1999) or the components of the IL-31 receptor might be involved in the pathogenesis of sporadic PCA. Although additional work is required to translate our current finding to disease management, modulation of MCP-1 level and function, through IL-31-dependent and -independent pathways, may offer a new approach for therapeutic development for FPCA.

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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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

Abbreviations: CMCs, circulating melanoma tumor cells; CTCs, circulating tumor cells; ICC, immunocytochemistry; IHC, immunohistochemistry; ISET, isolation by size of epithelial tumor cells

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 demonstrated 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 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).

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

	ICC anti-VE1, *n* (%)			
Pyrosequencing (*n*)	**Positive**	**Negative**	**κ-Index**	***P*-value[1]**
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.
[1]A χ^2 test was used. *P*-value significant at the 0.05 level.

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

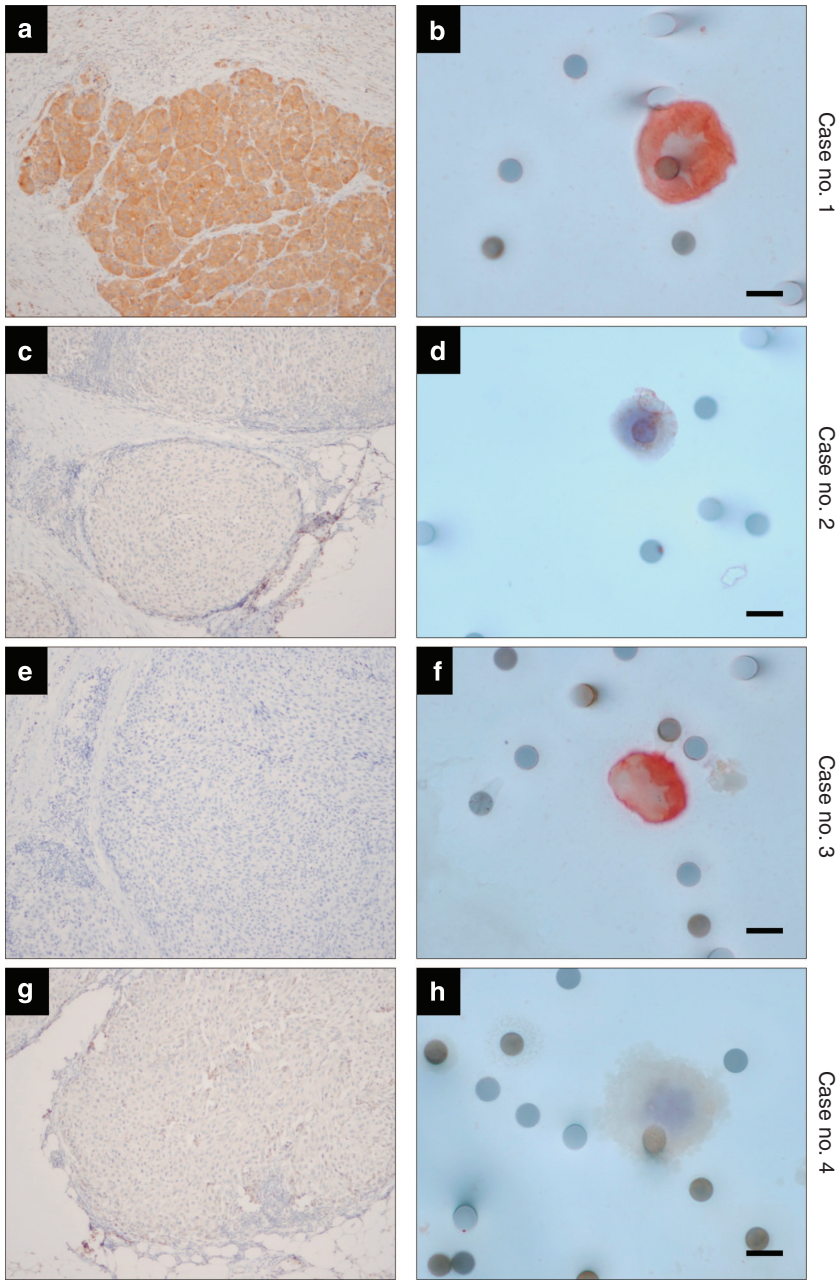

Figure 1. Immunochemical features of *BRAF*^{V600E}- or *BRAF*^{V600K}-mutated tumors and *BRAF* wild-type tumors. (Case no. 1) *BRAF*^{V600E}-mutated metastatic melanoma demonstrating positive immunostaining with the VE1 antibody on both (**a**) a tumor specimen and (**b**) circulating melanoma cells 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 ×200; left figures, immunoperoxidase, original magnification ×1,000; scale bar = 16 μm.

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

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Plasma MicroRNA-21 Is Associated with Tumor Burden in Cutaneous Melanoma

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TO THE EDITOR

In the wake of new treatments for advanced melanoma (Chapman *et al.*, 2011; Robert *et al.*, 2011), the identification of novel blood biomarkers to monitor therapeutic response and disease recurrence is timely. MicroRNAs (miRNAs) are promising because they can be assayed directly from blood. Over 1,000 of these exist (Griffiths-Jones *et al.*, 2008) showing alterations in both cancer tissue (Calin *et al.*, 2002) and blood (Mitchell *et al.*, 2008). MiR-21 is one of the most widely studied and is upregulated in many cancers (Volinia *et al.*, 2006). In melanoma, its genetic locus shows gains (Zhang *et al.*, 2006), and in histologically ambiguous melanocytic lesions it is associated with sentinel lymph node status (Grignol *et al.*, 2011) and correlates with prognosis (Jiang *et al.*, 2011). Our hypothesis was that plasma miRNAs are biomarkers of melanoma burden. We used miR-21 as a proof of concept to test this because it has been widely studied in cancer.

We analyzed 160 melanocytic tumors (Supplementary Table S1 online) and 56 blood samples. First, miR-21 expression was measured in 51 melanomas using quantitative PCR, finding a significant association with Breslow thickness and ulceration, two important prognostic features (Balch *et al.*, 2009), $P=0.02$, 0.024, respectively, Supplementary Table S2 online. To assess independent prognostic value, another set of 79 melanomas was analyzed (Supplementary Table S1 online), 40 having disease-free survival >5 years and 39 having metastasis within 5 years. Logistic regression showed that the stage (IB/IIA versus IIB/IIC) and miR-21 (dichotomized at median) both significantly predicted progression-free survival with an odds ratio of 4.83 (confidence interval (CI), 1.79–13.04), $P=0.002$ and 2.72 (CI, 1.01–7.34), $P=0.048$, respectively. The covariates explained between 16 and 21% of the total variation (Cox and Snell R^2 and Nagelkerke R^2, respectively). The addition of miR-21 to the American Joint Committee on Cancer (AJCC) stage increased the model accuracy ($\chi^2=4.10$, d.f. $=1$, $P=0.043$). These data suggest that tissue miR-21 has independent prognostic value. We next assessed miR-21 expression during tumor progression in 51 melanomas, 13 common nevi, and 11 congenital nevi using cultured melanocytes as calibrator (Figure 1a). Expression was significantly different ($F=5.65$, d.f. $=2$, $P=0.005$). *Post hoc* analysis revealed a trend of increasing expression from common nevi and congenital nevi to melanoma ($F=11.05$, d.f. $=1$, $P=0.001$). Colorimetric *in situ* hybridization confirmed tumor cell expression (Figure 1b–d). The relatively high expression in congenital nevi is intriguing, perhaps reflecting their increased risk of progressing to melanoma (Krengel *et al.*, 2006). These data fit well with recently reported data in melanoma tissues and cell lines (Satzger *et al.*, 2012). We next looked at whether miR-21 expression in metastatic tumor tissue related to plasma levels, finding a strong correlation, $n=5$, $r=0.997$, $P=0.0002$ (Figure 2a). These data confirm that miR-21 is an important tissue biomarker in melanoma and that tissue expression reflects plasma level.

We then looked at whether plasma miR-21 correlated with melanoma burden measured by the AJCC stage. We collected blood from 18 patients

Abbreviations: AJCC, American Joint Committee on Cancer; CI, confidence interval; miRNA, microRNA