

INVESTIGATIVE REPORT

Highly Concordant Results Between Immunohistochemistry and Molecular Testing of Mutated V600E BRAF in Primary and Metastatic Melanoma

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This study tested the sensitivity and specificity of VE1 antibody raised against BRAF^{V600E} protein, on 189 melanoma samples, compared with molecular testing. In addition, the therapeutic response to BRAF inhibitors was analysed in 27 patients, according to staining intensity (scored from weak to strong) and pattern (homogeneous or heterogeneous). BRAF^{V600E} status during melanoma progression was evaluated in a cohort of 54 patients with at least paired-samples. High sensitivity (98.6%) and specificity (97.7%) of VE1 were confirmed. During melanoma progression different samples showed concordant phenotypes. Heterogeneous VE1 staining was observed in 28.5% of cases, and progression-free survival was higher in patients with tumour samples displaying such staining. These findings suggest that only VE1-negative tumours would be genotyped to detect other BRAF^{V600} mutations, and that either primary melanoma or metastasis can be tested using immunohistochemistry, according to the material available. Key words: primary and metastatic melanoma; VE1 antibody; mutated BRAF protein; molecular testing; targeted therapy; survival.

Accepted Dec 16, 2015; Epub ahead of print Dec 22, 2015

Acta Derm Venereol 2016; 96: 630–634.

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Mutations at the V600 position of *BRAF*, conferring a constitutive kinase activity to BRAF protein, have been identified in approximately 50% of melanomas (1). Metastatic melanomas with activated mutant *BRAF*^{V600} are candidates for targeted therapy using inhibitors of BRAF kinase that improve patients' overall survival (2). The decision to treat metastatic melanoma with those molecules depends on the results of screening for *BRAF*^{V600} mutations. Several techniques, all based on molecular genotyping, are currently available to detect these mutations in formalin-fixed paraffin embedded (FFPE) samples, but these require specialized platforms. The production of a mouse monoclonal antibody (VE1 clone) against the

BRAF^{V600E} protein, which represents more than 80% of *BRAF*^{V600} mutations, is of great interest, as it appears to be highly sensitive and specific in *BRAF*^{V600E}-mutated melanomas (3–9). In contrast to DNA-based techniques, immunohistochemistry (IHC) could solve not only the problems of intra-tumoural heterogeneity previously questioned by Yancovitz et al. (10), but also overcome technical limitations in paucicellular samples.

The primary aim of our study was to determine whether this antibody could be a reliable surrogate for *BRAF*^{V600E} molecular testing in daily clinical management of metastatic melanoma. Thus, we investigated the expression of *BRAF*^{V600E} protein using VE1 antibody in melanoma samples, confronted with *BRAF*^{V600} genotyping. Then, because only a few studies have dealt with the discrepancy between primary and metastatic melanoma *BRAF*^{V600} status, we assessed *BRAF*^{V600} status after progression from primary melanoma to metastasis or between different metastases, in the same individual, then compared with to genotyping results. Finally, we determined whether VE1 staining intensity and/or homogeneity were correlated with therapeutic response to BRAF inhibitors.

MATERIALS AND METHODS

Patients and samples (Fig. S1¹)

From among the 411 melanoma samples sent from January 2011 and June 2013, either from our department of pathology or from regional departments of pathology, to the cancer molecular genetics centre at Toulouse Hospital to determine access to targeted therapy, only tissue blocks still available in our local archives were selected. A total of 189 unequivocal melanoma samples from 100 patients were retrieved from the archives of the Department of Pathology of Toulouse Hospital (Institut Universitaire du Cancer Toulouse Oncopole). These comprised 50 primary melanomas and 139 metastases. This retrospective study was conducted on American Joint Committee on Cancer stage IIIC unresectable or stage IV melanomas. For 46 patients, only 1 sample was available, while at least 2 samples were available for 54 patients (primary melanoma/metastasis or different metastases). Detailed information is given in Appendix S1¹.

All cases were FFPE samples. Patient samples were obtained after informed consent in accordance with the Declaration of

¹<http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-2326>

Helsinki and stored at the "CRB Cancer des Hôpitaux de Toulouse" collection. According to French law, CRB Cancer collection has been declared to the Ministry of Higher Education and Research (DC 2009–989) and obtained a transfer agreement (AC-2008-820) after approbation by ethics committees. Clinical and biological annotations of the samples have been declared to the Comité National Informatique et Libertés (CNIL).

Immunohistochemistry

Anti-BRAF^{V600E} staining was performed on the same block used for molecular analysis, using the VE1 mouse monoclonal antibody (Spring Bioscience Roche, ref E19294, Boulogne-Billancourt, France) diluted 1/100.

BRAF^{V600E} immunostaining was evaluated independently by two observers (one experienced dermatopathologist (LL) and one junior pathologist (LM)) blinded to mutational data. It was scored on a semi-quantitative scale: absent (0), weak (1+), moderate (2+), or strong (3+). Faint diffuse staining and weak staining of single interspersed cells were considered as negative. Samples with at least an area of 10% of tumour cells exhibiting a different staining intensity were considered heterogeneous. In cases with intra-tumoural heterogeneous staining, the percentage of immunostained tumour cells with different intensities was assessed. Then, the immunoreactive score (IRS) was analysed on the basis of intensity (1+ to 3+) and distribution (0–100% for each intensity): for example, a sample with 30% intensity 3+ and 70% intensity 2+ would be given a score of $(0.3 \times 3) + (0.7 \times 2) = 2.3$ (range 1–3). Cases that differed by a score >0.2 were reviewed by both observers together in order to achieve consensus. Weak positivity corresponds to an IRS ≤ 1.5 ; moderate > 1.5 and < 2.5 ; and strong ≥ 2.5 .

Mutational testing of BRAF^{V600}

Genomic DNA was extracted from 5 sections of FFPE block (each 5- μ m thick) using the QIAamp DNA FFPE extraction kit (Qiagen, Courtaboeuf, France), according to the manufacturer's protocol. Cases were enriched for tumour cell content by manual macrodissection following evaluation of the haematoxylin-eosin (H&E) section by a specialist pathologist (LL or ET). A brand new microtome blade was used for step sectioning each paraffin block to avoid DNA cross-contamination.

The optimized routine procedure consisted of a multistep analysis. Sequence alterations of exon 15 of the BRAF gene were scanned using high-resolution melting (HRM) analysis. Then, to identify the mutation precisely, variant cases were analysed by HRM using a TaqMan V600E specific polymerase chain reaction (PCR), since this sequence alteration is the most frequent, and submitted to direct sequencing only if this allele-specific PCR did not detect the BRAF^{V600E} mutation. Controls were conducted for all assays and included the absence of DNA and samples with known wild-type or mutant status. All assays were performed in duplicate. Details are given in Appendix S1¹.

Statistical methods

Progression-free survival (PFS) was analysed for patients with VE1-positive samples. PFS was defined as the time elapsed between the beginning of treatment by BRAF inhibitor to the first observation of progression of the disease. Patients were first separated into 2 groups: those with strong/moderate VE1 IRS and those with weak VE1 IRS; then patients with homogeneous or heterogeneous VE1 staining were compared. Median PFS in those groups were compared using Mann–Whitney test and log-rank test of Kaplan–Meier curves. Statistical analyses were performed using Graphpad prism V6.0 software.

RESULTS

Concordance between expression of the BRAF^{V600E} protein using VE1 IHC and BRAF^{V600} genotyping

Overall, 189 samples were immunostained in this study. Of these, 168 were genotyped for BRAF^{V600} mutation status, while 21 cases could not be tested because the proportion of residual tumour cells in the block was too small (Fig. S1¹, Table S1¹). Genotyping gave a non-interpretable result in only 7 cases, due to DNA quality. Therefore, the BRAF^{V600E} protein expression, assessed in a blinded fashion using the VE1 antibody in 189 samples, was confronted by BRAF^{V600} molecular testing in 161 cases (Table S1¹).

Eighty-eight samples showed positive cytoplasmic staining (46.5%). The staining was considered strong (IRS 2.5–3) in 45 cases, moderate (IRS 1.5–2.4) in 36 cases and weak (IRS 1–1.4) in 7 cases (Fig. S2A–C¹ and Table I). No isolate nuclear staining was observed in our series. The staining, assessed only in non-necrotic areas, was homogeneous in 71.5% of positive cases and heterogeneous in 28.5% (Table I and Fig. 1A, B). Cytoplasmic staining was easy to interpret in all cases, except for 2 heavily pigmented tumours. However, in those rare cases, we have always found some more easily interpretable areas that are less pigmented or achromic (Fig. 1 C, D).

Among the 88 VE1-positive samples, 74 had been genotyped with interpretable results: 72 harboured the BRAF^{V600E} mutation. One case (patient P31, Table S1¹) showed a BRAF^{V600} mutated profile by HRM, but the DNA quantity was too low to type the V600 mutation more precisely by Taqman assay or sequencing. Only one sample (from patient P32), with an IRS of 3, was found to be BRAF^{wild-type} by molecular testing, but this could be a molecular false-negative as the macrodissected tumour zone was extremely narrow.

BRAF^{V600} molecular status was determined in 87 VE1-negative cases (Fig. S2D¹): 76 cases (87%) had a BRAF^{wild-type} status, 7 cases (8%) harboured a BRAF^{V600K}, 1 case (1%) a BRAF^{V600R}, and 2 cases (2%) a synonymous BRAF^{V600V} mutation. Only one discrepant case was observed with negative VE1 staining and a BRAF^{V600E} molecular status (patient P69). The sensitivity of

Table I. Immunoreactive score of VE1 staining in the 88 positive samples and concordance with mutational BRAF status using DNA-based analyses

Immunoreactive score	Cases <i>n</i>	BRAF ^{V600E} mutated samples by
		genotyping/genotyped samples with interpretable results <i>n</i>
≥ 2.5	45	36/37
> 1.5 and < 2.5	36	29 ^a /30
≤ 1.5	7	7/7
Total	88	72/74

^aThe remaining case was BRAF^{V600} using high-resolution melting analysis, but could not be studied further due to low DNA quantity.

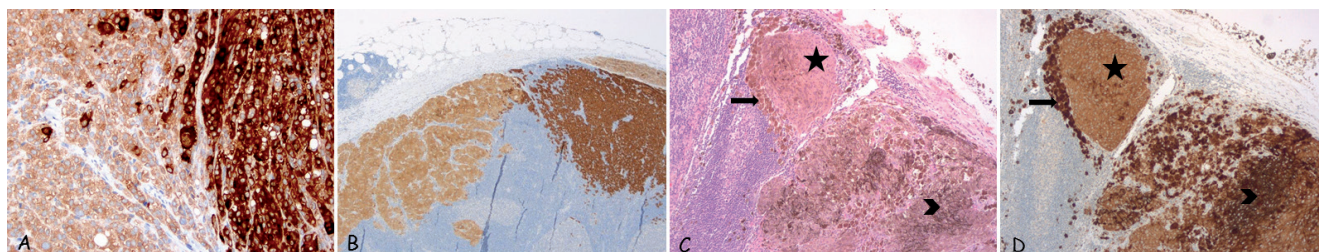


Fig. 1. (A, B) Representative images of intra-tumour heterogeneity of VE1 staining: 2 cases with distinct sub-clones, 1 being strongly positive (3+) for VE1 antibody, another 1 scored 2+ (A: magnification $\times 200$, Leica DM 3000 and B: magnification $\times 50$, Leica DM 3000). (C, D) Example of a heavily pigmented melanoma (arrowhead) metastatic to a lymph node and associated with melanin-laden histiocytes (arrow) (C: haematoxylin and eosin (H&E), magnification $\times 100$, Leica DM 3000) immunostained by VE1 (D: magnification $\times 100$, Leica DM 3000). Cytoplasmic staining is easier to detect in non-pigmented areas (★) than in pigmented tumour cells (arrowhead).

VE1 antibody for detecting the $BRAF^{V600E}$ mutation was 98.6% and the specificity was 97.7%.

Overall, concordance between IHC and molecular testing was 98% for $BRAF^{V600E}$ mutation detection.

Evaluation of $BRAF^{V600}$ status during melanoma progression

$BRAF^{V600E}$ status was evaluated by IHC in a cohort of 54 patients with at least paired-samples (from 2 to 6 samples per patient), either primary melanoma and metastasis or multiple metastases (Fig. S1¹). The same phenotype was found across different samples from the same individual: 31 patients presented with a VE1-negative melanoma; and 23 patients with a VE1-positive melanoma. During melanoma progression, metastatic melanoma samples harboured either the same IRS as the primary tumour (5 patients) or showed a higher IRS (6 cases), while in 2 patients, the IRS was higher in primary melanoma.

Genotyping analysis gave an interpretable result in at least 2 samples in 47 out of these 54 patients (Table SI¹). We observed a discrepancy between paired-samples in only 2 patients (P32 and P89) (Table SII¹).

Therapeutic response to $BRAF$ inhibitors

Therapeutic response was analysed for 28 patients with a VE1-positive tumour. Details of demographic data are summarized in Table II. For one patient, death occurred before the first evaluation, and therapeutic response to

$BRAF$ inhibitors was not analysed. Survival was evaluated for 27 patients. Median PFS was 173 days (interquartile range (IQR): 110–270) in the overall population. No significant difference in median PFS was observed between patients with samples displaying strong/moderate VE1 IRS staining and those with low IRS. However, median PFS was significantly higher in patients with samples displaying heterogeneous VE1 staining compared with samples displaying homogeneous VE1 staining ($n=8$; 330 days (IQR: 195–360) vs. $n=19$; 150 days (IQR: 106–210), respectively; $p=0.002$; hazard ratio = 2.84 (95% CI: 1.93–8.81); Fig. 2). No difference was observed in median IRS between patients with heterogeneous staining and patients with homogeneous staining (2.45 (IQR: 2.12–2.77) vs. 2.5 (IQR: 2.0–3.0); not significant (NS)).

DISCUSSION

This retrospective study confirms the high sensitivity and specificity of IHC using the VE1 antibody directed against the $BRAF^{V600E}$ mutated protein. Sensitivity was 98.6%, with only one discrepant case negative for VE1 and $BRAF^{V600E}$ by DNA analysis. Specificity was 97.7%, with only one case harbouring a strong VE1 staining and wild-type $BRAF^{V600}$ status, while the second case was $BRAF^{V600}$ mutated, but could not be more precisely genotyped. We can speculate that the former is a molecular false-negative, since the tumour content of the region to be macrodissected was very

Table II. Demographic data of patients treated with $BRAF$ inhibitor

Characteristics	
Age, years, median (IQR)	61 (45–68)
Sex ratio (M/F)	1.15
AJCC stage IIIc, <i>n</i>	4/28
IV M1a, <i>n</i>	1/28
M1b, <i>n</i>	2/28
M1c, <i>n</i>	21/28
Brain metastases (M1c + brain metastases), <i>n</i>	9/21
LDH at baseline, IU/ml; median (IQR)	428 (327–556)
$BRAF^{V600}$ inhibitor as a first-line therapy, <i>n</i>	17/28

IQR: interquartile range; LDH: lactate dehydrogenase; IU: international units; AJCC: American Joint Committee on Cancer.

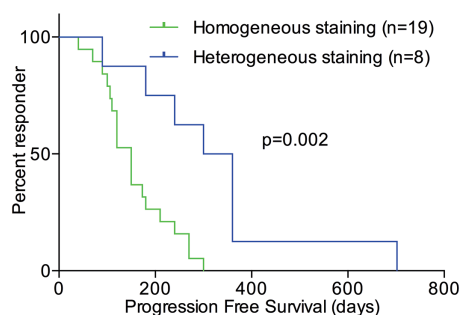


Fig. 2. Progression-free survival according to the VE1 staining pattern of pre-treatment samples in 27 patients treated with $BRAF$ inhibitor: homogeneous ($n=19$) vs. heterogeneous ($n=8$) ($p=0.002$).

low, possibly leading to false-negative result. This emphasizes that IHC has a higher potential compared with DNA-based analysis in samples of small size or with low tumour cell content. This technique, with its low cost and quick turnaround time, could be used as an ancillary screening tool for BRAF^{V600E} detection, while VE1-negative samples should be genotyped to detect another BRAF^{V600} mutation. In our study, 8% of VE1-negative cases had BRAF^{V600K} or BRAF^{V600R} mutations, which might respond to BRAF^{V600} targeted therapy.

In 3 cases, a discordant genotype was observed between primary and metastasis paired-samples or across different metastases from the same individual. For patient P32, the low number of metastatic cells was probably under the threshold of our technical sensitivity. For patient P89, the lymph node was massively involved and not macrodissected. However, the discordance could be ascribed to a molecular false-negative due to poor DNA quality. Other studies reported such a discrepancy at different rates, perhaps explained by the cohort design and the site of metastases (9, 11–13). In the study by Colombino et al. (11), more discrepancies were observed in patients with skin metastases compared with those with lymph node or visceral metastases. Furthermore, the identification of mutated metastasis from wild-type primary melanoma, raising the hypothesis of acquired mutations, suggests that the median time to metastasis might be important. However, although these discordant patterns could be of biological and clinical importance, the design of our study is not appropriate to address these questions.

Regarding the VE1 staining pattern, our experience is at variance with other studies that have reported a generally homogeneous staining (3, 6, 8, 9). Nearly 30% of our positive cases exhibited sub-clones differentially stained by VE1 within the same sample. Wilmott et al. (14) also reported heterogeneous staining in 22% of cases, despite methodological differences, since they considered heterogeneous expression only when distinct subpopulations of cells had an immune-reactive intensity score that differed by more than one scoring level. In their study, Feller et al. (15) did not emphasize the staining pattern; however, they showed clear heterogeneous staining in case number 9. In our study we did not observe VE1-positive areas within or adjacent to immune-negative areas as would be expected according to the study of Yancovitz et al. (10), who showed individual tumour specimens containing a mixture of BRAF mutant and wild-type melanoma areas using laser-capture microdissection combined with SNaPshot technology allowing semi-quantitative assessment of BRAF^{V600E} and BRAF wild-type alleles. Immunohistochemistry is only a semi-quantitative method, whose results can be influenced by endogenous or exogenous variables, such as necrosis or fixation. The strongest staining was not particularly observed in perivascular tumour cells, as described by

Capper et al. (3) and we were aware of possible negative staining in pre-necrotic or necrotic areas, as reported previously (3, 9). Furthermore, different zygosity status and copy number alterations involving mutant BRAF alleles, resulting in mutant allele-specific imbalance, which could lead to different levels of protein expression, have been described previously in melanoma (16). One could hypothesize that, in heterogeneous VE1-positive tumours, 2 different clones with different mutant allele imbalance coexist. However, only micro or single cell dissection of areas with different staining intensity and the use of quantitative methods, such as pyrosequencing, would answer this question. Single cell dissection would have the supplemental advantage of avoiding artefacts related to tightly intermingled stromal cells. Therefore, although immunohistochemistry could be a surrogate for molecular studies in V600E BRAF mutation screening, quantification of the BRAF-mutated protein cannot be assessed accurately using IHC. Wilmott et al. (17) have reported heterogeneous staining in a progressing BRAF^{V600E} mutated metastatic sample using IHC against the phospho-ERK1/2 downstream signalling protein, but in this case the weakly positive sub-clone was shown to harbour both BRAF^{V600E} and NRAS^{G13R} mutations. In our series, the NRAS mutational status of codons 12, 13 and 61 was always tested in parallel (data not shown) and we observed only one case of metastasis with both BRAF^{V600E} and NRAS^{Q61K} mutations, from a patient treated for 7 months with the BRAF inhibitor vemurafenib (P43). However, the VE1-staining was homogeneous, arguing against the reliability of IHC to suspect additional molecular aberrations in tumour sub-clones.

As already shown by Wilmott et al. (14), our study confirms that the level of BRAF^{V600E} protein expression determined by IHC did not predict the response or survival of patients receiving BRAF inhibitors. However, our results are at variance with Wilmott et al.'s regarding VE1 heterogeneity and outcome of patients. Our results suggest that the staining pattern (homogeneous vs. heterogeneous) might be associated with differences in therapeutic response to V600-mutated BRAF inhibitors. Patients with heterogeneous staining display longer PFS than patients with homogeneous staining. As there is no difference in median IRS between the 2 groups, this difference in PFS may result from tumour heterogeneity if we hypothesize that heterogeneous VE1 staining reflects tumour heterogeneity (10). It was reported that BRAF-mutated melanoma tumour cells have a poorer immune-related phenotype compared with others, suggesting that these cells are less prone to undergo immune-related control, and such association was stronger in BRAF mutant tumours displaying low expression levels of this gene (18). Even if in the latter study, patients were not treated with BRAF inhibitors, our results are counter to this hypothesis. It can also be extrapolated that cells with a low or moderate staining

express a low level of mutated *BRAF* gene. Fredericks et al. (19) recently reported that BRAF^{V600} inhibition was associated with a higher expression of melanoma antigens and an overall more favourable tumour micro-environment, but these results were not correlated with the level of *BRAF* mRNA. Lastly, it is well known that the use of vemurafenib stimulates the growth of BRAF non-mutated melanoma (20, 21), although we did not observe negative clones among positive tumours in our heterogeneous cases. Clearly, the design of our study is underpowered to speculate about physiopathological hypotheses. Nevertheless, despite the limited number of patients in our series, heterogeneous staining should not be overlooked or be considered as a “technical” artefact, because it might help to predict the variability observed in therapeutic response to BRAF^{V600} inhibitors. Further study on larger cohorts is needed.

The current study confirms the high sensitivity and specificity of VE1 clone to detect BRAF^{V600E} mutated protein in FFPE specimens, allowing rapid and reliable screening of patients eligible for BRAF inhibitor treatment. Furthermore, VE1 analysis may help to detect false-negative mutational analyses when tumour cells are rather rare. The low number of discrepant results obtained by IHC between primary and metastatic sites or in different metastases suggests that either primary melanoma or metastasis can be tested for BRAF^{V600E} mutant detection, according to available material. The algorithmic approach of molecular testing only ambiguous or VE1-negative cases could help to control costs and improve turnaround time.

ACKNOWLEDGEMENTS

The assistance of the staff of the Department of Pathology and the molecular genetics hospital platform, including Michel March, Laurence Jalabert, Valérie Thuries, Emmanuelle Devaux, Stéphanie Laffaure, and Sandy André, is gratefully acknowledged.

The authors declare no conflicts of interest.

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