Evaluation of the Afirma Gene Expression Classifier in Repeat Indeterminate Thyroid Nodules

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• ***Context.***—**Molecular testing in indeterminate thyroid nodules is a rapidly evolving field with variable reported outcomes.**

Objective.—**To report our experience at a tertiary thyroid referral center with the Afirma Gene Expression Classifier (Veracyte, San Francisco, California) in repeat fine-needle aspirations of thyroid nodules with a previous indeterminate cytologic result.**

Design.—**Results of cytopathology and the Afirma test were collected from August 2013 to March 2015, as were diagnoses from surgical resection when performed.**

Results.—**One hundred and fifteen thyroid nodules were evaluated by Afirma. The fine-needle aspiration diagnostic categories for these nodules were 100 (87%) Bethesda III, 10 (9%) Bethesda IV, 3 (2%) Bethesda II, 1 (1%) Bethesda V, and 1 (1%) Bethesda I. Afirma results for 52 of the nodules (45%) were benign, 57 (50%) were suspicious, and 6 (5%) specimens yielded no result because of low messenger RNA content. Three of the benign nodules (6%) were treated surgically, and all were benign on final surgical pathology. Forty-six (81%) of the suspicious nodules were treated surgically; final surgical pathology revealed 30 (65%) were benign and 16 (35%) malignant, yielding a positive predictive value of 35%.**

Conclusions.—**In our experience, 50% of the indeterminate nodules were classified as suspicious by Afirma, with a 35% rate of malignancy in these nodules at surgical resection, in comparison with a historical rate of malignancy at our institution of 11% for Bethesda III nodules and 23% for Bethesda IV. Our use of Afirma is consistent with prior reports in that it has a low positive predictive value in indeterminate thyroid nodules.**

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Thyroid nodules are a common clinical concern. Increasing use of diagnostic imaging likely explains a large part of the increased incidence of thyroid nodules and the subsequent diagnosis of thyroid cancer that has been observed during the last 3 decades.[1,2]

Ultrasound-guided fine-needle aspiration (FNA) is generally the first step for sampling a nodule that meets criteria for interrogation.[3,4] Currently, most thyroid aspirates are classified using the Bethesda System for Reporting Thyroid Nodules, which is a system with 6 major diagnostic categories, each associated with a different risk of malignancy (ROM). Applying the Bethesda System to thyroid FNA can successfully classify 62% to 85% of thyroid nodules as benign and 1% to 5% as malignant, thereby successfully stratifying patients for appropriate therapy.[5] However, 10% to 30% of nodules fall into an indeterminate category. These categories under the Bethesda System are category III (atypia of undetermined significance or follicular lesion of undetermined significance [AUS/FLUS]) or category IV (follicular neoplasm or suspicious for follicular neoplasm).[6] Prior to molecular assays, most patients with indeterminate cytology were referred for a diagnostic lobectomy (or total thyroidectomy, based on other risk factors for cancer or the presence of contralateral nodularity) immediately or after a rebiopsy demonstrating persistently indeterminate cytology results. However, most of the nodules that fall into an indeterminate category are benign on resection.[6]

Recently, the management of patients with indeterminate cytology has begun to incorporate molecular testing. The overall goal of molecular tests is to better categorize cytologically indeterminate nodules according to their ROM. ThyroSeq, developed by investigators at the University of Pittsburgh Medical Center in Pittsburgh, Pennsylvania, is a multigene panel that analyzes more than 1000 hotspots of 14 thyroid cancer–related genes and detects 42 types of gene fusion.[7,8] The Afirma Gene Expression Classifier (GEC) is a proprietary test developed in San Francisco, California, by Veracyte Inc that measures 167 gene transcripts and classifies the nodule as either benign or suspicious. The reported posttest probabilities of malignancy in the initial 2012 validation trial by Alexander et al[9] were 5% to 6% and 37% to 38% for benign and suspicious results, respectively.

Molecular testing in indeterminate thyroid nodules is a rapidly evolving field with variable reported outcomes in the

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Table 1. Patient Demographic Information	
Characteristic	**Value**
Sex, No. (%)	
Female	86 (78)
Male	24 (22)
Age, y	
Mean	56.5
Median (IQR)	59 (37.5–80.5)
Bethesda category, No. (%)	
I	1 (1)
II	3 (2)
III	100 (87)
IV	10 (9)
V	1 (1)
VI	0 (0)

Abbreviation: IQR, interquartile range.

literature. The aims of this study are 2-fold: to perform a review of the literature on the performance of this test, and to report our experience at a high-volume tertiary thyroid referral center with the Afirma GEC in repeat cytologically indeterminate thyroid nodules.

MATERIALS AND METHODS

The current study was approved by our institutional review board. A retrospective analysis was performed from September 2013 to March 2015 of all in-house thyroid FNAs sent to Veracyte Inc for Afirma GEC testing. The following information was collected from the patient's chart: age, sex, corresponding FNA cytologic diagnosis, Afirma GEC results, and the final surgical pathology diagnosis of the targeted nodule, where available. We included in this series all patients who had Afirma sent from our institution, and collected data on surgical follow-up. The collected data were stored, and all statistical analyses were performed using Excel 2014 (Microsoft, Redmond, Washington). A positive predictive value (PPV) with 95% CI was calculated. Any discrepancy in the sum of percentages in tabulation is due to the rounding of numbers for simplicity.

Each FNA was performed by palpation or with ultrasound guidance by board-certified radiologists, endocrinologists, surgeons, and cytopathologists. Immediate assessments for adequacy were performed with each biopsy. The diagnoses rendered for all specimens followed the Bethesda System for Reporting Thyroid Cytopathology. Thyroid nodules with a repeat Bethesda III or IV indeterminate diagnosis were sent for Afirma GEC testing at the request of the submitting physician. At our institution, Afirma GEC testing is generally not performed on the first biopsy and indeterminate cytologic result; rather, it is used at the second consecutive indeterminate cytologic diagnosis for the same thyroid nodule. We perform Afirma on the second biopsy because a portion of indeterminate nodules are cytologically indeterminate because of sampling issues and on rebiopsy are definitively benign or malignant. A second biopsy of a previously indeterminate nodule that reveals a definitive diagnosis is not sent for Afirma GEC testing, thus saving the patient the additional expense of molecular testing in these cases.

The surgical pathology results were correlated with the FNA and Afirma GEC findings by matching the biopsied nodule to the surgically resected nodule, which served as the gold standard. Incidental papillary microcarcinomas found elsewhere in the resected specimen were excluded when calculating the ROM. A total of 6 cytopathologists interpreted all of the thyroid cytology specimens. Six surgeons at our institution performed all thyroidectomies or diagnostic lobectomies.

In addition, we performed a review of the reported literature by searching related articles in PubMed from November 2012 to November 2015 to compare our experience with the Afirma GEC with other reported experiences.

RESULTS

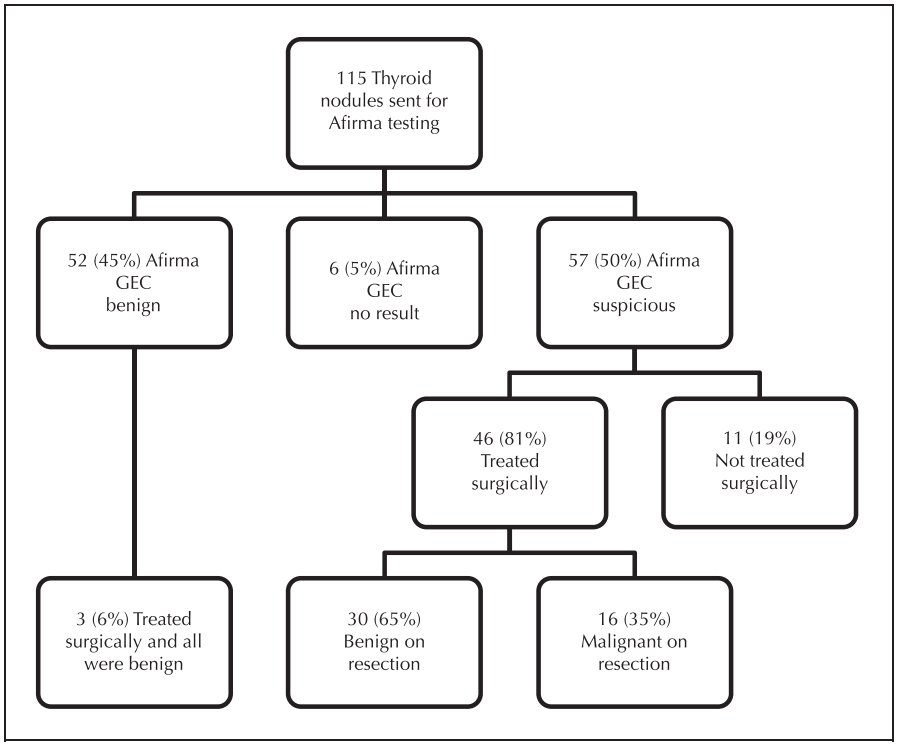
The study cohort comprised 115 thyroid nodules from 110 patients, including 86 females (78%) and 24 males (22%). The ages of the patients ranged from 16 to 87 years, with a mean age of 56.5 years at the time of FNA. Five patients had 2 different thyroid nodules that were simultaneously aspirated and sent for Afirma GEC testing, resulting in the total of 115 thyroid nodules examined. The FNA diagnostic categories for these nodules were 100 (87%) Bethesda III, 10 (9%) Bethesda IV, 3 (2%) Bethesda II, 1 (1%) Bethesda V, and 1 (1%) Bethesda I; none were Bethesda VI (Table 1). Five nodules were sent for Afirma GEC testing that were not indeterminate morphologically. Two samples were sent by request of the clinician despite the benign cytologic diagnosis; 1 Bethesda II sample sent for Afirma GEC testing was inadvertently ordered by the pathologist; another was sent for *BRAF* testing via the Afirma GEC panel because the residual cytologic sample contained too few cells to perform it in house; and 1 aspirate sent for Afirma GEC testing was cytologically nondiagnostic.

Afirma GEC results for 52 of the nodules (45%) were benign, 57 (50%) were suspicious, and 6 (5%) yielded no result because of low messenger RNA content. Among the benign nodules, 3 (6%) were treated surgically because of compressive symptoms, and all of these nodules were benign follicular/Hürthle cell adenomas on final surgical pathology. Forty-six of the suspicious nodules (81%) were treated surgically; final surgical pathology diagnoses for 30 (61%) were benign and for 16 (35%) were malignant, yielding an overall PPV of 35% (Figure). At surgical resection, the malignant diagnoses included 12 (75%) follicular variant of papillary thyroid carcinoma, 3 (19%) Hürthle cell carcinoma, and 1 (6%) classic variant of papillary thyroid carcinoma. The benign diagnoses included 14 (47%) nodular hyperplasia, 9 (30%) follicular adenoma, and 7 (23%) chronic lymphocytic thyroiditis (Tables 2 and 3).

Thirty-eight of the 46 suspicious nodules treated surgically (83%) were Bethesda III category nodules on cytology, and final surgical resection revealed that 25 (54%) were benign and 13 (28%) were malignant. Of the 4 Bethesda category IV suspicious nodules, only 1 was malignant. Only 1 Bethesda category V nodule was suspicious and found to be malignant on final surgical resection. The calculated PPV for Bethesda category III, IV, and V nodules that were suspicious on the Afirma GEC panel was 35% (95% CI, 25%–45%). The 2 Bethesda category II nodules that were suspicious were benign on surgical resection. One cytologically nondiagnostic specimen (Bethesda category I nodule) was suspicious and was malignant on final surgical resection (Table 2).

DISCUSSION

This study reports our experience with Afirma GEC testing in repeat cytologically indeterminate thyroid nodules at a large academic thyroid referral center. With its high reported negative predictive value, Afirma GEC testing was designed to rule out rather than rule in malignancy in thyroid nodules with indeterminate cytology. The retrospective nature of this study does not allow for accurate calculation of negative predictive value of the Afirma GEC



Flow highlighting the Afirma Gene Expression Classifier (GEC) results of the thyroid nodules, the percentage that were treated surgically, and whether these were benign or malignant on final surgical resection.

test because most of our patients with benign results were not treated surgically. Our calculated PPV of 35% is similar to that reported in a validation study published by Alexander et al.[9]

In our experience, using Afirma GEC testing at the time of the second indeterminate biopsy did not appear to increase the PPV of the assay, although there is some suggestion that implementation of molecular testing (including both Afirma GEC and University of Pittsburgh Medical Center panels at our institution) increased the ROM in Bethesda category III and IV nodules that went to surgery in comparison with our historical ROM for surgically resected lesions in these Bethesda category nodules. For comparison, the historical ROM at surgical resection at our institution from a time point prior to implementation of Afirma was 11% for Bethesda category III, 23% for Bethesda category IV, and 72% for Bethesda category V nodules. During a time point following implementation of molecular testing, ROM was 33% for Bethesda category III, 38% for Bethesda category IV, and 68% for Bethesda category V nodules. These data are not purely reflective of the impact of GEC testing, however, as both the GEC and multigene mutational panels are used at our institution. When looking at only GEC suspicious nodules that were excised, the rate of malignancy in Bethesda category III nodules rose to 35%. This is in contrast to the findings reported by Lastra et al,[10] who reported a GEC panel PPV of 61% in repeat FNA of AUS/FLUS nodules. Our experience shows that the overall rate of malignancy is increased for Bethesda category III/IV nodules, but this does not affect the overall PPV of the Afirma GEC panel in comparison with the Alexander et al[9] validation study (38%).

The Afirma GEC validation study was a prospective, noninterventional, multicenter trial that involved both

Table 2. Surgical Pathology Diagnoses at Resection of Afirma Suspicious Nodules[a]

		Surgical Pathology Diagnoses			
		Benign		Malignant	
FNA Diagnosis	No. (%)	Diagnosis	No. (%)	Diagnosis	No. (%)
Bethesda III (AUS/FLUS)	38 (83)	Follicular adenoma	6 (15.2)	FVPTC	11 (23.9)
		Nodular hyperplasia	11 (23.9)	Classic PTC	1 (2.2)
		Thyroiditis	7 (15.2)	HCC	1 (2.2)
Bethesda IV (FN/susp for FN)	4 (9)	Follicular adenoma	2 (4.3)	HCC	1 (2.2)
		Nodular hyperplasia	1 (2.2)		
Bethesda II (benign)	2 (4)	Nodular hyperplasia	2 (4.3)	None	0 (0)
Bethesda V (susp for malignancy)	1 (2)	None	0 (0)	FVPTC	1 (2.2)
Bethesda I (nondiagnostic)	1 (2)	None	0 (0)	HCC	1 (2.2)
All Categories[b]	**46** (100)	**30** (65.1)		**16** (34.9)	

Abbreviations: AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; FN, follicular neoplasm; FNA, fine-needle aspiration; FVPTC, follicular variant of papillary thyroid carcinoma; HCC, Hürthle cell carcinoma; PTC, papillary thyroid carcinoma; susp, suspicious.

[a] Afirma (Veracyte, San Francisco, California).
[b] Any discrepancy in sums of percentages is due to rounding.

Table 3. Summary of Final Surgical Pathology at Resection of Afirma Suspicious Nodules	
Diagnosis	**No. (%)**
Benign	
Follicular adenoma	9 (30)
Nodular hyperplasia	14 (47)
Chronic lymphocytic thyroiditis	7 (23)
Total	**30** (100)
Malignant	
Follicular variant of PTC	12 (75)
Hürthle cell carcinoma	3 (19)
Classic papillary thyroid carcinoma	1 (6)
Total	**16** (100)

Abbreviation: PTC, papillary thyroid carcinoma.

academic and community practices across 49 different US sites. They evaluated 265 cytologically indeterminate thyroid nodules (Bethesda category III–V) using the Afirma GEC, comparing the panel's findings with the final surgical pathologic diagnoses associated with those nodules. In that study, the Afirma GEC reported 100 of the nodules (38%) as benign and 165 (62%) as suspicious. Of the 265 indeterminate nodules, 180 (68%) were benign on final surgical resection, and 85 (32%) were malignant. It is noteworthy that the validation study used the Afirma GEC on the initial biopsy with indeterminate cytology and reported a negative predictive value of 94% and a PPV of 38%. Also, the validation study did not evaluate nondiagnostic or cytologically benign or frankly malignant nodules with the Afirma GEC, because evaluating these types of nodules is not clinically useful.[9]

Afirma GEC testing has been increasingly used during recent years. Now several high-volume institutions have reported their experiences using the GEC. Only 1 of 8 reporting institutions has used the Afirma GEC after the second biopsy associated with an indeterminate cytology result. This study, reported by Lastra et al[10] at the University of Pennsylvania in Philadelphia, included a retrospective review of all Bethesda category III and IV nodules sent for Afirma GEC testing during the course of 3 years. In all, 132 indeterminate thyroid nodules submitted for Afirma GEC testing resulted in 70 nodules (53%) being reported as benign and 62 (47%) as suspicious on the panel. Of the 48 cases that were suspicious based on the Afirma GEC and available for surgical pathology review, 22 (46%) were malignant and 26 (54%) were benign. Of the 22 malignant cases, 16 (73%) were the follicular variant of papillary thyroid carcinomas, 3 (14%) were classic papillary thyroid carcinomas, and 3 (14%) were follicular carcinomas. Furthermore, 18 suspicious nodules were cytologically AUS/FLUS and available for surgical follow-up; of these 11 (61%) were malignant and 7 (39%) benign on final surgical pathology, yielding an Afirma GEC PPV of 61% on repeat AUS/FLUS nodules. Historically, at that institution the rate of malignancy of repeat AUS/FLUS nodules was 39.4% before the implementation of the Afirma GEC.

The remaining reporting institutions have used Afirma following the first indeterminate biopsy and reported an Afirma GEC benign call rate ranging from 27% to 52%, a suspicious call rate of 47% to 73%, and a PPV that ranged from 14% to 57%.[11–15] Each institution's experience is unique. One explanation, proposed by Marti et al,[13] of the interinstitutional variation of the benign/suspicious call rates and PPVs is the different pretest rates of malignancy that have been observed at each of the individual institutions. Large academic referral centers may have a higher rate of malignancy in thyroid nodules in comparison with the general population. Marti et al[13] concluded that each institution using Afirma should investigate the cost versus benefits of using the Afirma GEC panel with respect to their specific patient population. Another explanation for interinstitutional variation is interobserver variability, not only in cytologically indeterminate thyroid nodules but also in the surgical pathology diagnoses rendered on the resected nodules. Specifically, follicular lesions are diagnostically challenging and have the greatest interobserver variability[16,17] (Table 4; Figure).

This data set represents only a small portion of our total volume of indeterminate thyroid nodules because we use multiple molecular tests at our institution depending on the unique clinical scenario, which includes the history and physical examination as well as the ultrasound results. Also, repeat biopsies in previously indeterminate nodules that reveal a definitive diagnosis are not sent for molecular testing. Thus, the samples sent for Afirma GEC testing represent only a select proportion of our repeat-indeterminate biopsies. Ten of our repeat-indeterminate biopsies were Bethesda category IV, which is traditionally treated with diagnostic lobectomy without molecular testing. However, each clinical scenario is unique, and for the cases in this series where we repeated FNA and performed

Table 4. Summary of Institutional Studies of Afirma Gene Expression Classifier[a]			
Source, y	**Afirma Benign Result: Benign/Total (%)**	**Afirma Suspicious Result: Suspicious/Total (%)**	**Positive Predictive Value (%)**
Current study	52/115 (45)	57/115 (50)	35
Alexander et al,[9] 2012	100/265 (37)	165/265 (62)	37–38
McIver et al,[14] 2014	16/60 (27)	44/60 (73)	16
Marti et al,[13] 2015			
MSK	18/47 (38)	29/47 (62)	57
MSBI	37/71 (52)	34/71 (48)	14
Lastra et al,[10] 2014	70/132 (53)	62/132 (47)	61
Harrell et al,[12] 2014	20/58 (34)[b]	36/58 (62)[b]	38
Yang et al,[15] 2016	93/217 (42)	107/217 (50)	51
Celik et al,[11] 2015	22/66 (33)[c]	34/66 (52)[c]	Not reported

Abbreviations: MSBI, Mount Sinai Beth Israel (New York, New York); MSK, Memorial Sloan Kettering (New York, New York).

[a] Any discrepancy of sums of percentages is due to rounding. Afirma (Veracyte, San Francisco, California).
[b] Two specimens (3%) were inadequate because of low messenger RNA content.
[c] Ten specimens (15%) were nondiagnostic.

Afirma, it was generally because of patient-specific management concerns, such as the patient's desire to avoid surgery or if the patient was a high-risk surgical candidate.

Limitations of our study are those shared with most of the other studies published in this area. A true negative predictive value was not calculated, because the majority of the nodules with benign Afirma GEC results were not treated surgically. Follow-up for our cytologically indeterminate nodules with benign Afirma GEC results is relatively short. Although early reports suggest GEC benign nodules are safely followed clinically, long-term follow-up data for the patients who have benign GEC results are still emerging given the relative novelty of the test.[18]

Molecular characterization of thyroid nodules is a rapidly evolving field, and our investigation has several potentially important implications. The strength of the Afirma GEC panel is its ability to stratify cytologically indeterminate thyroid nodules into a benign category, thereby potentially averting the need for unnecessary surgery. In our experience, 49 patients (43%) with repeat-indeterminate nodules by FNA and benign Afirma results avoided surgery. Our review of the literature revealed wide interinstitutional variability in the Afirma GEC panel's PPV and benign/suspicious call rates. Therefore, each institution should evaluate its own unique patient population to identify its own prevalence of malignancy in cytologically indeterminate thyroid nodules in order to discern if and how Afirma GEC panel use is appropriate for application in its own clinical practice setting.[13] Finally, this is the second largest study evaluating application of the Afirma GEC panel in repeat indeterminate nodules rather than at initial biopsy. Collecting additional material for Afirma GEC testing for the initial biopsy of every thyroid nodule carries with it extra time, expense (both of performance and storage of samples), and risk, which are not insignificant, given the volume of thyroid nodule procedures that are being performed in the United States today.[19] These considerations must be weighed in light of the potential for avoiding repeat biopsies. Our experience at a tertiary referral center is that when reserved for use in repeat-indeterminate nodules, the test has similar performance to that published at initial biopsy, thus avoiding the need to collect large numbers of additional passes for Afirma GEC testing at first biopsy, while also keeping the benefit of potentially reducing the number of operations performed for benign nodules.

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