Does Addition of BRAF V600E Mutation Testing Modify Sensitivity or Specificity of the Afirma Gene Expression Classifier in Cytologically Indeterminate Thyroid Nodules?

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Objective: The purpose of this study was to determine the frequency of BRAF mutation in cytologically indeterminate thyroid nodules and to investigate whether adding the BRAF test improves diagnostic accuracy of the Afirma Gene Expression Classifier (GEC).

Design: BRAF V600E mutational status was determined for DNA extracted from cytologically benign (n = 40), indeterminate (n = 208), and malignant (n = 48) fine-needle aspiration specimens previously categorized by GEC as molecularly Benign or Suspicious. Analytical performance of the BRAF assay was assessed to establish reproducibility and limits of detection. Molecular testing results were correlated with blinded expert histopathological diagnoses.

Results: The BRAF assay detected mutations reproducibly to 2.5% mutant allele frequency. The prevalence of BRAF mutations in cytologically benign specimens was 2 of 40 (5.0%, 95% confidence interval [CI], 0–16) and in cytologically malignant specimens was 36 of 48 (75.0%, 95% CI, 60–86). In the cytologically indeterminate category, 10.1% of specimens were BRAF+: 2 of 95 were subcategorized as atypia of undetermined significance or follicular lesion of undetermined significance (2.1%, 95% CI, 0–7); 1 of 70 as follicular neoplasm or suspicious for follicular neoplasm (1.4%, 95% CI, 0–9); and 18 of 43 as suspicious for malignancy (41.9%, 95% CI, 27–58). All BRAF+ specimens were classified as Suspicious by the GEC.

Conclusions: BRAF mutations are uncommon in nodules with atypia of undetermined significance or follicular lesion of undetermined significance or follicular neoplasm or suspicious for follicular neoplasm cytology. Most cytologically indeterminate nodules that proved to be malignant were also BRAF−, and all nodules that were false-negative by GEC were also BRAF−. Similarly, all BRAF+ specimens were also GEC Suspicious. Neither GEC test sensitivity nor specificity was improved by addition of BRAF mutation testing. (J Clin Endocrinol Metab 98: E761–E768, 2013)

Adoption of thyroid fine-needle aspiration (FNA) biopsy substantially reduced the need for thyroid surgery to exclude malignancy, and today find-needle aspiration is the recommended diagnostic approach to evaluate nodules greater than 1 cm in diameter (1). However, 15% to 30% of nodules cannot be conclusively diagnosed by FNA cytology alone (1) and are categorized as indeterminate. These indeterminate cytological categories...
include (1) atypia or follicular lesion of undetermined significance (AUS/FLUS) (The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) Category III); (2) follicular/Hürthle cell neoplasm or suspicious for follicular/Hürthle cell neoplasm (FN/SFN) (TBSRTC Category IV); and (3) suspicious for malignancy (SM) (TBSRTC Category V) (2, 3). Surgery is usually recommended for these patients to ensure that malignancy is not overlooked (1, 4, 5) and up to 74% of US patients with cytologically indeterminate nodules are operated upon (6). However, based on surgical histopathological results, in one meta-review only 34% (14%-48%) of cytologically indeterminate nodules were found to be histopathologically malignant, revealing significant rates of avoidable surgery, complications, and morbidity (6).

An RNA-based gene expression classifier (GEC) has been validated for the preoperative identification of benign nodules in patients with indeterminate FNA cytological results so that monitoring could be recommended in lieu of diagnostic thyroid surgery (7–9). The GEC sample is collected by FNA concurrently with the sample for cytology assessment and immediately stored in preservative solution. When FNA cytology is reported as indeterminate, processed samples are hybridized to a custom Afirma thyroid microarray and analyzed with a classification algorithm to categorize specimens as either “Benign” or “Suspicious.” The clinical validity of the GEC has been reported elsewhere with results from a large prospective and blinded multicenter trial (8). For AUS/FLUS nodules, sensitivity was 90%, specificity was 53%, and negative predictive value (NPV) was 95%, with a 24% prevalence of malignancy. For FN/SFN nodules, sensitivity was 90%, specificity was 49%, and NPV was 94%, with a 25% prevalence of malignancy. For SM nodules, sensitivity was 94%, specificity was 52%, and NPV was 85%, with a 62% prevalence of malignancy. Overall, the Afirma GEC raised specificity, ie, detection of benign nodules, from 0% for cytological analysis alone in indeterminate FNAs to 52% (8). Based on this specificity, approximately one half of patients with benign nodules resulting in indeterminate FNA cytology can be identified preoperatively. In clinical practice, 2 studies have shown that Benign GEC results enable a significant number of patients and physicians to elect clinical and sonographic follow-up in lieu of diagnostic surgery, with operative rates comparable to those of patients with cytologically benign FNAs (10, 11). Despite this impact, even more unnecessary surgical procedures could be avoided if a testing paradigm that could further raise specificity while maintaining high sensitivity and NPV existed (12).

The BRAF V600E mutation (BRAF+) is present in almost one half of papillary thyroid cancers, the most common thyroid malignancy, but is uncommon or absent in other thyroid malignancies. Similarly, it has high prevalence in the SM cytology group but low prevalence in the FN/SFN and AUS/FLUS groups (13, 14). Taken together, these findings account for its limited sensitivity among cytologically indeterminate nodules. However, BRAF mutations are absent in benign thyroid nodules (15). This high specificity, therefore, accurately predicts that a BRAF+ thyroid nodule is malignant (13). Consequently, we evaluated whether the combination of the highly sensitive GEC test with the highly specific BRAF test might provide an improved testing paradigm. Here we report the results of BRAF V600E mutation analysis in samples previously tested with the GEC, using a set of thyroid nodules with blinded surgical histopathological verification of their true character.

Materials and Methods

The 296 thyroid nodule samples analyzed in this study have been described previously (8) and represent a subset with ≥30 ng of DNA. In brief, cytologically indeterminate FNAs were prospectively collected from 49 study sites in a noninterventional multicenter study. Internal review board approvals were obtained from all applicable local or central internal review boards, including consent for validation of the GEC and additional molecular testing research (8). The extent of thyroid surgery was extracted from local surgery pathology reports. Data on whether patients were previously tested for BRAF mutations were not collected for this study. Gold standard histopathological diagnoses were determined for all patients by 2 thyroid pathology experts who were blind to both GEC and BRAF mutation testing results, as described previously (8). DNA was extracted from clinical FNAs collected in FNAprotect, using the AllPrep Micro Kit (Qiagen, Valencia, California). Yield was determined by a Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Grand Island, New York). The percent BRAF V600E mutation present was determined using the Mutation Detection Assays Competitive Allele-Specific TaqMan (castPCR) procedure (Life Technologies) and run on the ABI7900HT instrument platform. Each sample was tested in separate PCR reactions with wild-type and mutant assays using 5 ng of DNA. Three control samples were run on each plate: 1) BRAF mutation positive control (HT29 cell line), 1) BRAF mutation negative wild-type cell line control (HorizonDx), and a no-template control (water). Percent mutation present was determined per sample by comparing the cycle threshold (Ct) values (a predefined fluorescence intensity assay cycle number threshold) of the wild-type and mutant allele assays (∆Ct) using Mutation Detector Software (Life Technologies). Cutoffs for the Ct values were set at >35 cycles for both wild-type and mutant reactions and >30 cycles for the internal positive control. Analytical sensitivity experiments set the limit of detection at 2.5% mutation present, labeling all samples with ≥2.5% mutation as “BRAF-positive” (BRAF+). Samples with <2.5% mutation detected were called “BRAF-negative” (BRAF−) and samples with low/failing quality control values were designated “no call.” All cutoffs were determined before the testing of clin-
ical specimens. Sensitivity and specificity of the combined tests were computed by assigning samples that were either \textit{BRAF} or \textit{GEC} Suspicious to the “test positive” class, and samples that were \textit{both BRAF} and \textit{GEC} Benign to the “test negative” class. Refer to the Supplemental Materials and Methods published on the Endocrine Society’s Journals Online web site at http://jcem.endojournals.org for full details on the methods used.

**Results**

\textbf{BRAF} assay analytical sensitivity: total DNA input quantity, detection limit, and reproducibility

Analytical sensitivity and clinical performance of the castPCR assay depends on the DNA input amount for each wild-type and mutant assay. Some measurement variability around this nominal input amount can be expected in routine practice. Thus, we characterized performance of the assay across a range of DNA input amounts before setting specification for testing clinical samples. Control DNA from the \textit{BRAF} mutant HT29 cell line was titrated to 2, 5, and 10 ng of input and run in triplicate or higher (Figure 1, A and B, stock HT29 data). This study demonstrated no significant difference in \textit{ΔCt} values across input amounts above 5 ng (\(P = .11\), pooled \(SD = 0.83\)). Although the 2 ng data showed acceptable results, 5 ng was chosen as input for testing clinical samples.

To determine the lowest percentage of mutant \textit{BRAF} alleles reproducibly detected by the castPCR assay, we created a dilution series using \textit{BRAF} benign DNA. The experimentally derived 30% mutation detected in undiluted stock \textit{BRAF} HT29 DNA was diluted to 1.5%, 3.0%, 4.5%, 6.0%, and 9.0% mutation frequency (Figure 1C). Although the 2 ng data showed acceptable results, 5 ng was chosen as input for testing clinical samples.

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In a study of interrun repeatability, HT29 control DNA was tested in 19 different runs (same operator, same reagent lots) across 29 individual reactions. The average percent mutation detected was 32.7%, demonstrating high repeatability across different processing runs (Supplemental Materials and Methods and Supplemental Figure 1A). The final assay system was further tested on the intended use sample, ie, FNAs. The percent BRAF V600E mutation detected in 11 FNAs tested in triplicate was found to be reproducible across a wide range (3.3%–60%) with an average coefficient of variation of 20.8% (Supplemental Materials and Methods and Supplemental Figure 1B). The characteristics and performance of this BRAF V600E castPCR assay were compared to alternative assays (Supplemental Table 1).

Frequency of BRAF mutations in thyroid FNAs

The 296 FNAs with both valid GEC and BRAF results included 40 cytologically benign, 208 cytologically indeterminate, and 48 cytologically malignant samples. Three of the 40 (7.5%) cytologically benign samples were found to be malignant on surgical pathology and all 3 were GEC Suspicious, whereas 2 of 3 were BRAF+. All cytologically malignant nodules were confirmed to be cancers on surgical pathology and 48 of 48 (100%, 95% confidence interval [CI], 92.6–100), were categorized as GEC Suspicious, whereas 36 of 48 (75%, 95% CI, 60.4–86.4) were BRAF+.

Combined BRAF and GEC results in these indeterminate thyroid nodules are shown in Table 1. Among 208 indeterminate FNAs, 21 (10.1%) were found to be BRAF+. In the 95 specimens with AUS/FLUS indeterminate cytology only 2 of 95 (2.1%, 95% CI, 0–7) were BRAF+, in the FN/SFN group only 1 of 70 (1.4%, 95% CI, 0–8) was BRAF+, and in the SM group, 18 of 43 (41.9%, 95% CI, 27–58) were BRAF+.

Table 1 also shows the distributions of surgical pathological diagnoses within each cytology category. Among the 75 histopathologically malignant samples in the indeterminate FNA group, 25 were AUS/FLUS, 19 were FN/SFN, and 31 were SM. BRAF mutations were identified in 20 of 75 malignancies (27%), whereas 68 of 75 (91%) malignancies were GEC Suspicious. All BRAF+ samples were also classified as Suspicious by the GEC (Table 1). Among the 7 false-negative GEC cases described previously (8), none were BRAF+.

We examined BRAF and GEC results for each of the surgical pathology categories of thyroid cancer (Table 2). Of cases of follicular variant of papillary thyroid carcinoma (fvPTC), 88% (15 of 17) were BRAF+ as were 100% (0 of 14) of cases of follicular carcinoma (FC) and Hürthle cell carcinoma (HCC). All BRAF+ cases except 1 (see below) were conventional papillary thyroid carcinoma (PTC) or fvPTC. One BRAF+ result was observed in a sample designated by gold standard surgical histopathological analysis to be a Hürthle cell adenoma, which the local cytopathologist had described as a possible PTC. (The expert surgical histology review interpreted the Hürthle features as having arisen because of tumor necrosis.)

Utility of combined BRAF and GEC testing

We computed sensitivity and specificity for combined BRAF and GEC testing (Figure 2). The data show that a
combination of the 2 tests had no effect on sensitivity or specificity of the GEC test in this patient cohort, which is very similar to a cohort described in a recently published clinical validation study (8).

**Discussion**

Using a sensitive *BRAF* mutation detection assay analytically validated in thyroid FNAs, we show that the GEC correctly identified all *BRAF*+/*H11001* FNAs as GEC Suspicious. These FNAs were collected as part of a large, prospective multicenter trial among US academic and community-based practices using a variety of locally preferred fine-needle aspiration techniques. We believe the diverse practices captured by this study design extend the applicability of our results to a wide variety of clinical settings.

We tested the previously unexplored hypothesis that addition of *BRAF* mutation testing to GEC testing of indeterminate FNAs would improve overall performance, so that a greater proportion of true benign samples could be classified as benign (specificity), while striving to miss as few true malignancies as possible (sensitivity). We demonstrate a flaw in the assumption that a high specificity test such as *BRAF* can complement a high sensitivity test such as the GEC. Testing for *BRAF* mutations only confirmed a subset of malignancies already identified by the GEC; consequently, the performance of the GEC was not improved. All samples that were *BRAF*+ were also GEC Suspicious.

| Cytology Group | Histopathological Malignant Subtype | *BRAF*+ Result (%) | GEC Suspicious Result (%) |
|----------------|----------------------------------|---------------------|---------------------------|
| AUS/FLUS       | FC-c                             | 0/2 (0)             | 2/2 (100)                 |
|                | fvPTC                            | 0/5 (0)             | 5/5 (100)                 |
|                | HCC-c                            | 0/3 (0)             | 3/3 (100)                 |
|                | HCC-v                            | 0/1 (0)             | 1/1 (100)                 |
|                | ML                               | 0/1 (0)             | 1/1 (100)                 |
|                | PTC                              | 2/13 (15)           | 10/13 (77)                |
|                | FC-c                             | 0/1 (0)             | 1/1 (100)                 |
|                | FC-v                             | 0/1 (0)             | 1/1 (100)                 |
|                | fvPTC                            | 0/5 (0)             | 4/5 (80)                  |
|                | HCC-c                            | 0/4 (0)             | 4/4 (100)                 |
|                | HCC-v                            | 0/1 (0)             | 0/1 (0)                   |
|                | MTC                              | 0/1 (0)             | 1/1 (100)                 |
|                | PTC                              | 1/4 (25)            | 4/4 (100)                 |
|                | WDC-NOS                          | 0/2 (0)             | 2/2 (100)                 |
|                | fvPTC                            | 2/7 (29)            | 6/7 (86)                  |
|                | HCC-c                            | 0/1 (0)             | 1/1 (100)                 |
|                | ML                               | 0/1 (0)             | 1/1 (100)                 |
|                | MTC                              | 0/1 (0)             | 1/1 (100)                 |
|                | PTC                              | 15/21 (71)          | 20/21 (95)                |
|                | fvPTC                            | 1/3 (33)            | 3/3 (100)                 |
|                | MET                              | 0/1 (0)             | 1/1 (100)                 |
|                | PTC                              | 29/38 (76)          | 38/38 (100)               |
|                | PTC-TCV                          | 6/6 (100)           | 6/6 (100)                 |

Abbreviations: AUS/FLUS, atypia of undetermined significance or follicular lesion of undetermined significance; FC-c, follicular thyroid carcinoma, with capsular invasion; FC-v, follicular thyroid carcinoma, with vascular invasion; FN/SFN, follicular or Hürthle cell neoplasm or suspicious for follicular neoplasm; fvPTC, follicular variant of papillary carcinoma; HCC-c, Hürthle cell carcinoma, with capsular invasion; HCC-v, Hürthle cell carcinoma, with vascular invasion; MET, metastasis to thyroid; ML, malignant lymph node; MTC, medullary thyroid carcinoma; PTC, papillary thyroid carcinoma; PTC-TCV, papillary thyroid carcinoma-tall cell variant; SM, suspicious for malignancy; WDC-NOS, well-differentiated carcinoma, not otherwise specified.
Surgical decision making in patients with thyroid nodules (18). Tests with high NPV have been accepted to help decide whether surgery can be avoided (19), whereas tests with high positive predictive value may determine the extent of surgery indicated for patients needing surgery. FNA diagnostic tests must have high sensitivity to achieve a posttest risk of malignancy in an indeterminate nodule that approximates the 6% to 7% observed in nodules with benign cytopathology (6, 20). Panels that test for somatic DNA mutations, including BRAF, RAS, RET/PTC, and PAX8/PPARγ, have not demonstrated high enough sensitivity to avoid diagnostic surgery (18, 21, 22). Without the high NPV GEC, a large majority of patients with indeterminate FNA results undergo surgical resection to manage the approximately 25% pretest risk of malignancy (1, 4, 5). A combination of molecular marker testing and prescreening with BRAF mutation testing has been proposed by others as a means to avoid unnecessary surgery (23). For this approach to be applicable to clinical practice, the assay methodology should undergo analytical validation and appropriate reporting (24) as was done here. In addition, because of limitations in interobserver agreement between thyroid histopathologists, the molecular marker panel that distinguishes benign and malignant nodules must be validated clinically against an expert panel of blinded histopathologists, as was done for the GEC and now for the current castPCR BRAF assay (7–9).

The Afirma GEC has been shown in a recent large prospective multicenter study to have high sensitivity (93%) but lower specificity (52%) (8). This specificity potentially removes more than one half of patients with histologically benign thyroid pathology findings from surgical consideration and is a substantial improvement over an effective specificity of 0% for practice paradigms that recommended surgery for all patients with indeterminate cytology. Our current data show that although BRAF testing has very high specificity (>99%), its overall sensitivity of 27% and NPV of 71% are too low for use of this test alone to routinely avoid diagnostic surgery, an opinion shared by others (18, 22).

Although combining BRAF testing with GEC testing does not improve sensitivity or specificity over GEC testing alone, in selected cases where a hemithyroidectomy would have been considered, the high positive predictive value of BRAF testing might, in theory, allow clinicians to advise instead a total thyroidectomy (with or without prophylactic central neck dissection) when samples are BRAF+. Unfortunately, for those patients with BRAF− results, our study suggests that a significant proportion of those undergoing hemithyroidectomy will require a complete thyroidectomy when a malignant diagnosis is revealed by surgical histopathology results. BRAF testing is not justifiable in cytologically AUS/FLUS or FN/SFN indeterminate categories, because BRAF+ samples are present in only 2.1% and 1.4% of these samples, respectively. Low rates of BRAF mutations are consistent with a previously published retrospective analysis of prospectively collected samples in which 2.0% and 0.9% of the AUS/FLUS and FN/SFN samples, respectively, were BRAF+ (13). In contrast, our data show a much higher rate of BRAF mutations (42%) in the SM category than that observed in the aforementioned study, in which 19% of the SM samples were BRAF+ (13). Although this indeterminate category has a high frequency of BRAF mutation, the concomitantly high pretest risk of malignancy in SM samples (72% here and 62% in a recent meta-review) (6) alone may support consideration of a total thyroidectomy rather than a hemithyroidectomy (18). Within the SM category, we found that 37 of 43 (86%) of patients were treated with a total thyroidectomy, with a trend toward more hemithyroidectomies performed by academic sites compared with community sites (Supplemental Table 2). This figure is consistent with a larger cohort consisting only of patients with SM nodules (including those not tested with the GEC or BRAF) in whom 77 of 92 (84%) operations were total thyroidectomies (our unpublished data). In patients with SM nodules that proved to be cancer postoperatively, BRAF was only positive in 1 of 7 at academic centers and 16 of 24 at community sites. This result may reflect a tendency of academic cytopathologists to call a BRAF+ PTC as outright malignant on cytology, as opposed to community-based cytopathologists who may use the SM category more often in these cases. In academic centers, 73% of the SM indeterminate group underwent total thyroidectomies, whereas in community sites, 91% underwent total thyroidectomies (Supplemental Table 2). Thus, it appears that BRAF testing is not likely to benefit patients from academic centers because few of their SM tumors were BRAF+. BRAF testing is not likely to help patients from community centers because these patients already undergo total thyroidectomy for nearly all SM tumors. In our study, only 1 of 18 patients with BRAF+ SM tumors...
would have undergone a total thyroidectomy rather than a hemithyroidectomy had their \textit{BRAF} status been determined preoperatively, consistent with data reported by others (14). \textit{BRAF} testing alone in patients with SM tumors results in a high number of completion thyroidectomies, given the high prevalence of \textit{BRAF–} malignancies among them.

Overall, \textit{BRAF} testing alone left 73\% of malignancies undetected among cytologically indeterminate nodules and as many as 45\% in the SM category, in which PTC is more common. In our study, there were 35 PTC samples among the indeterminate cytology group; of these, 18 were \textit{BRAF+} (51.4\%), consistent with previous findings showing that 30\% to 60\% of PTCs carry a \textit{BRAF} V600E mutation (25–27). However, only 11.8\% of \textit{fvPTC} in the indeterminate cytology group were \textit{BRAF+}, and none of the FCs or HCCs were \textit{BRAF+} (0.0\%), again consistent with previous studies (28, 29). Finally, a single HCA was \textit{BRAF+}, leading to a false-positive result for both the \textit{BRAF} assay and the GEC. In this case, it is possible that these molecular markers reflect the tumor character at the time of the FNA collection more accurately than even histopathology.

It is not surprising that the GEC detects substantially more malignancies than \textit{BRAF} testing alone, because the GEC algorithm training included transcriptional profiles measured on samples from a wide variety of malignancies (8, 9), including both \textit{BRAF+} and \textit{BRAF–} neoplasms. The classifier was trained to recognize malignancy regardless of \textit{BRAF} status, and in fact, \textit{BRAF} status can be inferred directly from gene expression (ie, RNA) data (our unpublished data). The GEC yielded 7 false-negative results (Table 1), and a primary explanation is paucity of follicular cells in most of these samples (8). Hypothetically, this could also generate false-negative \textit{BRAF} results in the same 7 cases.

Our analytical validity studies of the \textit{BRAF} castPCR assay show that the test is accurate and precise and reported in accordance with STARD guidelines (24). We reliably and reproducibly detect the \textit{BRAF} V600E mutation at an analytical sensitivity of 2.5\% allele frequency in clinical thyroid FNAs. Titration experiments of FNA and control DNA established that within the recommended DNA input range of 1 to 20 ng, 5 ng was sufficient to produce consistent results >2 SD below the cutoff value of \textit{C}_\text{C}_{\text{S}} >35. Repeatability testing on control material also demonstrated that this assay yields consistent results in detected mutation proportion with a coefficient of variation of 12.5\%.

The castPCR and related assays (30, 31) have been shown to be as sensitive or more sensitive than other reported assays (Supplemental Table 1), this eliminates inadequate assay sensitivity as the explanation for the low rates of \textit{BRAF} mutation detection in the cytologically indeterminate samples studied here. More importantly, we have shown that a detection sensitivity of 2.5\% is reliable when using this method with FNAs and correspondingly lower input amounts of DNA, something not uniformly reported in previous studies. Among the \textit{BRAF} assay methods investigated (Supplemental Materials and Methods and Supplemental Table 1), only one was compliant with STARD guidelines and clearly stated the experimental methods and sensitivity obtained in working directly with FNAs (13), thus allowing comparisons to be made with this study (Supplemental Figure 2). Although there were differences between this study and our own, including the proportions of \textit{fvPTC} and PTC malignancies within each of the 3 indeterminate cytology subtypes (as well as differences in proportions of the cytology subtypes themselves), the overall percentage of \textit{fvPTC} and PTC malignancies was similar in the 2 studies: 26\% for the present study and 21\% for Nikiforov et al (13). Despite this similarity, however, there is a higher rate of \textit{BRAF} positivity among \textit{fvPTC} and PTC samples in our cohort of cytologically indeterminate samples. For example, whereas 36\% of the PTC/\textit{fvPTC} samples in our study were \textit{BRAF+}, only 15\% of the PTC/\textit{fvPTC} samples in the study by Nikiforov et al (13) were \textit{BRAF+}. We believe this higher \textit{BRAF+} detection rate is probably due to the analytical sensitivity and robustness of the castPCR system. Recently, Lee et al (32) demonstrated, using 3 \textit{BRAF} V600E mutation assays with sensitivities to detect 20\%, 2\%, and 0.1\% of mutated DNA alleles, that \textit{BRAF} mutations were detected in 54.8\%, 74.4\%, and 79.7\% of all PTCs, respectively; these were primarily derived from cytologically malignant tumors. Guerra et al (33) reported that the prevalence of the \textit{BRAF} V600E mutation among PTCs was 53.6\% or 36.9\% when 2 different \textit{BRAF} assays were applied to the same PTC samples (33). These studies highlight the lack of standardization of \textit{BRAF} testing methods, the uncertainty regarding the allelic threshold to define a positive test, and the challenges of tumor heterogeneity (15).

Our findings demonstrate no benefit of \textit{BRAF} testing in GEC Benign nodules. \textit{BRAF} testing did not improve GEC test performance. All \textit{BRAF+} samples were GEC Suspicious; thus, sensitivity for cancer detection was not increased. Conversely, the addition of \textit{BRAF} testing did not improve GEC specificity because \textit{BRAF–} status is not pathognomonic for benignity. This same conclusion would hold for \textit{RAS}, \textit{PAX8/PPARγ}, \textit{RET/PTC}, and the other known mutations with low sensitivity for malignancy (34) because their absence (alone or in a panel combination) cannot exclude cancer and therefore cannot move a GEC Suspicious sample from the test positive to the test negative.
category. Thus, no test with low sensitivity can improve GEC specificity. Finally, the role of mutational and GEC testing in the minority of patients with cytologically SM remains to be clarified.

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