Afirma Benign Thyroid Nodules Show Similar Growth to Cytologically Benign Nodules During Follow Up

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Context: The Afirma gene expression classifier (GEC) is a molecular diagnostic test that has a high negative predictive value for ruling out malignancy in thyroid nodules with indeterminate cytology. Many patients with a cytologically indeterminate and GEC benign (Cyto-I/GEC-B) nodule undergo monitoring instead of diagnostic surgery, but few data describe their follow-up.

Objective: To determine the sonographic changes and clinical outcomes for patients with Cyto-I/GEC-B nodules compared to patients with cytologically benign (Cyto-B) nodules.

Design: A retrospective analysis of consecutive Cyto-I/GEC-B nodules evaluated at Brigham and Women’s Hospital compared to Cyto-B nodules.

Main Outcomes: Nodule growth ≥20% in 2-dimensions or ≥50% in volume, change in sonographic features, and rates of repeat FNA, thyroidectomy, and malignancy.

Results: Ninety-five Cyto-I/GEC-B nodules in 90 patients were identified. Five patients underwent primary surgical resection. Of the remaining 90 nodules, 58 (64.4%) had sonographic follow up available at a median of 13 months (range 4–40 months). Cyto-I/GEC-B nodules showed similar growth compared to 1224 Cyto-B nodules using either criterion: ≥20% in 2 dimensions (8.6% vs. 8.3%, P = .80) or ≥50% in volume (17.2% vs. 13.8%, P = .44). Thyroidectomies were more frequent in the Cyto-I/GEC-B group (13.8% vs. 0.9%, P < .0001), but cancer was only found in one patient, with no evidence of persistent disease after initial treatment.

Conclusions: Cyto-I/GEC-B nodules demonstrate similar growth to Cyto-B nodules during follow up. Though Cyto-I/GEC-B nodules were more frequently resected, only one malignancy was found. These data suggest that reassessment of Cyto-I/GEC-B nodules may be performed similarly to those with benign cytology.

Thyroid nodules are common and although usually benign, 10%–15% are malignant (1–3). Cytopathologic evaluation by ultrasound (US)-guided fine needle aspiration (FNA) is the principal means to evaluate malignancy risk (4, 5). Unfortunately, FNA yields an indeterminate result in 15%–25% of cases, with an overall cancer risk of 20%–30% in these nodules (6, 7). Surgical resection is often recommended but most prove benign (3, 6–8).

To standardize reporting and eliminate ambiguity, The
Bethesda System for Reporting Thyroid Cytology (TBS) replaced the indeterminate category and instead defined and endorsed diagnostic categories of escalating malignancy risk (9). Regardless of category however, the cancer risk remains such that surgical resection is frequently recommended (4, 5). For patients with benign disease, superfluous surgery carries unnecessary morbidity and cost (10, 11), whereas for those with malignancy, initial diagnostic surgery may be suboptimal (12, 13).

More recently, molecular testing to improve the diagnostic assessment of indeterminate nodules has become commercially available (14–16). The Afirma gene-expression classifier (GEC) (Veracyte, Inc.) analyzes the mRNA expression of 167 genes in aspiration material and provides a “benign” or “suspicious” result to improve preoperative risk stratification (17). In a prospective, multicenter, observational trial, the negative predictive values (NPV) of the GEC were 95% and 94% for TBS categories of atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS) and suspicious for follicular neoplasm/follicular neoplasm (SFN/FN), respectively (18), suggesting a malignancy rate low enough to allow surveillance rather than surgical resection. One subsequent investigation has shown that most patients with cytologically indeterminate-GEC benign (Cyto-I/GEC-B) nodules do not undergo surgery (19), but there are few data on the follow-up of such patients. To verify the appropriateness of conservative management for Cyto-I/GEC-B nodules, it is imperative to assess the outcomes of patients with these nodules.

The purpose of this study is to specifically assess the growth and change in sonographic appearance of Cyto-I/GEC-B nodules over time, as well as the frequency of repeated FNA, surgical resection, and histologically proven malignancy (false negatives). The assumption that these nodules are similar to those with benign cytology (Cyto-B) is tested by comparison to a large population of Cyto-B nodules with similar timing of sonographic follow-up.

Materials and Methods

Data for all adult patients (age ≥ 21 years) with a thyroid nodule ≥ 1 cm in greatest dimension and a benign Afirma GEC result between November 23, 2010 and April 1, 2014 at the Brigham and Women’s Hospital (BWH) Thyroid Nodule Clinic were retrospectively analyzed. The study period encompassed the earliest clinical use of the Afirma GEC at BWH and allowed ≥ 1 year for subsequent US reassessment to be obtained.

The control population was obtained from the prospectively collected database of consecutive patients evaluated at the BWH Thyroid Nodule Clinic (3, 20, 21). Adult patients with a thyroid nodule ≥ 1 cm, benign cytology (Cyto-B) at initial aspiration from 1/1/2001 to 12/31/2010, and subsequent sonographic assessment were included. This 10-year range did not overlap with the implementation of Afirma GEC testing to avoid nodule crossover between groups. Sonographic reassessment after one year was routinely recommended for Cyto-B nodules, though the exact interval varied. To more closely match the groups and facilitate the most relevant comparison, only Cyto-B nodules with initial US reassessment within the follow-up range of the Cyto-I/GEC-B nodule cohort (4–40 months) were included. Additionally, because no Cyto-I/GEC-B nodules were predominantly cystic, Cyto-B nodules with a > 50% cystic component were excluded.

For all patients in either group, thyroid US evaluation was performed by a radiologist with expertise in thyroid sonography, using a 6–15 mHz transducer, to confirm the presence of a clinically relevant nodule. Nodule location and composition as solid or cystic (<25%, 25%–50%, 50%–75%, or > 75% cystic) were reported, and size was measured in three dimensions. FNA was performed by a thyroidologist under US guidance, most often using a 25-gauge needle and involving two to four aspirations. All aspirates were processed using a liquid-based cytology preparation (ThinPrep; Hologic, Marlborough, MA). In each case, one or two Thin Prep slides were prepared, with a selected minority of cases (<5%) also including preparation of a cell block. Each specimen was read by a cytopathologist experienced in thyroid cytopathology using TBS. Though many cytologic assessments predated this system (circa 2009), the criteria and nomenclature used at BWH over the entire study period was similar to that later defined in TBS.

For nodules with indeterminate cytologies evaluated after the clinical introduction of the Afirma GEC, a separate secondary aspiration was performed and processed for Afirma GEC analysis at the discretion of the treating thyroidologist. For Cyto-I/GEC-B nodules, the timing of sonographic reassessment and clinical decisions regarding continued surveillance, repeat FNA biopsy, or surgical resection were made by the patient and treating thyroidologist.

For all included subjects, demographic, cytological, sonographic, and histopathological data were collected. Significant nodule enlargement was defined as a ≥ 20% increase of ≥ 2 nodule dimensions, or a ≥ 50% increase in volume (4, 22), where nodule volume was calculated using the rotational ellipsoid formula (length x width x depth x \pi/6).

Assessment of US characteristics was performed by a single radiologist with expertise in thyroid sonography blinded to clinical and Afirma GEC results. Nodule characteristics evaluated included echogenicity (hyperechoic, isoechoic, hypoechoic, very hypoechoic), shape (round, taller than wide), calcifications (not present, rim, fine punctate) and relative color Doppler blood flow (none, minimal internal flow, peripheral flow, hypervascular internal flow). Suspicious characteristics were defined as hypo- or very hypo-echogenicity, taller than wide shape, presence of any calcifications, increasing solid compared to cystic component, or hypervascular internal blood flow. All Cyto-I/GEC-B nodules were included irrespective of their baseline sonographic features, and were evaluated for change. The appearance of any new suspicious sonographic feature was sufficient to categorize the nodule as suspicious during follow up.

For cases that included surgical resection, the final diagnosis was based on histopathological analysis. False negatives were defined as malignant histopathology for the specified Cyto-I/GEC-B nodule. If present, the type and extent of thyroid cancer...
was recorded and available follow-up data collected to assess the implications of a false negative result on patient outcome.

Data are presented as median (+ range) as appropriate based on the D’Agostino & Pearson normality test. Analyses were performed using the Mann-Whitney U-test and χ²-test for continuous and dichotomous variables, respectively. Further, the comparisons of growth between Cyto-I/GEC-B and Cyto-B nodules were corrected for covariates including age, nodule size, nodule composition, the presence of multinodularity, and duration of follow up, using logistic regression analysis. P-values < 0.05 were considered statistically significant. Analyses were performed with GraphPad Prism v6.0c for Mac OS X (La Jolla, CA), and SPSS version 22 (IBM, NY) and figures produced using GraphPad Prism and Adobe Photoshop (San Jose, CA).

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Results

During the study period (11/23/2010 to 4/1/2014), there were 90 patients with 95 Cyto-I/GEC-B thyroid nodules. All nodules had a cytologic diagnosis of either AUS/FLUS or SFN/FN. The rates of AUS/FLUS and SFN/FN cytology at BWH over this time were 9.7% and 2.9%, respectively.

The Suspicious for Hurthle Cell Neoplasm rate was 1.6%, however no Cyto-I/GEC-B nodule had this cytologic description. Five patients with Cyto-I/GEC-B nodules proceeded to surgical resection without further evaluation and were performed due to patient preference (2 cases), or cytological findings from a different nodule that necessitated surgical intervention at which time the Cyto-I/GEC-B nodule was also resected (3 cases). These five nodules were all histologically benign. In the remaining cases, ultrasound follow-up was available for 58 of 90 (64.4%) Cyto-I/GEC-B nodules in 56 patients.

For the comparison group, there were 1678 Cyto-B nodules evaluated from 2001–2010, of which 1379 had repeat US assessment from four to 40 months after initial cytologic evaluation. There were 155 Cyto-B nodules with > 50% cystic component that were excluded, leaving a final cohort of 1224 nodules in 873 patients.

Patient and nodule data are shown in Table 1. For Cyto-I/GEC-B nodules, median patient age was 58.4 (21–88) years, 46 of 56 (82%) patients were female, median nodule size was 1.7 cm (1.0 - 5.3 cm), and median follow up was 13 months. These characteristics were similar when compared to the Cyto-B nodule group (Table 1). Multinodu-

| Table 1. Patient and Nodule Characteristics for Cytologically Indeterminate / GEC Benign and Cytologically Benign Groups. |
|---|---|---|
| **Cyto-I / GEC-B** | **Cyto-B** | **P** |
| **Patient Characteristics, n** | | |
| Median age (range), years | 58.4 (21 – 88) | 53.9 (21 – 95) | 0.19 |
| Female, n (%) | 46 (82.1%) | 780 (89.3%) | 0.20 |
| **Nodule Characteristics, n** | | |
| Median nodule size (range), cm | 1.7 (1.0 – 5.3) | 1.7 (1.0 – 8.4) | 0.50 |
| Multinodularity, n (%) | 29/58 (50.0%) | 806/1224 (65.8%) | 0.02 |
| Composition, n (%) | | |
| Solid | 35 (60.4%) | 449 (36.7%) | <0.01 |
| Complex (<50%) | 23 (39.6%) | 775 (63.3%) | |
| Cystic Cytologic Classification, n (%) | | |
| AUS/FLUS | 31 (53.5%) | n/a | |
| SFN/FN | 27 (46.5%) | n/a | |

Cyto-I/GEC-B = Cytologically indeterminate Afirma GEC benign, Cyto-B = cytologically benign, AUS/FLUS = atypia of undetermined significance / follicular lesion of undetermined significance, SFN/FN = suspicious for follicular neoplasm / follicular neoplasm, n = number, n/a = not applicable.
larity, defined as more than one nodule measuring ≥ 1 cm, was present for 29 of 58 (50%) Cyto-I/GEC-B nodules compared to 806 of 1224 (66%) Cyto-B nodules (P = .02). Nodules were described as solid in 35 of 58 (60.4%) Cyto-I/GEC-B nodules compared to 449 of 1224 (36.7%) Cyto-B nodules (P < .001). For Cyto-I/GEC-B nodules, baseline patient and nodule characteristics were similar between those that had available sonographic follow up and those that did not.

Nodule Growth

The growth of Cyto-I/GEC-B and Cyto-B nodules is shown in Figure 1. Using the growth criteria of ≥ 20% in ≥ 2-dimensions, 5 of 58 (8.6%) Cyto-I/GEC-B nodules and 102 of 1224 (8.3%) Cyto-B nodules grew (P = .80). Using a ≥ 50% volume increase to define growth, 10 of 58 (17.2%) Cyto-I/GEC-B nodules met this criterion compared to 169 of 1224 (13.8%) Cyto-B nodules (P = .44). When comparing Cyto-I/GEC-B nodule growth by cyto-logic class, four of 31 AUS/FLUS nodules and six of 27 SFN/FN nodules demonstrated ≥ 50% volume growth during follow up (P = .49).

The comparison of nodule growth was further analyzed using logistic regression analysis to adjust for differences between the groups. After adjusting for multinodularity, cystic content, and duration of follow-up, Cyto-I/GEC-B nodules showed a similar risk of growth compared to Cyto-B nodules using either ≥ 20% in ≥ 2-dimensions [OR 1.17 (0.45–3.10), P = .75] or ≥ 50% volume [OR 1.37 (0.67–2.82), P = .39]. When extending this analysis to include additional covariates of age, sex and initial nodule size, there remained no difference in growth risk between groups (data not shown).

Sonographic Characteristics

There was no development of suspicious sonographic nodule features for 53 of the 58 (91%) Cyto-I/GEC-B nodules. Three nodules were described as taller than wide at follow-up assessment, of which two were resected and were both histologically benign. Two additional nodules initially described as isoechoic were later described as hypoechoic. These nodules did not show the concurrent appearance of other suspicious features or interval growth. No nodules demonstrated new calcifications during monitoring. The technical settings for color Doppler flow assessment frequently varied between US studies, precluding evaluation of this parameter.

Repeat FNA’s, thyroidectomies, and thyroid cancer diagnoses

To evaluate further the clinical course of Cyto-I/GEC-B nodules, occurrences of repeat FNA, thyroidectomy, and thyroid cancer were assessed. Repeat FNA biopsy was performed in four patients (6.9%) from six to 37 months after Afirma GEC assessment. In two cases, repeat cytology was benign, while in the other two cases cytology remained indeterminate (SFN/FN for both). There were 206 repeat FNA assessments in the 1224 Cyto-B nodules (16.8%), (P < .05 compared to Cyto-I/GEC-B nodules, Figure 1). The cytologic results from the repeated FNA biopsy of Cyto-B nodules were again benign for 190 of 206 nodules. In the remaining nodules the cytologic findings were nondiagnostic (n = 5), atypical (n = 6), suspicious for follicular neoplasm (n = 2), suspicious for papillary thyroid cancer (n = 2), and positive for papillary thyroid carcinoma (n = 1).

During follow-up, surgical resection was performed in 8 of 56 (14.3%) Cyto-I/GEC-B nodule patients (Table 2), at a median of 9 months (6–19 months) post-Afirma GEC result. Thyroid malignancy was identified in one case (patient 8). This was a 3.1 cm minimally-invasive follicular thyroid carcinoma (FTC) resected after sonographic assessment revealed an increase in maximal diameter from 3.0 to 3.5 cm (volume increase = 46%). Pathologic evaluation showed a focal area of capsular invasion without extrathyroidal extension, lymphovascular invasion, or involved margins. Postoperative, post-131I therapy scanning...
was negative for iodine-avid metastases and thyroglobulin was undetectable with a negative thyroglobulin antibody assessment six months postoperatively.

Including the five Cyto-I/GEC-B nodules removed as initial management, thyroid malignancy was identified in 1 of 13 (7.7%) Cyto-I/GEC-B nodules. During a similar follow-up interval, thyroidectomy was performed for only 12 of 873 (1.4%) patients with Cyto-B nodules ($P < .0001$, Figure 1). In this group, indications for surgery were abnormal repeat cytology ($n = 7$), compressive neck symptoms ($n = 2$), and nodule growth ($n = 3$). Of these 12, four (33%) were malignant ($P = .16$ compared to Cyto-I/GEC-B groups). Eighty-five of the 95 patients (89.5%) with a Cyto-I/GEC-B nodule are still followed at our institution with no mortalities observed during up to 4 years of follow-up.

## Discussion

The management of thyroid nodules with indeterminate cytology is rapidly evolving as the use of molecular markers to improve preoperative risk stratification gains wider use. For nodules with AUS/FLUS and SFN/FN cytologies, a benign Afirma GEC is suggested to have a sufficiently low malignancy risk to allow nonoperative monitoring (18, 19, 23). Data describing these nodules during observation, however, are lacking.

This study is the first to provide a comprehensive description of the growth and sonographic characteristics of Cyto-I/GEC-B nodules during follow-up. Previous reports assessing the outcomes of Afirma GEC-tested nodules have focused little on those with a benign result (19, 24–28). One previous study followed 71 GEC benign nodules, but the median follow-up was only 8.5 months and evaluation of nodule growth and sonographic features was not included (19).

In the current study, significant growth during surveillance was assessed by two widely accepted criteria: ≥20% change in ≥2-dimensions or ≥50% change in volume (4, 22). When comparing Cyto-I/GEC-B nodules to a control group of Cyto-B nodules over similar follow-up duration, there was no observed difference in the proportion of nodules demonstrating growth. Multinodularity and cystic contents, previously shown to influence nodule growth (22, 29), were dissimilar between these groups, but logistic regression analysis adjusting for these factors again indicated that the risk of nodule growth was similar.

The proportion of Cyto-I/GEC-B nodules with growth in this study is similar to previous studies evaluating growth of benign nodules. We observed that 8.6% of Cyto-I/GEC-B nodules grew ≥20% in ≥2-dimensions, which is comparable to the 11.1% rate found for 1567 cytologically or sonographically benign nodules in a 5-year prospective study (22). Over that 5-year period, the average nodule growth was 4.9 mm (roughly 1 mm per year), which suggests a rate of growth that may be hard to assess after only one year of follow up. A ≥50% volume increase was found in 17.2% of Cyto-I/GEC-B nodules in this study similar to the rate of 16.6% found in 249 benign nodules evaluated retrospectively, with the majority meeting this criterion within 36 months (30).

Repeat FNA cytology was performed in 6.9% of Cyto-I/GEC-B nodules, but did not provide information to change clinical management in any case. While more patients with Cyto-B nodules underwent repeat FNA, fewer had thyroidectomy performed during this period of follow-up. These differences likely convey the relatively low threshold for recommending thyroidectomy for patients with Cyto-I/GEC-B nodules, given the elevated concern for malignancy based on prior abnormal cylocytic results, whereas repeat FNA was preferred for those with previously benign cytology.

### Table 2. Characteristics and Histopathologic Diagnosis of Cytologically Indeterminate / GEC Benign Nodules that Underwent Resection.

<table>
<thead>
<tr>
<th>n</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Nodule Size (initial), cm</th>
<th>Cytology</th>
<th>Time to resection, months</th>
<th>Nodule Size (final), cm</th>
<th>Reasons for Resection</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>F</td>
<td>2.2</td>
<td>AUS</td>
<td>0</td>
<td>n/a</td>
<td>Patient decision</td>
<td>Follicular adenoma</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>F</td>
<td>1.7</td>
<td>AUS</td>
<td>0</td>
<td>n/a</td>
<td>2nd nodule requiring surgery</td>
<td>Nodular hyperplasia</td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>F</td>
<td>1.3</td>
<td>AUS</td>
<td>0</td>
<td>n/a</td>
<td>2nd nodule requiring surgery</td>
<td>Adenomatous nodule</td>
</tr>
<tr>
<td>4</td>
<td>77</td>
<td>F</td>
<td>1.1</td>
<td>SFN</td>
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<td>n/a</td>
<td>History of EBRT, Patient decision</td>
<td>Adenomatous nodule</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>M</td>
<td>2.0</td>
<td>SFN</td>
<td>0</td>
<td>n/a</td>
<td>History of EBRT with FDG-PET avidity</td>
<td>Adenomatous nodule</td>
</tr>
<tr>
<td>6</td>
<td>42</td>
<td>F</td>
<td>1.4</td>
<td>AUS</td>
<td>7</td>
<td>1.7</td>
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<tr>
<td>7</td>
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<td>AUS</td>
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<td>Nodular hyperplasia</td>
</tr>
<tr>
<td>8</td>
<td>52</td>
<td>F</td>
<td>3.0</td>
<td>SFN</td>
<td>14</td>
<td>3.5</td>
<td>Nodule Growth</td>
<td>Follicular adenoma</td>
</tr>
<tr>
<td>9</td>
<td>21</td>
<td>F</td>
<td>2.2</td>
<td>AUS</td>
<td>7</td>
<td>2.8</td>
<td>Nodule Growth. Repeat FNA = benign</td>
<td>Follicular adenoma</td>
</tr>
<tr>
<td>10</td>
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<td>4.3</td>
<td>SFN</td>
<td>8</td>
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<td>Nodule Growth</td>
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<tr>
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<td>2.7</td>
<td>SFN</td>
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<td>2.9</td>
<td>Nodule Growth. Repeat FNA = SFN</td>
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<tr>
<td>13</td>
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<td>2.3</td>
<td>SFN</td>
<td>19</td>
<td>2.6</td>
<td>Nodule Growth</td>
<td>Follicular adenoma</td>
</tr>
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</table>

n = number, F = female, M = male, AUS = atypia of undetermined significance, SFN = suspicious for follicular neoplasm, EBRT = external beam radiation treatment, FDG-PET = fluorodeoxyglucose positron emission tomography, FTC = follicular thyroid carcinoma, FNA = fine-needle aspiration
Out of the 13 resected Cyto-I/GEC-B nodules, one false negative result was found. The presence of thyroid cancer in Afirma GEC benign nodules that underwent resection was previously reported in 2 of 5 cases (both papillary cancer) by Harrell and Bimston (24), and 1 of 4 cases (an FTC exhibiting focal capsular and vascular invasion) by McIver et al (25). In contrast, no malignancies were found in the studies by Lastra et al (26), Marti et al (27), and Brauner et al (28), though these studies contained very few resected Afirma GEC benign nodules. Together, these studies suggest a small but persistent concern for false negative Afirma GEC results. None of these studies however, include further data on cancer treatment or outcome for these patients. In our study, complete cancer resection,11 scanning without evident metastases, and an undetectable thyroglobulin, are consistent with an excellent response and suggest that the potential delay in diagnosis was not harmful to this patient.

We recognize several limitations to the present study. The assessment of nodule growth over the relatively short follow up time of one year is limited given the slow growth of most benign nodules (22). However, since a common interval of follow up for benign thyroid nodules is 6–18 months, our data report the change in Afirma GEC benign nodules during a clinically relevant monitoring period. The sample size in this study is small and encompasses 64% of the Cyto-I/GEC-B nodules not referred for initial surgery, introducing the possibility of selection bias. There may have been a less rigorous effort to follow up patients whose Cyto-I/GEC-B nodules were smaller or of lower risk, leading to an overestimation of risk in the study population. It is unlikely that patients at higher risk for a malignant nodule based on clinical or ultrasound findings were disproportionately excluded from follow up at our institution.

The Cyto-B nodule control group was a retrospective cohort that is subject to potential confounding or bias, but this effect was likely limited since all patients were evaluated by the same physicians at the same institution, and most characteristics were similar between groups. Furthermore, subsequent correction for potential differences using logistic regression did not change our results. We were unable to evaluate the degree to which nodule growth was due to an increase in cystic component in Cyto-B nodules. Isolated cystic enlargement was likely rare given that all nodules were predominantly solid, but we cannot exclude this possibility in some instances. We also did not evaluate every possible suspicious sonographic characteristic. Though suspicious features may predict thyroid malignancy despite a lack of nodule growth (31, 32), there is suboptimal reproducibility (33) and their usefulness has recently been questioned (34, 35).

We attempted to mitigate these by focusing on higher risk features (36), and using a single blinded evaluator. Similar sonographic review of all Cyto-B nodules was not feasible, but given the low rate of new suspicious features in the Cyto-I/GEC-B group, a statistical difference between groups is unlikely.

In conclusion, Cyto-I/GEC-B and Cyto-B nodules show similar growth during follow up, suggesting comparable clinical behavior. Only one malignancy was detected, for which an excellent response was achieved despite any potential delay in diagnosis. These data provide a strong indication that treating Cyto-I/GEC-B nodules similarly to those with benign cytology is appropriate.

Acknowledgments

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