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Validation of the VE1 Immunostain for the *BRAF* V600E Mutation in Melanoma

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Abstract

BACKGROUND—*BRAF* mutation status, and therefore eligibility for BRAF inhibitors, is currently determined by sequencing methods. We assessed the validity of VE1, a monoclonal antibody against the *BRAF* V600E mutant protein, in the detection of mutant BRAF V600E melanomas as classified by DNA pyrosequencing.

METHODS—The cases were 76 metastatic melanoma patients with only one known primary melanoma who had had *BRAF* codon 600 pyrosequencing of either their primary (n=19), metastatic (n=57) melanoma, or both (n=17). All melanomas (n=93) were immunostained with the BRAF VE1 antibody using a red detection system. The staining intensity of these specimens was scored from 0 – 3+ by a dermatopathologist. Scores of 0 and 1+ were considered as negative staining while scores of 2+ and 3+ were considered positive.

RESULTS—The VE1 antibody demonstrated a sensitivity of 85% and a specificity of 100% as compared to DNA pyrosequencing results. There was 100% concordance between VE1 immunostaining of primary and metastatic melanomas from the same patient. V600K, V600Q, and V600R *BRAF* melanomas did not positively stain with VE1.

CONCLUSIONS—This hospital-based study finds high sensitivity and specificity for the BRAF VE1 immunostain in comparison to pyrosequencing in detection of *BRAF* V600E in melanomas.

INTRODUCTION

Forty to sixty percent of all cutaneous melanomas harbor mutations in the *BRAF* oncogene, which regulates cellular growth signals.(1, 2) Alterations within *BRAF* often occur as somatic point mutations in the activating segment at amino acid 600, with the V600E alteration resulting in a missense substitution of valine by glutamic acid.(1, 3–5) This V600E mutation accounts for 69 – 94% of *BRAF* mutations in melanoma.(1, 6, 7) Two BRAF inhibitors are FDA approved for treatment of unresectable or metastatic melanoma patients; vemurafenib in patients with *V600E* mutant melanoma and dabrafenib in patients

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with a *V600E* or *V600K* mutant melanoma.(8–10) Current methods of detection of a *BRAF* mutation are DNA-based assays.(11, 12) These methods often take weeks for completion and require meticulous selection of a specimen with predominantly viable tumor.(12–14) Treatment with BRAF inhibitors often results in rapid clinical improvement, and a delay in therapy could be detrimental to patient care.(13)

Treating patients without a known mutation status with BRAF inhibitors carries the risk of further acceleration of melanoma tumor growth in *NRAS* mutant cases due to paradoxical activation of MAPK signaling.(15–18) With the use of current molecular methods, the potential for enhanced tumor growth must be weighed against harmful delays in treatment. Recently, a monoclonal antibody against mutant BRAF V600E protein (VE1) has been developed.(11, 19–22) Initial studies indicate high sensitivity and specificity of this antibody as compared to DNA sequencing.(11, 14,19–24) Use of immunohistochemistry for VE1 could potentially allow for a quick and efficient method of detection of *BRAF* mutation status. In this study, we attempt to validate the VE1 antibody using a different immunostaining platform and protocol as compared to previous investigators, test the antibody against different *BRAF* mutations, measure interobserver differences in scoring VE1 staining, examine the heterogeneity of VE1 staining within melanomas, and determine concordance of BRAF V600E status between primary and metastatic lesions.

MATERIALS AND METHODS

Case Selection

Following institutional review board approval, 97 primary and metastatic melanomas were retrieved from a case series of 79 patients treated at UNC Healthcare with known *BRAF* mutational status determined for clinical purposes in the UNC Molecular Genetics Laboratory using a CLIA-certified method of DNA pyrosequencing.(9, 25) H&E slides from these cases were reviewed for presence of sufficient tumor. One primary and three metastatic melanomas were excluded because of insufficient melanoma tissue in the block for recuts as determined by the study dermatopathologist. The remaining 93 primary and metastatic melanomas from 76 patients with a sufficient amount of tumor tissue for immunohistochemistry were analyzed.

Immunohistochemistry

Immunohistochemistry for mutant BRAF V600E protein was performed using the monoclonal mouse antibody VE1 (Spring Bioscience, Pleasanton, CA). Immunostaining was performed in the UNC Department of Dermatology Dermatopathology Laboratory. In this study, all tissue was fixed in neutral buffered formalin purchased commercially. Most samples had between 6 and 48 hours of total formalin fixation time prior to tissue processing. Our routine overnight tissue processing cycle includes the following: formalin for 60 minutes, 70% alcohol for 55 minutes, 95% alcohol for 35 minutes, 95% alcohol for 55 minutes, 100% alcohol for 30 minutes, 100% alcohol for 40 minutes, 100% alcohol for 55 minutes, xylene for 45 minutes, xylene for 55 minutes, paraffin for 30 minutes, paraffin for 30 minutes, paraffin for 30 minutes, and paraffin for 45 minutes. The original block used for genetic analysis was accessible and immunostained for all but 3 of the specimens. A

tissue block adjacent to the original block was chosen for these three specimens. Freshly cut 4-μm thick sections of formalin-fixed and paraffin-embedded melanoma tissue blocks were stained using the fully automated Leica Bond III system. Pretreatment was performed using an onboard heat-induced epitope retrieval in EDTA buffer (ER2) for 30 minutes. Incubation with the VE1 antibody at a 1:100 dilution was done for 30 minutes at room temperature. Chromogenic detection was performed using the Leica Refined Red polymer detection system (Leica Microsystems). Incubation with hematoxylin for 10 minutes was used for counterstaining. Melanomas with documented *BRAF* mutational status were used as internal controls.

Pathology scoring

Immunostained slides were subsequently evaluated by a dermatopathologist (D.C.Z.) blinded to all genetic and clinical data. Specimens were analyzed for their degree of cytoplasmic immunostaining (0–3+). When cytoplasmic staining was scored as 0 or 1+ (with 1+ representing a weak cytoplasmic blush of staining), cases were scored as negative; while scores of 2+ and 3+ (indicating strong cytoplasmic staining) were considered positive results. Additionally, the dermatopathologist commented on whether there was VE1 nuclear staining independent of cytoplasmic staining. The percent of tumor that stained was also scored. To measure interobserver differences, a second dermatopathologist (P.A.G) similarly scored the metastatic melanomas with pyrosequencing results (n=57) independently.

Statistical analysis

The sensitivity and specificity of the VE1 immunostain as compared to DNA pyrosequencing were determined. A Pearson chi-squared test was used to determine the relationship of VE1 status with patient sex. An unpaired Student’s t-test was used to determine the association of VE1 status with age at diagnosis of the primary melanoma. *P*-values are two-sided. Statistical analyses were implemented in SAS (SAS Institute, Cary, NC) version 9.3.

RESULTS

Patients

The study included a series of 76 melanoma patients with known *BRAF* mutational status in their metastatic or primary melanoma and sufficient tumor for immunostaining with the VE1 antibody. Of these 76 patients, 27 had a *BRAF* V600E mutation and another 9 had an alternate *BRAF* V600 mutation, (V600K, V600R, or V600Q) on initial analysis. Of these specimens with pyrosequencing results, 19 were primary melanomas while 57 were metastatic melanomas. The age at diagnosis of the primary melanoma, sex, and AJCC TNM stage of these patients at the time of pyrosequencing of their melanoma is provided in Table 1. Additionally, 17 patients in this series with sufficient tumor had an additional matched primary (n=13) or metastatic melanoma (n=4) that was stained for VE1.

Interobserver Differences of BRAF VE1 Staining and Concordance of VE1 Immunohistochemistry Between Matched Primary and Metastatic Lesions

The kappa statistic for agreement for VE1 positivity between the two dermatopathologists was 1.00. All 17 matched pairs of metastatic and primary melanomas with sufficient tissue for immunohistochemistry demonstrated 100% concordance for VE1 staining. Three of these matched cases had positive immunohistochemistry results while 14 stained negatively.

VE1 Immunohistochemistry Compared with Genetic Analysis and Re-review of Pyrosequencing on Melanomas with Discrepant Results

According to DNA pyrosequencing, 40 of the 76 cases were determined as *BRAF* wild type while 36 cases had a *BRAF* mutation, comprised of *BRAF* V600E (n=26), V600K (n=8), V600R (n=1), or V600Q (n=1) mutations (Table 2). Twenty-two specimens immunostained positively (scores of 2+ and 3+) for VE1, while 54 specimens immunostained negatively (scores of 0 and 1+) (Figures 1, 2).

In this study there were 5 (6.6%) discordant cases, all of which were 0 (n=3) or 1+ (n=2) by VE1 immunostaining but DNA pyrosequencing showed a V600E mutation (Table 2). Three of the discordant cases were metastatic lesions while the remaining 2 were primary lesions. Two of the discordant metastatic lesions had a matched primary lesion that was also tested. In both of those cases, the VE1 immunostain results for the primary and metastatic lesions were concordant with each other but were discordant with the positive DNA pyrosequencing result.

The pyrograms and interpretations from the five discrepant cases that were *BRAF* V600E mutation positive by pyrosequencing, but negative by IHC, were reviewed. Two of the cases showed a *BRAF* V600E mutation at a high allele frequency, and the high level of mutant DNA was consistent with the high estimated tumor percentage on the reviewed H&E section. Two of the cases showed a *BRAF* V600E mutation at an allele frequency lower than expected, possibly indicating tumor heterogeneity for the mutation. These two cases may represent true false negatives by IHC or false positives by pyrosequencing. The fifth case was found on review to contain a *BRAF* V600K mutation that was originally misinterpreted as a V600E mutation.

Sensitivity and Specificity

Compared to pyrosequencing (which was used as the gold standard for this study) after rereview of the discrepant cases, one case was reclassified as V600K rather than V600E. After reclassification of this case, immunohistochemical analysis with VE1 demonstrated a sensitivity of 85% (22/26) and a specificity of 100% (50/50) for the *BRAF* V600E mutation (Table 3). Specimens with the V600K, V600R, and V600Q mutations were not immunoreactive with the VE1 antibody.

Heterogeneity of VE1 Staining Within Melanomas

Only 2 specimens were determined to be heterogeneous in immunohistochemical staining. One of these cases stained 99% of the tumor. Upon initial scoring, the other case was scored as homogeneously negative; however, upon re-examination, this tumor was determined to be

10% positive. This specimen was also one of the 5 discordant cases reported, and we did not change its classification because it was originally scored as negative. No other heterogeneity in cytoplasmic staining of the melanoma tumor cells was found.

Primary Melanomas

Including the matched pairs, 33 of the 93 specimens with VE1 immunostaining results were primary melanomas. Ten of these primaries stained positively while 23 stained negatively. These melanomas varied in stage, site, and classification. In this study, there was positive staining for superficial spreading (6/12), nodular (2/11), acral lentiginous (1/3), and spitzoid (1/1) melanoma subtypes. Primary melanoma subtypes tested with negative immunostaining results included mucosal (n=4), desmoplastic (n=1), and lentigo maligna (n=1) melanoma. Additionally, primary melanomas of different AJCC clinical stages were tested with the VE1 antibody. AJCC Stage T1 (0/2), Stage T2 (1/4), Stage T3 (5/9), and Stage T4 (4/18) melanomas demonstrated positive immunostain results. Primary melanomas with and without ulceration were used in this study, showing positive immunostaining in ulcerated (3/17) and non-ulcerated (7/16) melanomas (see Fig. 3).

Metastatic Melanomas

Our results demonstrated associations between immunostaining with VE1 in metastatic melanomas and certain clinical characteristics. Younger age at diagnosis of the primary melanoma was found to be associated with increased positive immunostaining ($p=0.002$). There was a borderline but non-significant association with sex between positive and negative VE1 results ($p=0.06$). Melanoma metastases from a variety of locations were tested with the VE1 immunostain, with positive results in cutaneous (6/19), lymph node (9/28), and mucosal (1/4) locations. Other metastases with negative immunostaining results had soft tissue (n=2), brain (n=2), muscle (n=1), or lung (n=1) location.

DISCUSSION

Our study demonstrated a sensitivity of 85% and a specificity of 100% for the BRAF VE1 immunohistochemistry using a red chromagen as a method of detection of BRAF V600E mutant protein as compared to DNA pyrosequencing for detection of *BRAF* V600E mutant DNA in routinely processed formalin-fixed paraffin-embedded melanoma tumor tissues. No melanoma with a *BRAF* V600K, V600R, or V600Q mutation stained with the VE1 antibody. Also the antibody had little variability in strength or intensity across our 93 specimens. After gaining experience with the antibody, we noticed that in nearly every case the tumor is either strongly positive or it is negative/very weak blush. The majority of specimens had homogeneous VE1 melanoma staining. Our study also demonstrated 100% concordance between matched primary and metastatic melanomas. We also demonstrated high interobserver agreement for scoring of the VE1 antibody staining, similar to Marin et al.(26) We demonstrated the utility of VE1 on a variety of metastatic and primary melanomas, including different primary melanoma histologic subtypes and metastases from a variety of cutaneous, lymph node, and visceral locations. Furthermore, the VE1 immunostain was associated with younger patient age at diagnosis of the primary melanoma, supporting the association of *BRAF* mutated melanoma and younger age.(27)

There are several differences in our methods as compared to most previous investigators. These include the use of the Mixed Red Refine reagent from Leica Microsystems as opposed to the Ventana OptiView and Ultraview Universal DAB Detection kits. This difference allows for improved differentiation of staining in melanoma specimens with a red chromagen as opposed to a brown chromagen. Additionally, our study is unique in using the Leica Bond III system as opposed to the Ventana Benchmark system.(11, 19,22, 28, 29) Other differences in protocol as compared to previous investigators include dilution of the antibody used, method of epitope retrieval, and time and temperature of incubation with the immunostain. We used a 1:100 dilution of the antibody, while other studies used higher concentrations including dilutions of 1:50, 1:5, or undiluted solutions.(11, 19,24, 29) We also performed epitope retrieval with EDTA for 30 minutes as opposed to Ventana Cell Conditioning solution for 64 minutes.(24) Additionally, we incubated the antibody for 30 minutes at room temperature as opposed to 37 degrees Celsius for 32 minutes.(11, 19, 29) Therefore, our study serves to provide additional validation of this antibody with a different immunostaining platform and protocol.

We used a graded system of staining as a method of assessment, similar to initial studies (11, 28), to determine the variability in the strength and intensity of VE1 staining. Other investigators did not use a graded system.(19, 20, 23) Our results showed only occasional heterogeneity of BRAF VE1 staining, similar to some other studies.(19, 24) Like us, some previous investigators scored isolated nuclear staining as negative.(19, 20, 24) We characterized the nuclear staining as part of the process of validating the antibody but did not find any clinical significance of nuclear staining in retrospective analysis. In accordance with the antibody specifications sheet, the VE1 immunostain is a cytoplasmic stain, consistent with BRAF being a cytoplasmic protein.

Our analysis is consistent with prior studies indicating a high sensitivity and specificity of the VE1 antibody for identifying *BRAF* V600E mutant melanomas, although some studies have achieved higher sensitivity (Table 4).(11, 19–22) Several reasons could account for the differences in sensitivity. Methods of VE1 staining may have played a major role. Additionally, previous investigators have used different gold standards to determine the presence of the *BRAF* mutation. Colomba et al. compared different methods of detection of *BRAF* mutations in melanoma specimens and found pyrosequencing to be the most efficient. We used pyrosequencing as our reference. It is also possible that preanalytical variables played a part in the discrepancy between our results and previous investigators. However, our results might also approximate what will be found in widespread clinical practice as many different labs start offering this test. Commonly, when immunostains are first published, it is often difficult for subsequent investigators to achieve the same high results as the initial publications.

Five of our cases were discordant on initial review, where DNA pyrosequencing was positive for the *BRAF* V600E mutation but immunostaining of the tissue with the VE1 antibody was negative. However on reanalysis of the pyrosequencing results, one case that was originally interpreted as V600E was found on review to represent a V600K mutation. The pyrosequencing assay used in the UNC Clinical Molecular Genetics Laboratory is designed to quantitatively interrogate mutations at position 1799. While the assay is only

semiquantitative when applied to tumors with varying amounts of mixed stromal cells, the percentage of T or A alleles at that position can be estimated. This assay is meant to identify the V600E c.1799T>A mutation and other clinically important variants at or near nucleotide position 1799, such as variants at position 1798 or 1800. While surrounding base cells are represented by peaks on the pyrogram, the level of those nucleotide peaks are not quantitatively interrogated by the software. The alternate peak patterns may be difficult to interpret, especially if the mutant allele frequency is low. Previous investigators have also indicated discrepant results in molecular analysis.(14, 19) While BRAF inhibitors have demonstrated improved rates of overall and progression-free survival in patients with the *BRAF* V600E mutation in a phase 3 randomized clinical trial, some sensitivity has been shown against other mutations.(30) Of the five discordant cases, only one patient has been treated with targeted BRAF treatment. This metastatic melanoma patient is currently being treated with BRAF/MEK combination therapy (dabrafenib and trametinib) and, while improved since baseline, demonstrates slow progression.

Mismatches between immunohistochemistry and DNA pyrosequencing may also be due to sampling errors, tissue necrosis, or a decreased sensitivity of immunohistochemistry for the *BRAF* mutation. These differential results may also be due to intratumoral heterogeneity regarding the presence of a *BRAF* mutation,(31) although the majority of the melanomas in our study had homogenous VE1 staining. Generally homogenous VE1 staining with occasional or no heterogeneity in BRAF V600E expression has been demonstrated in previous studies.(11, 20,22, 32, 33) This heterogeneity has not been shown to correlate with survival and may be due to pre-analytical factors.(11, 32, 34) Heterogeneity within a tumor for the presence of the *BRAF* mutation status must be further studied.

Our results corroborate preservation of the *BRAF* V600E mutation between paired primary and metastatic melanomas from the same patient,(35) and we extend these results to concordance of BRAFV600E at the protein level. These findings are consistent with the early occurrence of *BRAF* mutations within melanoma pathogenesis.(35) Additionally, we show strong VE1 staining of a capsular melanocytic nevus in Figure 1. This finding supports the presence of BRAF V600E mutant protein in nevi, indicating that *BRAF* mutations alone are not sufficient for melanoma pathogenesis.(3) Therefore, this immunostain should not be considered diagnostic for melanoma. Together, these results suggest that in cases where metastatic lesions are inaccessible or unavailable, the primary lesion could potentially be used for testing. In this situation, patients with the *BRAF* mutation may have improved access to BRAF inhibitors.

Immunostaining with the VE1 antibody for the *BRAF* V600E mutation did not produce any false positive results. This finding combined with the high sensitivity of VE1 for this *BRAF* mutation supports the use of an algorithm incorporating both the VE1 antibody and DNA mutational analysis. In this model, immunohistochemistry initially could be used in patients with insufficient melanoma tissue for genetic analysis. This subset of patients would otherwise not be analyzed for the *BRAF* mutation using existing methods of detection. In patients with sufficient melanoma tissue, immunohistochemistry first could be used to quickly and inexpensively detect *BRAF* V600E mutations. Cases with negative VE1 results should be tested by a DNA mutational analysis assay to rule out a possible false negative

result or a different *BRAF* mutation. The sequential use of these methods should allow for a highly sensitive and specific detection of the *BRAF* V600E mutation.(9, 14) Alternatively, if immunostaining were done in addition to mutational analysis on melanomas, the combined results might increase overall diagnostic accuracy for V600 mutational subtype if discrepant cases were reevaluated for their mutational status.

In the era of personalized medicine, *BRAF* mutation status has become a key piece of information in the clinical management of melanoma patients. In this study, we found the VE1 monoclonal antibody as method of detection of the *BRAF* V600E mutation in our institution to have high sensitivity and specificity with generally homogenous staining. Based on these results, immunostaining with the VE1 antibody seems to be an effective and efficient screening tool in the assessment of *BRAF* V600E mutant status in melanoma patients. In addition, VE1 staining seems to be complementary with mutational screening, as reevaluation of discrepant cases may improve diagnostic accuracy.

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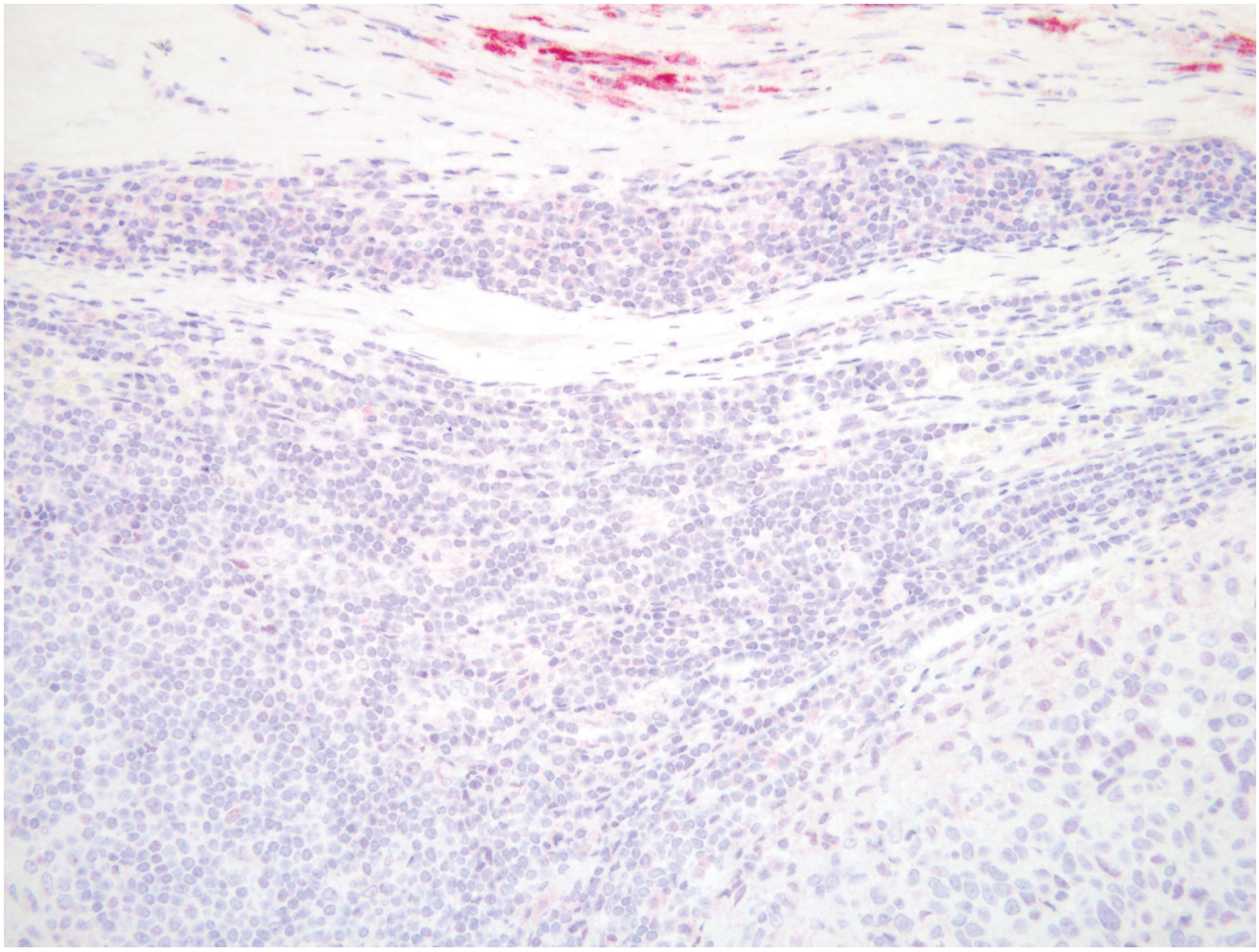


Figure 1.
BRAF wild type melanoma in a lymph node that is negative for VE1 immunostain alongside a 3+ positive capsular melanocytic nevus (Original magnification ×100).

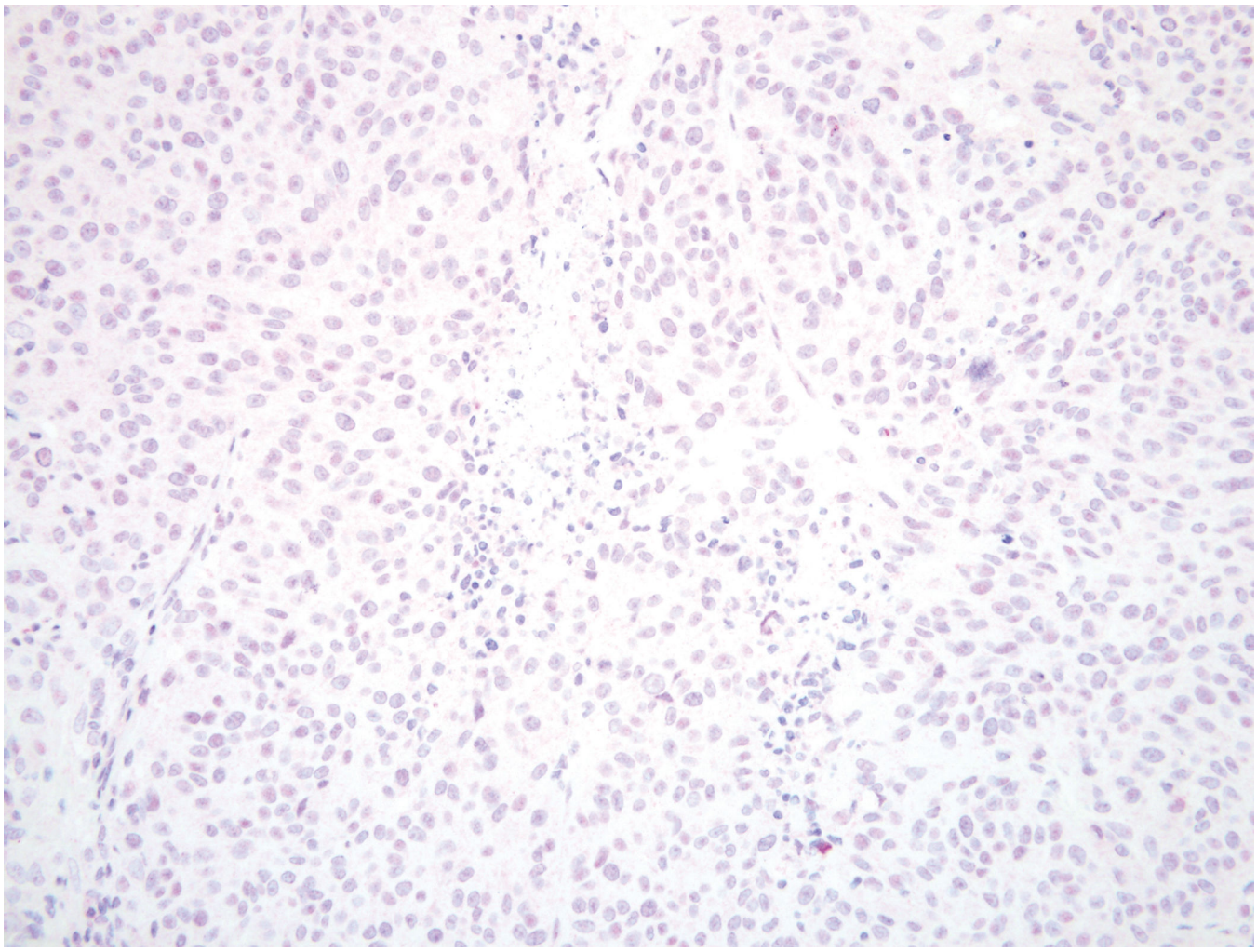
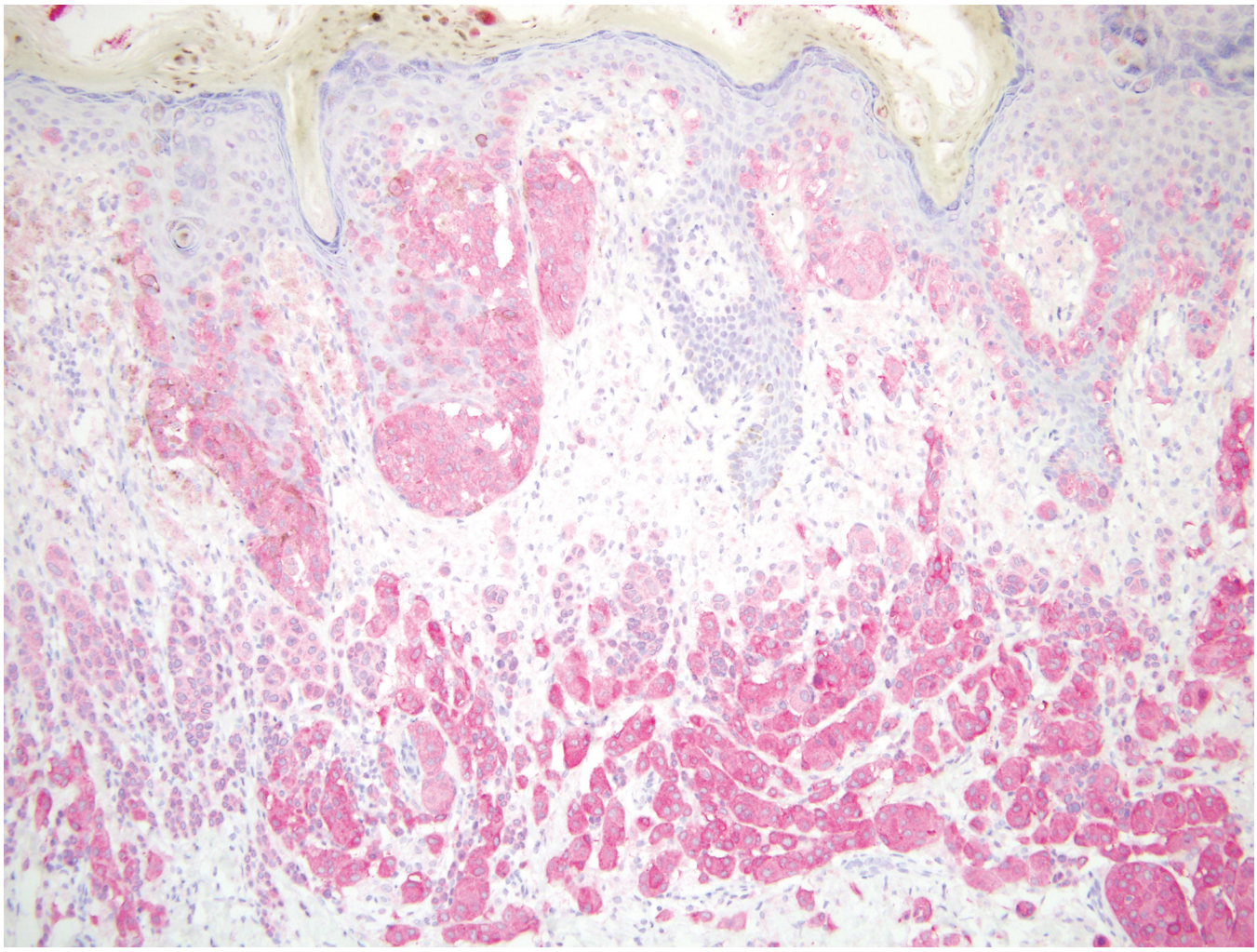


Figure 2.
BRAF wild type melanoma with 1+ weak cytoplasmic and nuclear blush on VE1 immunostain (Original magnification ×200).



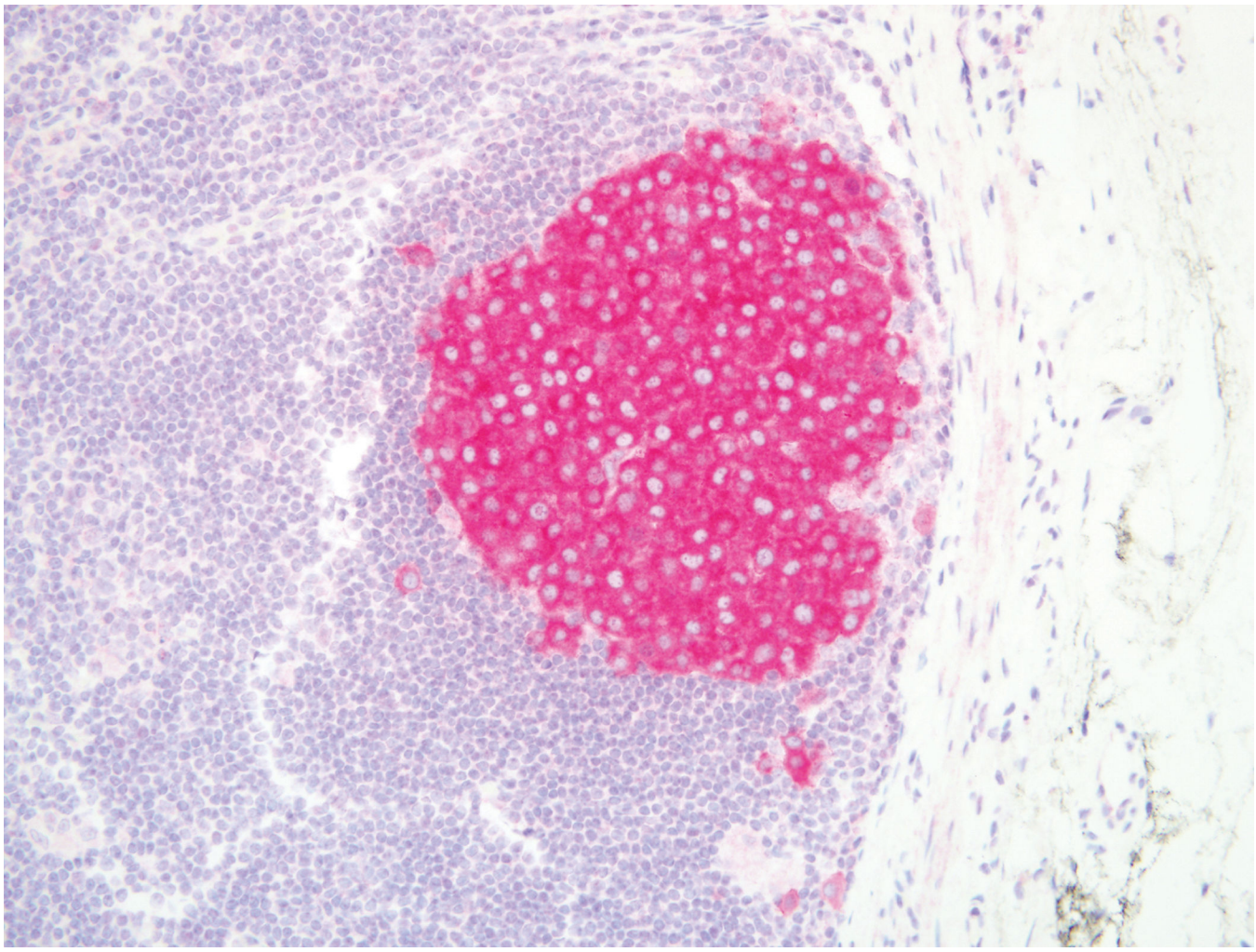


Figure 3.

A, *BRAF* V600E mutant primary melanoma with positive staining for VE1 (×200). B, Corresponding *BRAF* V600E metastatic melanoma with positive staining for VE1 in the same patient (Original magnification ×100).

Table 1

Patient Case Series

Characteristic	Value	N (%)	VE1+ (%)	VE1− (%)
Total patients		76 (100)	22(29)	54(71)
Age at Diagnosis of Primary Melanoma				
	Mean	59		
	Median	60		
	Range	19–92		
Sex				
	Male	44 (58)	9 (20)	35 (80)
	Female	32 (42)	13 (41)	19 (59)
Overall AJCC TNM Stage at Pyrosequencing				
	I	9 (14)	4 (44)	5 (55)
	II	20 (31)	3 (15)	17 (85)
	III	33 (51)	12 (36)	21 (64)
	IV	3 (5)	0 (0)	3 (100)
DNA Pyrosequencing Results				
	Wild type	40 (53)		
	V600E	27 (36)		
	V600K	7 (9)		
	V600R	1 (1)		
	V600Q	1 (1)		
Melanoma Type Tested for Pyrosequencing				
	Metastatic	57 (75)	16 (28)	41 (72)
	Primary	19 (25)	6 (32)	13 (68)
Primary Location				
	Skin	16 (84)	6 (38)	10 (63)
	Mucosal	3 (16)	0 (0)	3 (100)
Metastatic Location				
	Skin	19 (33)	6 (32)	13 (68)
	Lymph node	28 (49)	9 (32)	19 (68)
	Muscosal	4 (7)	1 (25)	3 (75)
	Soft tissue	2 (4)	0 (0)	2 (100)
	Brian	2 (4)	0 (0)	2 (100)
	Muscle	1 (2)	0 (0)	1 (100)
	Lung	1 (2)	0 (0)	1 (100)
VE1 Cytoplasmic Staining Intensity				
	0	48 (63)		
	1+	6 (8)		
	2+	9 (12)		
	3+	13 (17)		

Staining Intensity scored from 0 to 3+ (0=negative staining, 1+=weak background staining, 2+=moderately positive staining, 3+ strongly positive staining) with 0 and 1+ considered negative scores and 2+ and 3+ considered positive scores.

Table 2

Discordant Cases of Immunohistochemical Analysis and Genetic *BRAF* Analysis Results

DNA Pyrosequencing Result	BRAF Cytoplasmic VE1 Staining Intensity	Percent Tumor Stained in Cytoplasm	BRAF Nuclear VE1 Staining Intensity	Percent Tumor Stained in Nucleus	Pyrogram Results for *BRAF* V600E mutation
V600E*	0	100	0	0	V600K
V600E	0	100	0	0	Low Allele Frequency
V600E	0	100	0	0	High Allele Frequency
V600E	1+	100	1+	50	High Allele Frequency
V600E	1+	100	3+	75	Low Allele Frequency

* This case indicates the discrepant case on re-review of molecular analysis.

Table 3

Comparison of VE1 Immunostain Results and Pyrosequencing Results After Pyrosequencing Re-review

BRAF Mutation Type	Pyrosequence Positive	Pyrosequence Negative	VE1 Positive	VE1 Negative	Sensitivity	Specificity
V600E	26*	40	22	44	85%	100%
V600K	8*	0	0	8	-	-
V600R	1	0	0	1	-	-
V600Q	1	0	0	1	-	-

* After molecular reanalysis, one discrepant case was determined to be V600K by pyrosequencing as opposed to V600E. Therefore, this case was counted as a V600K metastatic melanoma.

Table 4

Comparison of Our Results with Initial VE1 Studies

Study	Sensitivity	Specificity	Number of Primary Melanomas Tested	Number of Metastatic Melanoma Tested	Evaluation of VE1 Staining*	Method of Molecular Analysis
Our Study	22/26 (85%)	50/50 (100%)	19	57	Quantitative	Pyrosequencing
Long, 2013	37/38 (97%)	58/59 (98%)	10	87	Qualitative	PCR-HRM, PCR-mass spectrometry
Colomba, 2013	40/40 (100%)	39/39 (100%)	0	79	Qualitative	Sanger Sequencing, Real-time PCR, Pyrosequencing
Menzies et al. (2014)	30/30 (100%)	34/34 (100%)	7	57	Quantitative	PCR-HRM
Busam, 2012	22/22 (100%)	22/22 (100%)	7**	44	Quantitative	PCR-mass spectrometry
Capper, 2011	16/16 (100%)	27/27 (100%)	0	43	Quantitative	Direct Sequencing

*Quantitative assessment of VE1 staining indicates a graded system for the evaluation of the VE1 staining while qualitative assessment indicates either the presence or absence of staining.

**These 7 cases were not included in the statistical analysis published in this article.