Uncommon *BRAF* Mutations in the Follicular Variant of Thyroid Papillary Carcinoma: New Insights

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BACKGROUND: Mutational analysis is reshaping the practice of fine-needle aspiration cytology for the diagnosis of thyroid nodules. The v-Raf murine sarcoma viral oncogene homolog B1 (*BRAF*) valine (V) to glutamic acid (E) substitution at codon 600 ($BRAF^{V600E}$) is the most effective diagnostic/prognostic marker and is used mainly for papillary thyroid carcinomas (PTCs). Although $BRAF^{V600E}$ represents 95% of all *BRAF* mutations, uncommon *BRAF* mutations have been identified in thyroid carcinomas. For the current study, the authors evaluated morphologic (plump pink cells and sickle-shaped nuclei) anti-$BRAF^{V600E}$ antibody (VE1) immunocytochemical and molecular findings of *BRAF* mutations in PTCs and in the follicular variant of PTC (FVPC). **METHODS:** Between January 2013 and June 2014, there were 150 cytologic samples with surgical follow-up at the authors' institution. *BRAF* mutations, which were identified using liquid-based cytology, were classified into wild-type *BRAF*, $BRAF^{V600E}$, and uncommon *BRAF* mutations. All clinicopathologic correlations between *BRAF* and FVPCs were analyzed. **RESULTS:** Forty-four of 150 samples were identified as benign histologic lesions, and the authors focused on the 106 cytologic samples from patients who had malignant outcomes (60 PTCs and 46 FVPCs). The series included 16 follicular neoplasms, 36 samples diagnosed as suspicious of malignancy, and 54 samples diagnosed as positive for malignancy. The $BRAF^{V600E}$ mutation was detected in 17.4% of FVPCs and in 66.6% of PTCs, whereas uncommon *BRAF* mutations were detected only in FVPCs. Plump pink cells and VE1 expression were not identified in samples that had uncommon *BRAF* mutations. VE1 immunocytochemistry yielded positive results in all 36 samples that had the $BRAF^{V600E}$ mutation. **CONCLUSIONS:** Uncommon *BRAF* mutations were observed only in FVPCs and were linked to less aggressive behavior. Negative/weak VE1 expression was observed in both wild-type and uncommon *BRAF* mutations. The current investigation did not reveal any plump cells or morphologic *BRAF* findings in samples that had uncommon *BRAF* mutations. In the authors' experience, *BRAF* mutations detected by DNA methods were more accurate in identifying FVPCs. ***Cancer (Cancer Cytopathol)* 2015;123:593-602**. © *2015 American Cancer Society.*

KEY WORDS: *BRAF* mutations; follicular neoplasms; follicular variant of papillary thyroid carcinoma; liquid-based cytology; papillary thyroid carcinoma.

INTRODUCTION

Fine-needle aspiration (FNA) cytology (FNAC) of thyroid nodules represents an invaluable diagnostic tool that leads to a correct diagnosis in greater than 70% of all thyroid lesions.[1,2] The remaining 30% of lesions are categorized into the indeterminate category of *follicular neoplasm* (FN), which represents the most challenging category for achieving a definitive and correct diagnosis.[1,2] In recent years, although several authors have

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encouraged the application of ancillary techniques (immunocytochemistry [ICC] and molecular testing) to refine diagnoses in the FN category, their questionable utility is still being debated.[3–14]

Because ICC, carried out either as a single test or as a panel, does not reach 100% diagnostic accuracy and specificity, the aid of molecular testing has been strongly recommended. In this perspective, mutations in v-Raf murine sarcoma viral oncogene homolog B1 (*BRAF*) represent a well known feasible marker in thyroid carcinoma, accounting for 45% to 70% of the prevalence mainly in the classic variant of papillary thyroid carcinoma (PTC).[3–5] Therefore, the *BRAF* hotspot mutation, with a 95% prevalence mainly in PTC, is recognized as an activating somatic mutation on exon 15 of the B isoform of the RAF-kinase gene and is frequently related to more aggressive behavior involving lymph nodes and/or distant metastases as well as extrathyroid infiltration.[4,9–13]

A new perspective on morphologic detection of the *BRAF* valine (V) to glutamic acid (E) substitution at codon 600 (the $BRAF^{V600E}$ mutation) through specific findings (ie, plump pink cells and sickle-shaped nuclei) has recently gained popularity as a foreseeable diagnostic and predictive sign of mutation also on FNAC, regardless of the classification systems or categories used.[14,15] Furthermore, a new monoclonal antibody directed against the mutated $BRAF^{V600E}$ protein (clone VE1) has demonstrated high diagnostic accuracy in detecting this mutation among several malignant neoplasms, including PTC.[16]

In contrast to the highly prevalent $BRAF^{V600E}$ mutation in PTC, as largely underlined in the literature, the follicular variant of PTC (FVPC), which is frequently diagnosed as FN on FNAC, has an expression rate that varies between 0% and 35%, with an average of 15% in different studies.[8,17] Not only does this latter histotype exhibit a mixed pattern, including PTC nuclei and follicular growth without papillae patterned as diffuse or encapsulated variants, but the molecular profile appears to be somewhere in between the profiles of PTC and follicular carcinoma (FTC). Detection of the *BRAF* lysine (K) to glutamic acid (E) substitution at codon 601 ($BRAF^{K601E}$) mutation and of the subset of complex and less common *BRAF* mutations has been documented in FVPCs and has been correlated with less tumorigenic potential than that of the $BRAF^{V600E}$ mutation, as described in the limited literature.[8,17–27] In this report, we retrospectively focus on morphologic and molecular findings in 106 cytologic thyroid lesions that were diagnosed as PTC and FVPC on histology.

MATERIALS AND METHODS

During the period between January 2013 and June 2014, 6578 thyroid FNACs (including 80 nondiagnostic samples, 5918 benign lesions, 300 FNs, 100 samples that were suspicious for malignancy [SM], and 180 samples that were positive for malignancy [PM]) were collected in the Division of Anatomic Pathology and Histology of the Catholic University, "Agostino Gemelli" Hospital (Rome, Italy). Among them, all samples categorized as FN, SM, and PM (including 580 samples) were evaluated for molecular *BRAF* testing on FNAC. In total, 150 thyroid nodules had a histologic follow-up diagnosis of benign histologic lesion (goiter; n = 44), 60 were diagnosed as PTCs (including 3 that were diagnosed as the tall cell variant [TCV] of PTC), and 46 were diagnosed as FVPC. Because the 44 benign histologic samples were categorized as wild-type *BRAF*, the data discussed here include only the 106 cytologic samples that had malignant histologic outcomes.

All FNAC procedures were carried out under sonographic guidance (ultrasound [US]), mostly by surgeons and endocrinologists, and were processed with the liquid-based cytology (LBC) method (ThinPrep 5000; Hologic Co, Marlborough, Mass). Our patients were studied with US during a thyroid check-up performed in the Center for Thyroid Diseases at our hospital. The series included 42 men and 64 women, and their median age was 49 years (age range, 19-73 years). All aspirations (usually 2 passes for each lesion) were performed with 25-gauge to 27-gauge needles, and no rapid on-site assessment of the adequacy of the material was done. All patients had been appropriately informed regarding use of the LBC method for processing their samples, and a written informed consent was signed. Our study followed the tenants of the Declaration of Helsinki, and we received the internal ethical approval for the study.

The technical steps for liquid-based preparation have been clearly described in some previous articles by our group.[9,10] The resulting slides were fixed in 95% ethanol and stained with Papanicolaou, and the remaining material was stored in PreservCyt solution (Hologic Co) to be used for the preparation of additional slides for

further investigations (including both ICC and molecular analyses). The lower limit for the adequacy of each sample was established according to the British Royal College of Pathologists' classification into 6 groups of thyroid epithelial cells within the submitted slides, each of which had at least 10 well observed epithelial cells.[28]

Our cytologic samples were all classified according to the morphologic criteria adopted by the Italian Consensus Working Group (Italian Society for Anatomic Pathology and Cytology-International Academy of Pathology [SIAPEC-IAP]) classification, which is similar to the Bethesda System for thyroid cytology.[1,2,29,30] The above-mentioned categories are defined as follows: thyroid 1 (TIR1, inadequate or hemorrhagic; TIR2, nonneoplastic lesion; TIR3, follicular lesion/suspected FN, also including atypical lesions with undetermined significance (FN/AUS); TIR4, SM; and TIR5, PM. Our global cytologic series included the following distribution of diagnoses for the reference years: 1.2% TIR1 (nondiagnostic), 90% TIR2 (nonneoplastic), 4.6% TIR3 (indeterminate), 1.5% TIR4 (suspicious), and 2.7% TIR5 (malignant). All cytologic and histologic sections were reviewed by 2 expert pathologists (Esther Diana Rossi and Guido Fadda), and samples for which the interpretation was equivocal were submitted to the diagnostic judgment of the other pathologists until a final agreement was achieved. The percentage of disease-specific cells for molecular analysis was $\geq$50% in all the LBC samples but 30% for the ICC VE1 evaluation. Our morphologic evaluation of the $BRAF^{V600E}$ mutation was characterized by evidence of cells with eosinophilic, large cytoplasm and nuclear features of PTC (plump cells) and the additional finding of sickle-shaped nuclei, as previously reported.[15]

Histology

All surgical specimens were fixed in 10% buffered formaldehyde, embedded in paraffin, and the 5-micron-thick microtomic sections were stained with hematoxylin and eosin. All perithyroid adipose tissue was embedded and examined for lymph node research. The diagnosis of PTC was based on the presence of true papillary structures and distinctive nuclear features; whereas the diagnosis of FVPC relied on the detection of the nuclear features of PTC in multiple foci within the tumor, including both diffuse and encapsulated variants.[1,2] The diagnosis of TCV was characterized by the predominance of neoplastic cells with a height at least 3 times their width and the presence of classic PTC nuclear features. All samples were classified according to the seventh edition of the tumor-lymph lymph node-metastasis (TNM)-based staging system recommended by the American Joint Commission on Cancer.[31]

Molecular Analysis

DNA was extracted from LBC samples stored in PreservCyt solution (Hologic Co) and from paraffin-embedded tissues, as described in several of our previous articles.[9,10,15,16] LBC samples were centrifuged, the supernatant was discarded, and the cellular pellet was processed. The pellet was incubated at 56°C for 3 hours in 180 μL ATL lysis buffer and 20 μL Proteinase K (20 mg/mL) from the QIAamp DNA mini-kit (QIAGEN, Hilden, Germany). For histologic samples, 10 mm of slide tissue was deparaffinized and, after ethanol treatment, was incubated at 56°C overnight in 180 μL ATL lysis buffer and 20 μL Proteinase K (20 mg/mL) from the QIAamp DNA mini kit (QIAGEN). DNA was extracted according to the manufacturer's protocol, and we used spectrophotometry to assess the quantity and quality of the DNA (A260; A260/280 ratio; spectrum, 220-320 nm; Biochrom, Cambridge, UK) and by separation on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, Calif). Low-purity or insufficient DNA samples were extracted a second time. BRAF genes (codons 464, 466, 469, and 599) were amplified using the same primers and polymerase chain reaction (PCR) conditions previously described.[32] Briefly, DNA (100-200 ng) was amplified in a mixture containing 1 × PCR buffer (20 mM Tris, pH 8.3; 50 mMKC_l; 1.5 mM $MgCl_2$), deoxyribonucleotide triphosphates (200 mM each), primers (20 pM each), and 0.5 U GoTaq (Promega, Milan, Italy) in a final volume of 25 μL. PCR conditions were as follows: initial denaturation at 95°C for 8 minutes followed by 35 cycles at 95°C for 40 seconds, 55°C for 40 seconds, and 72°C for 40 seconds. After visualization onto agarose gels, PCR products were treated with ExoSAP-IT (USB Corp, Cleveland, Ohio) following the manufacturer's protocol, amplified with the BigDye Terminator cycle-sequencing kit (version 3.1; Applied Biosystems; Waltham, Mass) using forward and reverse primers, and sequenced with an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Water was used as a negative control. The sensitivity of this method is 15% in our laboratory.[33] The percentage of disease-specific cells for molecular analysis was at least

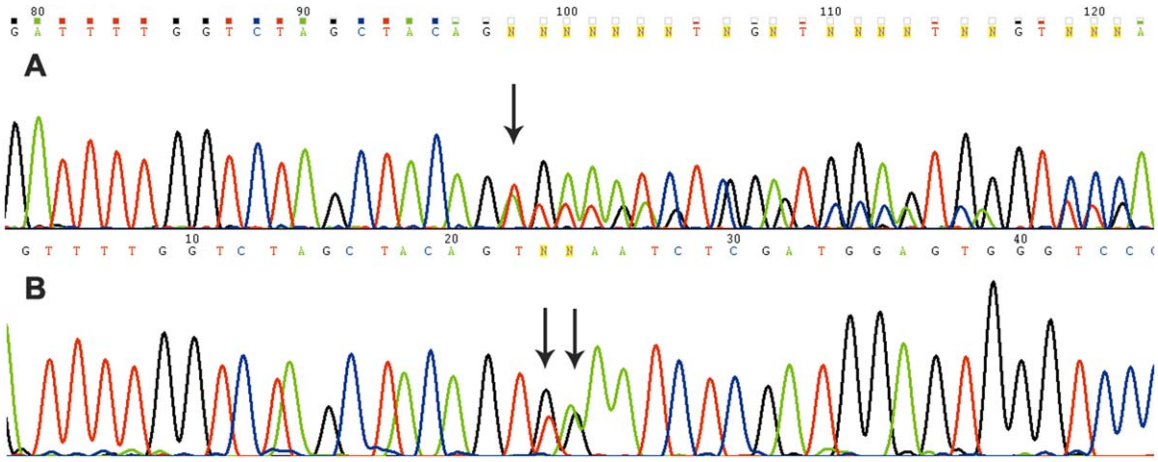

Figure 1. (A) The v-Raf murine sarcoma viral oncogene homolog B1 (*BRAF*) c.1799_1811>ATTT sequence is illustrated. (B) The *BRAF* lysine to glutamic acid mutation at codon 6001 (K6001E) is illustrated (arrows indicate the mutations).

50% in all LBC samples. *BRAF* mutational analyses also were performed on DNA extracted from surgical specimens that contained at least 70% of the tumor. The concordance of mutational analyses between the surgical and LBC samples was 100%. All *BRAF* mutations were mutually exclusive.

ICC and Immunohistochemistry

Retrospectively, of the total 106 samples, 55 (52%) that underwent a previous analysis of VE1 antibody on LBC were enrolled from our previous series.[16] Immunohistochemistry for the VE1 antibody was run on LBC cytologic specimens using the standard protocol reported in our previous article and was evaluated by 2 pathologists (Esther Diana Rossi and Guido Fadda) who were blinded to the *BRAF* molecular status.[16] Briefly, the cytoplasm positivity in each sample was graded between 0 and 3+ and was performed on both cytologic and histologic samples. An intensity of 0 was defined as negative, 1+ was defined as positivity in <30% of cells, 2+ was defined as positivity in >30% and <80% of cells, and 3+ was defined as positivity in >80% of cells. For our convenience, we report this arbitrary distribution in 4 categories (0, 1+, 2+, and 3+) of VE1 intensity, which also are based on the percentage of stained cells for each category. The results for each category were homogenous and irrespective of the number of tumor cells stained. We did not observe any discrepancy in the intensity or percentage of VE1-positive cells between cytologic and histologic samples.

Statistical Analysis

Statistical analyses were performed using GraphPad-Prism 5 software (GraphPad Software, San Diego, Calif) and MedCalc version 10.2.0.0 (MedCalc Software, Mariakerke, Belgium). Statistical comparisons of continuous variables were performed using the Mann-Whitney *U* test or the *t* test for paired data, as appropriate. Comparisons of categorical variables were performed using the chi-square statistic with the Fisher exact test. P values $< .05$ were considered statistically significant.

Bioinformatics Analysis

Rarer *BRAF* mutations were predicted and compared using different computational tools, all of which indicated protein damage (Fig. 1). In detail, the Scale-Invariant Feature Transformation Algorithm (SIFT) score (University of British Columbia, Vancouver, British Columbia, Canada) ranged from 0 (damaging) to 1 (tolerated; 0.03), the Polymorphism Phenotyping Version 2 (PolyPhen-2) score (Harvard Medical School, Brigham & Women's Hospital, Boston, Mass) ranged from 0 (benign) to 1 (damaging; 0.998), the Single Nucleotide Polymorphisms sing Gene Ontology (GO) annotation (SNPs&GO) to predict whether a given variation can be classified as disease-related or neutral (Computational Molecular Biology Unit, University of Alabama at Birmingham, Birmingham, Ala) was scored as either *disease-related* or *neutral*, with a reliability index ranging from 0 to 10 (disease), and the Protein Variation Effect Analyzer (PROVEAN) score (J. Craig Venter Institute, Rockville,

TABLE 1. Analysis of the Current Series of 106 Samples With Cytohistologic Correlation

Variable	FN	SM	PM
Males/females	6/10	12/24	24/30
PTC/FVPC	2/14	20/16	38/16
BRAF mutations[a]	4	11	39
Angioinvasion	1	4	4
Lymph node metastases	1	3	10
Extrathyroid invasion	3	9	13
Multifocality	6	13	29

Abbreviations: *BRAF*, v-Raf murine sarcoma viral oncogene homolog B1; FN, follicular neoplasms; FVPC, follicular variant of papillary thyroid carcinoma; PM, positive for malignancy; SM, suspicious for malignancy; PTC, papillary thyroid carcinoma.
[a] These include the *BRAF* valine to glutamic acid substitution at codon 600 (V600E) mutation and uncommon mutations.

Md) ranged from less than −2.5 (deleterious) to greater than 2.5 (neutral; 5.798).

RESULTS

The 150 cytologic samples were diagnosed as 44 benign lesions (nodular goiters), 46 FVPCs, and 60 PTCs (including the 3 TCVs) on histology. We excluded the 44 LBC samples that had both benign histology and wild-type *BRAF*, as reported above (see Materials and Methods). In detail, all of the remaining 106 cytologic samples were processed with LBC and were diagnosed as FN (n = 16), SM (n = 36), and PM (n = 54). Specifically, and considering the histologic outcomes, the 46 FVPCs had been diagnosed as 14 FNs, 16 SMs, and 16 PMs; whereas the 60 PTCs had been diagnosed as 2 FNs, 20 SMs, and 38 PMs (Table 1). In Table 1, we also provide the data concerning some aggressive parameters (angioinvasion, lymph node metastases, multifocality, and extrathyroid infiltration) regardless of the *BRAF* mutational status (Table 1).

In Table 2, we have divided the series based on specific *BRAF* mutational profiles and correlated the results with the cytologic categories and histologic histotypes: these included 52 wild-type tumors, 48 with the $BRAF^{V600E}$ mutation, and 6 with uncommon *BRAF* mutations. We observed that the PTCs included 20 wild-type tumors and 40 with the $BRAF^{V600E}$ mutation (the 3 TCVs were mutated); whereas the FVPCs included 32 wild-type tumors, 8 with the $BRAF^{V600E}$ mutation, and 6 with uncommon *BRAF* mutations. The $BRAF^{V600E}$ mutation was related to PTCs with respect to the FVPC category ($P < .0001$; odds ratio [OR], 0.125; 95% confidence interval [CI], 0.049-0.321). It is noteworthy that,

TABLE 2. Correlation of Molecular Assessment With 106 Cytologic Samples

Variable	Total No. of Samples	FN	SM	PM
WT *BRAF* (PTC/FVPC)	52 (20/32)	2/10	13/12	5/10
$BRAF^{V600E}$ (PTC/FVPC)	48 (40/8)	0/1	7/3	33/4[a]
Uncommon *BRAF* mutations (PTC/FVPC)	6 (0/6)	0/3	0/1	0/2

Abbreviations: *BRAF*, v-Raf murine sarcoma viral oncogene homolog B1; FN, follicular neoplasms; FVPC, follicular variant of papillary thyroid carcinoma; PM, positive for malignancy; SM, suspicious for malignancy; PTC, papillary thyroid carcinoma; V600E, valine to glutamic acid substitution at codon 600; WT, wild type.
[a] The 3 tall cell variant PTCs are included.

when we correlated the *BRAF* mutations with the PTC or FVPC category, we observed that the $BRAF^{V600E}$ mutation had a significant association with PTCs, whereas uncommon *BRAF* mutations were significantly associated with FVPCs ($P = .0001$; OR, 61.94; 95% CI, 3.176-1208). Furthermore, all of the *BRAF* mutations were correlated with the cytologic categories, as clearly outlined in Table 2: 12 FNs were wild type, only 1 had the $BRAF^{V600E}$ mutation, and 3 displayed uncommon *BRAF* mutations. In the SM category, there were 25 wild-type samples, 10 samples with the $BRAF^{V600E}$ mutation, and 1 with uncommon *BRAF* mutations; whereas, for the PM category, there were 15 wild-type samples, 37 samples with the $BRAF^{V600E}$ mutation, and 2 with uncommon *BRAF* mutations (Table 2).

Table 3 lists the aggressive disease parameters (representing critical points for disease-free status) combined with the different *BRAF* mutations. Patients with the $BRAF^{V600E}$ mutation displayed a predominance of multifocality (8 specimens), extrathyroid infiltration (13 specimens), and lymph node metastases (11 specimens) compared with those who had uncommon *BRAF* mutations. Also, the presence of vascular invasion was reported in patients who had the $BRAF^{V600E}$ mutation but was absent in the remaining patients (Table 3). Significantly, all of the uncommon *BRAF* mutations were associated with encapsulated FVPCs ($P = .0097$; OR, 0.03; 95% CI, 0.001-0.745), whereas a higher rate of $BRAF^{V600E}$ mutation was noted among diffuse FVPCs.

In Table 4, a detailed description of our 6 uncommon *BRAF* mutations is provided based on different computational tools, all of which indicate protein damage. The table demonstrates that all of these uncommon mutations are damaging except for the neutral serine to

threonine substitution at codon 614 (S614T) mutation, as reported with the PROVEAN tool (Table 4, Fig. 1A,B).

Table 5 combines an analysis of the $BRAF^{V600E}$ mutation with analyses of VE1 expression and the morphologic findings, as depicted in our previously published reports[21,22] and in Figures 2A,B and 3A,B. Because we retrospectively analyzed all cytohistologic series, 55 samples that had analyses of the VE1 antibody on LBC were enrolled from our previous studies.[21,22] Specifically, all 6 of our samples with the uncommon *BRAF* mutations were negative for VE1 (0 intensity) (Fig. 3A,B). Moreover, the analysis of morphologic features in those 6 samples did not reveal the presence of plump cells or the peculiar sickle-shaped nuclei. In addition, among our 33 samples with the $BRAF^{V600E}$ mutation, 27 had a diffuse plump component and sickle-shaped nuclei (including 16 with 2+ positivity and 11 with 3+ positivity), and 6 had focal plump cells and sickle-shaped nuclei with 2+ intensity. The remaining 16 wild-type samples exhibited negative expression, which was distributed in 13 samples with 0 positivity (including 2 with a focal plump cell component) and in 3 samples with 1+ positivity (1 in the group without plump cells and 2 in the group with <20% plump cells). Concerning the evaluation of sickle-shaped nuclei, we observed that they were present in 100% of the samples with the $BRAF^{V600E}$ mutation and absent in the samples with wild-type *BRAF*.

TABLE 3. Correlation of *BRAF* Mutations With Parameters of Aggressiveness in the Current Series

Parameter	$BRAF^{V600E}$	Uncommon *BRAF* Mutation	Wild-Type *BRAF*
Diffuse/encapsulated FVPC	6/2	0/6	9/23
Angioinvasion	5	0	4
Lymph node metastases	11	0	3
Extrathyroid invasion	13	0	12
Multifocality	28	1	19

Abbreviations: *BRAF*, v-Raf murine sarcoma viral oncogene homolog B1; FVPC, follicular variant of papillary thyroid carcinoma; V600E, valine to glutamic acid substitution at codon 600.

DISCUSSION

The cytologic diagnosis of FVPC is still an unsolved issue, and the objective of our current study was to identify a possible correlation between cytohistologic features and the application of ancillary techniques (ie, ICC and molecular testing; namely, *BRAF* mutations) to empower the diagnostic accuracy of FNAC specimens. We did identify some correlations between FVPC and uncommon *BRAF* mutations in our 106 cytologic samples with malignant histologic follow-up. To the best of our knowledge, this study is the largest series to date in which all *BRAF* mutations were analyzed using morphologic, ICC, and molecular tools.

Although cytology has been doing an excellent job in achieving high diagnostic and prognostic results, it is highly likely to fall short of a correct cytologic diagnosis of FVPC, because some of these belong to the cytologic category of follicular/indeterminate neoplasms (FN) and exhibit either the absence of evidence or subtle evidence of the malignant nuclear features of PTC.[1,2,34,35] From this perspective, although the cytologic features of nuclear pseudoinclusions in samples diagnosed as *positive for malignancy-favoring PTC* and the morphologic features of *benign lesions* are straightforward and unequivocally recognized by cytopathologists, the categories of FN/AUS and SM represent a *gray zone* that includes both benign and malignant lesions with different diagnoses (including FVPC) and treatments.[1,2,34,35]

TABLE 4. Analysis of Uncommon *BRAF* Mutations With 4 Different Computational Tools

Mutation	SIFT	PolyPhen-2	SNPs&GO	PROVEAN
S614T	1.00, Tolerated	0.003, Benign	Neutral	−0.75, Neutral
P.k601_S602>T	—	—	—	−14.776, Deleterious
K601E	0.03, Damaging	0.784, Possibly damaging	Disease	−3.705, Deleterious
p.V600_W604>DL	—	—	—	−39.552, Deleterious
T599E	0.16, Damaging	0.467, Possibly damaging	Disease	−5.465, Deleterious
ΔV600	—	—	—	−12.370, Deleterious

Abbreviations: −, deletion; ins, insertion; E, glutamic acid; K, lysine; PolyPhen-2, Polymorphism Phenotyping Version 2 (Harvard Medical School, Brigham & Women's Hospital, Boston, Mass); PROVEAN, Protein Variation Effect Analyzer (J. Craig Venter Institute, Rockville, Md); S, serine; SIFT, Scale-Invariant Feature Transformation Algorithm (University of British Columbia, Vancouver, British Columbia, Canada); SNPs&GO, server for predicting single nucleotide polymorphisms (SNPs) using Gene Ontology (GO) annotation to predict whether a given variation can be classified as disease-related or neutral (Computational Molecular Biology Unit, University of Alabama at Birmingham, Birmingham, Ala); T, threonine; V, valine; W, tryptophan.

TABLE 5. Evaluation of Cytologic Parameters and VE1 in 55 Samples[a]

Mutational Findings	VE1 Negative (0 and 1+ Intensity)	VE1 Positive (2+ and 3+ Intensity)
$BRAF^{V600E}$ mutated (33 samples)		
Plump cells ≤20%	0	6[b]
Plump cells >20%	0	27
Uncommon *BRAF* mutations (6 samples)	6	0
Plump cells absent	6	0
$BRAF^{V600E}$ wild type (16 samples)		
Plump cells absent	12[c]	0
Plump cells ≤20%	4[d]	0
Plump cells >20%	0	0

Abbreviations: $BRAF^{V600E}$, v-Raf murine sarcoma viral oncogene homolog B1 valine to glutamic acid substitution at codon 600; VE1, anti-$BRAF^{V600E}$ antibody.
[a] Zero intensity indicates negative; 1+, positivity in <30% of cells; 2+, positivity in >30% and <80% of cells; 3+, positivity in >80% of cells.
[b] All 6 focal plump cells were VE1-positive with 2+ intensity.
[c] These 12 samples included all wild type without plump cells among papillary thyroid carcinomas and follicular variant papillary thyroid carcinomas.
[d] Only 2 samples (from the group with <20% plump cells) expressed VE1 with 1+ intensity.

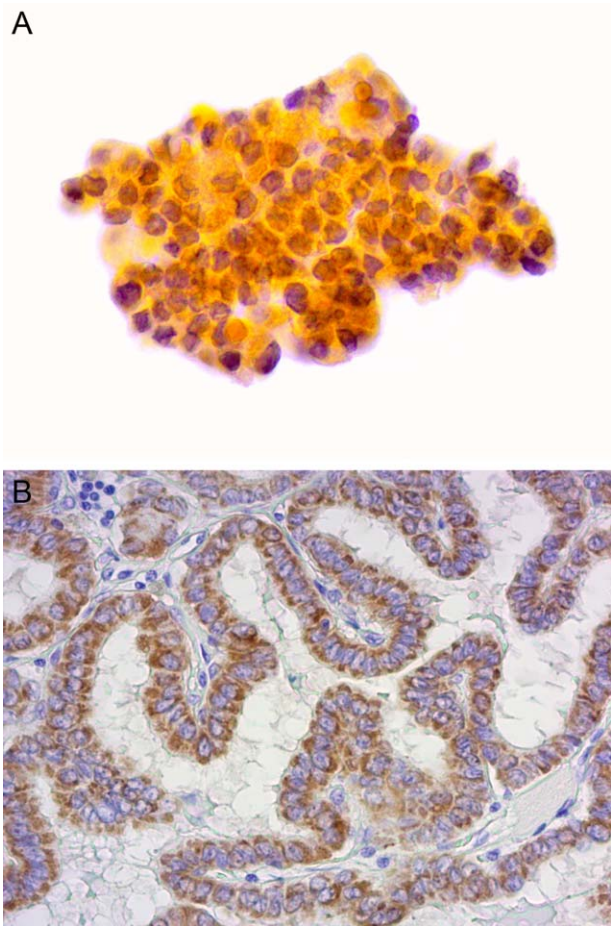

Figure 2. (A) Cytologic details of positivity (3+, strong cytoplasmic positivity) for VE1 (the anti v-Raf murine sarcoma viral oncogene homolog B1 [*BRAF*] valine to glutamic acid substitution at codon 600 [$BRAF^{V600E}$] antibody) are observed in a sample of papillary thyroid carcinoma (avidin-biotin complex; original magnification ×60). (B) Histologic details of VE1 positivity are observed in the same sample (avidin-biotin complex; original magnification ×60).

The awareness of these morphologic limitations and knowledge of the molecular mechanisms of cancer have paved the way for the application of ancillary techniques (both ICC and molecular analysis) on cytologic samples to provide useful insights into malignancy regardless of diagnostic categorizations.[6,7,9,10,36,37] Taking these findings together, the high specificity of *BRAF* mutations (also including the uncommon *BRAF* mutations) suggests their use as a strong hallmark of cancer also on thyroid FNAC.[36–40] Some authors have reported that the $BRAF^{V600E}$ mutation even exhibits a prognostic role, in which less favorable biologic behavior is defined by extrathyroid extension, advanced tumor stage at presentation, and lymph node or distant metastases.[5,6,8,10–13] Conversely, more recently, this association has represented a hot and controversial topic that will require additional and larger series to be confirmed or denied.

Since discovery of the implications of somatic mutations in thyroid tumorigenesis, *BRAF* mutations have emerged as the most prevalent oncogenic alteration in PTC, with $BRAF^{V600E}$ representing 95% of all such alterations.[3–13] In fact, according to the literature, the $BRAF^{V600E}$ mutation is typically identified in 29% to 69% of classic PTCs, which are the most common thyroid malignancy; is identified in 80% to 100% of TCVs; and is less commonly identified in FVPCs.[8,18–20] In this regard, our 17.5% rate of $BRAF^{V600E}$ mutation in FVPCs is in perfect alignment with the literature, and the rate slightly doubled when we considered all *BRAF* mutations in FVPCs (30.5%); this rate of mutated FVPC is probably because of the bias of our series, which had a limited 2-year-span, as well as our use of strict parameters for the diagnosis of FVPC.

It is noteworthy that recent studies, mainly single case reports, have appraised the detection of uncommon *BRAF* mutations in FVPC histotypes associated with less tumorigenic potential than $BRAF^{V600E}$ and $BRAF^{K601E}$ mutations.[19–21,24–26] Our study is in keeping with data reported by Trovisco et al[21] on their solid variant of PTC, in which they reported a novel triplet deletion of the

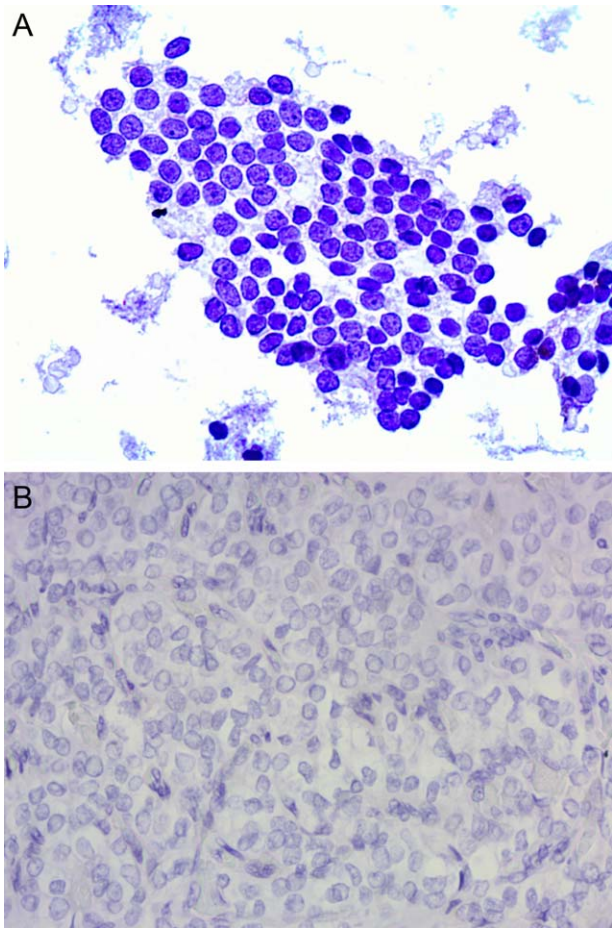

Figure 3. (A) Cytologic details of negativity for VE1 (the anti v-Raf murine sarcoma viral oncogene homolog B1 [*BRAF*] valine to glutamic acid substitution at codon 600 [$BRAF^{V600E}$] antibody) are observed in a sample of the follicular variant of papillary thyroid carcinoma with an uncommon *BRAF* mutation (avidin-biotin complex; original magnification ×60). (B) Histologic details of VE1 negativity are observed in the same sample (avidin-biotin complex; original magnification ×60).

coding nucleotides 1799 through 1801 (TGA1799-1801 deletion) involving the 2 targeted codons (600 and 601). Conversely, Barollo et al demonstrated a 3.2% rate of uncommon *BRAF* mutations mainly in the classic variant of PTC.[8,17] Whereas Trovisco et al reported that 9% of FVPCs had the $BRAF^{K601E}$ mutation, our series included only 1 $BRAF^{K601E}$ mutation in a sample that had an FVPC histotype.[21]

The current series highlights a 100% correlation between FVPC and uncommon *BRAF* mutations (all 6 of our uncommon *BRAF* mutations were from the group of FVPCs, and 3 belonged to the FN category). Moreover, we report a slightly more favorable prognostic outcome in patients with uncommon *BRAF* mutations in terms of lymph node metastases and multifocality, although we are aware that our findings may represent a limiting bias in terms of reaching definitive conclusions and that larger series are needed to confirm our results. In keeping with the report by Chen et al, we observed that all 6 of our uncommon *BRAF* mutations were detected in encapsulated FVPCs, whereas most diffuse FVPCs (6 of 8 tumors) expressed the $BRAF^{V600E}$ mutation ($P = .0097$).[40] However, conversely, it has been observed that encapsulated FVPCs without vascular invasion behave like low-risk lesions, and it is also doubtful that an uncommon *BRAF* mutation may be responsible.[40] From this perspective, very recent meetings and studies have documented an indolent behavior of noninvasive FVPCs, and some pathologists have begun to question whether the definition of carcinoma is warranted.[41]

Supported by computational tools, our current analysis demonstrates that all of these uncommon mutations are close to the *BRAF* 600 position (in the binding pocket named A-loop), which is involved in switching between active and inactive conformation, affecting protein function. These findings were correlated with all active mutations except the neutral S614T mutation, although all 6 of our samples with uncommon mutations exhibited significantly less aggressive parameters than the $BRAF^{V600E}$-mutated samples. The awareness of an activated mutational mechanism linked to less aggressive disease and prognostic outcome may be the consequence of a different conformational loop in the mutated protein.[27]

In fact, according to Dibb et al, the oncogenic activity of *BRAF* may be linked to the alteration of amino acids within the P-loop and the activating segment (A-loop), which flip into the active conformation. Although these rarer mutations induce partial kinase activity, they also alter amino acids, which are essential for the catalysis that leads to an oncogenic *BRAF* activation.[27]

Several authors have underlined the technical difficulties with detecting these uncommon *BRAF* mutations, because traditional screening was initially restricted to the most frequent hotspot region, which may hinder the detection of other mutations of *BRAF* on exon 15.[19–21,37,38] However, the correct detection of all possible *BRAF* mutations offered by the high-throughput platforms may be helpful in guiding any further tailored therapy for patients with mutated thyroid carcinomas. From that perspective, awareness of all this evidence has

aroused interest in the molecular evaluation of cytologic thyroid lesions for providing potential risk stratification, and excellent results also can be obtained when LBC is adopted.[6,7,38–40]

Nevertheless, some of the issues raised with these molecular DNA-based methods have encouraged enthusiasm for either immunohistochemical $BRAF^{V600E}$ evaluation or the new emerging insights of its specific morphologic features. According to Virk et al and our recent studies, some specific morphologic features on thyroid FNACs that are positive for malignancy (eosinophilic, large, "plump" cytoplasm with the nuclear features of PTC) have been correlated with the presence of the $BRAF^{V600E}$ mutation, exhibiting high specificity and a high positive predictive value.[14,15] Our findings establish that these plump cells and specific, smaller, and eccentric-located nuclei (called sickle-shaped nuclei) were present in all samples that harbored the $BRAF^{V600E}$ mutation but were completely absent in our wild-type *BRAF* specimens; therefore, we explored possible correlations of the $BRAF^{V600E}$ mutation both with FVPC histotype and with uncommon *BRAF* mutations.

Hence, we yielded both negative VE1 expression and the absence of any $BRAF^{V600E}$ morphologic features (plump cells and sickle-shaped nuclei) in all 6 samples that had uncommon *BRAF* mutations, leading to the conclusion that molecular analysis alone remains the gold standard for this specific research.[15,16] Furthermore, the search for specific morphologic features, as in our recently published series on the $BRAF^{V600E}$ mutation, did not correlate with plump cells or sickle-shaped nuclei, demonstrating the strong and unequivocal association of these features only with the $BRAF^{V600E}$ mutation regardless of histotype.[15]

In conclusion, only the molecular assay helped in identifying the uncommon *BRAF* mutations on thyroid cytology regardless of the categories used. Not only may these uncommon *BRAF* mutations have diagnostic involvement, but they even seem to be associated with a possibly less aggressive behavior, especially in terms of multifocality and lymph node metastases, which may consequently justify a less aggressive surgical approach even in the presence of a malignant cytologic diagnosis. We are aware that the major bias of our original research is in the limited numbers of these uncommon mutations, and larger series still wait to be proposed for achieving conclusive and univocal results.

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