

Mutational Analysis of *BRAF* in Fine Needle Aspiration Biopsies of the Thyroid: A Potential Application for the Preoperative Assessment of Thyroid Nodules

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ABSTRACT

Background: Fine needle aspiration (FNA) is routinely used in the preoperative evaluation of thyroid nodules, but subsequent patient management is often complicated by the inability to decisively recognize malignancy on cytologic grounds alone. Activating mutations of the *BRAF* oncogene commonly occur in papillary thyroid carcinomas (PTCs) but not in other types of benign and malignant thyroid lesions. Mutational analysis of FNAs could enhance selection of thyroid nodules for surgical removal.

Methods: Ninety-five excised PTCs along with 49 corresponding FNAs were evaluated for *BRAF* mutations by a newly developed assay that uses a novel primer extension method (MutectorR assay) and by direct sequencing. An additional 42 FNAs from thyroid nodules that were excised based on a suspicion of malignancy were also evaluated.

Results: *BRAF* mutations were identified in 36 of the 95 (38%) excised PTCs. By histological subtype, *BRAF* mutations were more common in conventional PTCs than in the follicular variant (67% versus 12%; $P < 0.0001$; χ^2). Analysis of the preoperative FNAs accurately reflected *BRAF* status of the resected PTC in 46 of the 49 paired samples (94% concordance). In FNA samples grouped according to the preoperative cytologic findings (malignant, $n = 25$; benign, $n = 11$; and indeterminate, $n = 55$), a *BRAF* mutation confirmed the diagnosis of PTC in 72% of carcinomas within the malignant group, and it established the diagnosis of PTC in 16% of carcinomas within the indeterminate group. *BRAF* mutations were not detected in FNAs from 32 benign thyroid lesions. Direct sequencing and the MutectorR assay yielded completely concordant results.

Conclusions: *BRAF* mutations are common in conventional PTCs, and they are specific for PTC. A *BRAF* mutation can be reliably detected in cells aspirated from a thyroid nodule suggesting a role for this marker in the preoperative evaluation of thyroid nodules.

INTRODUCTION

Thyroid carcinoma accounts for only 1,200 deaths in the United States each year (1), but the cost associated with thyroid carcinoma detection is far from trivial. Largely reflecting the inability of current diagnostic tests to discern thyroid carcinoma from the overwhelming background of benign thyroid nodules, the estimated 75,000–80,000 thyroid surgeries performed each year in the United States is disproportionate to the prevalence of clinically relevant thyroid cancers (2). Fine needle aspiration (FNA) is highly touted as the best available diagnostic tool, but as a guide for subsequent management of thyroid nodules it is often indecisive. In most series, between 15% and 20% of FNAs yield indeterminate results, results that are suspicious for but not diagnostic of malignancy (3). Moreover, only 17–51% of thyroids resected in the FNA era actually harbor carcinoma (4). Recognizing that most nodules that are suspicious on cytologic grounds ultimately prove to be benign, intraoperative frozen section analysis has long been used as a means of guiding the extent of surgical resection. Frozen section analysis, however, rarely impacts on the intraoperative management of suspicious nodules, bringing into question this once hallowed practice (5, 6). Clearly, novel strategies to discern benign from malignant thyroid nodules are much needed.

Activating mutations of the *BRAF* gene occur in a broad range of human cancers including thyroid cancer (7–9). *BRAF* mutations induce constitutive activation of the RAS/RAF/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase signal transduction pathway, providing a potent promitogenic force that drives malignant transformation (10). Not only is the *BRAF* gene of biological interest, but it may provide an ideal target for addressing the malignant potential of thyroid nodules in the clinical arena. *BRAF* mutation is found in up to 70% of papillary thyroid carcinomas (PTCs), and it is highly specific for PTC (8, 9). This recent information places the *BRAF* gene as an attractive marker for assessing the nature of a thyroid nodule, and sets the stage for its application in the preoperative setting. We examined whether *BRAF* mutational status could be established from cells aspirated from a thyroid nodule and, thus, help discriminate malignant from benign nodules.

MATERIALS AND METHODS

Sample Selection and DNA Isolation. Study approval was obtained from the Institutional Review Board. Patients with

Received 9/23/03; revised 12/2/03; accepted 12/5/03.

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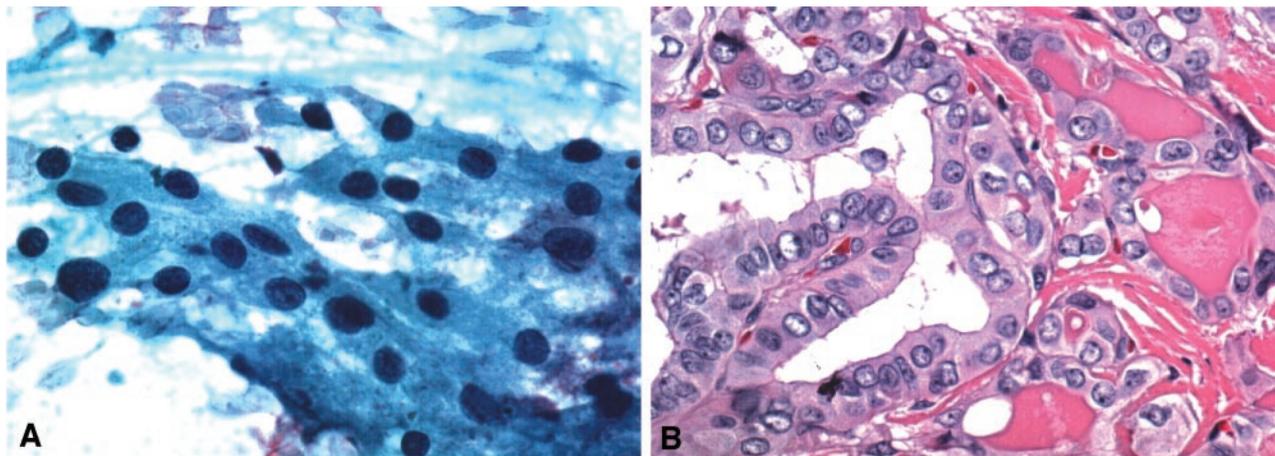


Fig. 1 *BRAF* mutated papillary thyroid carcinoma (PTC). A, cytologically, this PTC showed some atypical features including nuclear enlargement and elongation, but overtly malignant features were not identified in this “indeterminate” fine needle aspirate. B, the histological features of PTC were well developed in the resected tumor nodule.

surgically resected papillary thyroid carcinomas were identified from a search of archival surgical pathology files of The Johns Hopkins Hospital during a 2-year period (January, 2001 to January, 2003). To help address the utility of *BRAF* analysis in those PTCs that elude preoperative diagnosis, selection of cases was biased toward those patients who had undergone preoperative FNA of their nodules where the cytologic findings were indeterminate for malignancy. Case selection was otherwise arbitrary. After initial patient selection, all of the original histological slides were reviewed to confirm the diagnosis and establish the subtype of PTC. For those patients who had also undergone a preoperative FNA at our institution, the cytologic slides were retrieved for *BRAF* analysis. A second group of preoperative FNAs was retrieved from a group of 42 patients who had undergone surgical resections of a more diverse spectrum of benign and malignant thyroid nodules. Selection of these FNAs was again weighted toward the indeterminate group. On the basis of the preoperative cytologic diagnoses, FNAs were categorized as malignant, benign, or indeterminate. The “indeterminate” category encompassed those samples demonstrating hypercellularity suggestive of a follicular neoplasm (inclusive of follicular variant of papillary carcinoma), and/or atypical cytoarchitectural features suggestive of, but not diagnostic for, malignancy (Fig. 1).

For the tumor resections, archival formalin-fixed and paraffin-embedded tissues were sectioned, the sections were reviewed by the study pathologist (W. H. W.) to identify cellular areas of the tumor, and the sections were carefully microdissected under a dissecting microscope to obtain >75% neoplastic cells. DNA was extracted using standard protocols as published previously (11). For the FNA samples, coverslips were detached in xylene, and the slides were destained. The cells were then suspended in 100–200 μ l TE buffer [10 mM Tris-HCl and 1 mM EDTA (pH 7.5)], incubated with 20 μ l proteinase K (600 mAu/ml) at 48°C for 10 h, and then boiled for 5 min. DNA was then tested without additional purification.

Detection of *BRAF* Mutations. PCR primer sequences were designed to amplify a 224-bp fragment of exon 15 (5'-TCA TAA TGC TTG CTC TGA TAG GA-3' and 5'-GGC CAA AAA TTT AAT CAG TGG A-3') and a 102-bp fragment of exon 15 (5'-GAA GAC CTC ACA GTA AAA ATA GGT GA-3', and 5'-CCA CAA AAT GGA TCC AGA CA-3'). PCR amplification was performed using 100 ng of tumor sample DNA as template or an aliquot of 1–3 μ l from the cytology samples. The PCR reactions were carried out in a 96-well thermocycler. Cycling conditions were as follows: a denaturation step at 95°C for 5 min was followed by 2 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, primer extension at 72°C for 1 min, 2 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, primer extension at 72°C for 1 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, primer extension at 72°C for 1 min, and one final extension at 72°C for 5 min. Amplified fragments were separated on an agarose gel and visualized by ethidium bromide staining.

Analysis of the PCR products for a *BRAF* mutation at nucleotide position 1796 was performed using direct automated sequencing and/or the Mutector assay (TrimGen, Sparks, MD). The Mutector assay is designed for the detection of any type of known DNA mutation (12). In brief, a detection primer is designed that does not permit primer extension when the target base is not mutated. As a result, primer extension does not occur, labeled nucleotides are not incorporated, and a color reaction is not observed. When the target base is mutated (e.g., T \rightarrow A transversion at *BRAF* T1796), primer extension continues and a strong color reaction is observed. We used as a template 10 μ l of PCR products of the 102-bp fragment of *BRAF* exon 15. The assay was performed according to the manufacturer's instructions.

RESULTS

BRAF mutations were identified in 36 of the 95 (38%) surgically excised PTCs from 92 patients, but the frequency of

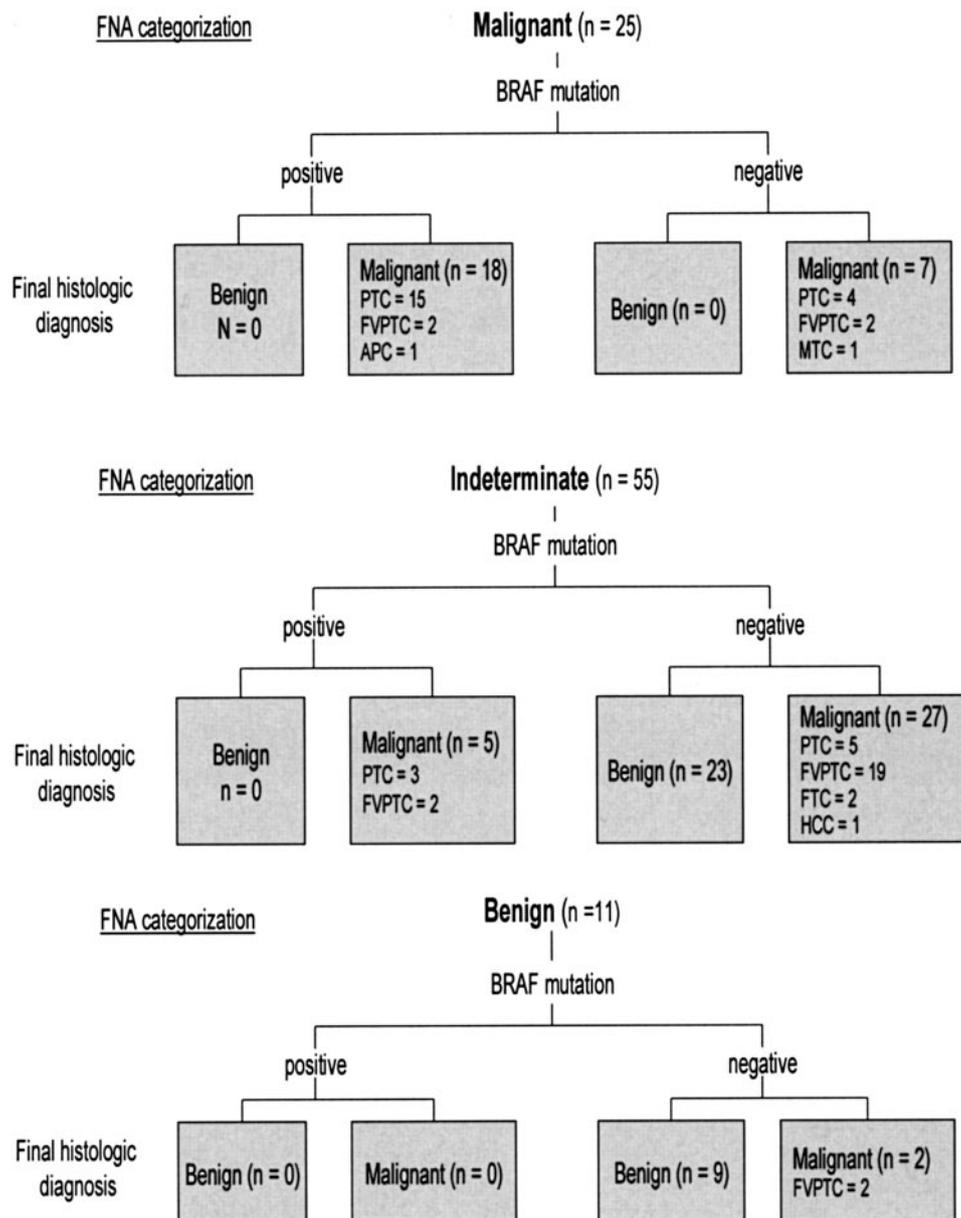


Fig. 2 BRAF status of fine needle aspiration specimens. The thyroid nodules are categorized according to the preoperative cytologic findings as malignant, indeterminate, or benign. For each category, the final histologic diagnosis is indicated (boxes). FNA, fine needle aspiration; PTC, papillary thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; APC, anaplastic carcinoma; MTC, medullary carcinoma; FTC, follicular thyroid carcinoma; HCC, Hurthle cell carcinoma.

mutations varied across the subtypes of PTCs. BRAF mutations were much more common in the conventional PTCs (28 of 42; 67%) than in the follicular variant of PTC (6 of 51; 12%). In 2 patients with an undifferentiated (*i.e.*, anaplastic) carcinoma arising in association with conventional PTC, BRAF mutations were present in both the differentiated and undifferentiated components. In the other patient with more than one tumor, a BRAF mutation was present in one of the conventional PTCs but not in the other conventional PTC.

Corresponding preoperative FNA specimens were available for 49 (52%) of the 95 resected PTCs. When compared with

the paired tumor resection specimens, there was 94% concordance for BRAF status. Discordant results were noted in only 3 of the paired samples. In 2 of the resected tumors that harbored a BRAF mutation, a mutation was not detected in the FNAs. In these 2 cases, the FNA material was sparsely cellular. Conversely, a BRAF mutation was detected in 1 FNA, although the mutation was not identified in the resected tumor.

Altogether, 91 preoperative FNAs from a spectrum of thyroid nodules with an established histological diagnosis were evaluated for BRAF mutations. On the basis of the preoperative cytopathologic findings, 25 of the FNAs were categorized as

malignant, 11 as benign, and 55 as indeterminate (Figure 2). Within the indeterminate group, 33 of the FNAs demonstrated a hypercellular follicular pattern suggestive of a follicular neoplasm, 16 showed cytologic atypia in addition to a microfollicular pattern thus raising the possibility of a follicular variant of papillary carcinoma, and 6 demonstrated cytoarchitectural atypia suggestive of but not entirely diagnostic of PTC. *BRAF* mutations were not detected in any (0%) of the 32 nodules that ultimately proved to be benign, but mutations were detected in 23 of 59 (39%) of nodules that proved to be malignant. Mutations were only noted in PTCs. They were not detected in any of the four non-PTC thyroid malignancies (medullary carcinoma, $n = 1$; follicular carcinoma, $n = 2$; or Hurthle cell carcinoma, $n = 1$). By FNA category, a *BRAF* mutation was detected in 18 of 25 (72%) carcinomas within the malignant group, in none of the 2 (0%) carcinomas within the benign group, and in 5 of 32 (16%) carcinomas within the indeterminate group. Within the indeterminate group, a carcinoma was more likely to harbor a *BRAF* mutation if the preoperative FNA sample was interpreted as suspicious for but not diagnostic of PTC (2 of 6, 33%), than if it was interpreted as a follicular neoplasm (2 of 33; 6%) or a follicular neoplasm with cytologic atypia suspicious for follicular variant of PTC (1 of 16; 6%).

Of the 186 total samples evaluated for *BRAF* mutations, 97 (52%) were evaluated by both direct sequencing and the Mutector[®] assay (Fig. 3). There was a 100% correlation rate with these two methods. In a single case, a mutation detected using the Mutector assay was initially missed by automated sequencing but then confirmed on resequencing.

DISCUSSION

About half of all adults in the United States harbor a thyroid nodule, but only ~5% of these nodules represent thyroid carcinoma (3). FNA is now widely accepted as the most reliable diagnostic method for discerning thyroid carcinoma from the overwhelming background of benign nodules, yet 5–10% of FNAs are inadequate and another 15% to 20% are indeterminate (3, 13). Repeat FNA, core biopsy, and even intraoperative frozen section are often not successful in clarifying the nature of these ambiguous nodules. As a result, most thyroid nodules removed on the suspicion of malignancy do not harbor carcinoma, even in the current era of FNA diagnosis. Enhanced selection of thyroid nodules for surgical removal could certainly occur if diagnostic strategies incorporated detection of genetic markers of malignancy. In the present study, we present data indicating that: (a) *BRAF* mutations are a specific marker for PTC, by far the most common type of thyroid carcinoma; (b) *BRAF* mutations can be detected in cells aspirated from a thyroid nodule; and (c) detection of *BRAF* mutations in FNAs is useful in confirming or establishing the preoperative diagnosis of PTC.

The *BRAF* oncogene is a good candidate marker because it represents the most commonly targeted gene in the development of PTC. Two previous studies reported a mutation rate of 40% and 70% (8, 9). We found that variation in reported prevalence rates is likely related to the composition of the study cases. *BRAF* mutations are common in the conventional form of PTC, but are uncommon in the follicular variant of PTC. This discrepancy may reflect fundamental differences in genetic pathways underlying phenotypic

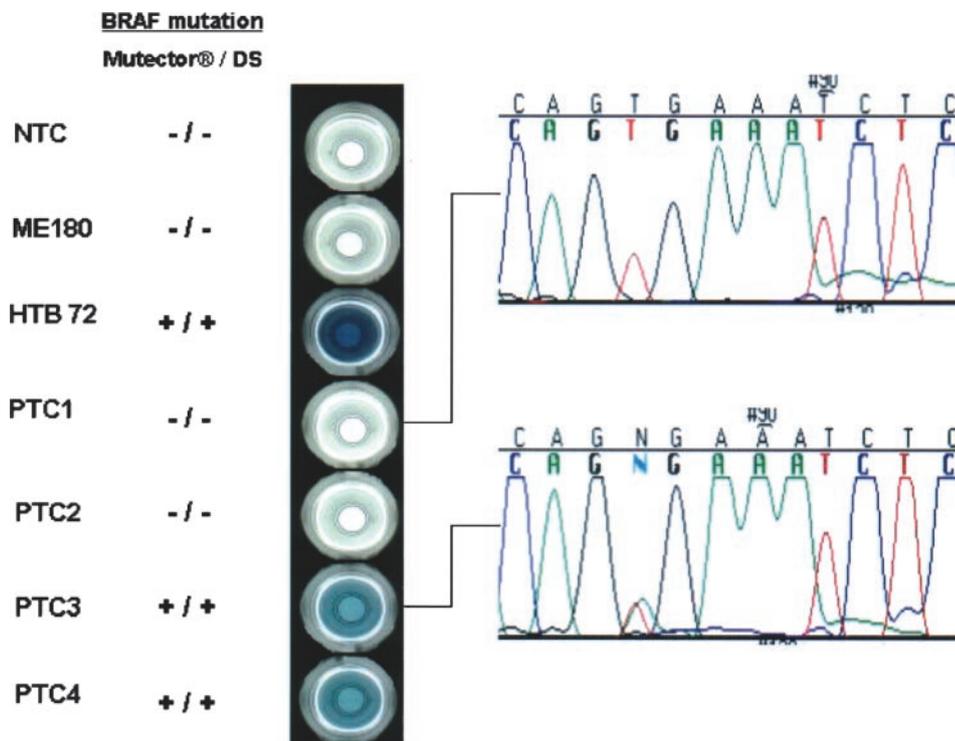


Fig. 3 *BRAF* mutation analysis of four papillary thyroid carcinomas (PTCs 1–4). Using the Mutector assay, the presence of a *BRAF* mutation is indicated by a colorimetric change from clear to green (PTC 3 and PTC 4). In the absence of a mutation, a color signal is not visible (PTC 1 and PTC 2). In all 4 tumors, the Mutector assay result was confirmed by direct sequencing (DS). Examples of direct sequencing are shown for PTC 1 (wild-type) and PTC 3 (mutated). The controls used include the ME 180 cervical cell line (wild-type for *BRAF*), the HTB 72 melanoma cell line (homozygous T → A transversion at *BRAF* T1796), and a water sample without a DNA template (NTC, no template control).

variation. The follicular variants of PTC harbor ras mutations at a frequency much higher than noted in conventional PTC, additionally indicating that these two tumor subtypes are genetically distinct (14).

Not only are *BRAF* mutations common in conventional PTC, but they are highly specific for PTC. A *BRAF* mutation was not detected in any of the 32 benign nodules of this study or in any of the 46 benign nodules reported previously (8, 9). Moreover, *BRAF* mutations are not encountered in other types of thyroid gland malignancies (8, 9). Because follicular carcinomas account for a mere 2% of thyroid malignancies by some recent estimates (15), this restricted distribution to PTCs does not offset the value of *BRAF* mutation as a common marker of thyroid malignancy. In some instances, this tumor specificity may even be diagnostically advantageous by clarifying tumor type and origin. In two cases of PTCs harboring *BRAF* mutations, for example, the mutations persisted in areas that were histologically undifferentiated.

BRAF mutational status can be reliably established from cells aspirated from a thyroid nodule. In 46 of 49 (94%) cases where matching samples were evaluated, the *BRAF* status of the resected PTC and the preoperative FNA were identical. The ability to reliably establish *BRAF* status from FNAs suggests a potential application in the preoperative evaluation of thyroid nodules. Traditional morphological assessment of FNA stratifies thyroid nodules as benign, malignant, or indeterminate. On average, the benign and malignant groups are associated with error rates of 5% and 3%, respectively (4, 16); but it is the indeterminate group that poses the most substantial management problem. About 15–20% of indeterminate nodules prove to be malignant (3), a rate that is too high to surgically ignore, yet so low as to offset the value of FNA as a means of eliminating unnecessary thyroid surgery or limiting the extent of thyroid surgery.

Using *BRAF* mutations as a definitive marker of malignancy in FNAs, we confirmed the preoperative diagnosis of PTC in 72% of carcinomas within the malignant group, and established a diagnosis of malignancy in 16% of carcinomas that could not be conclusively diagnosed by FNA alone. The presence of a *BRAF* mutation established the diagnosis of carcinoma even for those nodules that were resected based on the cytologic suspicion of a follicular neoplasm, but was most helpful in establishing the diagnosis of carcinoma when the cytologic findings were suspicious for but not diagnostic of PTC. Given the high prevalence of thyroid nodules and the widespread popularity of FNA, even a modest gain in diagnostic precision would translate into a definite diagnosis for many patients. Preoperative detection of a *BRAF* mutation, regardless of the cytologic findings, would streamline the management of patients with thyroid nodules. It would permit formulation of an unambiguous surgical plan whereas foregoing the need for other less specific diagnostic tests such as repeat FNA, radionuclide thyroid scintigraphy, and intraoperative frozen section evaluation.

Because the activating mutation involves a specific bp substitution (*i.e.*, a thymine-to-adenine transversion) and

consistently targets a specific nucleotide site (*i.e.*, nucleotide position 1796), *BRAF* mutational analysis is amenable to automation and high throughput analysis. Using the newly developed Mutector assay (12), we found that automated *BRAF* analysis is accurate, objective, and rapid. The results are concordant with those obtained by direct sequencing; moreover, the colorimetric assay is easy to interpret, and the entire assay, from aspiration of the thyroid nodule to interpretation of color reaction, can be completed in less than a day. In effect, *BRAF* mutational analysis of FNAs is straightforward and amendable to transfer from the research bench to the diagnostic laboratory. *BRAF* analysis holds great promise as a diagnostic tool to supplement traditional FNA cytology in the selection of thyroid nodules for surgical resection.

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Clin Cancer Res 2004;10:2761-2765.

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