Immunohistochemistry Is Highly Sensitive and Specific for the Detection of V600E *BRAF* Mutation in Melanoma

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Abstract: This study investigated the sensitivity and specificity of immunohistochemical (IHC) analysis using an anti-*BRAF* antibody to detect the presence of the *BRAF* V600E mutation in patients with metastatic melanoma. A total of 100 patients with American Joint Committee on Cancer stage IIIC unresectable or stage IV melanoma and who underwent tumor DNA *BRAF* mutation testing were selected. Paraffin-embedded, formalin-fixed melanoma biopsies were analyzed for the *BRAF* mutation status by independent, blinded observers using both conventional DNA molecular techniques and IHC with the novel *BRAF* V600E mutant-specific antibody, VE1. The antibody had a sensitivity of 97% (37/38) and a specificity of 98% (58/59) for detecting the presence of a *BRAF* V600E mutation. Of the *BRAF*-mutated cases, none of the non-V600E cases (including V600K) stained positive with the antibody (0/11). There were 5 cases with discordant *BRAF* mutation results. Additional molecular analysis confirmed the immunohistochemically obtained *BRAF* result in 3 cases, suggesting that the initial molecular testing results were incorrect. Two of these patients would not have received a *BRAF* inhibitor on the basis of the initial false-negative mutation testing result. Two cases remained discordant. The reported IHC method is an accurate, rapid, and cost-effective method for detecting V600E *BRAF* mutations in melanoma patients. Clinical use of the V600E *BRAF* antibody should be a valuable supplement to conventional mutation testing and allow V600E mutant metastatic melanoma patients to be triaged rapidly into appropriate treatment pathways.

Key Words: *BRAF*, immunohistochemistry, mutation testing, human melanoma, targeted therapies, personalized medicine, V600E, *BRAF* mutation, V600K, *BRAF* inhibitor

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The RAS/RAF/MEK/ERK, mitogen activated protein kinase pathway regulates cellular proliferation, survival, and migration and is aberrantly activated in the majority of melanomas and solid tumors. Mutation of the *BRAF* gene is one mechanism of aberrant activation and occurs in many human cancers including cutaneous melanoma (50%), papillary thyroid cancer (46%), borderline ovarian tumor (34%), pleomorphic xanthoastrocytomas (66%), biliary tract tumor (11%), colorectal cancer (10%), non–small cell lung cancer (2%), and hairy cell leukemia (100%) among others.[1–3] The most common *BRAF* mutation is a single amino acid substitution of valine for glutamic acid at residue 600 (V600E), and occurs in approximately 75% of patients with *BRAF*-mutant metastatic melanoma.[4,5] In melanoma patients less than 40 years of age, >80% have a *BRAF* mutation of which >85% are V600E.[5] *BRAF* mutations have been reported to be associated with poor prognosis in

melanoma,[5] colorectal cancer,[6,7] and papillary thyroid carcinoma.[8,9] Potent inhibitors of V600 mutant *BRAF* have revolutionized the treatment of metastatic melanoma as a result of high response rates and their rapid mode of action and have recently been proven to improve progression-free and overall survival.[10,11] Delays in the diagnosis and treatment of cancer patients may compromise their quality of life and survival, particularly with the emergence and availability of fast-acting and effective targeted therapies. Accurate and rapid detection of *BRAF* mutations at the time of diagnosis of metastatic melanoma (and other tumors) is therefore essential for optimal patient care.

Tests to determine the tumor *BRAF* genotype include real-time polymerase chain reaction (RT-PCR),[10] coamplification at low denaturation temperature PCR,[12] locked nucleic acid PCR sequencing,[13] mismatch ligation assay,[14] allele-specific PCR,[15] array analysis,[16] high-resolution melting curve analysis (HRM),[5] pyrosequencing,[17] and the most commonly used, Sanger sequencing.[2] In most laboratories, combinations of methods are utilized to identify the specific *BRAF* genotype. Each of the aforementioned methods has differing sensitivities (80% to 99%), specificities, and costs. Furthermore, they require the use of specialized and expensive equipment and techniques (which are currently not readily available in most diagnostic pathology laboratories) and rigorous quality control. Hence, in routine clinical practice tumor tissue may need to be sent to specialized laboratories for molecular testing, which inevitably results in a delay in defining the patient's optimal management. Immunohistochemical (IHC) analysis is a technique that is widely utilized in routine diagnostic pathology laboratories and, in contrast to the molecular techniques described above, is more rapid and potentially cheaper and more sensitive. It also has the added advantage of allowing visualization of individual antigen-bearing tumor cells—for example, single metastatic tumor cells in lymph nodes. Thus, development of IHC methods to detect *BRAF* mutations would improve melanoma patient care by providing rapid diagnosis, conserving patient tissue, and being cost-effective and accessible to most pathology departments. Here we report the sensitivity and specificity of an anti-*BRAF* antibody to detect V600E mutations in patients with metastatic melanoma.

PATIENTS AND METHODS

Patients

One hundred consecutive patients with American Joint Committee on Cancer stage IIIC unresectable or stage IV melanoma[18] and who underwent tumor DNA *BRAF* mutation testing at the Peter MacCallum Cancer Centre, Department of Diagnostic Molecular Pathology (Melbourne, Australia) via Melanoma Institute Australia (MIA) and had available tissue were included in this study. This study was conducted with human ethics review committee approval.

Immunohistochemistry

Anti-*BRAF* V600E immunostaining was performed on the same tissue block used for mutation testing (as described below), using the monoclonal mouse antibody VE1 as described previously on 4-μm-thick tissue sections of formalin-fixed, paraffin-embedded (FFPE) tumor tissue blocks (Heidelberg, Germany).[19] Briefly, sections were dried at 80°C for 15 minutes and stained with the undiluted hybridoma supernatant of *BRAF* V600E-specific clone VE1 (provided by A.v.D., also commercially available at Spring Bioscience, Pleasanton, CA) on a Ventana BenchMark XT immunostainer (Ventana Medical Systems, Tucson, AZ). The Ventana staining procedure included pretreatment with cell conditioner 1 (pH 8) for 60 minutes, followed by incubation with the VE1 antibody at 37°C for 32 minutes. Antibody incubation was followed by standard signal amplification with the Ventana amplifier kit, ultra-Wash, and counterstaining with 1 drop of hematoxylin for 4 minutes and 1 drop of bluing reagent for 4 minutes. For chromogenic detection, ultraView Universal DAB detection kit (Ventana Medical Systems) was used. Subsequently, slides were removed from the immunostainer, washed in water with a drop of dishwashing detergent, and mounted. No chromogen was detected when primary antibody *BRAF* V600E clone VE1 was omitted.

All histologic slides were freshly cut before IHC analysis. All immunostained slides were evaluated twice by 2 independent observers (D.C. and M.P.) blinded to all clinical, histopathologic, and genetic data. The VE1 antibody staining was scored as positive when the majority of viable tumor cells showed clear cytoplasmic staining. The VE1 antibody staining was scored as negative when there was no staining or only isolated nuclear staining, weak staining of single interspersed cells, staining of monocytes/macrophages, or faint diffuse staining. Cases were scored as not determinable when no tumor could be identified on the slide.

Mutation Testing

BRAF mutation testing was performed on sections from archival FFPE tissue blocks and samples were tested at the Peter MacCallum Cancer Centre, Department of Diagnostic Molecular Pathology (Melbourne, Australia). All samples were locoregional or distant metastases except in 10 patients, for whom the primary cutaneous melanoma was used. Samples were macrodissected and subjected to HRM 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 HRM traces were subjected to bidirectional DNA sequencing using the primers described above.

More extensive sequencing was performed on samples from patients considered possible candidates for the GlaxoSmithKline (GSK) phase 1 clinical trial of the selective *BRAF* inhibitor Dabrafenib (GSK2118436). Samples were amplified with M13-tagged primers flanking

exon 15 of *BRAF* and sequenced using M13 primers. Sequence data were obtained for the whole of exon 15 comprising nucleotides c.1742 to c.1860 in reference sequence NM_004333.4, corresponding to codons 581 to 620.

Discordant IHC-negative and *BRAF* V600E mutation-positive results were retested at the Peter MacCallum Cancer Centre as mentioned above. Discordant IHC-positive and *BRAF* V600E mutation-negative results were retested for the presence of a *BRAF* mutation utilizing a different molecular methodology at Healthscope Pathology (Clayton, Australia). Hematoxylin and eosin-stained sections of FFPE tissue were reviewed by a pathologist, followed by macrodissection to ensure the percentage of tumor cells was enriched to at least 60%. DNA was then extracted using the Qiagen QIAmp mini extraction columns. Exon 15 of the *BRAF* gene was amplified by PCR. A single-base extension assay was performed using a forward primer that interrogated the nucleotide at position 1798, as well as a reverse primer that interrogated the nucleotide at position 1799. The primers were designed using the Sequenom Assay Designer software. Standard protocols based on the Sequenom Typlex kit were then followed according to the manufacturer's instructions. Products of the extension reaction are fired and analyzed on a Sequenom Massarray mass spectrometer.

RESULTS

The 2 independent observers were concordant in VE1 antibody staining assessment in the 100 cases, although concordance was achieved after blinded reevaluation in 2 cases. Three cases (3%) were found to contain no tumor cells for evaluation, which was subsequently confirmed on independent pathologic examination.

Of the 97 assessable cases, 47 cases were *BRAF* mutant on initial DNA sequencing (V600E = 37, V600K = 6, and other *BRAF* mutations = 4). Of the *BRAF* mutant cases, none of the non-V600E stained positively with the VE1 antibody (Fig. 1B). Thirty-five of the 37 V600E *BRAF* mutant tumors stained positively for the VE1 antibody (Fig. 1C; Table 1). Both V600E-mutated primary tumors and metastases stained positively with VE1, but the staining was more easily assessed in metastatic lesions. The tumor for 1 of the 2 discordant cases was obtained by fine-needle biopsy, and a cell block was produced using plasma and fibrinogen to clot the aspirate and an alcohol-formalin-acetic acid solution to fix the sample (Fig. 1F). The other discordant case was resequenced (the same block that was used for initial sequencing and VE1 staining), and a K601Q mutation was detected instead of the initial reported V600E *BRAF* mutation. Of the 50 *BRAF* wild-type melanomas, 47 were negative for the VE1 antibody (Fig. 1A), and 3 stained positively (Fig. 1D). Molecular retesting for the *BRAF* mutation status of the 3 discordant cases with PCR-mass spectrometry (ie, a more sensitive technique compared with that originally used) identified a V600E mutation in 2 cases (Fig. 1D; Table 1). The 1 case that remained *BRAF* wild type on retesting was a lymph node that contained only scattered single and small clusters of

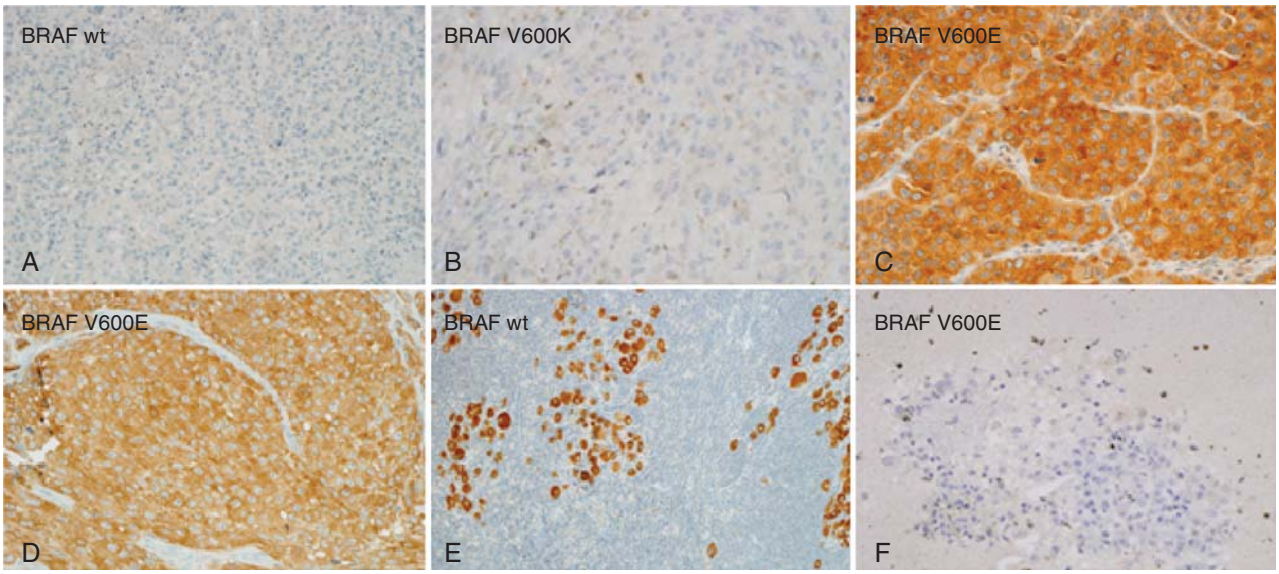

FIGURE 1. IHC staining with the VE1 antibody visualized using the chromogen diaminobenzene (brown staining) in metastatic melanoma. A, *BRAF* wild-type (wt) melanoma, which is negative for VE1. B, *BRAF* V600K-mutated melanoma, which is negative for VE1 (brown melanin pigment is present in a few melanoma cells). C, *BRAF* V600E-mutated melanoma strongly positive for VE1. D, Discordant case that was strongly VE1 positive and *BRAF* wt on original mutation testing. Upon retesting, a *BRAF* V600E mutation was detected. E, Lymph node containing scattered single and small clusters of strongly VE1-positive melanoma cells in a background of numerous lymphocytes. This case was *BRAF* wt on original and repeat mutation testing. F, Discordant fine-needle biopsy case showing negative VE1 staining in the cell block preparation. Mutation testing detected a *BRAF* V600E mutation. The cell block was prepared and fixed using techniques that differed from those used for all other cases (which utilized FFPE tissues).

TABLE 1. Discordant Cases of Genetic *BRAF* Analysis and VE1 IHC and Reanalysis Results

Initial Genetic *BRAF* Analysis	*BRAF* VE1 Antibody	Genetic Reanalysis	Conclusion
Wild type	Positive	V600E*	Initial genetic testing false negative
Wild type	Positive	V600E*	Initial genetic testing false negative
Wild type	Positive	Wild type*	Probable false-negative genetic testing‡
V600E	Negative	K601Q†	Initial genetic testing false positive
V600E	Negative	V600E*†	VE1 antibody false negative§

*Retesting using PCR-mass spectrometry.
†Retesting using PCR-HRM sequencing.
‡Specimen contained only scattered tumor cells in a background of numerous lymphocytes (Fig. 1E) (see text for details).
§Fine-needle biopsy cytology specimen fixed and processed differently from all other specimens (see text for details).

melanoma cells in a background of numerous small lymphocytes (Fig. 1E). It is therefore highly probable that the ratio of tumor to nontumor nuclei in the specimen used for mutation testing was very low.

Using the mutational status from the retested discordant cases (Table 2), the sensitivity of this VE1 antibody was 97% (37/38) and specificity was 98% (58/59). The positive and negative predictive values were 97% (37/38) and 98% (58/59), respectively.

DISCUSSION

Metastatic melanoma carries a poor prognosis, with a median overall survival of 9 to 10 months,[18] and patients often have extensive and rapidly progressing disease. V600E *BRAF* mutant metastatic melanoma is highly sensitive to *BRAF* inhibition with vemurafenib (PLX 4032, RG7204, RO5185426) and dabrafenib (GSK2118436), with high response rates[20] and improved progression-free and overall survival.[10,11] Rapid analysis of the *BRAF* genotype is critical for treatment decisions that significantly impact patient outcomes. Because testing for *BRAF* mutations currently requires the use of molecular techniques that are not currently accessible or available in most diagnostic pathology laboratories, testing may require the laborious process of block retrieval, sectioning, postage, macrodissection, and DNA extraction before *BRAF* testing can commence. Furthermore, these molecular techniques are time consuming and expensive to perform. As a consequence, it may be a number of weeks before the result of a patient's *BRAF* mutation test is known. In contrast, mutation testing with IHC offers many potential advantages; it can be performed and reported within 24 to 48 hours of tumor excision, is inexpensive, is widely available in (almost all) pathology departments, and requires minimal tissue (a single 4-μm-thick section from a tissue block compared with up to 60 μm required for traditional PCR/sequencing methods).

TABLE 2. Summary of the Mutation Testing and VE1 IHC Analysis of *BRAF* V600E Mutation Status

Genotype	*BRAF* Sequencing (After Reanalysis of Discordant Cases) (n)	*BRAF* VE1 Antibody Positive (n)	*BRAF* VE1 Antibody Negative (n)
V600E	38	37	1*
V600K	6	0	6
Other†	5	0	5
Wild type	48	1	47

Sensitivity = 37/38 (97%), positive predictive value = 37/38 (97%), specificity = 58/59 (98%), and negative predictive value = 58/59 (98%).
*This case was the only cell block of the series and was processed differently.
†Other mutations = D594N, K601E, T599dup, V600_K601E, and K601Q.

This study compares an IHC antibody-derived method of identification of the V600E *BRAF* mutant protein with PCR and sequencing-based mutation tests in melanoma patients. Our results confirm that the VE1 antibody is highly sensitive (97%) and specific (98%) for the presence of a *BRAF* V600E mutation, consistent with a previous study of a range of solid tumors, which included 50 brain melanoma metastases.[19,21] Unlike previous studies, the current study includes a consecutive cohort of 100 melanoma patients with metastases from a large number of different anatomic sites. Sequencing was performed on all specimens, which enabled the detection of all relevant *BRAF* mutations in the V600 region (11% were non-V600E mutations in the current study) in contrast to the prior study in which all *BRAF* mutant cases were V600E *BRAF* mutants. In addition, discordant cases were subjected to multiple methods of DNA *BRAF* mutation testing, which permitted an accurate analysis of the positive and negative predictive values of the VE1 antibody. There were 5 discordant cases initially, but after repeating the DNA mutational analysis, only 2 remained discordant, suggesting that the antibody may be more sensitive for the V600E mutation than traditional sequencing techniques. Of the 2 persistent discordant cases, the first stained negatively with a V600E sequencing result; however, this was the only case in which IHC analysis was carried out on a cell block preparation collected with a fine-needle biopsy, which was processed with a clotting agent and a fixative different from those used in the other cases. Biopsy and fixation methods have been found to affect the accuracy of the IHC analysis in such cases.[19] The second stained positively but was *BRAF* wild type on both PCR-HRM sequencing and PCR-mass spectrometry. The tissue block had a very low tumor to normal cell ratio (less than approximately 5%), which may have resulted in a false-negative result in the mutation testing, because the tumor DNA content was below the threshold required to detect a *BRAF* mutation using the molecular techniques.

The VE1 antibody was able to discriminate with 100% accuracy the V600E mutant cases from the other *BRAF* mutant forms, as it did not show positive staining in other *BRAF* genotypes, such as V600K, K601E, K601Q, or T599dup. *BRAF* inhibitors have shown clinical activity in melanoma patients harboring a *BRAF* mutation other

than V600E, such as V600K.[20,22] Therefore the identification of *BRAF* mutations other than V600E will still be required. Nevertheless, V600E testing by IHC will facilitate the rapid commencement of optimal care for the vast majority of *BRAF* mutant melanoma patients.

Intratumoral heterogeneity of *BRAF* mutation was not observed in this study, in which all wild-type and mutant cases showed either negative or positive VE1 staining in virtually all cells within the tumors examined. This differs from reports utilizing small numbers of cases using single-cell RT-PCR that suggest the majority of nevi, primary, and metastatic melanomas contain both wild-type and mutant *BRAF* cells.[23,24] Because of the potential importance of polyclonality to models of melanomagenesis and ultimately for developing effective therapeutic regimes for metastatic melanoma, further studies correlating VE1 histochemistry with single-cell RT-PCR in nevi and melanomas are urgently needed.

In conclusion, this VE1 antibody is highly sensitive and specific for the detection of V600E mutant *BRAF* melanoma in FFPE tissue when compared with *BRAF* genetic testing. This IHC method may be used to evaluate tissues with very low tumor content. It is cost effective, uses minimal tissue, and provides a result at the time of pathologic diagnosis. Use of the VE1 antibody is a valuable supplement to traditional mutation testing and allows V600E mutant patients to be triaged rapidly into appropriate treatment pathways; genetic testing would only be required for VE1-negative patients to detect non-V600E *BRAF* mutations.

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