

VE1 Antibody Is Not Highly Specific for the *BRAF* V600E Mutation in Thyroid Cytology Categories With the Exception of Malignant Cases

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ABSTRACT

Objectives: We evaluated the utility of the VE1 antibody that can detect a mutant protein resulting from the *BRAF* V600E mutation as a diagnostic tool for thyroid fine-needle aspiration cytology (FNAC).

Methods: We performed VE1 immunocytochemistry on 202 FNAC specimens from surgically confirmed thyroid nodules. The results were compared with the molecular analyses of the *BRAF* mutation in these specimens matched with their corresponding histology.

Results: Diagnoses of FNAC specimens included benign (9.4%), atypia of undetermined significance/follicular lesion of undetermined significance (11.4%), follicular neoplasm/suspicious for follicular neoplasm (2.0%), suspicious for malignancy (9.4%), and malignancy (65.8%). VE1 immunostaining was positive in 71.3% of FNAC specimens. The overall sensitivity of the VE1 antibody was 88.8%, specificity was 71.2%, positive predictive value was 88.2%, negative predictive value was 72.4%, and diagnostic accuracy was 83.7%.

Conclusions: VE1 immunocytochemistry in thyroid FNAC as a screening test for *BRAF* mutations is highly specific for malignant category cases but can be suboptimal due to its high false-positive rate for the nonmalignant cases.

Fine-needle aspiration cytology (FNAC) is the most reliable and commonly used method for the diagnosis of thyroid nodules. However, in up to 30% of thyroid nodules, FNAC has limited accuracy in distinguishing between benign and malignant follicular lesions.^{1,2}

However, reports have recently described a potential diagnostic alternative for the evaluation of thyroid FNAC through the use of somatic mutation molecular analysis.²⁻⁵ Moreover, as a potential target in thyroid malignancies, B-type Raf kinase (*BRAF*) was explored, being the most common somatic mutation among thyroid cancers, especially papillary thyroid carcinoma (PTC), while it is almost never identified in benign thyroid nodules.⁴⁻⁷ *BRAF* mutations are related to the development of thyroid cancer and have been associated with poorer prognostic factors of thyroid carcinoma.⁸⁻¹¹ Among all *BRAF* alterations detected in thyroid cancers, valine-to-glutamate substitution at codon 600 (V600E) constitutes the vast majority.¹² The *BRAF* V600E mutation has been reported in approximately 45% of PTCs,^{9,13} but with a wide prevalence range (29%-84%) reported among different types of PTCs.^{5,7,9} Detection of the *BRAF* V600E mutation could be of great value in the diagnosis of PTC, especially in those in whom PTC and the *BRAF* V600E are highly prevalent.^{5,7} The detection of the *BRAF* V600E mutation has been performed using a variety of DNA-based methods, such as mutation-specific polymerase chain reaction (PCR), pyrosequencing, and direct sequencing.^{14,15}

Recently, a mouse monoclonal antibody (VE1), which can detect a mutant protein resulting from the *BRAF* V600E mutation, has been developed, and some studies suggested that diffuse and/or strong immunopositivity for VE1

was correlated strongly with the *BRAF* V600E mutation in PTC.¹⁶⁻¹⁸ However, these results were obtained using surgically resected thyroid specimens. Even though these results indicated that VE1 was a promising candidate for somatic mutation analysis, the diagnostic application of VE1 immunocytochemical staining in thyroid FNAC is not well established.¹⁹

In this study, we investigated immunocytochemical expression using the VE1 antibody in 202 cases of histologically confirmed FNAC, ranging from benign to malignant thyroid nodules, and evaluated the usefulness of this antibody as a screening tool for detection of the *BRAF* V600E mutation in thyroid FNAC specimens.

Materials and Methods

Case Selection

In this study, we screened 3,013 cases with thyroid FNAC slides from the Ajou University Hospital from July through December 2012, which were processed using the liquid-based cytology method BD SurePath (TriPath, Burlington, NC). Among this set, we retrospectively analyzed 202 FNAC thyroid nodule samples from patients who underwent thyroidectomy and whose pathology specimens were available for review and ancillary tests. Approval to link laboratory data with clinical and pathologic data was obtained from the Institutional Review Board of the Ajou University Hospital. All cases of FNAC were classified into diagnostic categories using the Bethesda System for Reporting Thyroid Cytopathology. All cytologic and histologic slides were reviewed independently by two experienced pathologists (S.-R.L. and J.-H.K.).

VE1 Immunocytochemical Staining

Prior to immunostaining, all liquid-based thyroid FNAC slides collected for the study were immersed in xylene overnight to remove the coverslips and for mounting. Slides were then dehydrated and destained for 10 minutes each using successive solutions of 99%, 95%, and 70% ethyl alcohol; 5% HCl in 70% ethyl alcohol; and 95% ethyl alcohol. Immunostaining was performed using the Ultravision LP Detection System (Thermo Fisher Scientific, Fremont, CA) according to the manufacturer's protocol. Briefly, the endogenous peroxidase activity was blocked using 0.3% hydrogen peroxidase in distilled water and washed with Tris-buffered saline (TBS), pH 7.4, for 5 minutes. Slides were treated with Ultra V Block (Thermo Fisher Scientific) for 5 minutes and incubated at room temperature for 1 hour with the anti-*BRAF* V600E mouse monoclonal antibody VE1 (1:50; Spring Bioscience, Pleasanton, CA). Slides were washed with TBS and

incubated with Primary Antibody Enhancer (Thermo Fisher Scientific) for 15 minutes. Slides were washed with TBS and incubated with HRP Polymer (Thermo Fisher Scientific) for 20 minutes without light exposure. The reaction products were developed with the Vector NovaRED Substrate Kit for peroxidase (Vector Laboratories, Burlingame, CA) for 5 minutes. Slides were counterstained with hematoxylin, dehydrated, and mounted.

Two pathologists (S.-R.L. and J.-H.K.) who were blinded to the molecular results independently assessed the immunocytochemical staining results. Any differences in interpretation were resolved by consensus. According to the cytoplasmic staining of follicular cells, the intensity of VE1 immunostaining was graded from 0 to 3, with 0 as negative staining, grade 1 as weak, grade 2 as moderate, and grade 3 as strong cytoplasmic staining (Image 1). The results were considered positive for VE1 immunostaining only if the cells displayed grade 2 or 3 cytoplasmic staining, regardless of the extent of staining. Thus, the presence of any follicular cells, even a single cell, with greater than moderate cytoplasmic immunostaining was interpreted as positive.

Detection of the *BRAF* V600E Mutation

For genomic DNA isolation, one representative formalin-fixed, paraffin-embedded tissue block of surgical specimens corresponding to thyroid FNAC cytology was selected and cut at 10- μ m thickness. Genomic DNA was extracted from the manually microdissected tumor area of each tissue section using the QIAamp DNA FFPE Tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. To detect the *BRAF* V600E mutation, we performed PCR–restriction fragment length polymorphism (RFLP) analysis as described previously.¹⁴ Mismatched PCR primers were designed to create a restriction site for *Xba*I. The forward primer was 5'-TAA AAA TAG GTG ATT TTG GTC TAG CTC TAG CTC TAG-3', and the reverse primer was 5'-ACT ATG AAA ATA CTA TAG TTG AGA-3'. PCR was performed at 95°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds, followed by 70°C for 10 minutes. After purification, the PCR products were digested with *Xba*I for 90 minutes, electrophoresed, and stained with ethidium bromide. The presence of the *BRAF* V600E mutation was confirmed when PCR-RFLP showed two distinct bands visible with UV transillumination. In the case of negative or equivocal RFLP results for *BRAF* V600E mutation, we also performed mutant enrichment with 3'-modified oligonucleotides (MEMO) sequencing to confirm the presence or absence of the *BRAF* V600E mutation using primers and PCR conditions described previously.²⁰ The results were analyzed using Sequencher 4.10 software (Gene Codes, Ann Arbor, MI).

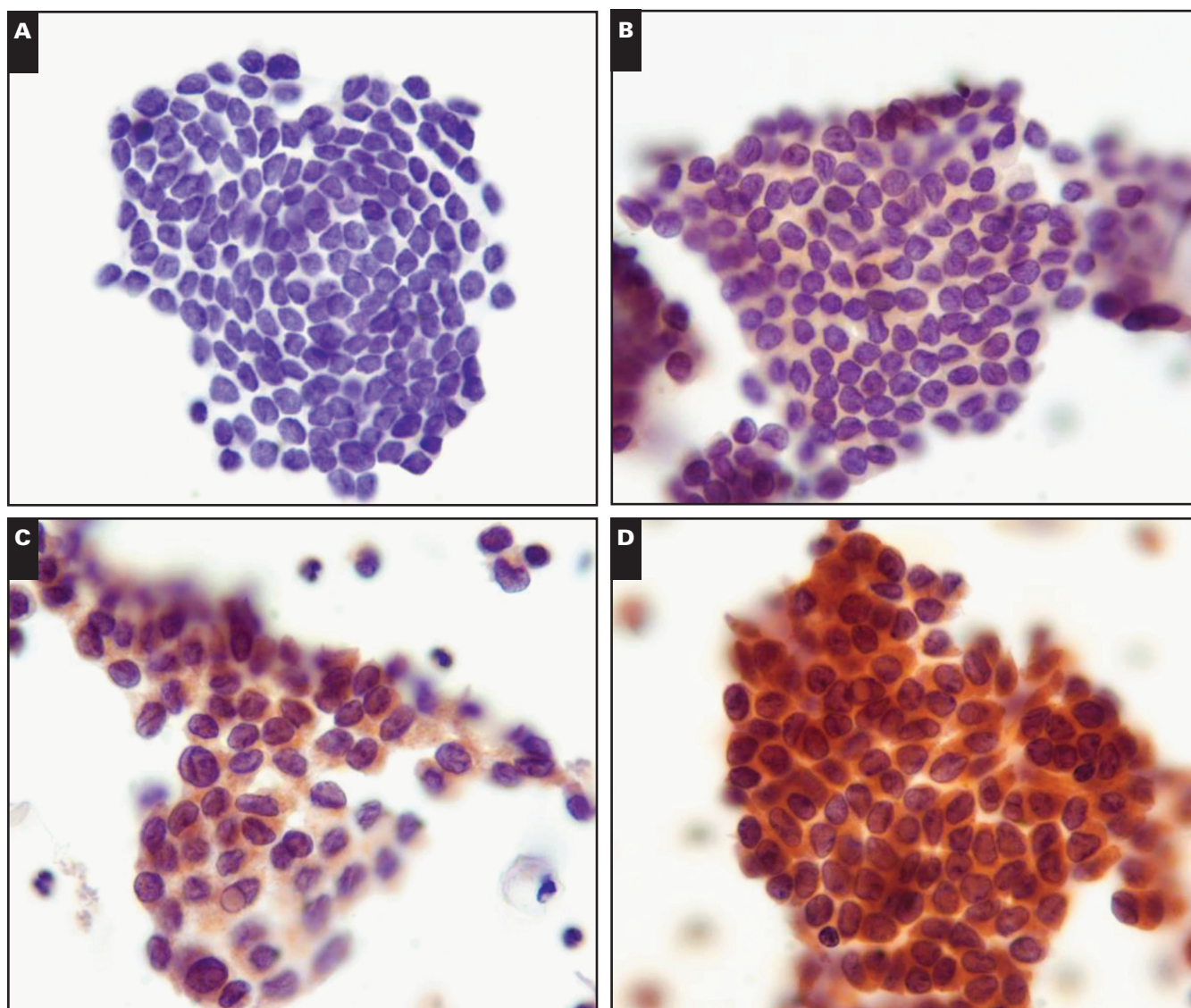


Image 1 Examples of VE1 immunostaining grades in liquid-based thyroid fine-needle aspiration cytology specimens (×400). Grade 0, no staining (**A**); grade 1, weak staining (**B**); grade 2, moderate staining (**C**); and grade 3, strong staining in the cytoplasm of follicular cells (**D**).

A true positive was defined as a case in which the *BRAF* V600E mutation was confirmed by any of the molecular methods used in a corresponding lesion of the surgical specimen. A true negative was defined as a case in which the absence of the *BRAF* V600E mutation was confirmed by MEMO sequencing.

Results

FNAC and Final Pathology

The FNAC diagnoses were benign in 19 (9.4%) cases, atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS) in 23 (11.4%)

cases, follicular neoplasm/suspicious for follicular neoplasm in 4 (2.0%) cases, suspicious for PTC in 19 (9.4%) cases, and definitive PTC in 133 (65.8%) cases. Four (2.0%) cases were nondiagnostic smears because of insufficient cellularity.

The FNAC diagnosis and final pathology are summarized in **Table 1**. The clinical indications for total thyroidectomy or lobectomy of benign lesions included an associated finding identified in the patients with PTC as a separate lesion ($n = 15$), accompanying parathyroid lesion ($n = 2$), and large size of the mass ($n = 2$).

VE1 Immunocytochemical Staining

Among the 202 cases of thyroid FNAC, 144 (71.3%) were positive for VE1 immunostaining; 36 cases of grade

Table 1
Preoperative Cytologic Categories and Corresponding Pathologies

Cytologic Category	Final Pathology (No.)	Total, No. (%)
I. Nondiagnostic or unsatisfactory	Adenomatous hyperplasia (1) Papillary carcinoma (3)	4 (2.0)
II. Benign	Adenomatous hyperplasia (18) Follicular adenoma (1)	19 (9.4)
III. AUS/FLUS	Adenomatous hyperplasia (8) Follicular/Hürthle cell adenoma (4) Follicular carcinoma (3) Papillary carcinoma (8)	23 (11.4)
IV. Follicular neoplasm/suspicious for follicular neoplasm	Adenomatous hyperplasia (1) Hürthle cell adenoma (3)	4 (2.0)
V. Suspicious for malignancy	Papillary carcinoma (19)	19 (9.4)
VI. Malignant	Papillary carcinoma (133)	133 (65.8)
Total		202 (100)

AUS, atypia of undetermined significance; FLUS, follicular lesion of undetermined significance.

0, 22 cases of grade 1, 83 cases of grade 2, and 61 cases of grade 3. The positive cases were diagnosed as benign (8 cases, 5.6%), AUS/FLUS (10 cases, 6.9%), follicular neoplasm/suspicious for follicular neoplasm (2 cases, 1.4%), suspicious for PTC (11 cases, 7.6%), and definitive PTC (113 cases, 78.5%). All positive cases with benign cytology were related to benign follicular nodules containing colloid. Occasionally, colloids and nonfollicular cells, such as stromal cells, macrophages, or giant cells, showed moderate or strong immunostaining **Image 2**. All positive cases having cytologic features suspicious for a follicular neoplasm were follicular adenomas of the Hürthle cell type.

Molecular Analysis of BRAF V600E

The *BRAF* V600E mutation was detected in 143 (70.8%) of 202 lesions in the surgical specimens corresponding to thyroid FNAC. All *BRAF* V600E mutant tumors were PTCs **Table 2**. Neither nodular hyperplasia nor follicular neoplasms showed the *BRAF* V600E mutation. In PTCs, the prevalence of the *BRAF* V600E mutation was 87.7%.

Correlation Between Immunocytochemical Staining and Molecular Analysis for the BRAF V600E Mutation

Among the 143 cases with the *BRAF* V600E mutation identified by molecular analysis, 127 cases were positive for VE1 immunohistochemical staining. There were 16 false-negative cases of VE1 staining **Table 3**. The 17 cases that were false positive for immunostaining included benign cytology in eight cases, AUS/FLUS in seven cases, and follicular neoplasm/suspicious for follicular neoplasm in two cases **Table 4**. None of the false-positive cases showed strong staining. The overall sensitivity of the VE1 antibody

was 88.8%, specificity was 71.2%, positive predictive value was 88.2%, negative predictive value was 72.4%, and diagnostic accuracy was 83.7%.

In the indeterminate cytologies, including categories III and V, the sensitivity of the VE1 antibody was 82.3%, specificity was 72.0%, positive predictive value was 66.7%, negative predictive value was 85.7%, and diagnostic accuracy was 76.2%.

In cytologic category VI, the sensitivity of the VE1 antibody was 89.7%, specificity was 100.0%, positive predictive value was 100.0%, negative predictive value was 35.0%, and diagnostic accuracy was 90.2%.

According to the final pathology, false-positive cases included nodular hyperplasia in eight cases, follicular adenoma in six cases, minimally invasive follicular carcinoma in one case, and PTC in two cases. The two cases of PTC that were false positive for VE1 staining had been preoperatively diagnosed as cytologic category III (AUS/FLUS).

Discussion

The *BRAF* V600E mutation, the most common genetic alteration in patients with PTC, has been studied intensively because its detection in thyroid specimens could help the diagnostic confirmation of PTC and may predict a more aggressive disease course.^{4,5,9,13} To improve detection sensitivity for the *BRAF* V600E mutation, various methods, mostly DNA based, have been developed, and thyroid FNAC combined with these methods showed a diagnostic value for PTC.^{5,14,15} All of these molecular methods, however, have limitations, especially in thyroid FNAC specimens with scant cellularity or with abundant nonneoplastic cells. Moreover, these methods require expensive equipment and techniques with rigorous quality control.^{18,19}

In pathologic practice, immunocytochemical methods for detecting the *BRAF* V600E mutation in thyroid FNAC have several advantages over molecular analysis. Immunohistochemistry is a less expensive and less laborious method than molecular testing. Moreover, it is a widely used routine test that has a short turnaround time in general pathology laboratories.^{16,18,21} Recently, a novel monoclonal antibody, VE1, directed against the BRAF mutant protein has been developed,¹⁶ and some studies showed a high sensitivity and specificity for its detection of the *BRAF* V600E mutation in PTCs.^{18,21} All of these studies, however, investigated the effectiveness of VE1 immunohistochemical analysis in surgically resected thyroid tissues. Studies on the diagnostic application of VE1 immunocytochemical staining in thyroid FNAC are very few and limited.^{19,22} Zimmermann et al¹⁹ demonstrated the diagnostic applicability of VE1 using cell blocks of thyroid FNAC from 55 patients with

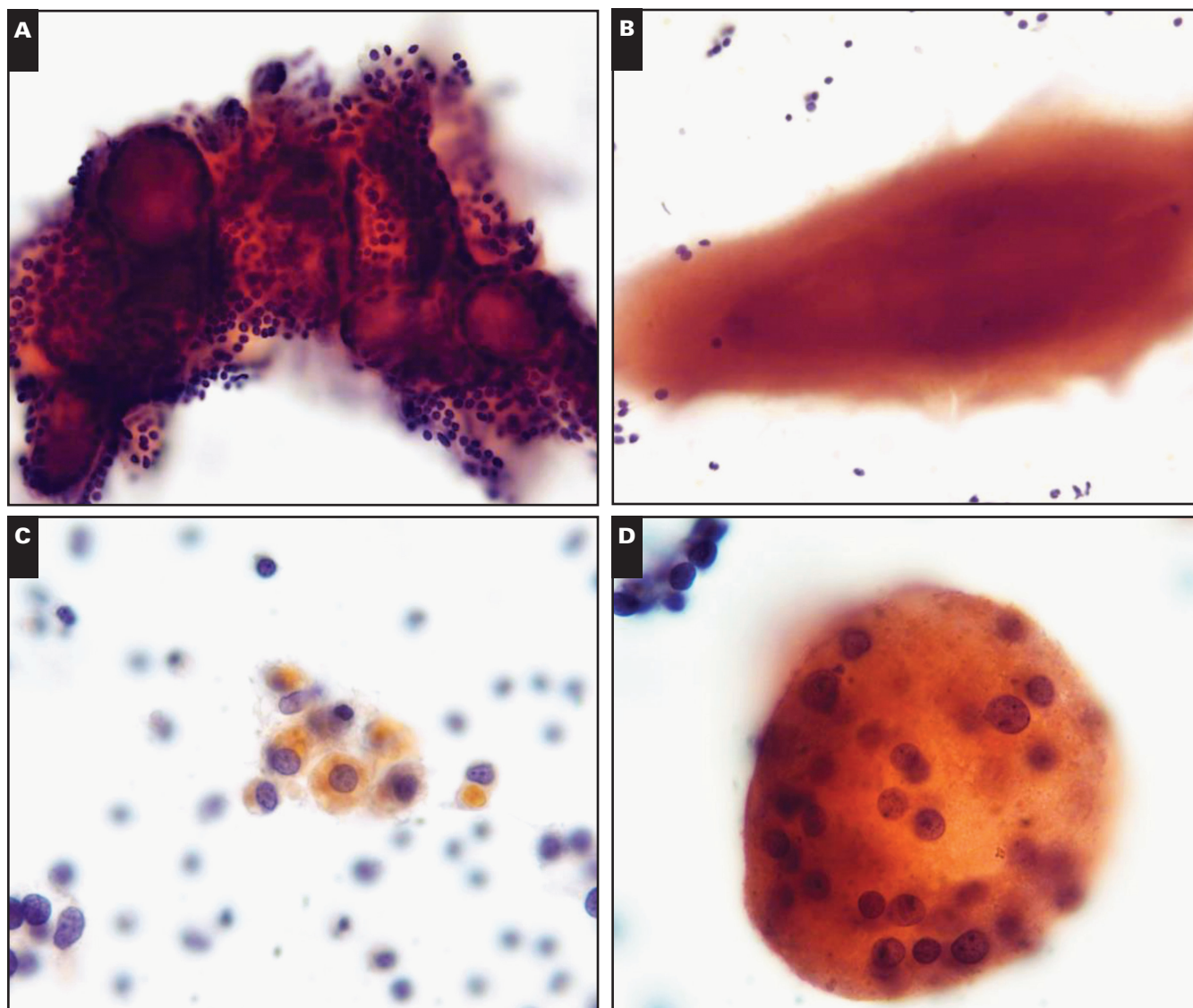


Image 2 Immunoreexpression of VE1 for benign cytologic category cases. Thick clusters of follicular cells (×200) (A), colloid (×200) (B), macrophages (×400) (C), and multinucleated giant cells (×400) (D).

PTCs. Recently, Rossi et al²² evaluated VE1 as an immunomarker using liquid-based thyroid FNAC compared with corresponding final histologic specimens from 55 patients with PTCs. Although these studies showed a relatively high sensitivity and specificity for VE1 in thyroid FNAC, these results were very limited because they focused on specimens only from patients with a definitive diagnosis of PTC and did not include those from patients diagnosed by other cytology categories.

In the present study, we performed immunostaining with VE1 on 202 liquid-based thyroid FNAC slides, including benign and malignant categories. The overall sensitivity and specificity of the VE1 antibody in our study were 88.8% and 71.2%, respectively. Our results were lower compared with those of previous studies using surgically resected thyroid specimens.^{18,21} These differences might be related to

several factors. First, the overall sensitivity and specificity of thyroid FNAC are lower than corresponding immunohistochemical methods, because thyroid FNAC could not represent all of the same features.^{1,2,19,23} Recent studies evaluating VE1 staining using thyroid FNAC and comparing with the corresponding histology showed similar results to those of our study.^{19,22} The detection method used for the *BRAF* V600E mutation in the present study may have influenced our results. In the equivocal and negative cases for *BRAF* V600E mutation detection by the RFLP method, to confirm the results, we also performed MEMO sequencing, which is more sensitive than direct sequencing.⁵ Second, the suboptimal conditions of the FNAC slides in the present study may have influenced the immunohistochemical staining of VE1. Immunodetection by VE1 can be affected by various factors, such as the type and condition of the

Table 2
BRAF V600E Mutation According to Final Pathology

Final Pathology	Total, No. (%)	BRAF V600E Mutation, No.
Adenomatous hyperplasia	28 (13.9)	0
Follicular/Hürthle cell adenoma	8 (4.0)	0
Follicular carcinoma	3 (1.5)	0
Papillary carcinoma	163 (80.7)	143
Total	202 (100)	143

Table 3
Comparisons of VE1 Immunostaining and BRAF V600E Mutation Status

VE1 Immunostaining	BRAF V600E Mutation		Total, No. (%)
	Positive	Negative	
Positive	127	17	144 (71.3)
Negative	16	42	58 (28.7)
Total, No. (%)	143 (70.8)	59 (29.2)	202 (100.0)

Table 4
Comparisons of VE1 Immunostaining and BRAF V600E Mutation Status According to the Cytologic Category

Cytologic Category/ VE1 Immunostaining	BRAF V600E Mutation, No.		Total, No. (%)
	Positive	Negative	
I. Nondiagnostic or unsatisfactory			
Positive	0	0	0 (0.0)
Negative	0	4	4 (2.0)
II. Benign			
Positive	0	8	8 (4.0)
Negative	0	11	11 (5.4)
III. AUS/FLUS			
Positive	3	7	10 (5.0)
Negative	1	12	13 (6.4)
IV. Follicular neoplasm/suspicious for follicular neoplasm			
Positive	0	2	2 (1.0)
Negative	0	2	2 (1.0)
V. Suspicious for malignancy			
Positive	11	0	11 (5.4)
Negative	2	6	8 (4.0)
VI. Malignant			
Positive	113	0	113 (55.9)
Negative	13	7	20 (9.9)
Total, No. (%)	143 (70.8)	59 (29.2)	202 (100.0)

AUS, atypia of undetermined significance; FLUS, follicular lesion of undetermined significance.

specimen and immunohistochemical method.^{16,19,21} Because of insufficient liquid-based cytology material stocks, we had to perform VE1 immunostaining on archival liquid-based thyroid FNAC slides after destaining. Nonetheless, our data in the cytologic malignant category were similar to or even better than those of the recent study performed by Rossi et al²² evaluating thyroid FNAC, which used high-quality unstained liquid-based cytology slides. Third, we have investigated thyroid FNAC specimens, including those of the malignant category, as well as others. Although all *BRAF*

V600E mutations of the surgically resected thyroid specimens were identified in PTCs, positive immunostaining for VE1 was found in all categories of thyroid cytology, except for the nondiagnostic and unsatisfactory categories.

In the present study, we found high specificity of VE1 immunostaining in the malignant and suspicious for malignancy cytologic categories. Therefore, in those cytologic categories, a negative result of VE1 immunostaining might help to save time and money, restricting the molecular test to antibody-positive cases only. The other categories, however, showed lower sensitivity and specificity than those of previous reports.^{18,19,21} We found 17 cases of thyroid FNAC with false-positive immunoexpression by VE1. All of these cases showed moderate intensity to VE1. Among these cases, 13, including eight benign category and five AUS/FLUS category cases, showed focal or multifocal immunoexpression in small follicles, including colloid, or in thick clusters of follicular cells with fibrous stroma. These types of immunoreaction were not unique to thyroid FNAC specimens but have been reported in previous studies using paraffin-embedded, formalin-fixed thyroid tissue.^{18,19,21} We also performed VE1 immunohistochemistry using the corresponding histologic specimens of false-positive cases and found a similar immunostaining pattern in some of the corresponding lesions. To rule out the possibility of false-positive VE1 antibody signals related to the detection system, we also tested some of these cases using a different detection system, the OptiView DAB Kit (Ventana Medical Systems, Tucson, AZ), together with a BenchMark XT automated immunohistochemistry stainer (Ventana Medical Systems), and similar findings were also identified (data not shown). Therefore, careful interpretation is needed when immunoreaction is detected in follicular-patterned lesions, which contain colloid or fibrous stroma.¹⁸ The remaining four cases, including two AUS/FLUS category and two follicular neoplasm or suspicious follicular neoplasm category cases, showed moderate cytoplasmic immunostaining, and the final pathology of all four cases was Hürthle cell (oncocyctic) adenoma. In thyroid neoplasms with oncocyctic and/or tall-cell features, VE1 immunoexpression has been detected more commonly than in other types of thyroid neoplasms.¹⁸ However, VE1 immunopositivity in oncocyctic thyroid neoplasms may be false, especially in Hürthle cell (oncocyctic) adenoma, as in our results.¹⁹ Another serious issue in interpreting VE1 immunostaining was the background immunostaining, which made discrimination between grades 1 and 2 VE1 staining difficult. We also performed a pilot test using 30 high-quality unstained liquid-based cytology slides, but this issue was also detected in approximately one-third of the cases (data not shown). These findings may create clinical issues related to the risk of false-positive interpretation.²² Recently, Kim et al²⁴ proposed a scoring system according to the proportion and intensity of the VE1-positive cells in

surgical specimens, using an algorithmic approach according to the score, which was able to reduce the false-positive and false-negative results from the molecular tests. However, the application of their algorithm to thyroid FNAC should be validated in further studies.

This study had several limitations, mostly related to its retrospective design, lack of a direct correlation between VE1 immunostaining and the *BRAF* V600E mutation in liquid-based cytology materials, the suboptimal liquid-based slides for immunocytochemistry, and the small proportion of indeterminate category cases. Therefore, further prospective studies on thyroid FNAC are needed to establish a diagnostic value for VE1 immunostaining in pathologic practice. Nonetheless, the high false-positive rate of VE1 immunocytochemical staining in benign and indeterminate cytologic categories and the low overall specificity in the present study cannot be ignored, since *BRAF* V600E mutation analysis is often used to aid the diagnosis of cytologically equivocal/indeterminate cases in clinical practice.

In conclusion, although VE1 immunocytochemical staining is highly predictive of the presence of the *BRAF* V600E mutation in liquid-based thyroid FNAC cases of the malignant cytologic category, it can be suboptimal as a routine screening tool for the *BRAF* V600E mutation because of the high false-positive rate in nonmalignant cytologic categories.

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