

Usefulness of Immunocytochemistry for the Detection of the *BRAF*^{V600E} Mutation in Circulating Tumor Cells from Metastatic Melanoma Patients

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TO THE EDITOR

Metastatic melanoma patients harboring a *BRAF* gene mutation on codon 600 can be treated with targeted therapies (Flaherty, 2012). Depending on the content of tumor cells and on the analytical sensitivity, *BRAF* mutations are found in 50–70% of metastatic melanoma patients (Davies *et al.*, 2002). Around 80% display a valine-to-glutamic acid substitution (V600E) and ~16% harbor a valine-to-lysine substitution (V600K) causing constitutive kinase activation (Wan *et al.*, 2004; Rubinstein *et al.*, 2010). *BRAF*^{V600E} mutation analysis is currently performed in daily clinical practice on tissue samples using various molecular biology technologies. Moreover, the detection of the *BRAF*^{V600E} mutation in blood samples from melanoma patients in the context of translational research and clinical trials has been described (Board *et al.*, 2009; Sakaizawa *et al.*, 2012). Metastatic dissemination correlates with the presence of circulating tumor cells (CTCs) detected in blood samples (Paterlini-Brechot *et al.*, 2011; Alix-Panabières *et al.*, 2012). The detection of circulating melanoma tumor cells (CMCs) can be performed using different technologies, in particular by the isolation by size of epithelial tumor cells (ISET) method, a direct method that allows cytopathological analysis of CMCs (De Giorgi *et al.*, 2010). Moreover, ancillary methods for CTC characterization can be performed on cells isolated by ISET (De Giorgi *et al.*, 2010; Ilie *et al.*, 2012). Recent studies highlighted the value of immunohistochemistry (IHC) using the

VE1 antibody for the detection of the *BRAF*^{V600E} mutation in melanoma (Capper *et al.*, 2012). The aim of this work was to combine ISET and immunocytochemistry (ICC) using the VE1 antibody to investigate the presence of *BRAF*^{V600E} in CMCs from metastatic melanoma patients.

Therefore, 98 metastatic melanoma patients were screened for *BRAF*^{V600E} both by pyrosequencing and by IHC anti-VE1. Concomitantly and blindly, ICC for the *BRAF* mutation was performed on CMCs isolated by ISET (See Supplementary Data). Population data are shown in Supplementary Table S1.

Of 98 patients, 53 (54%) had a *BRAF*^{V600E} mutation detected by pyrosequencing in tissue samples. Among these patients, 51/53 (96%) showed strong immunostaining with the VE1 antibody in tissue sections (Supplementary Table S2). Homogenous intracytoplasmic staining without associated nuclear staining was demon-

strated in melanoma cells only (Figure 1). Among the tumors negative for the *BRAF* mutation by pyrosequencing, none had positive VE1 immunostaining (Supplementary Table S2; Figure 1). An excellent concordance was found between these two methods (Supplementary Table S2). The IHC anti-VE1 demonstrated 96% sensitivity and 100% specificity when compared with the sequencing results. CMCs were isolated in 87/98 (89%) patients. Of 87 patients, 54 (62%) demonstrated positive immunostaining on ISET filters as detected by VE1 ICC (Table 1; Figure 1). Forty-six out of fifty-four (85%) patients with CMCs positively stained by ICC had a *BRAF*^{V600E} mutation detected in tissue specimen by pyrosequencing (Table 1; Figure 1). Eight out of fifty-four (15%) patients with positive VE1-immunostained CMCs lacked *BRAF*^{V600E} in tumor tissues, analyzed both by pyrosequencing and IHC (Table 1; Figure 1). The ICC VE1 CMC-based assay

Table 1. Correlation between the mutational status of the *BRAF* gene detected by pyrosequencing on tumor specimens and *BRAF*^{V600E} expression detected by ICC with the VE1 antibody on circulating melanoma cells isolated by ISET from 87 metastatic melanoma patients

Pyrosequencing (n)	ICC anti-VE1, n (%)		κ -Index	P-value ¹
	Positive	Negative		
V600E (46)	46 (85%)	0 (0%)	0.62	<0.001
V600K (5)	0 (0%)	5 (15%)		
Wild-type (36)	8 (15%)	28 (85%)		
Overall	54 (62%)	33 (38%)		

Abbreviations: ICC, immunocytochemistry; ISET, isolation by size of epithelial tumor cells.

¹A χ^2 test was used. P-value significant at the 0.05 level.

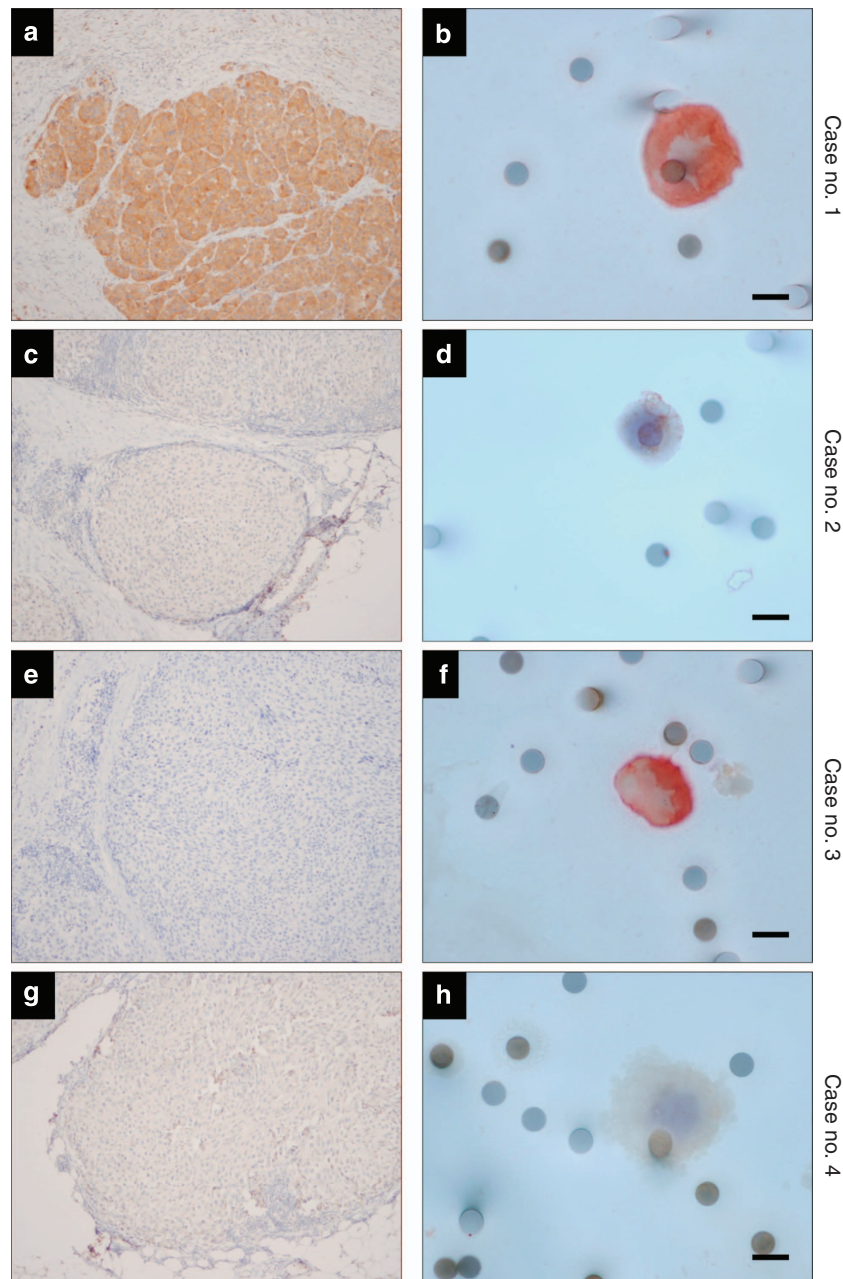


Figure 1. Immunohistochemical features of *BRAF*^{V600E}- or *BRAF*^{V600K}-mutated tumors and *BRAF* wild-type tumors. (Case no. 1) *BRAF*^{V600E}-mutated metastatic melanoma demonstrating positive immunostaining with the VE1 antibody on both (a) a tumor specimen and (b) circulating melanoma cells detected by isolation by size of epithelial tumor cells (ISET). (Case no. 2) *BRAF* wild-type metastatic melanoma showing no staining with the VE1 antibody on both a tumor specimen (c) and (d) circulating melanoma cells. (Case no. 3) *BRAF*^{V600E}-mutated metastatic melanoma displaying negative immunostaining with the VE1 clone on the tumor tissue (e) and (f) intense positive cytoplasmic staining on the circulating melanoma cells detected by ISET. (Case no. 4) *BRAF*^{V600K}-mutated metastatic melanoma demonstrating negative immunostaining with the VE1 antibody on both a tumor specimen (g) and (h) circulating melanoma cells isolated by ISET. Right figures, immunoperoxidase, original magnification $\times 200$; left figures, immunoperoxidase, original magnification $\times 1,000$; scale bar = 16 μm .

revealed 100% sensitivity and 81% specificity when compared with the pyrosequencing results on the corresponding tumor specimens. Among the 87 patients with CMCs isolated by ISET, 5 had *BRAF*^{V600K} mutation in melanoma tissue, without positive staining with the

VE1 antibody, both in tissue sections and in CMCs (Figure 1). Control immunostaining on CMCs using anti-CD45 was negative (not shown).

This study shows that CMCs isolated by ISET can be used to detect the *BRAF*^{V600E} mutation in patients with

advanced melanoma by using the VE1 antibody. We demonstrated that this noninvasive approach is highly sensitive and relatively specific for the detection of *BRAF*^{V600E} in CMCs, having high level of concordance with results in tissue samples. In comparison with

other approaches used for the detection of BRAF^{V600E} from blood samples, ISET allows cytopathological detection of CMCs before the analysis for a mutation, affording correlation of cytomorphological and ICC data and avoiding interpretation bias (Paterlini-Brechot *et al.*, 2007). Moreover, ISET is a rapid and low-cost method that can easily be repeated, thereby allowing the monitoring of CMC detection in patients on targeted therapy. The use of ICC for the detection of the BRAF^{V600E} mutation on CMCs has advantages, but also a few potential drawbacks. Interestingly, eight patients included in the present series showed CMCs positively stained by ICC using the VE1 antibody, whereas BRAF^{V600E} was not found in the corresponding tumor tissue samples. As molecular heterogeneity is a common event in tumors, it is possible that the tissue sample used for both pyrosequencing and IHC analysis may not harbor the BRAF^{V600E} mutation (Longo, 2012). In these cases, BRAF^{V600E}-mutated CMCs derived from other parts of the tumor would have invaded the blood stream, initiating metastatic dissemination. Second, even if pyrosequencing is a sensitive technology (~5%), the presence of a small amount of mutated cells in the tissue sample may give a false negative result (Gonzalez de Castro *et al.*, 2012). It has been described previously that VE1 immunostaining may be useful for the detection of smaller amounts of BRAF^{V600E}-mutated cells in tissue sections (Capper *et al.*, 2012). The hypothesis of a false positive ICC result on CMCs can be reasonably eliminated, as negative controls made in parallel on CMCs isolated by ISET did not show any staining. Future developments on the investigation of the BRAF^{V600E} mutation in CMCs isolated by ISET, both by ICC and DNA sequencing, should add more information to this issue. For now, the low number of detected CMCs (from two to eight CMCs) in these eight patients did not allow us to obtain conclusive results by pyrosequencing performed on

extracted DNA from CMCs. Larger studies are now needed to determine whether the detection of BRAF^{V600E} in CMCs using VE1 immunostaining could allow the selection of patients for a targeted therapy, despite the absence of detection in tissue sample. In conclusion, CMCs can be detected by ISET in patients with advanced melanoma, and can be analyzed by using ICC with the VE1 antibody for the identification of the BRAF^{V600E} mutation in melanoma cells. This approach is noninvasive, rapid, very sensitive, and specific, and opens new options for taking care of metastatic melanoma patients in the era of innovative targeted treatments.

All patients enrolled in the study provided written, informed consent. The study was approved by the Ethics Committee of the Nice University Hospital Centre and was performed in adherence to the Helsinki Guidelines.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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