

INVESTIGATIVE REPORT

High Incidence of Naevi-associated BRAF Wild-type Melanoma and Dysplastic Naevi under Treatment with the Class I BRAF Inhibitor Vemurafenib

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There is growing evidence that not only malign keratinocytic but also melanocytic tumours can arise during treatment with vemurafenib. During an on-going early access trial, 13 patients harbouring a BRAF-V600E mutation received vemurafenib (Zelboraf®) 960 mg twice daily to test the safety, tolerability, efficacy and response rate for advanced melanoma. Clinically or dermatoscopically suspicious cutaneous tumours under treatment with vemurafenib were excised. The BRAF-V600E status of confirmed new primary melanoma and dysplastic naevi was tested using a genetic mutation assay and immunohistochemistry. Four of the 13 patients (31%) developed 4 new naevi-associated malignant melanomas and 5 dysplastic naevi between 6 weeks and 6 months after the start of treatment. With the exception of one *in situ* melanoma, all tumours were BRAF wild-type. Immunohistochemistry revealed increased expression of ERK, pERK and active Rac1-GTP in the naevi-associated melanoma and dysplastic naevi. Careful and continuous skin examination, including dermoscopy, appears to be required during treatment with vemurafenib. Key words: Vemurafenib, new primary melanoma, naevi-association, Rac1.

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To develop a more stratified therapy to treat the clinically and genetically diverse subgroups of malignant melanoma, vemurafenib (a type I BRAF inhibitor) was the first targeted therapy to achieve a response rate of approximately 50% in patients with metastatic disease (1–3). BRAF V600E is a key mutation of the MAPK signalling cascade. It is found in 80–90% of all BRAF-mutated melanomas (1). However, during treatment with vemurafenib, recent research identified a paradoxical activation of the MAPK pathway in the presence of wild-type BRAF as a possible cause of the cutaneous side effects. These include the formation of follicular

hyperkeratosis, papillomas, keratoacanthomas and squamous cell carcinomas (3, 4). Further reports suggest that benign pre-existing BRAF wild-type naevi experience dynamic changes with the potential for malignant transformation (4–7).

Herein, we report 4 cases of BRAF wild-type naevi-associated melanomas and 5 atypical naevi among 13 patients with metastatic melanoma under treatment with vemurafenib.

METHODS

Thirteen patients with advanced inoperable malignant melanoma participated in a still on-going open-label, multicentre expanded access study (protocol number MO25515, Eudract 2010-023526-21) at the Department of Dermatology and Venereology at Otto-von-Guericke University in Magdeburg, Germany. Newly formed accessible metastases were excised and analysed for their BRAF-V600 mutation status using the Roche Cobas® Mutation Assay (Roche AG, Basel, Switzerland). Patients carrying a BRAF V600E mutation were included and received vemurafenib at 960 mg twice daily until the development of progressive disease, protocol violation, consent withdrawal, unacceptable toxicity or death. Tumour responses were assessed using RECIST (Response evaluation criteria in solid tumours) for CT and MRI at screening and thereafter every 8 to 16 weeks until the end of the study. At every visit, all patients underwent a full-body dermatological examination, including dermoscopy following the excision of suspicious tumours when necessary. All skin tissue samples were embedded in paraffin and stained with haematoxylin and eosin. Melanocytic tumours were stained with melan-A, S100B, HMB-45 and p16 using immunohistochemistry. For further analysis, skin sections of human melanoma or naevi were stained with anti-human BRAF V600E antibody (1:100, clone VE1, Zytomed, Berlin Germany), anti-human pERK (1:50, clone E4, sc7383, Santa Cruz Biotechnology, Heidelberg, Germany), anti-human ERK1 (1:200, sc-94, Santa Cruz Biotechnology, Heidelberg, Germany) and anti-human active Rac1-GTP (1:200, New East Biosciences, US). Paraffin skin sections (3 µm) were deparaffinised and heated for antigen retrieval in either a citrate buffer, pH 6 (for Rac1-GTP IHC) or an EDTA buffer, pH 9 (for BRAF, ERK and pERK IHC) for 1 h. Sections were then quenched using 0.25% Tween 20 (Sigma, Schnellendorf, Germany) in 1x phosphate buffer solution (PBS) for 10 min, then blocked using blocking solution containing 10% foetal calf serum (Invitrogen, Heidelberg, Germany), 1% bovine albumin and 0.25% fish skin gelatine (both Sigma, Schnellendorf, Germany) in PBS for 1 h. This blocking was followed by incubation with the primary antibodies listed above diluted in blocking solution for 3 h at room temperature. After washing, the Zytomed red immunohistochemistry detection kit (Zytomed, Berlin, Germany) was used

according to the manufacturer's instructions to detect the binding of primary antibodies. For anti-human BRAF V600E and pERK antibody detection, an ultraView Universal Alkaline Phosphate Red Detection Kit (Ventana, Roche AG, Basel, Switzerland) was applied to achieve Zytomed red immunohistochemistry detection for 5 min. During the course of the study, the BRAF V600 mutational statuses of newly detected melanocytic tumours were again tested using the Roche Cobas® BRAF V600E Mutation Assay. For expression analysis, we used a semi-quantitative immunohistochemistry score (H-score) (8, 9).

RESULTS

Patients with new melanocytic tumours appearing during therapy

Four out of 13 patients in an on-going clinical trial of vemurafenib developed secondary melanocytic tumours. With the exception of one patient with melanoma of unknown primary (MUP) origin, all of the other 3 patients presented superficial spreading melanoma as the primary tumour, which progressed into the lung (Table I).

Prior to vemurafenib, 3 patients had no adjuvant therapy, including no PEGylated, high or low dose interferon. In one case, chemotherapy with dacarbazine had been administered prior to treatment with vemurafenib. Only one patient had a previously known atypical mole syndrome in addition to intensified sun exposure during adolescence. None of the patients showed any sign of pre-malign or malign cutaneous tumours on the full-body examination performed prior to initiation of the study.

All 4 patients showed an objective tumour response in terms of stable disease or partial remission according to the RECIST criteria during the first 6 to 12 weeks after initiating treatment with vemurafenib. However, eventually all of the patients died, mainly due to progressive refractory brain metastasis and meningiosis melanoblastoma, despite receiving additional radiation or subsequent chemotherapy. One patient showed an intermittent full remission of the previously known lung metastasis, while a newly, emerged refractory skin metastasis had to be surgically removed (patient D). However, a rapid tumour response and complete remission of 12 months was achieved in another patient (patient B).

Arthralgia was reported as the most common systemic side effect by 3 of the 4 patients, followed by constant fatigue in 2 cases. A grade 4 polyneuropathy with subsequent abasia in patient D required hospitalisation and

a constant reduction of vemurafenib twice daily to a dosage of 460 mg.

During the course of this clinical trial, a total of 20 suspicious skin tumours were excised from the 4 reported patients. Histopathology confirmed one bowenoid keratosis, 5 papillomas, one invasive and well-differentiated squamous cell carcinoma, 4 naevi-associated malignant melanomas and 5 dysplastic naevi. Most of the keratinocytic tumours had developed *de novo*. All of the melanocytic tumours were associated with pre-existing naevi that had clinically changed between 6 weeks and 6 months treatment with vemurafenib and had shown malignant dermoscopic changes, such as an irregular, darkened or distorted pigmentation network and prominent unilateral globules and dots, mainly in the centre of the tumour. However, they were not preferentially located in sun-exposed areas. The cutaneous and systemic side effects are summarised in Table II.

Genotyping and immunohistochemistry

Genetic profiling confirmed *Braf* exon 15 and *K-ras* exon 12/13 wild-type in all examined melanocytic malignant tumours and naevi (one *in situ* melanoma in patient B and a metastasis in patient D were not examined). The expression levels of ERK 1, pERK and Rac1-GTP were strong in all tested melanocytic and keratinocytic tumours (Table III, Fig. 1 and Fig. S1¹). BRAF V600E was absent in all melanocytic tumours except the distant metastasis in patient C, confirming the mutation assay results (Table III, Fig. 1 and Fig. S1¹). In the distant metastasis, pERK expression was low, indicating only a minor paradoxical stimulation of the MAPK pathway (Fig. S1¹). The dysplastic naevi exhibited lower H-scores for pERK, ERK 1 and Rac1-GTP than the melanomas (Table III, Fig. 1 and Fig. S1¹). We also observed that notable expression of Rac1-GTP was confined to the dysplastic rather than the non-dysplastic area of the naevi.

DISCUSSION

Vemurafenib (Zelboraf®) has proven to achieve higher response rates and longer progressive-free intervals, as well as improved overall survival, relative to standard

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Table I. Demographics and patient-specific information

Patient	Age at start of trial, years	Melanoma subtype	Tumour thickness of the primary melanoma, mm	Metastases (at start of trial)	Progression (metastases during treatment)	Vemurafenib treatment, months
A	57	SSM	0.8	liver, lung brain	brain, lung, LN	5
B	61	MUP	MUP	LN, skin	brain	13
C	41	SSM	0.7	lung	brain, skin	9
D	71	SSM	3	lung	brain skin	14

SSM: superficial spreading malignant melanoma; MUP: melanoma of unknown primary; LN: lymph node.

Table II. Cutaneous and non-cutaneous side effects

Patients	Cutaneous side effects	Non-cutaneous side effects
A	Melanoma (0.5 mm, CL3), melanoma <i>in situ</i>	None
B	Melanoma (0.5 mm, CL3), SCC, skin papilloma, verruca, dysplastic naevi, palatal hyperplasia, dysplastic naevus, alopecia, photosensitivity	Arthralgia
C	Melanoma (0.6 mm, CL4), hyperkeratosis	Arthralgia
D	Dysplastic naevus, papillomas	Polyneuralgia

CL: Clark level; SCC: squamous cell carcinoma

chemotherapy in patients with advanced malignant melanoma (1, 8). Moreover, its efficacy and side effects have facilitated the identification of key mutations and their functions in the complex interplay of various kinase pathways, particularly in the malignant primary tumour and its metastases (9). Common cutaneous keratinocytic adverse events include not only pre-cancerous and malignant skin tumours, such as papilloma, keratoacanthoma, and squamous cell carcinoma, but also benign skin tumours. Both oncogenes and pre-existing innocuous mutations can lead to faulty stimulation, proliferation and tumour growth under treatment with vemurafenib (10). There is evidence that melanocytes may follow similar mechanisms to develop rapidly, as dysplastic naevi or already thin malignant melanomas as a consequence of treatment with BRAF-inhibitors (1, 4–6). In both keratinocytic and melanocytic skin tumours, a paradoxical activation of the MAPK pathway in RAS-mutated or BRAF wild-type cells might explain these severe side effects (4, 5, 11–14). In this process, vemurafenib presumably does not act as a

Table III. H-score for immunohistochemistry expression of BRAF V600E, ERK, pERK and Rac1-GTP

Patient	Tumour	H-score			
		BRAF V600E	ERK	p-ERK	Rac1-GTP
A	Melanoma (0.5 mm)	0	246	132	261
A	Melanoma <i>in situ</i>	0	222	225	n.d.
B	Melanoma (0.5 mm)	0	219	213	282
B	Squamous cell carcinoma	n.d.	256	212	210
B	Papilloma	n.d.	269	233	n.d.
C	Melanoma (0.6 mm)	0	244	235	267
C	Dysplastic naevus	0	202	147	n.d.
C	Metastasis (<i>in transit</i>)	178	190	130	108
D	Dysplastic naevus	0	228	110	227
D	Papilloma	n.d.	200	220	n.d.
D	Papilloma	n.d.	194	236	n.d.

n.d.: not determined.

direct tumour promoter but rather as a stimulator of pre-existing RAS-mutated BRAF wild-type subclinical skin tumours (11, 15). The paradoxical upregulation of wild-type BRAF has been proposed to potentiate the activity of the MAPK pathway in the presence of a UV-induced RAS mutation (13–17). Secondary malignant melanoma seems to develop preferentially in association with pre-existing naevi on sun-damaged areas (5). However, our patients did not exclusively develop secondary malignant melanoma on sun-exposed areas, nor were they K-Ras mutated. Interestingly, we were able to detect not only strong upregulation of ERK but also activation of the small GTPase Rac1 in its active form as Rac1-GTP (18, 19) in both secondary malignant melanomas and keratinocytic tumours. In melanoma with mutant BRAF, ERK as an effector of

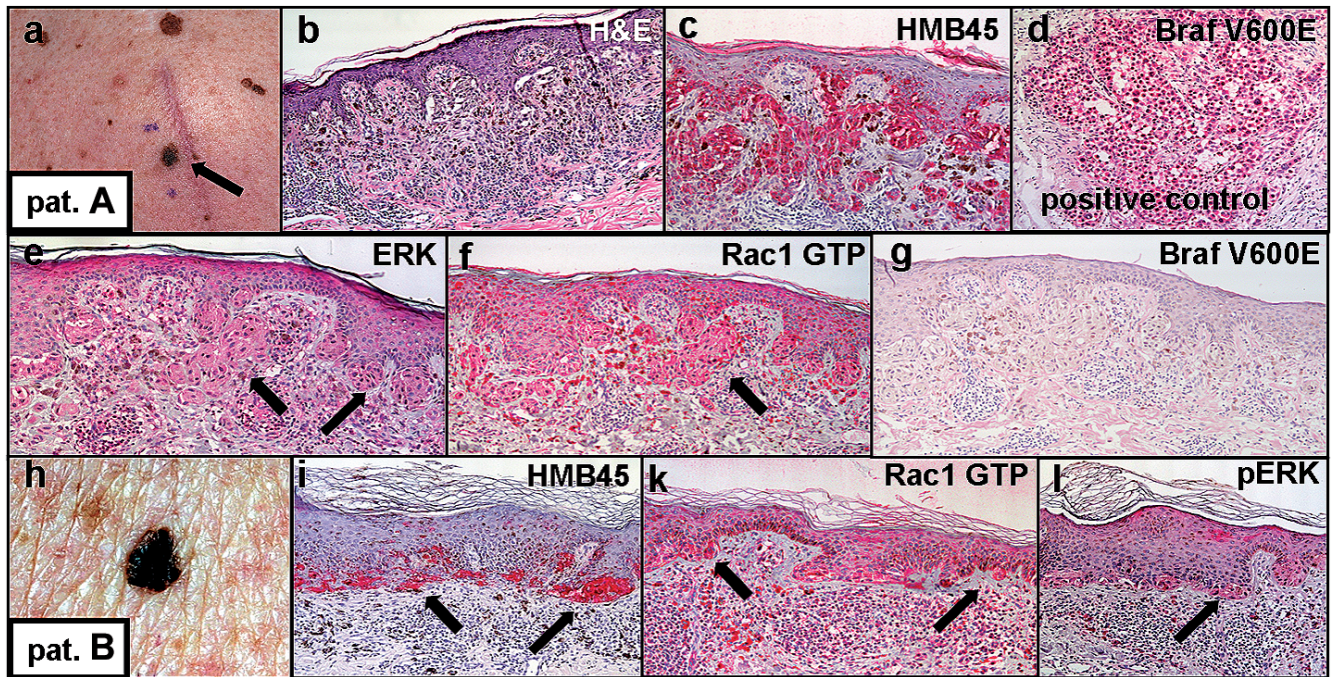


Fig. 1. A new primary melanoma (0.5 mm Clark level III) appearing during vemurafenib treatment in patient A (a); H&E (b) and immunohistochemistry, for HMB45 (c), ERK (e), Rac1-GTP (f) and Braf V600 E (g) in the excised tumour indicated in (a). Braf-positive control (d). A new primary melanoma (0.5 mm, Clark level III) in patient B (h); immunohistochemistry for HMB45 (i), Rac1-GTP (k) and p-ERK (l) (arrows indicate localisation of melanoma cells).

the MAPK pathway (15, 20, 21), and Rac1 is known to be constitutively activated in dysplastic naevi, primary melanomas, and melanoma metastases but absent from adjunct benign naevi (16–19, 22) and overexpressed in squamous cell carcinoma (23). Rac1 plays an important pathogenetic role in melanoma, as it is not only constitutively activated when mutated (Rac1^{P29S}) in approximately 9.2% of sun-exposed melanomas (17) but also leads to increased cycling of melanoma cells (20, 21). However, constitutive activation of Rac1 was not sufficient to initiate a melanocytic neoplasia but was able to accelerate the progression to melanoma in an animal model (19), possibly by mediating a cadherin switch from E- to N-cadherin (24). Rac1 is known to relay signals from growth receptors, junctional proteins and mutated NRasQ61K to downstream effectors via PI3K, including MAPK and ERK (22, 23). Interestingly, Ras and Rac1 mutations are both induced by UV irradiation.

In conclusion, performing particularly careful inspection of the whole skin to ensure early detection and removal of potential *de novo* tumours under long-term vemurafenib treatment is important, together with extensive patient information to avoid excessive sun exposure. Additionally, combination therapies with BRAF and MEK inhibitors may prove beneficial to retard melanocytic or keratinocytic secondary tumour growth (1).

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