 24(3):127–36. doi:10.1530/ERC-16-0512
55. Shrestha RT, Evavoch MR, Amin K, Radulescu A, Sanghvi TS, Nelson AC, et al. Correlation between histological diagnosis and mutational panel testing of thyroid nodules: a two-year institutional experience. Thyroid (2016) 26(8):1068–76. doi:10.1080/10432560.2016.0048
56. Khatami R, Heimann A, Hwang S, Shroyer K, Wu M. ThyroSeq v2 application study for indeterminate thyroid FNAs with surgical follow-up; experience at a university hospital. Am J Clin Pathol (2016) 135(5):S80–1. doi:10.1093/ajcp/aqw165.018
57. Tol eradof G, Godley FA, Cerda SR, Mcaneny D, Dougherty GM, Braverman L, et al. Large Independent Prospective Study to Evaluate the Performance of ThyroSeq2 Multigene Next Generation Sequencing Panel Analysis on Cancer Diagnosis in Thyroid Nodules with Indeterminate Cytology. ThyroSeq Press Release: ThyroSeq (2016). Report No.: 1050–7256.
75. Vivero M, Kraft S, Barletta JA. Risk stratification of follicular variant of papillary thyroid carcinoma. *Thyroid* (2013) 23(3):273–9. doi:10.1089/thy.2013.0360

76. Gandy I, Wang L, Tuttle RM, Katabi N, Ceballos GA, Harach HR, et al. Invasion rather than nuclear features correlates with outcome in encapsulated follicular tumors: further evidence for the reclassification of the encapsulated papillary thyroid carcinoma follicular variant. *Hum Pathol* (2015) 46(5):657–64. doi:10.1016/j.humpath.2015.01.010

77. Rosario PW, Penna GC, Calsolari MR. Noninvasive encapsulated follicular variant of papillary thyroid carcinoma: is lobectomy sufficient for tumours ≥1 cm? *Clin Endocrinol* (2014) 81(4):630–2. doi:10.1111/cen.12387

78. Baloch ZW, LiVolsi VA. Encapsulated follicular variant of papillary thyroid carcinoma with bone metastases. *Mod Pathol* (2000) 13(8):861–5. doi:10.1038/modpathol.3880153

79. Howitt BE, Paulson VA, Barletta JA. Absence of BRAF V600E in non-infiltrative, non-invasive follicular variant of papillary thyroid carcinoma. *Histopathology* (2015) 67(4):579–82. doi:10.1111/his.12680

80. Liu Z, Zhou G, Nakamura M, Koike E, Li Y, Ozaki T, et al. Encapsulated follicular thyroid tumor with equivocal nuclear changes, so-called well-differentiated tumor of uncertain malignant potential: a morphological, immunohistochemical, and molecular appraisal. *Cancer Sci* (2011) 102(1):288–94. doi:10.1111/j.1349-7006.2010.01769.x

81. Sahli ZT, Umbricht CB, Schneider EB, Zeiger MA. Thyroid nodule diagnostic markers in the face of the new diagnosis, NIFT-P: time for a reset? *Thyroid* (2017) 27(11):1393–9. doi:10.1089/thy.2017.0238

82. Jiang X, Harrison GP, Datto MB. Young investigator challenge: molecular features. *Cancer Cytopathol* (2016) 124(12):893–900. doi:10.1002/cncy.21802

83. Nikiforov YE, Ohori NP, Hodak SP, Carty SE, LeBeau SO, Ferris RL, et al. Impact of mutational testing on the diagnosis and management of patients with cytologically indeterminate thyroid nodules: a prospective analysis of 1056 FNA samples. *J Clin Endocrinol Metab* (2011) 96(11):3390–7. doi:10.1210/jc.2011-1469

84. Najafian A, Nourredine S, Azar F, Atallah C, Trinh G, Schneider EB, et al. RAS mutations, and RET/PTC and PAX8/IPPAR-gamma chromosomal rearrangements are also prevalent in benign thyroid lesions: implications thereof and a systematic review. *Thyroid* (2017) 27(1):39–48. doi:10.1089/thy.2016.0348

85. Duh Q-Y, Busaidy NL, Rahilly-Tierney C, Gharib H, Randolph G. A systematic review of the methods of diagnostic accuracy studies of the Afirma gene expression classifier. *Thyroid* (2017) 27(10):1215–22. doi:10.1089/thy.2016.0656

86. Angell BE, Frates MC, Medi ci M, Liu X, Kwong N, Cibas ES, et al. Afirma benign thyroid nodules show similar growth to cytologically benign nodules during follow-up. *J Clin Endocrinol Metab* (2015) 100(11):E1477–83. doi:10.1210/jc.2015-2658

87. Durante C, Costante G, Lucisano G, Bruno R, Meringolo D, Paciar oni A, et al. The natural history of benign thyroid nodules. *JAMA* (2015) 313(9): 926–35. doi:10.1001/jama.2015.0956

88. Alexander EK, Hurwitz S, Heering JP, Benson CB, Frates MC, Doubilet PM, et al. Natural history of benign solid and cystic thyroid nodules. *Ann Intern Med* (2003) 138(4):315–8. doi:10.7326/0003-4819-138-4-200302180-00010

89. Kloos RT, Hanna JW, Haroldson J, Barth N. Systematic review of clinical utility and longer term follow up of Afirma GEC testing. *Endocr Soc* (2016). doi:10.1210/endo-meetings.2016.THPTA.14.SAT-284

90. Labourier E. Utility and cost-effectiveness of molecular testing in thyroid nodules with indeterminate cytology. *Clin Endocrinol* (2016) 85(4):624–31. doi:10.1111/cen.13096

91. Li H, Robinson KA, Anton B, Saldanha JJ, Ladenson PW. Cost-effectiveness of a novel molecular test for cytologically indeterminate thyroid nodules. *J Clin Endocrinol Metab* (2011) 96(11):E1719–26. doi:10.1210/jc.2011-0459

92. Yip L, Apfel A, Singh A, Yoo J, McCoy K, Stang MT, et al. Comprehensive cost analysis of available molecular tests for thyroid nodules with follicular neoplasm cytology. *International Thyroid Congress and American Thyroid Association. Lake Buena Vista, FL* (2015).

93. Steward D, Carty S, Sippel R, Yang P, Sosa J, Sipos J, et al. Clinical validation of ThyroSeq V3® performance in thyroid nodules with indeterminate cytology: a prospective blinded multi-institutional validation study. *American Thyroid Association. Victoria, Canada* (2017).

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Copyright © 2018 Sahli, Smith, Umbricht and Zeiger. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*