Multicenter Clinical Experience With the Afirma Gene Expression Classifier

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Background: Increasingly, patients with thyroid nodule cytology labeled atypical (or follicular lesion) of undetermined significance (AUS/FLUS) or follicular neoplasm (FN) undergo diagnostic analysis with the Afirma gene expression classifier (GEC). No long-term, multisite analysis of Afirma GEC performance has yet been performed.

Methods: We analyzed all patients who had received Afirma GEC testing at five academic medical centers between 2010 and 2013. Nodule and patient characteristics, fine needle aspiration cytology, Afirma GEC results, and subsequent clinical or surgical follow-up were obtained for 339 patients. Results were analyzed for pooled test performance, impact on clinical care, and site-to-site variation.

Results: Three hundred thirty-nine patients underwent Afirma GEC testing of cytologically indeterminate nodules (165 AUS/FLUS; 161 FN; 13 suspicious for malignancy) and 174 of 339 (51%) indeterminate nodules were GEC benign, whereas 148 GEC were suspicious (44%). GEC results significantly altered care recommendations, as 4 of 175 GEC benign were recommended for surgery in comparison to 141 of 149 GEC suspicious ($P < .01$). Of 121 Cyto Indeterminate/GEC Suspicious nodules surgically removed, 53 (44%) were malignant. Variability in site-to-site GEC performance was confirmed, as the proportion of GEC benign varied up to 29% ($P = .58$), whereas the malignancy rate in nodules cytologically indeterminate/GEC suspicious varied up to 47% ($P = .11$). Seventy-one of 174 GEC benign nodules had documented clinical follow-up for an average of 8.5 months, in which 1 of 71 nodules proved cancerous.

Conclusions: These multicenter, clinical experience data confirm originally published Afirma GEC test performance and demonstrate its substantial impact on clinical care recommendations. Although nonsignificant site-to-site variation exists, such differences should be anticipated by the practicing clinician. Follow-up of GEC benign nodules thus far confirm the clinical utility of this diagnostic test. **(*J Clin Endocrinol Metab* 99: 119–125, 2014)**

The diagnostic strategy for evaluating thyroid nodules has rapidly evolved. Addressing inherent limitations to fine needle aspiration (FNA) cytology, molecular analysis of aspirated tissue has emerged as an important tool for assessing cytologically indeterminate nodules (1). Although many molecular markers have been proposed, only a small minority have proven robust enough to modify clinical decision-making and thus impact care. This is

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Abbreviations: AUS/FLUS, atypical (or follicular lesion) of undetermined significance; FN, follicular neoplasm; FNA, fine needle aspiration; GEC, gene expression classifier; SUSP, suspicious for malignancy.

especially true with regard to diagnostic markers designed to confidently predict a benign diagnosis despite abnormal cytology (2–4). Traditionally, indeterminate FNA cytology raises concern for thyroid cancer and, because of this, patients are often referred for diagnostic surgery. However, over half of such patients prove to have benign disease after histopathological interpretation (5). For these patients, surgery was unnecessary, yet they were subjected to morbidity, operative risk, and excess cost.

The Afirma diagnostic test is a gene expression classifier (GEC) measuring the expression of 167 gene transcripts, recommended for use in cytologically indeterminate thyroid nodules. It classifies aspirated material from thyroid nodules as either benign or suspicious and was designed a priori to maximize test sensitivity and negative predictive value. Results of a prospective, multicenter, blinded validation demonstrated the test's ability to identify many benign thyroid nodules accurately even when cytologically indeterminate (6). A benign Afirma GEC result, when applied to nodules with FNA cytology labeled atypical (or follicular lesion) of undetermined significance (AUS/FLUS) or follicular neoplasm (FN), proved benign in 95% and 94% cases, respectively. Such accuracy approaches that of a benign cytological result (7) and allows consideration of nonsurgical management. A follow-up study demonstrated that nonsurgical management is preferred in the vast majority of cytologically indeterminate–Afirma GEC benign cases (8).

However, the above findings were obtained as part of a blinded validation trial, where protocol and enrollment are tightly managed. Understanding how the Afirma GEC performs in a clinical setting remains unclear. For this reason, continued clinical surveillance is paramount. After completion of the initial validation trial, five academic centers tracked their experience with the Afirma GEC among its intended use population. To date, 346 consecutive samples have been collected and represent the focus of this analysis. We sought to analyze the diagnostic performance and use of the Afirma GEC in the clinical setting, and its impact on clinical decision-making.

Materials and Methods

We retrospectively collected data from all patients with cytologically indeterminate thyroid nodules who underwent testing with the Afirma GEC between September 1, 2010 and January 10, 2013 at one of five U.S. medical centers, each with expertise in thyroid nodule evaluation. These medical centers included the Brigham and Women's Hospital, the University of Colorado Hospital, the University of Pennsylvania Hospital, the Ohio State Medical Center, and the University of Cincinnati Medical Center. Patients were referred for the evaluation of thyroid nodules $\geq$1 cm in maximal diameter. All underwent clinical and sonographic evaluation. Ultrasound was first performed and confirmed the presence of a clinically relevant thyroid nodule. FNA was performed with ultrasound guidance, most often using a 25-gauge needle and involving two to four needle passes. FNA cytology was prepared by smear- or liquid-based processes and read internally by experienced cytopathologists at each separate institution. In some but not all instances, smear-based cytology preparation allowed for rapid on-site assessment to determine the need for Afirma testing during the same visit. Cytology results were classified according to the Bethesda System for Reporting Thyroid Cytopathology (9). When classified as AUS/FLUS, FN, or (rarely) Suspicious for malignancy (SUSP), separate secondary aspirations were processed for Afirma GEC analysis at the discretion of the treating physician. [Currently, Veracyte, Inc recommends the Afirma GEC be performed only on nodules with cytology classified as AUS/FLUS or FN.] Clinical decisions, including recommendations for surgical resection, were made thereafter by the treating physician based on all available data.

For each subject, we collected demographic, sonographic, cytological, histopathological, and Afirma GEC data. Nodule size and cystic content were assessed sonographically. We documented whether thyroid surgery was recommended by the treating physician, and if surgery was performed by the time of the analysis. If surgery was performed, final histopathology interpretation was obtained and the date and extent of surgery were documented. Specifically, we queried if patients underwent hemi-thyroidectomy vs near-total thyroidectomy. If surgery was not performed, we queried all subsequent clinical and follow-up data in an attempt to determine the status of each individual and thyroid nodule.

For this investigation, our prespecified endpoints were to first independently validate the proportion of benign vs suspicious Afirma results obtained from cytologically indeterminate nodules, and investigate how these results modified clinical care recommendations and resource use. If surgery was performed, we sought to determine the proportion of "cytologically indeterminate/Afirma suspicious" nodules that proved malignant on post-surgical histopathological assessment. Finally, we investigated the clinical utility of the Afirma GEC by assessing follow-up status of patients with indeterminate cytology but a benign Afirma GEC result.

Institutional review board approval was obtained for this investigation. Statistical analysis was performed using the χ^2 or Student's t test, as applicable. P values $<.05$ were considered significant. This study did not receive any financial support, approbation, or review by Veracyte, Inc or any other commercial entity. Dr Alexander has served or currently serves as a consultant for Asuragen, Inc, Veracyte, Inc, and Genzyme, Inc (past), with Veracyte stock options.

Results

Three hundred forty-six Afirma GEC analyses were performed between March 1, 2010 and January 10, 2013. On review, seven cases (2%) did not meet our entry criteria and were excluded before knowledge of their Afirma GEC result. Specifically, four nodules were first considered AUS on rapid on-site evaluation, although final cytopathology was interpreted as "benign," and three nodules had non-

Table 1. Nodule and Patient Characteristics[a]

	Total Cohort n = 339	Site 1 130	Site 2 72	Site 3 70	Site 4 37	Site 5 30
Nodules						
Size, cm (mean)	2.2	2.4	2.2	1.8	2.4	2.3[b]
Cystic content						
<50% cystic	331	126	70	68	37	30[c]
>50%cystic	6	3	2	1	0	0
Unknown	2	1	0	1	0	0
Patients						
Age, y (mean)	55	54	57	55	54	58[d]
Sex						
Female	267 (79%)	103 (79%)	60 (83%)	50 (71%)	29 (78%)	25 (83%)[e]
Male	72 (21%)	27 (21%)	12 (17%)	20 (29%)	8 (22%)	5 (17%)

[a] Data from the total cohort is shown at the left, with subanalysis by specific site to the right.

[b] $P = .79$ for difference across sites.

[c] $P = .76$ for difference across sites.

[d] $P = .59$ for difference across sites.

[e] $P = .47$ for difference across sites.

diagnostic FNA cytology. The remaining 339 cases define our study cohort. The enrollment from each institution, as well as patient and nodule characteristics, are shown in Table 1.

One hundred sixty-five of 339 (49%) nodules were cytologically AUS/FLUS. Afirma GEC analyses of these nodules were "benign" in 91 of 165 (55%) cases, "suspicious" in 66 of 165 (40%), and "nondiagnostic" in 8 of 167 (5%) cases. Separately, 161 of 339 (47%) nodules were cytologically classified as FN. Afirma GEC analyses of these nodules were "benign" in 79 of 161 (49%) cases, "suspicious" in 73 of 161 (45%), and "nondiagnostic" in 9 of 161 (6%) cases. A "nondiagnostic" GEC result is generally due to insufficient or poor quality RNA. Finally, 13 of 339 nodules were cytologically SUSP. Afirma GEC analyses of these nodules were "benign" in 4 of 13 (31%) cases, and "suspicious" in 9 of 13 (69%) cases. In total, 174 of 339 (51%) cytologically indeterminate nodules were Afirma "benign," whereas 148 (44%) of the cytologically indeterminate nodules were Afirma "suspicious." Seventeen of 341 (5%) were Afirma "nondiagnostic." These data are shown in Table 2.

Among patients with cytologically indeterminate nodules, the Afirma GEC result substantially impacted clinical care recommendations. Patients with indeterminate cytology and "suspicious" Afirma GEC results were recommended for surgery in 141 of 148 (95%) cases. In contrast, patients with indeterminate cytology but a "benign" Afirma GEC result were recommended for surgery in only 4 of 174 (2%) cases ($P < .01$), supporting previous findings regarding the clinical impact of this test on care recommendations (8). Patients with indeterminate cytology and "nondiagnostic" Afirma GEC results were recommended for surgery in 4 of 17 (34%) cases. After an intention-to-treat assumption in which thyroid surgery is typically recommended for patients with cytologically in-

Table 2. Afirma Gene Expression Classifier Results[a]

		Afirma Results		
	n (% total)	GEC Benign	GEC Suspicious	GEC Nondiagnostic[b]
All nodules	339	174 (51%)	148 (44%)	17 (5%)
FNA cytology				
Atypical (follicular lesion) of Undetermined significance	165	91 (55%)	66 (40%)	8 (5%)
Follicular neoplasm	161	79 (49%)	73 (45%)	9 (6%)
SUSP	13	4 (31%)	9 (69%)	0

[a] Data are shown for the entire cohort, with subanalysis based on FNA cytology.

[b] Of the 17 patients with nondiagnostic Afirma GEC results, six were lost to further follow-up, three underwent surgical resection, whereas two had repeat Afirma testing at a later date. Two others had repeat FNA cytology that was benign, whereas two underwent ultrasound follow-up showing no change. Finally, one nodule was deemed a pseudonodule on repeat imaging and no further intervention recommended, whereas one patient refused further testing.

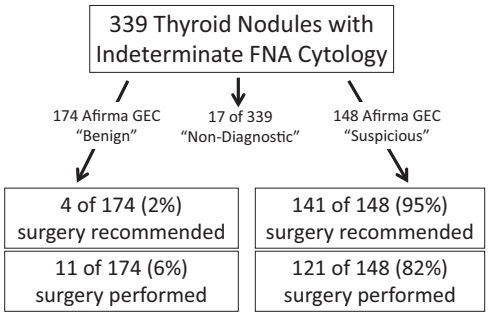

Figure 1. Clinical care recommendations and surgical outcome in 339 patients with indeterminate FNA cytology who underwent Afirma GEC testing.

determinate nodules, the Afirma GEC modified care recommendations in 171 of 339 (50%) patients (Figure 1).

Of the 141 patients with indeterminate cytology and "suspicious" Afirma GEC results who were recommended for thyroid surgery, 121 (86%) completed surgery. Specifics of the 20 patients who did not complete surgery are as follows: seven patients declined the recommendation; five separate patients were lost to follow-up; and seven have committed to future surgery that has not yet been performed. One additional patient died of a separate cause. We next sought to validate the malignancy rate among patients with cytologically indeterminate and "suspicious" Afirma GEC nodules. These data are shown in Table 3. Together, 53 of 121 (44%) cytologically indeterminate/"suspicious" Afirma GEC nodules proved malignant after histopathological assessment. As expected, most the malignant lesions were papillary thyroid carcinoma (87%).

Of the 174 patients with indeterminate cytology and "benign" Afirma GEC results, only four patients were immediately recommended for surgery (2%). Corresponding FNA cytology was AUS/FLUS in three nodules and SUSP in one nodule. Of the remainder, 71 (41%) had documented follow-up at a mean of 8.5 months (median 8 mo; range 1–24 mo) after GEC testing. In 10 of these 71 patients, a follow-up clinical examination was performed, whereas the remaining 61 underwent repeat sonographic assessment. Ultimately, 11 of these patients (inclusive of the four immediately recommended for surgery above) underwent thyroid surgery, most because of personal preference or compressive symptoms in the neck. Ten of these 11 cases proved benign histologically, whereas one was confirmed malignant (1.0-cm sonographic nodules, which proved a 0.6-cm papillary carcinoma histologically). Uniquely, a separate patient's nodule histology revealed a micropapillary carcinoma (0.8 cm) confirmed within a 3.2-cm nodule, although most nodular tissue was assessed as benign. Importantly, 17 patients with indeterminate FNA cytology, but benign Afirma GEC results, had clinical follow-up of 12 months or more (range 12–24 mo). Three of these 17 patients ultimately underwent surgical removal of the nodule due to compressive symptoms (n = 2) or nodule growth (n = 1). All three nodules were benign histologically. The remaining 14 patients were assessed with ultrasound, and no nodule change or evidence of malignancy was detected over this 12- to 24-month time period.

Finally, we investigated the performance of the Afirma GEC by individual site, to better understand practice variation. These data are shown in Table 4 and also depict the site-specific estimates of malignancy prevalence for each cytological category. The proportion of nodules cytologically AUS/FLUS but subsequently found to be Afirma GEC benign ranged from 42% to 71%, whereas those nodules cytologically FN but subsequently found to be Afirma GEC benign ranged from 38% to 67%. The proportion of cytologically indeterminate/Afirma GEC suspicious nodules that proved cancerous after histopathological assessment ranged from 33% to 55% between sites. These differences were not statistically significant.

Discussion

Molecular diagnostic tests are increasingly recommended for the evaluation of cytologically indeterminate thyroid

Table 3. Afirma Gene Expression Classifier Results[a]

		Histopathology Malignant	
ALL Cyto Indeterm + Afirma SUSP, n	148		
Surgery recommended	141		
Surgery performed	121	53 (44%)	
FNA cytology			
Atypical (follicular lesion) of undetermined significance	48	23 (48%)	21 Papillary carcinoma 1 Follicular carcinoma 1 Other
Follicular neoplasm	65	24 (37%)	19 Papillary carcinoma 4 Follicular carcinoma 1 Hurthle cell carcinoma
SUSP	8	6 (75%)	6 Papillary carcinoma

[a] Data are shown for the entire cohort, with subanalysis based on FNA cytology.

Table 4. Variance in Test Performance by Study Site[a]

	Total Cohort	Site 1	Site 2	Site 3	Site 4	Site 5	*P* Value
Total Cohort	339	130	72	70	37	30	
I. Distribution of Afirma GEC results:							
Cytology:							
AUS/FLUS[b]	165	69	39	24	21	12	
Afirma "benign"		32 (46%)	24 (62%)	15 (63%)	15 (71%)	5 (42%)	.58
Afirma "suspicious"		31 (45%)	15 (38%)	7 (29%)	6 (29%)	7 (58%)	
Afirma "nondiagnostic"		6 (9%)	0	2 (8%)	0	0	
FN[c]	161	58	30	45	16	12	
Afirma "benign"		29 (50%)	17 (57%)	19 (42%)	6 (38%)	8 (67%)	.69
Afirma "suspicious"		27 (47%)	12 (40%)	22 (49%)	9 (56%)	3 (25%)	
Afirma "nondiagnostic"		2 (3%)	1 (3%)	4 (9%)	1 (6%)	1 (8%)	
Suspicious for malignancy	13	3	3	1	0	6	
Afirma "benign"		0	0	1 (100%)	0	3 (50%)	.11
Afirma "suspicious"		3 (100%)	3 (100%)	0	0	3 (50%)	
Afirma "nondiagnostic"		0	0	0	0	0	
II. % Cancer if Afirma GEC "suspicious"							
AUS/FLUS + GEC "suspicious"	48	23	9	5	6	5	
Malignant histopathology		8 (35%)	7 (78%)	4 (80%)	2 (33%)	2 (40%)	.11
FN + GEC "suspicious"	65	24	9	20	9	3	
Malignant histopathology		9 (38%)	3 (33%)	7 (35%)	3 (33%)	2 (67%)	.87
SUSP + GEC "suspicious"	8	3	2	0	0	3	
Malignant histopathology		2 (67%)	2 (100%)	0	0	2 (67%)	.64

[a] Two variables are shown: the distribution of Afirma GEC results according to FNA cytology (top), and the proportion cancerous when Afirma GEC "suspicious" (bottom).

[b] Prevalence of malignancy in nodules AUS/FLUS: site 1: 10–15%, site 2: 15–20%; site 3: 10–20%; site 4: 5–15%; site 5: 25–30%. Percentages are the best internal estimates based on currently available experience, although the range reflects acknowledged uncertainty.

[c] Prevalence of malignancy in nodules FN: site 1: 15–20%, site 2: 20–30%; site 3: 20–30%; site 4: 5–15%; site 5: 25–30%. Percentages are the best internal estimates based on currently available experience, although the range reflects acknowledged uncertainty.

nodules. Although analytic (laboratory) validity has been established for most such markers, clinical validity and clinical use are lacking for all but a few. This point is worthy of emphasis, because blinded, multicenter clinical validation, as well as postmarketing surveillance, is necessary for a full understanding of a test's clinical impact within a diverse and heterogeneous population. Presently, few data exist that describe Afirma GEC performance in the live clinical setting. We investigated 346 consecutive patients with indeterminate thyroid nodule cytology who underwent Afirma analysis in one of five expert centers. Overall, 51% of cytologically indeterminate nodules were "benign" by Afirma GEC testing. As might be expected, the proportion of "suspicious" Afirma results increased when applied to cytologically AUS/FLUS (40%), FN (45%), and SUSP nodules (69%), respectively. In total, "cytologically indeterminate/Afirma GEC suspicious" nodules proved cancerous in 44% of patients after surgical removal. Most notably, a benign Afirma GEC result dramatically altered clinical care recommendations, as 95% of Afirma GEC "suspicious" nodules were referred for thyroid surgery in comparison to only 2% of Afirma GEC "benign" nodules. Follow-up clinical assessment confirms a low rate of false negativity among Afirma benign nodules. Together, these independent data externally validate many of the findings from the initial blinded investigation, while demonstrating the power of thyroid nodule molecular analysis to modify clinical practice. Our findings support continued recommendations for a conservative approach toward most patients with cytologically indeterminate nodules when Afirma GEC testing returns benign. This recommendation holds true across a wide range of geographic regions and patient demographics.

In the initial multicenter validation (6), 100 of 265 (38%) cytologically indeterminate nodules were benign on Afirma analysis. Although our study documented 174 of 339 (51%) such nodules as benign on Afirma analysis, it is worth noting important study differences. The original validation sought to enroll patients inclusive of all cytologically indeterminate subtypes and demonstrated the expected distribution of AUS/FLUS, FN, and SUSP findings. However, it is notable that Afirma GEC testing was thereafter only recommended for use in nodules with AUS/FLUS and/or FN cytology. Our study demonstrates adherence to this recommendation within clinical practice, while also explaining an increased proportion of benign Afirma results. Three hundred twenty-six of 339 (96%) cytologically indeterminate samples were labeled AUS/FLUS and/or FN. Because these are indeterminate categories that imply a lower malignant risk in compari-

son to SUSP cytology, it is logical to anticipate a higher proportion of Afirma GEC benign results in these populations.

Our investigation demonstrates variability in cytology distribution as well as Afirma GEC performance across the five different participating study sites. This finding is worthy of discussion. Although caution should be taken in direct site-to-site comparison when enrollment is variable across locations, our data nonetheless demonstrate that the proportion of samples that prove to be Afirma "benign" may vary up to 25%. Nationally, we also note that many thyroid nodule FNA samples are cytologically evaluated at a central cytology practice in Austin, Texas (and not internal to each medical center) before Afirma GEC analysis. Performance at such a practice may be similar or vary in comparison to our data. Unfortunately, no published results exist to allow such analysis. Most importantly, these data clarify that site-to-site variation regarding the implied meaning of each cytology diagnosis may occur. We note that many prior publications have also demonstrated poor interrater (and intrarater) cytology concordance even among experts (10, 11). Thus, it is increasingly thought that such modest variation appears unavoidable (12) and supports the need for synergistic cytological and molecular analysis of thyroid nodules within this population. These data also support a recommendation that pretest probability of malignancy be individually assessed at each practice locale, and to the best extent possible, before Afirma GEC interpretation.

Our data demonstrate a substantial change in practice patterns after the availability of the Afirma GEC (13). Among five major medical centers, clinical recommendations for surgery dropped 93% among patients with cytologically indeterminate thyroid nodules. We note some patients (n = 11) nonetheless pursued surgery even if Afirma GEC was benign, whereas others (n = 20) did not comply with surgical recommendations. Nonetheless, in an academic clinical setting, a 76% reduction in surgery was observed when the Afirma GEC was applied to patients in whom surgery would otherwise have been typically performed. Follow-up assessment of those with Afirma GEC benign results confirms a very low rate of false negative results and provides support for the clinical utility of this test. We acknowledge, however, that neither cytological nor Afirma GEC results alone should mandate an exact clinical recommendation for all patients. Individualized clinical risk assessment and personalized care recommendations should always be pursued (14), because clinical symptoms, nodule size, and/or sonographic findings can at times be enough to warrant intervention regardless of molecular analysis.

We acknowledge limitations to our study. Our data are retrospective in nature, allowing for associated referral and sample bias. However, this study was purposefully designed with such intent to best analyze Afirma GEC performance in a clinical environment. We also acknowledge these data do not capture all outcome measures. Only 41% of patients with Afirma GEC benign results have documented assessment of their subsequent clinical status. It is notable, however, that such variability is inevitable as there exist no data confirming a specific strategy of recommended repeat assessment. At present, expert opinion of this matter remains highly variable, leading to diverse practice patterns (13, 15, 16). Nonetheless, only 1 of 71 patients with an Afirma GEC benign result demonstrated subsequent malignancy. We also note that many thyroid nodule FNA samples are cytologically evaluated at a central cytology practice in Austin, Texas (and not internal to each medical center) before Afirma GEC analysis. Performance at such a practice may be similar or vary in comparison to our data.

In summary, these data provide the first extensive analysis of the Afirma GEC diagnostic test applied to a live, clinical environment. Analysis confirms test performance similar to that of the initial blinded validation and engenders confidence that such data can be effectively translated into the everyday care of patients with cytologically indeterminate (AUS/FLUS, FN) nodules. We also demonstrate variation in site-to-site performance metrics surrounding thyroid nodule evaluation. Such findings are increasingly notable and provide insight into the acknowledged limitations of clinical or cytological risk assessment for thyroid cancer and further support the need for synergistic molecular analysis. These data can assist the practicing clinician with integrating the clinical, radiologic, cytology, and molecular information necessary for improving individual care.

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