Intrapatient Homogeneity of BRAF^{V600E} Expression in Melanoma

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Abstract: Concern regarding the presence of intertumoral heterogeneity of *BRAF* mutation status in patients with metastatic melanoma has led to uncertainty surrounding which specimens should preferentially undergo *BRAF* testing. We sought to examine the extent of intrapatient heterogeneity of BRAF^{V600E} protein expression in patients with multiple tumors. Sixty-four patients with 171 tumors at various stages of disease progression had tumor BRAF^{V600E} protein expression immunohistochemically (IHC) assessed using the BRAF^{V600E} mutant–specific antibody VE1. Melanoma sections were examined for staining intensity (score 0 to 3), the presence of intratumoral heterogeneity, and concordance with molecular *BRAF* genotype. Intrapatient, intertumoral heterogeneity of BRAF^{V600E} expression was also assessed by comparing VE1 staining on different tumors within the same patient. All specimens from 64 patients displayed complete intertumoral homogeneity of BRAF^{V600E} expression status, and all tumors had concordant molecular and IHC *BRAF* status. Only 1 patient demonstrated >1 level of staining intensity heterogeneity between specimens. Intratumoral heterogeneity of staining intensity was not observed in any specimen. IHC-measured BRAF^{V600E} protein expression displays complete intertumoral homogeneity, minimal intertumoral intensity heterogeneity, and no intratumoral heterogeneity in metastatic melanoma patients in various stages of disease progression. Our results suggest that, provided there is adequate quantity of viable tumor cells and minimal admixture of nontumor cells, testing any melanoma sample from a patient with metastatic disease will accurately determine *BRAF* status for treatment planning.

Key Words: BRAF, melanoma, VE1, V600E, heterogeneity, homogeneity, immunohistochemistry, mutation testing

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Constitutive activating mutations in the *BRAF* gene occur in approximately 50% of melanomas, of which 70% to 90% result in a single amino acid substitution of valine with glutamic acid at residue 600 (V600E).[1–4] The selective BRAF inhibitors vemurafenib and dabrafenib are highly effective in targeting the oncogenic BRAF protein in *BRAF*-mutant metastatic melanoma patients, with rapid and high response rates and an improvement in survival compared with dacarbazine.[5–9] These treatments are now Food and Drug Association approved for patients with *BRAF*-mutant metastatic melanoma.

At present there is uncertainty regarding which melanoma specimen (ie, whether a distant metastasis, locoregional metastasis, or primary melanoma) should be used to determine the *BRAF* status of metastatic melanoma in a patient. For patients with metastatic melanoma, common clinical practice is to test the most recently obtained, available, and suitable specimen, usually distant metastatic tissue, provided there is adequate quantity of viable tumor cells and minimal admixture of nontumor cells in order to obtain an accurate result. In some cases this requires rebiopsy of the patient for the sole purpose

of *BRAF* testing. Evidence to support this approach comes from data suggesting that intertumoral heterogeneity may exist in 15% to 44% of patients, between a primary melanoma and metastases or between metastases.[10,11] This is despite the fact that it would be unlikely for melanoma to lose the *BRAF* driver mutation through disease progression.

The rapid and accurate determination of melanoma *BRAF* status is vital in planning treatment for patients with metastatic melanoma, and adjuvant clinical trials of BRAF inhibitors have also recently commenced (NCT01667419, NCT01682083). Immunohistochemistry (IHC) using the VE1 antibody is highly sensitive and specific for the $BRAF^{V600E}$ protein but does not detect non-V600E BRAF–mutated proteins such as $BRAF^{V600K}$ or $BRAF^{V600R}$.[12–14] IHC has advantages over molecular techniques in that it uses minimal tissue, can be used in specimens with low tumor content, and can provide a result at the time of pathologic diagnosis. It has therefore been argued that IHC using VE1 should be the first test used to determine *BRAF* status, with additional molecular tests for less prevalent, but clinically relevant, *BRAF*-mutant genotypes like V600K[15] and V600R,[16] and other mutations (such as *NRAS* or *C-KIT*) only in those with a negative or inconclusive result.[14]

We sought to examine the intrapatient heterogeneity of $BRAF^{V600E}$ protein expression among patients with multiple tumor specimens. We hypothesized that $BRAF^{V600E}$ mutation status, as determined by $BRAF^{V600E}$ protein expression, displays intertumoral and intratumoral homogeneity throughout all stages of melanoma disease progression.

MATERIALS AND METHODS

Patients and Specimens

The study was undertaken with Human Ethics Review Committee approval and patient informed consent. The Melanoma Institute Australia database and archival files of the Department of Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, were used to indentify stage IIIC/IV melanoma patients with known molecularly determined BRAF mutation status and with multiple formalin-fixed paraffin-embedded (FFPE) melanoma specimens available at various stages of disease progression. In patients with a history of multiple primary melanomas, the culprit primary melanoma was selected for examination based on a predefined algorithm.[17]

Molecular Mutation Testing for the *BRAF* Gene

BRAF mutation testing was performed on sections from archival FFPE tissue blocks, and specimens were tested either at the Department of Diagnostic Molecular Pathology, Peter MacCallum Cancer Centre (Melbourne, Vic., Australia) or the Department of Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital (Sydney, NSW, Australia). At the Peter MacCallum Cancer Centre, specimens were macrodissected from FFPE sections, and extracted DNA was subjected to high-resolution melt analysis using primers flanking codon 600 in the *BRAF* gene. These primers identify variations in exon 15 of the *BRAF* gene between nucleotides c.1788 and c.1823 in reference sequence NM_004333.4, corresponding to codons 597 to 607. All abnormal high-resolution melt traces were subjected to bidirectional DNA sequencing using the primers described above. At the Royal Prince Alfred Hospital, specimens were macrodissected from FFPE sections, and extracted DNA was amplified for 238 variant targets in a 24 multiplex polymerase chain reaction using the OncoCarta Panel v1.0 Kit and analyzed on the basis of the matrix-assisted laser desorption ionization-time of flight mass spectrometry technology on the Sequenom MassArray platform.

VE1 IHC for the $BRAF^{V600E}$ Protein

The FFPE melanoma specimens used for IHC included the blocks used for molecular mutation testing in all patients. Two 4-μm-thick sections were cut, the first for hematoxylin and eosin (H&E) staining to confirm the presence of melanoma and the second for VE1 staining. IHC was performed using an automated IHC system (Ventana BenchMark ULTRA; Ventana Medical Systems Inc., Tucson, AZ) utilizing the OptiView DAB IHC Detection Kit. After deparaffinization of FFPE sections, heat-induced epitope retrieval was applied using CC1 for 64 minutes. The sections were incubated with anti-BRAF mouse antiserum VE1 (1:50 dilution; Spring Bioscience, Pleasanton, CA) for 1 hour, followed by detection and visualization with the Ventana OptiViewTM Universal DAB Detection Kit (Optiview HQ Linker 8 min, Optiview HRP Multimer 8 min, Optiview H_2O_2/DAB 8 min, Optiview Copper 4 min). Counterstaining was performed using the hematoxylin II counter stain for 4 minutes and then with a blueing reagent for 4 minutes. A positive control was included in each IHC round.

IHC Staining Evaluation

All H&E and immunostained slides were evaluated twice by 2 independent observers (A.M.M. and R.A.S.) blinded to all clinical, histopathologic, and mutation data. H&E slides were reviewed to confirm the presence of melanoma. The $BRAF^{V600E}$ VE1 antibody staining in melanoma cell cytoplasm was scored for the percentage of immunoreactive cells. Intensity of staining was judged on a semiquantitative scale of 0 to 3+: no staining (0), weakly positive staining (1+), moderately positive staining (2+), and strongly positive staining (3+). Slides that differed in intensity between the 2 observers were then viewed by both observers together to reach agreement on any discordant scores. In addition, sections were assessed for intratumoral homogenous or heterogenous expression of $BRAF^{V600E}$ protein. Heterogenous intensity was defined as the presence of distinct subpopulations of cells that had an immunoreactive intensity score that differed by 1 or more scoring level (eg, one population of cells with 3+ and another with 2+). Any type of isolated nuclear staining, weak staining of single interspersed cells, or staining of monocytes/macrophages was scored negative. Heavily pigmented areas were avoided. Melanoma

cells undergoing early necrosis were excluded, as this has previously been shown to affect the antigenicity of the VE1 epitope.[13,18]

RESULTS

Patients and Tumor Specimens

A total of 171 melanoma specimens from 64 patients were included for analysis (Table 1). The 64 specimens (1 for each patient) that underwent prior molecular testing consisted of primary melanomas (n = 7, 11%), lymph node metastases (n = 24, 37%), and distant metastases (n = 33, 52%). The 171 specimens that underwent IHC with VE1 consisted of primary melanomas (n = 41, 24%), lymph node metastases (n = 47, 28%), and distant metastases (n = 83, 49%). Distant metastatic sites included skin/subcutaneous (n = 47), viscera (n = 25), soft tissue and bone (n = 6), and brain (n = 5). Of the 41 patients with a primary melanoma used for VE1 staining, 7 (17%) had a history of multiple primary melanomas before advanced disease. The culprit primary melanoma corresponding to the source of the metastases was determined as previously described.[17]

Nineteen (30%) patients had primary, lymph node, and distant metastasis triplicate specimens, 11 (17%) had a primary melanoma and at least 1 distant metastasis, 11 (17%) had a primary melanoma and lymph node metastasis, 16 (25%) had lymph node and distant metastases, and 7 (11%) had multiple distant metastases (Table 2). The maximum number of specimens tested in a patient was 5 (n = 6 patients). Thirty (47%) patients had *BRAF*^{V600E} melanoma on the basis of molecular testing, 7 (11%) patients had non-V600E *BRAF* mutations (V600K, n = 5; K601E, n = 1; V600_K601E, n = 1), and 27 (42%) had wild-type *BRAF* melanoma (Table 2).

VE1 IHC Characteristics

All 171 specimens contained melanoma on H&E slides. Of these, 98 specimens (57%) showed no immunoreactivity with VE1 (intensity = 0, VE1-negative). Of the 73 (43%) specimens with VE1 reactivity (VE1-positive), most stained strongly. One (1%) sample had an intensity of 1+, 21 (29%) had 2+, and 51 (70%) had 3+ intensity. There was no intratumoral heterogeneity, as all melanoma cells in all sections stained homogenously, with no populations of VE1-positive and VE1-negative cells observed, and uniform staining intensity throughout. Artifactual heterogeneity of staining was observed, influenced by fixation, necrosis, and folding of the sections. Coarse and fine dust brown/black melanin pigment appeared distinct from the diffuse brown cytoplasmic staining in VE1 immunoreactive cases.

TABLE 1. Melanoma Specimens Tested for BRAF^{V600E} Expression By IHC

Specimen	No. Tested By IHC (No. Molecularly Tested)
Primary melanoma	41 (7)
Lymph node metastasis	47 (24)
Distant metastasis	83 (33)
Total	171 (64)

TABLE 2. Patients Included for Analysis on the Basis of Types of Melanoma Specimens (Primary, Lymph Node Metastasis, Distant Metastasis) Available for VE1 IHC, and by Molecular Assessment of *BRAF* Status

	Total (n [%])	V600E (n)	Non-V600E (n)
PLM	19 (30)	5	14
PM	11 (17)	4	7
PL	11 (17)	5	6
LM	16 (25)	11	5
Multiple M	7 (11)	5	2
Total	64 (100)	30	34

L indicates lymph node metastasis; M, distant metastasis; P, primary melanoma.

Comparison of BRAF^{V600E} IHC and Genomic Mutation Testing

All specimens with *BRAF*^{V600E} melanoma on molecular testing (n = 30) also were VE1 positive. All cases with wild-type or non-V600E *BRAF* melanoma on mutation testing (n = 34) were VE1 negative.

Intrapatient Intertumoral Concordance

All specimens from every melanoma patient displayed intertumoral concordance of BRAF^{V600E} immunoreactivity status (Fig. 1). Of the 30 patients with VE1-positive tumors, 21 (70%) had concordant VE1 staining intensity between specimens. Nine patients had discordant staining intensity; however, only in 1 patient was this >1 level (lymph node metastasis 2+, first distant metastasis 1+, second distant metastasis 3+). Seven of the 9 patients had specimens at different stages of melanoma progression. Of these, 4 demonstrated an increase in intensity from primary melanoma or lymph node metastasis (2+) to distant metastasis (3+). One case displayed a reduction in intensity from lymph node (3+) to distant metastasis (2+), whereas another had variable staining from lymph node (2+) to 2 distant metastases (1+ and 3+). In 2 patients with multiple distant metastases (one patient with 5 metastases, another with 3), specimens had immunoreactivity scores of 2+ or 3+, and no temporal association was seen regarding the date of metastasis resection and intensity score.

DISCUSSION

The *BRAF* status of a patient's melanoma is a critical factor when determining clinical management for patients with metastatic disease. Several clinical correlates exist to help predict *BRAF* status,[1,2,15] and several accurate methods for determining *BRAF* status have been developed, including molecular methods[14,19,20] and, most recently, VE1 IHC.[12–14] Clinicians ordering *BRAF* tests for patients with metastatic melanoma must decide on the selection of available

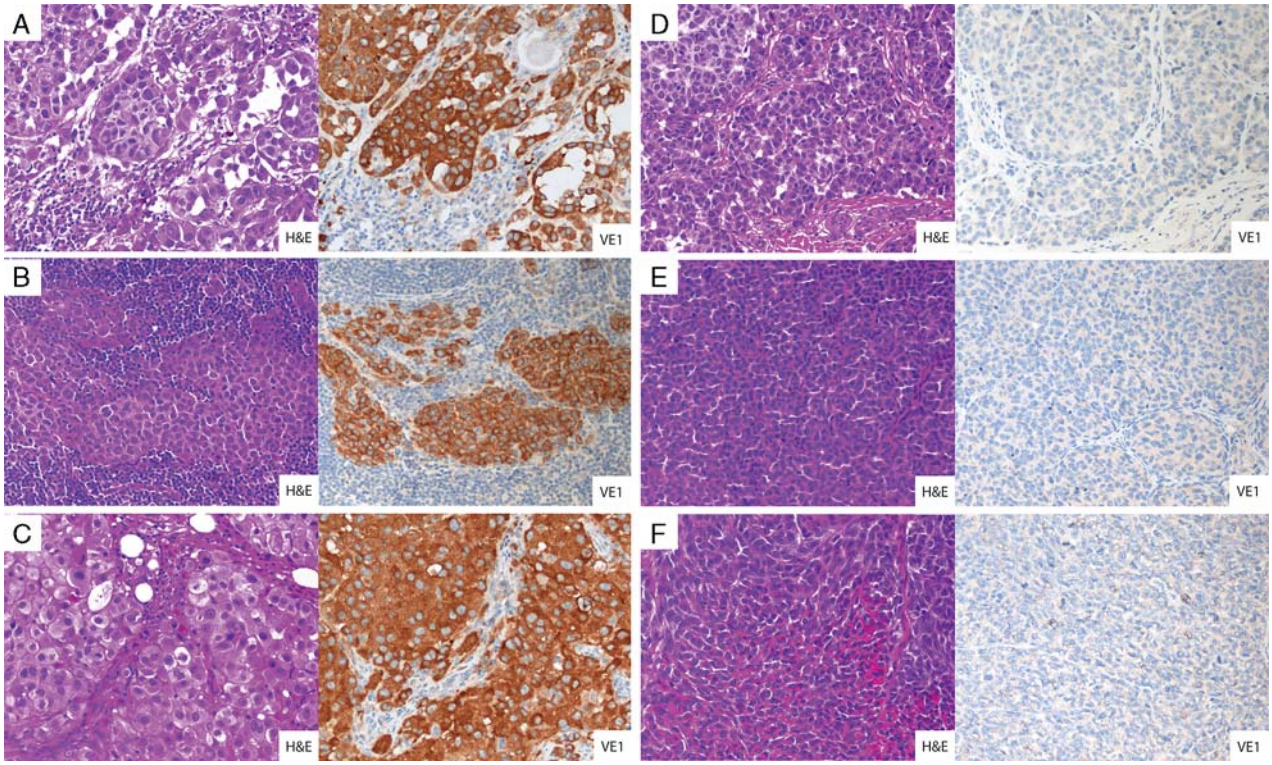

FIGURE 1. Examples of VE1-positive (A–C) and VE1-negative (D–F) triplicate biopsies from patients. VE1-positive (A) primary melanoma, (B) lymph node metastasis, and (C) distant metastasis. VE1-negative (D) primary melanoma, (E) lymph node metastasis, and (F) distant metastasis.

specimens for testing and whether patients should undergo biopsy of a metastatic lesion for the specific purpose of *BRAF* testing if earlier-stage archival tissue exists. Results from this study suggest that melanoma $BRAF^{V600E}$ status, as determined by $BRAF^{V600E}$ expression using IHC with the VE1 antibody, is 100% concordant between and within tumors of an individual and that, therefore, any tumor from an individual may undergo *BRAF* mutation testing, provided there is adequate quantity of viable tumor cells and minimal admixture of nontumor cells. Because no normal structures contain mutant $BRAF^{V600E}$ protein, it is important that appropriate positive controls be utilized when performing VE1 IHC.

The use of IHC in this study has a major advantage compared with molecular techniques as it was possible to directly visualize and determine $BRAF^{V600E}$ status in individual tumor cells throughout the whole specimen. Additional strengths of this study include the use of a standardized IHC technique in FFPE specimens, performance and scoring of IHC staining blinded to clinical and molecular data, the independent determination of protein expression by 2 separate investigators, correlation of molecular and IHC data, the inclusion of patients with multiple melanoma specimens (up to 5), a large proportion of whom had triplicate primary, lymph node, and distant metastatic specimens, and the inclusion of patients with several molecularly determined *BRAF* genotypes.

The demonstration of intertumoral homogeneity of $BRAF^{V600E}$ status in all cases included in this study suggests that a clinician may test any melanoma lesion for *BRAF* mutation, and the need for a biopsy for the sole purpose of *BRAF* testing may not be necessary, provided there is adequate quantity of viable tumor cells and minimal admixture of nontumor cells. Previous studies have reported discordance of *BRAF* status between primary melanoma and metastases and between metastases, in up to 44% of patients.[10,11] These results may have been influenced by the molecular method of testing in small primary or lymph node melanoma metastases, whereby nonmelanoma DNA may have been sampled or sufficient cell purity could not be obtained (some molecular techniques are unable to precisely detect the presence of mutations if they are present in $< 25\%$ of the DNA in the sample),[21,22] or by testing primary melanomas that were not the culprit tumors related to the subsequent disease.

Most patients with *BRAF*-mutant metastatic melanoma develop resistance to BRAF inhibitors after 6 to 7 months.[8,9] The presence of wild-type *BRAF* clones within the de novo metastatic population would strongly influence the pattern of response to BRAF inhibitors. However, clinical evidence with BRAF inhibitors is consistent with the findings in this study, as tumors have a uniform initial metabolic response to BRAF inhibition by 18F-fluorodeoxyglucose-positron emission tomography imaging,[23]

and resistant lesions resected from patients still contain mutant *BRAF*.[24]

Care is required when selecting the primary melanoma for *BRAF* testing if multiple primary melanomas exist or if the primary melanoma was of low stage, lymphatic recurrence was not regional, or the time period from primary to metastasis was not as expected. In this study, 17% of patients with a primary melanoma used for VE1 staining had a history of multiple primary melanomas before advanced disease. By assigning the culprit primary melanoma using a predefined algorithm[17] and selecting it for VE1 assessment, no discordance with later metastases was seen.

Intertumoral heterogeneity of VE1 staining intensity was observed in a minority of BRAF^{V600E} immunoreactive cases. When present, most specimens demonstrated an increase in staining through tumor progression; however, the number of cases was small, and this finding was not consistent. The use of the VE1 antibody, optimized to maximize sensitivity and specificity for detecting BRAF^{V600E} protein, may have prevented the relative assessment of expression in immunoreactive specimens from a patient.

No intratumoral heterogeneity of BRAF^{V600E} expression was observed in this study, including in primary melanomas. A minority of specimens that initially appeared to display intratumoral heterogeneity had artifactual staining patterns, such as a lack of staining in necrotic areas, which has been previously described,[25] and nonspecific staining next to areas of folding of the tissue specimen. The observation of intratumoral homogeneity is in contrast to a previous study using laser microdissection and a mutation-specific Snapshot assay[10] but confirms previous reports of intratumoral homogeneity of VE1 BRAF^{V600E} status.[13,25,26] As previously stated, molecular techniques may be influenced by tumor cell purity, and nonmelanoma DNA can be inadvertently assessed. Furthermore, the clinical relevance of molecularly discovered wild-type *BRAF* minor subclones in a predominantly *BRAF*-mutant tumor (or vice versa) may be of little clinical significance, as treatment decisions would likely be made on the basis of the dominant clone in the tumor, and BRAF inhibitor resistance does not involve the emergence of wild-type *BRAF* clones. Other molecular aberrations present in subclones that may lead to the development of resistance to BRAF inhibitor therapy may be important, however, and warrant further investigation.

Adjuvant clinical trials in high-risk early-stage *BRAF*-mutant melanoma have recently commenced (NCT01667419, NCT01682083), enrolling patients with AJCC stage IIC (thick ulcerated primary, lymph node negative) and III disease.[27] Demonstration of homogeneity of the *BRAF* genotype in various stages of disease progression in this study validates this treatment approach, as the distant metastatic disease *BRAF* genotype appears concordant with the primary and locoregional disease.

This study only assessed for the presence of BRAF^{V600E}, not other V600 *BRAF* mutations or other mutations (eg, *NRAS*, *C-KIT*) that are also suitable for targeted therapies.[16,28–32] Although it is likely that non-V600 *BRAF* mutation status is similarly conserved throughout tumor progression, it is not known whether other mutations may share this trait.

In summary, BRAF^{V600E} expression assessed with IHC using the VE1 antibody displays complete intertumoral homogeneity, minimal intertumoral intensity heterogeneity, and no intratumoral heterogeneity in metastatic melanoma patients through all stages of disease progression. The results suggest that, provided there is adequate quantity of viable tumor cells and minimal admixture of nontumor cells, testing any melanoma sample from a patient with metastatic disease will accurately determine *BRAF* status for treatment planning.

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