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BRAF^{V600E} protein expression and outcome from BRAF inhibitor treatment in *BRAF^{V600E}* metastatic melanoma

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Background: To examine the association between level and patterns of baseline intra-tumoural BRAF^{V600E} protein expression and clinical outcome of *BRAF^{V600E}* melanoma patients treated with selective BRAF inhibitors.

Methods: Fifty-eight *BRAF^{V600E}* metastatic melanoma patients treated with dabrafenib or vemurafenib on clinical trials had pre-treatment tumour BRAF^{V600E} protein expression immunohistochemically (IHC) assessed using the BRAF V600E mutant-specific antibody VE1. Sections were examined for staining intensity (score 1–3) and percentage of immunoreactive tumour cells, and from this an immunoreactive score (IRS) was derived (intensity × per cent positive/10). The presence of intra-tumoural heterogeneity for BRAF^{V600E} protein expression was also assessed. BRAF^{V600E} expression was correlated with RECIST response, time to best response (TTBR), progression-free survival (PFS) and overall survival (OS).

Results: Expression was generally high (median IRS 28 (range 5–30)) and homogeneous (78%). Expression of mutated protein BRAF^{V600E} as measured by intensity, per cent immunoreactive cells, or IRS did not correlate with RECIST response, TTBR, PFS or OS, including on multivariate analysis. Heterogeneity of staining was seen in 22% of cases and did not correlate with outcome.

Conclusion: In the current study population, IHC-measured pre-treatment BRAF^{V600E} protein expression does not predict response or outcome to BRAF inhibitor therapy in *BRAF^{V600E}* metastatic melanoma patients.

Constitutive activating mutations in the *BRAF* gene occur in ~50% of melanomas, of which 70–90% result in a single-amino acid substitution of valine for glutamic acid at residue 600 (V600E) (Thomas *et al*, 2007; Long *et al*, 2011; Jakob *et al*, 2012; Moreau *et al*, 2012). The selective BRAF inhibitors vemurafenib and dabrafenib have proved to be highly effective in targeting the oncogenic BRAF protein in metastatic melanoma patients, with a rapid mode of action, high response rates of ~50%, and an

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improvement in progression-free (PFS) and overall survival (OS) compared with dacarbazine (Flaherty *et al*, 2010; Chapman *et al*, 2011; Falchook *et al*, 2012; Hauschild *et al*, 2012; Sosman *et al*, 2012).

There is a wide spectrum of response to BRAF inhibitors. The minority of *BRAF*^{V600E} melanoma patients (3–5%) have no response, while a few have a complete response (3–5%) (Chapman *et al*, 2011, 2012; Hauschild *et al*, 2012). The majority of patients have a rapid early response but subsequently progress with a median progression-free interval of 6–7 months (Chapman *et al*, 2011; Hauschild *et al*, 2012). No biomarker assessed to date is able to predict the clinical outcome (response, PFS and OS) of patients treated with BRAF inhibitors. Such a marker would not only be of use to guide expectations of outcomes from treatment, but may also assist in the selection of patients who may be more appropriately managed with other therapies (e.g., combined BRAF inhibition with other targeted/immunological drugs) at initial diagnosis, rather than after initial treatment failure.

Mutated BRAF protein is an attractive biomarker to examine for several reasons; First, it is the protein product of the driver oncogene in this melanoma subgroup, and is the specific drug target of BRAF inhibitors. Second, *BRAF*^{V600E} copy number gain has been shown to result in *BRAF*^{V600E} protein overexpression which can confer aquired resistance to BRAF inhibitors (Shi *et al*, 2012). Direct assessment of mutant BRAF protein expression therefore, may be predictive of clinical outcome after BRAF inhibitor therapy.

We have previously shown that immunohistochemistry (IHC) with the BRAF^{V600E} VE1 antibody is highly sensitive (97%) and specific (98%) for the detection of genomic *BRAF*^{V600E} mutation status (Long *et al*, 2013). Detection of the target protein of the BRAF inhibitor using IHC has a number of advantages over other molecular mutation testing techniques. Not only can it provide a result very quickly utilising a technique readily available in most pathology laboratories, but it also allows accurate determination of BRAF mutation status in specimens containing a very low number of tumour cells and has the advantage of quantification and cellular localisation of the mutated BRAF^{V600E} protein within a lesion. Additionally, we have observed heterogeneity of mutated BRAF^{V600E} protein expression in *BRAF*^{V600E} mutant melanoma biopsies from patients (Long *et al*, 2013).

Therefore, we sought to examine whether the level and patterns of expression of BRAF^{V600E} mutant protein could predict the outcome in *BRAF*^{V600E} melanoma patients treated with a BRAF inhibitor. We hypothesised that patients with low or heterogeneous expression of mutant BRAF^{V600E} protein would have a poor response to treatment with BRAF inhibitors and reduced survival, compared with patients with high and homogeneous expression.

PATIENTS AND METHODS

Patient selection and study design. This study comprised a cohort of metastatic melanoma patients who received a BRAF inhibitor as part of clinical trials at Melanoma Institute Australia between 2009–2011. Patients eligible for these clinical trials had American Joint Committee on Cancer (AJCC) stage IV BRAF-mutant melanoma and were treated on the Phase 1/2, 2 and 3 trials with dabrafenib (Trefzer *et al*, 2011; Falchook *et al*, 2012; Hauschild *et al*, 2012), the phase 2 brain metastasis trial with dabrafenib (Long *et al*, 2012), or the Phase 2 and 3 and expanded access trials with vemurafenib (Chapman *et al*, 2011; Larkin *et al*, 2012; Sosman *et al*, 2012). The selection criteria for the current study included all patients diagnosed with a *BRAF*^{V600E} mutation via somatic mutation testing, who received a BRAF inhibitor on the abovementioned clinical trials, and had sufficient archival tissue to perform IHC. Patients who did not receive the recommended phase 2 doses (RP2D) of vemurafenib (960 mg BD) or dabrafenib (total daily dose of 300 mg) were excluded. Patients who discontinued therapy before best response were also excluded. Seven non-*BRAF*^{V600E} patients were selected to serve as controls. Clinical and follow-up details were collected and analysed on all patients as approved by the Westmead and Royal Prince Alfred Hospital Research Ethics Committees.

Drug treatment. All patients treated with vemurafenib received 960 mg twice daily. All patients treated with dabrafenib received 150 mg twice daily from study commencement, or received $\geqslant$ daily RP2D of 300 mg after first tumour assessment as per the respective clinical trial protocols (Trefzer *et al*, 2011; Falchook *et al*, 2012; Hauschild *et al*, 2012; Long *et al*, 2012).

Response to treatment and clinical outcome. Objective response to BRAF inhibitor treatment was assessed with computed tomography (CT) imaging 6–9 weekly as part of the aforementioned trials, using RECIST 1.0 (Therasse *et al*, 2000) for those enroled in the phase 1/2 dabrafenib trial, and RECIST 1.1 (Eisenhauer *et al*, 2009) for the other trials. Clinical outcome was assessed using time to best response (TTBR), PFS and OS from commencement of BRAF inhibitor. Upon disease progression, patients were allowed to continue BRAF inhibitor therapy at the discretion of the treating physician if it was determined they were receiving ongoing clinical benefit.

Immunohistochemistry (IHC). Anti-BRAF^{V600E} immunostaining was performed on the same tissue block used for mutation testing, using the monoclonal mouse antibody VE1 (Heidelberg, Germany) as described previously on 4 μm-thick tissue sections of formalin-fixed, paraffin-embedded (FFPE) tumour tissue blocks (Capper *et al*, 2011, 2012; Long *et al*, 2013). Briefly, sections were stained with undiluted hybridoma supernatant of BRAF^{V600E}-specific clone VE1 (provided by Andreas von Deimling, now commercially available at Spring Bioscience, Pleasanton, CA, USA) on a Ventana BenchMark XT immunostainer (Ventana Medical Systems, Tucson, AZ, USA). The Ventana staining procedure included pre-treatment with cell conditioner 1 (pH 8) for 60 min, followed by incubation with the VE1 antibody at 37 °C for 32 min. Antibody incubation was followed by standard signal amplification with the Ventana amplifier kit, ultra-Wash, counterstaining with one drop of haematoxylin for 4 min and one drop of bluing reagent for 4 min. 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 the primary antibody BRAF V600E clone VE1 was omitted. Cases with BRAF wild-type ($n=2$) and non-V600E BRAF mutations ($n=5$) were included as quality controls.

Immunohistochemical staining evaluation. All immunostained slides were evaluated twice by two independent observers (JSW and RAS) blinded to all clinical, histopathological and mutation data. The BRAF^{V600E} VE1 antibody staining was scored for the percentage of immunoreactive cells. Intensity of staining was judged on a semi-quantitative scale of 0–3+: no staining (0), weakly positive staining (1+), moderately positive staining (2+) and strongly positive staining (3+) (Figure 1A–D). An immunoreactive score (IRS) was derived by multiplying the percentage of positive cells with staining intensity divided by 10 (i.e., IRS potenital range: 0–30). Slides that differed by an IRS of >5 between the two observers were then viewed by both observers together to reach agreement on any discordant scores. Additionally, sections were assessed for homogenous or heterogeneous expression of BRAF^{V600E} protein. Heterogeneous expression was defined as the presence of distinct subpopulations of cells that had

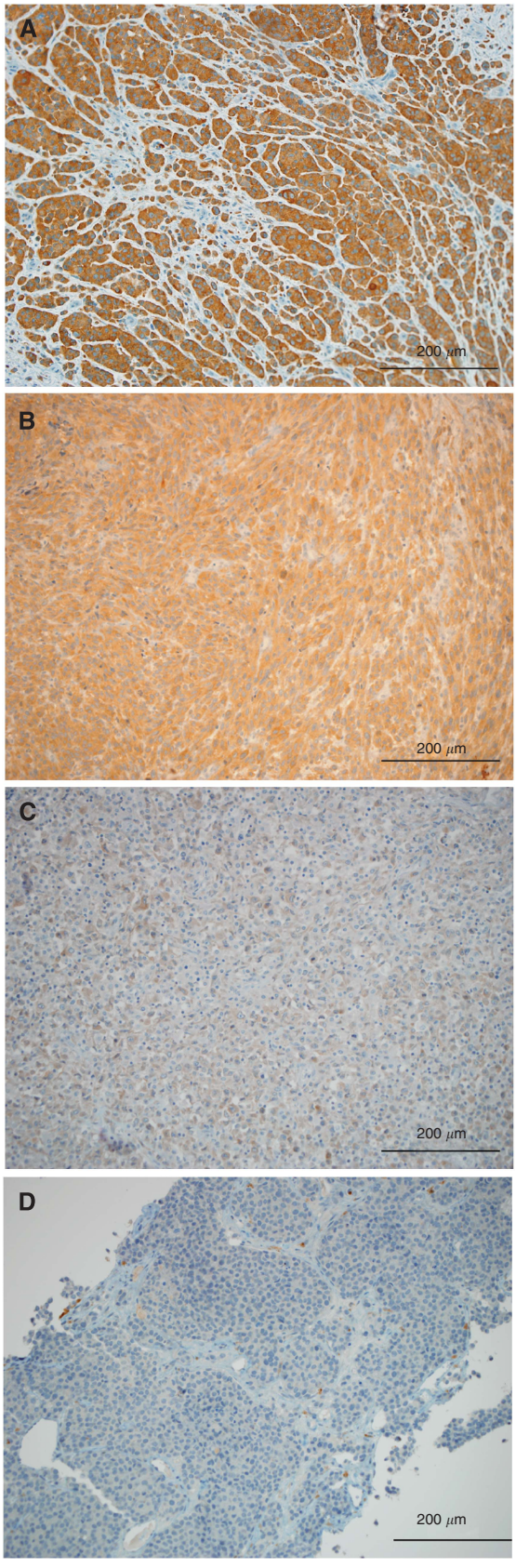

Figure 1. BRAF^{V600E} VE1 immunoreactivity in melanoma sections: (**A**) strongly positive homogeneous staining, (**B**) moderately positive homogeneous staining, (**C**) weakly positive homogeneous staining and (**D**) negative staining of a patients biopsy that was originally thought to be *BRAF^{V600E}*, subsequent repeat genomic retesting of this tumour identified a *BRAF^{V600D}* mutation.

an immunoreactive intensity score that differed by greater than one scoring level (e.g., one population of cells with 3+ and another with 1+ Figure 3A). Cases not fullfilling the above definition of heterogeneous were classified as homogenous expression. Cases with only isolated single interspersed cells with different intensity scores from the majority of the tumour were scored as homogenous. The slides were assesed for cytoplasmic staining only. Any type of isolated nuclear staining, weak staining of single interspersed cells, or staining of monocytes/macrophages was scored negative. Heavily pigmented areas were avoided. Melanoma cells undergoing early necrosis were excluded, as this has previously been shown to affect the antigenicity of the VE1 epitope (Capper *et al*, 2011, 2012; Long *et al*, 2013).

Statistical methods. Statistical analyses were carried out using IBM SPSS Statistic v19.0. Immunohistochemical results tested for association with survival outcomes using Cox regression or Kaplan–Meier (log-rank test) included; continuous IRS, continuous percentage of stained cells, categorical staining intensity and categorical heterogeneity (also classified as 100% staining *vs* <100% staining). Three survival outcomes were tested; TTBR, PFS and OS. All time intervals were measured in relation to the commencement of BRAF inhibitor. Follow-up for patients who subsequently received dabrafenib and trametinib combination (CombiDT) therapy ($n=5$) was censored at date of cross-over to CombiDT therapy, as response rates of 19% and a median PFS of 3.6 months have been reported in this patient population (Flaherty *et al*, 2011). Follow-up for one patient who discontinued therapy after best response but before progressive disease was censored at date of BRAF inhibitor cessation. Multivariate survival models were also tested for PFS and OS in the overall cohort adjusting for age, sex, presence of active (untreated, or previously treated but relapsed) brain metastases (BM), lactate dehydrogenase (LDH) level, Eastern Cooperative Oncology Group (ECOG) performance status and ongoing treatment with BRAF inhibitor after RECIST-defined disease progression. Pearson's correlation and Mann–Whitney *U*-methods were used to test all staining parameters for association with best CT response in terms of best per cent change (sum of diameters target lesions baseline to best response) and also coded categorically as a partial response; no patient experienced a complete response. A subgroup response and survival correlate analysis was conducted excluding patients who had BM at trial commencement.

RESULTS

Comparison of BRAF^{V600E} IHC and genomic mutation testing. Immunohistochemistry was performed on a total of 66 patient biopsies for this study. All cases with wild-type or non-V600E *BRAF* on mutation testing ($n=7$) did not show immunoreactivity to the BRAF^{V600E} antibody. One of 59 *BRAF^{V600E}* patient biopsies displayed no immunoreactivity, and subsequent repeat genomic retesting of this tumour confirmed a *BRAF^{V600D}* mutation (Figure 1D). This patient was excluded from all subsequent analyses.

Patient demographics and tumour tested. Fifty-eight *BRAF^{V600E}* patients were included in the analysis (Table 1), of whom 47 (81%) received dabrafenib, and 11 (19%) received vemurafenib. Eighteen (31%) patients had active (untreated, or previously treated but progressive) BM at trial entry. Fifty-two (90%) patients had AJCC Stage M1c disease, 41 (71%) achieved a partial response to therapy and 5 (8%) patients received subsequent CombiDT as part of the phase 1/2 clinical trial (Flaherty *et al*, 2011). Mutation testing and IHC was performed on 50 metastatic melanoma tissues and in eight patients the primary melanoma tissue was used.

Table 1. Overall cohort characteristics

Factor	Value	N	%
Total patients	*N*	58	100
Patient sex	Female	20	34
	Male	38	66
Age at trial start (years)	Mean/median (range)	53/58 (21–83)	—
BRAF inhibitor	Dabrafenib	47	81
	Vemurafenib	11	19
Trial	BREAK-1(6)	27	47
	BREAK-2(14)	2	3
	BREAK-3(9)	4	7
	BREAK-MB(15)	14	24
	BRIM2(7)	3	5
	BRIM3(8)	3	5
	Vemurafenib EAP(16)	5	9
Tissues tested	Primary melanoma	8	14
	Metastatic melanoma	50	86
Brain metastases (BM) at trial start	No	40	69
	Yes	18	31
M stage	M1a	3	5
	M1b	3	5
	M1c	52	90
Baseline sum of diameters (mm)	Mean/median (range)	106/84 (9–317)	—
ECOG	0	38	66
	1	20	34
LDH	Normal	29	50
	Elevated	29	50
Best CT response	PD	1	1.7
	SD	16	27.6
	PR	41	70.7
	CR	0	0
Progression status	Progressed	50	86
	Not progressed	8	14
BRAF inhibitor status	BRAF inhibitor ceased	47	81
	Ongoing	11	19
Treatment beyond progression[a]	No	38	66
	Yes	20	34
Treatment beyond progression (months)	Mean/median (range)	4.0/2.6 (1.1–12.5)	—
Subsequent CombiDT[b]	No	53	91
	Yes	5	9
Last follow-up status	Dead	36	62
	Alive	22	38
Follow-up (months)[a]	Mean/median (range)	11.6/9.6 (1.7–35.2)	—

Abbreviations: CombiDT = dabrafenib and trametinib combination; CR = complete response; CT = computed tomography; ECOG = Eastern Cooperative Oncology Group; LDH = lactate dehydrogenase; PD = Progressive disease; PR = partial response; SD = Stable disease. Table excludes 1 patient initially *BRAF^{V600E}* on genetic testing, with negative IHC staining, and subsequent restesting found to be *BRAF^{V600D}*.

[a]Treatment beyond progression classified as cessation of BRAF inhibitor >1 month after date of progression.

[b]Follow-up for subsequent CombiDT patients ($n = 5$) was censored at date of cross-over to CombiDT therapy.

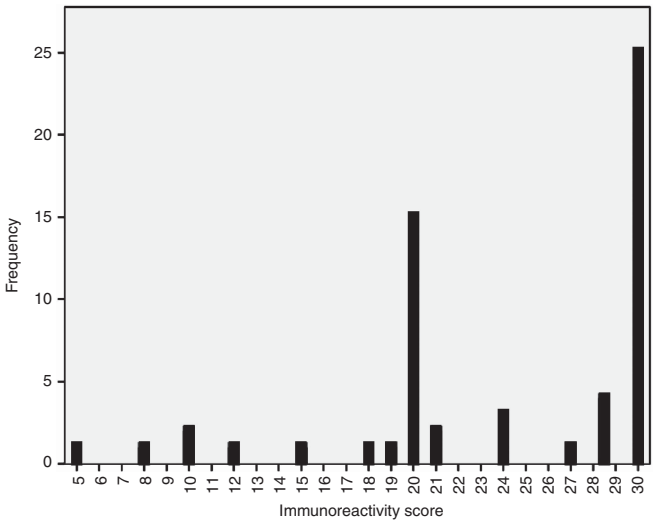

Figure 2. Histogram of immunoreactivity scores (IRS) for *BRAF^{V600E}* patients on BRAF inhibitor.

VE1 immunoreactivity characteristics. The IRS score from each observer differed by a score of >5 in five of the 58 cases. These discordent cases were assessed by both obersvers together to reach an agreement on the score. In general, most *BRAF^{V600E}* melanomas stained intensely and homogeneously. Mean and median IRS were 24 and 28, respectively (range 5–30, out of a maximum score of 30) (Figure 2). Staining intensity was strong in 36 cases (62.0%), moderate in 20 (34.5%) and weak in two cases (3.5%). Forty one cases (71%) displayed 100% of tumour cells staining, 10 cases (17%) had 80–99% staining, and 7 (12%) had <80% staining (the lowest staining was 40%) (Table 2). The IRS score of primary and metastatic melanoma cases was similar, with a median IRS of 29 and 28, respectively. Tumour cell subpopulations with heterogeneous immunoreaction were noted in 13 cases (25% of primary and 22% of metastatic melanomas) (Figure 3A–C).

Association of VE1 immunoreactivity and patient outcome. No immunoreactivity variable correlated with TTBR, PFS or OS, and no trends were seen on Kaplan–Meier analysis (Table 3). Immunoreactive score, staining intensity, percentage of stained cells and heterogeneity did not correlate with PFS or OS, including on multivariate analysis adjusting for age, sex, ECOG, LDH, M stage, the presence of BM and ongoing BRAF inhibitor treatment after disease progression Figure 4. No immunoreactivity variable correlated with RECIST response, when examined either categorically or continuously (Table 3).

DISCUSSION

This study suggests that IHC BRAF^{V600E} protein expression does not predict the response or survival of patients receiving BRAF inhibitors. It was found that BRAF^{V600E} protein is usually highly expressed in *BRAF^{V600E}* mutant melanomas but occasionally in a heterogeneous fashion. These results strongly imply that there are more important modifiers of patient response and outcome than BRAF^{V600E} protein expression.

Strengths of the current study include the performance and scoring of IHC staining blinded to clinical data, the independent determination of protein expression by two separate investigators, the various measures of expression used, and detailed clinical data in a large patient cohort recruited from contemporary prospective therapy trials. In addition, the analysis accounted for subsequent

Table 2. Immunohistochemistry scoring results for BRAF^{V600E} expression

	N (%)
Staining intensity	
Strong (3+)	36 (62)
Moderate (2+)	20 (35)
Weak (1+)	2 (3)
Negative	0 (0)
Percentage of immunoreactive tumour cells	
100%	41 (71)
<100%	17 (29)
Heterogeneous	13 (22)
Homogenous	45 (78)

active therapies received that may have confounded survival analysis. Furthermore, the IHC technique was standardised and utilised archival paraffin pre-treatment samples.

Although we demonstrated that protein expression varied between patients, there was no correlation between the level of mutant BRAF protein expression and response to BRAF inhibitors or survival in treated patients. One possible explanation for this relates to the method of detecting BRAF expression. The concentration of the VE1 antibody used in this study was optimised to maximise the sensitivity and specificity for BRAF^{V600E} protein detection. As a consequence, the vast majority of cases produced a strong degree of staining intensity, which resulted in reduced variation of the staining intensity between the cases. It is unlikely that the use of an alternate scoring systems would produce significant results, as none of the individual scoring parameters (percentage positive and staining intensity) were correlative with any response or outcomes data. An increased dilution of the VE1 antibody may provide a greater separation of those cases with very high expression (albeit at the risk of not detecting any expression in the lower expressing tumours) and requires further assessment as a tool for predicting responses to treatment with BRAF inhibitors. Another explanation could be that the heterogeneity in staining signal intensity seen between individual cases is influenced by pre-analytical parameters, for example, tissue handling during and after resection, length of tissue fixation in formaldehyde or tissue storage conditions. Previous studies using the VE1 antibody have shown loss of BRAF^{V600E} expression in pre(semi)-necrotic tissue (Capper *et al*, 2012). Other techniques to quantify BRAF expression levels, such as RNA expression microarray analysis, RT–PCR or proteomics, may provide improved predictive power.

A more likely explanation for the lack of correlation of BRAF^{V600E} protein expression and clinical outcome is that melanoma proliferation and survival is determined by a complex array of molecular events, and the effect of BRAF inhibition is similarly complex, so that examination of the level of expression of a single biomarker (even though it is thought to be the main driver of tumour growth and survival) at a single time point, in only one tumour manifestation, cannot account for differences in overall tumour response and survival seen in patients. Analysis of activity of multiple aspects of cell signalling pathways and processes (e.g., MAPK, PI3K, Wnt, NFKB, apoptotic, cell cycling, tumour microenvironment, immune regulation) may be required to predict the clinical effect of subsequent BRAF inhibition.

Heterogeneity of BRAF^{V600E} staining intensity was seen within tumours in a subgroup of patients in this study, often corresponding to areas of morphological heterogeneity. Previous studies of heterogeneity focused on the *BRAF* genotype, comparing various mutation testing techniques in whole tumours, microdissection of tumours, or single-cell analyses (Lin *et al*, 2009; Wilmott *et al*, 2012; Yancovitz *et al*, 2012). The latter techniques are time consuming, costly and cannot be readily performed on a large number of tumour samples, as was done in our study. Quantifying the degree of BRAF protein expression seen in a tumour may therefore be used as a first step to assess for intra-

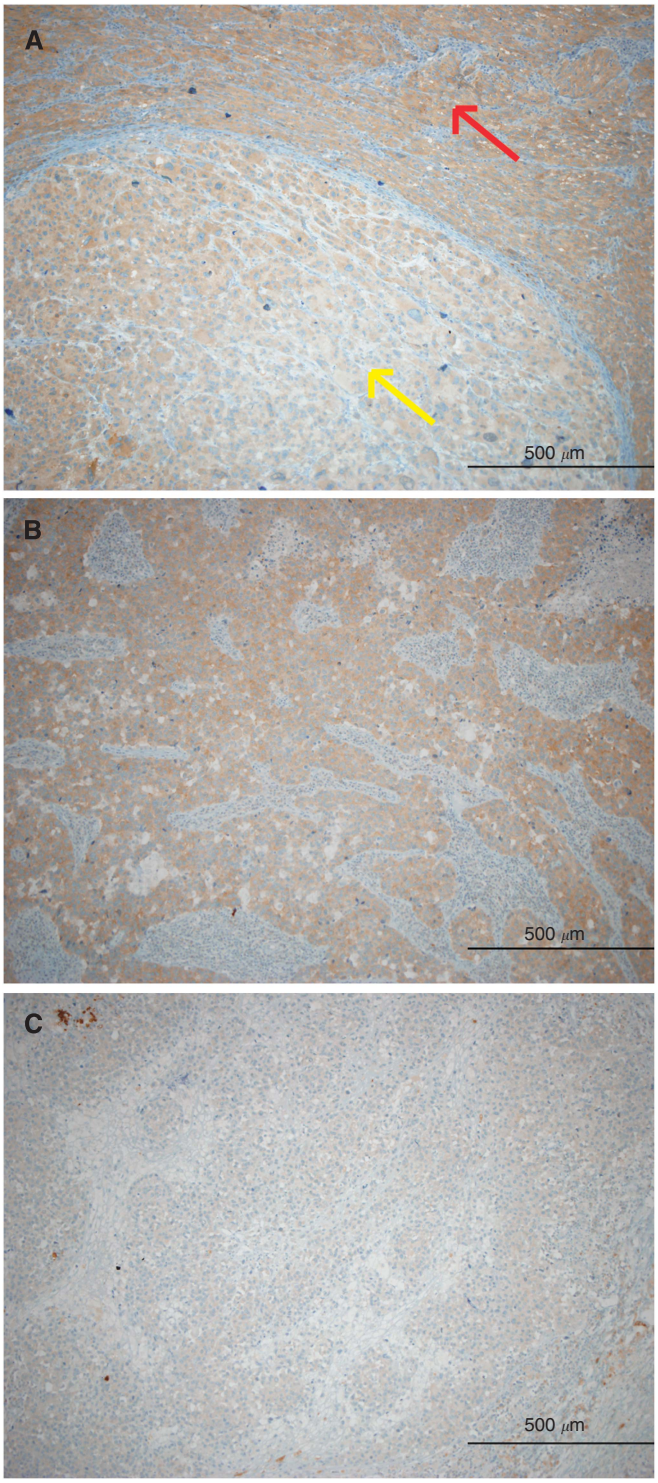

Figure 3. Examples of heterogeneous BRAF^{V600E} VE1 staining: (**A**) primary melanoma with strongly stained cells (red arrow) adjacent to a region of morphologically distinct weakly stained tumour cells (yellow arrow), (**B**, **C**) metastatic melanoma specimen with areas of differing staining intensity in the same tumour specimen (strongly stained cells in (**B**) and weakly stained cells in (**C**)).

Table 3. Correlation of BRAF^{V600E} antibody immunohistochemical staining with patient response and outcome data

BRAF^{v600E} measures tested	Time to best response, disease-free and overall survival		RECIST-defined response	Change in CT lesion (%)	Summary of results
IRS (continuous)[a]	Kaplan–Meier	Cox Regression	Mann–Whitney *U*	Pearson's correlation	Not significant
Per cent reactive cells (continuous)[a]	Kaplan–Meier	Cox Regression	Mann–Whitney *U*	Pearson's correlation	Not significant
Per cent reactive cells (categorical $<100\%$ *vs* 100%)[a]	Kaplan–Meier	Cox Regression	Mann–Whitney *U*	Mann–Whitney *U*	Not significant
Intensity (continuous)[a]	Kaplan–Meier	Cox Regression	Mann–Whitney *U*	Pearson's correlation	Not significant
Intensity (categorical 1and 2+ *vs* 3+)[a]	Kaplan–Meier	Cox Regression	Mann–Whitney *U*	Mann–Whitney *U*	Not significant
Heterogeneity (categorical)[a]	Kaplan–Meier	Cox Regression	Mann–Whitney *U*	Mann–Whitney *U*	Not significant

Abbreviations: CT = computed tomography; IRS = immunoreactive score; RECIST = response evaluation criteria in solid tumours.
[a]Results unchanged when analysed for patients with no active brain metastases ($N = 40$).

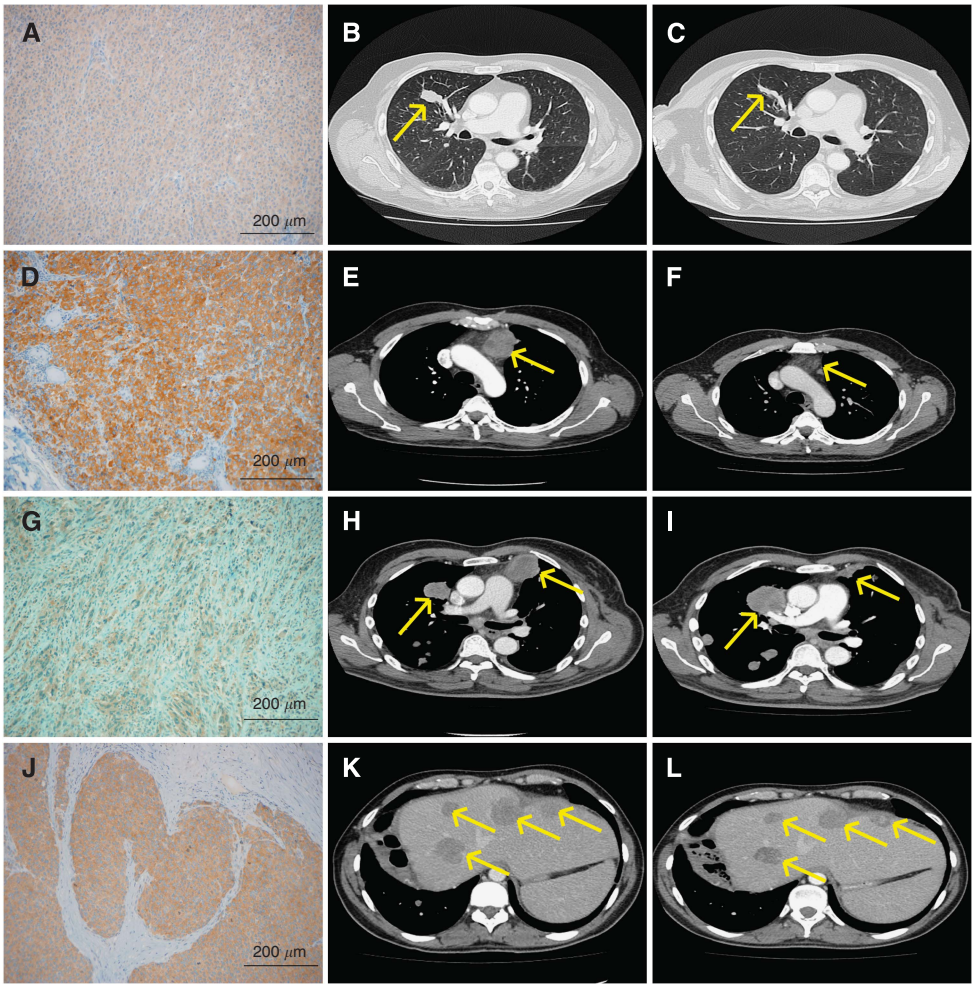

Figure 4. Examples of BRAF^{V600E} VE1 immunoreactivity in melanoma sections and clinical response: (**A–C**) section with low, homogeneous staining and CT images demonstrating a good response, (**D–F**) section with high, homogeneous staining and CT images demonstrating a good response, (**G–I**) section with low, homogeneous staining and CT images demonstrating a poor response and (**J–L**) section with high, homogeneous staining and CT images demonstrating a poor response.

tumoural heterogeneity, allowing subsequent intra-tumoural sampling to occur, such as microdissection, followed by detailed molecular testing.

One case in this study highlighted the importance of IHC assessment of genomic *BRAF*^{V600E} mutation status. In this case, a tumour initially thought to be *BRAF*^{V600E} on allele-specific PCR testing displayed no immunoreactivity with the BRAF^{V600E} antibody and was found to carry the *BRAF*^{V600D} mutation on subsequent exon 15 DNA sequencing. This patient had a poor response to BRAF inhibition and short survival, worse than that expected in *BRAF*^{V600E} melanoma, and more in keeping with non-V600E *BRAF*^{V600} mutations (such as V600K/R/D) (Trefzer *et al*, 2011; Long *et al*, 2012). IHC assessment may therefore be a useful tool in combination with genomic testing to accurately predict tumour *BRAF*^{V600E} status.

The results of this study require confirmation in subsequent studies. The issue of inter-tumoral heterogeneity was not assesed in the current study. Multiple tumours within a patient have been shown to respond heterogenously to BRAF inhibitor treatment (Carlino *et al*, 2013), therefore future studies that account for inter-tumoral heterogeneity by assesing multiple patient tumours should be conducted. If a lack of predictive power of the BRAF^{V600E} antibody or additional measures of BRAF protein expression is confirmed, it suggests there may be other strong modulators of BRAF inhibitor response, over and above BRAF^{V600E} protein expression, and these may be readily targeted with drugs. Further research into mutant BRAF protein expression at baseline, during treatment, and upon development of disease progression, as well as a search for other modulators of response to BRAF inhibitors, is therefore likely to be important.

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CONFLICT OF INTEREST

Menzies AM has received travel support from Roche, GlaxoSmithKline. Thompson JF is a consultant to Roche, GlaxoSmithKline. Kefford RF is a consultant and has received honoraria from Roche, GlaxoSmithKline; he has received travel support from GlaxoSmithKline. Scolyer RA is a Consultant to Roche, GlaxoSmithKline and has received honoraria from Abbott Molecular. Long GV is a Consultant to Roche, Bristol-Myers Squibb, GlaxoSmithKline and has received honoraria from Roche; he has received travel support from GlaxoSmithKline, Roche and research support from Roche. Preusser M has received honoraria from Roche; he has received travel support from Roche and research support from Roche. The remaining authors declare no conflict of interest. Under a licensing agreement between Ventana Medical Systems, Inc., Tucson, Arizona, and the German Cancer Research Centre, Dr Capper and Dr von Deimling are entitled to a share of royalties received by the German Cancer Research Centre on the sales of VE1 antibody. The terms of this arrangement are being managed by the German Cancer Research Centre in accordance with its conflict of interest policies.

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