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A UK feasibility and validation study of the VE1 monoclonal antibody immunohistochemistry stain for BRAF-V600E mutations in metastatic melanoma

Michelle Chin I Lo^{1,7}, Anna Paterson^{2,7}, Jane Maraka^{1,7}, Richard Clark^{1,8}, Joseph Goodwill³, Jenny Nobes^{4,5}, Jennifer Garioch^{5,6}, Marc Moncrieff^{1,5,9}, Ed Rytina^{2,9} and Laszlo Igali^{*3,5,9}

¹Department of Plastic and Reconstructive Surgery, Norfolk and Norwich University Hospitals NHS Foundation Trust, Norwich NR4 7UY, UK; ²Department of Histopathology (Box 235), Cambridge University Hospitals NHS Foundation Trust, Cambridge CB2 2QQ, UK; ³Department of Cellular Pathology, Norfolk and Norwich University Hospitals NHS Foundation Trust, Norwich NR4 7UY, UK; ⁴Department of Clinical Oncology, Norfolk and Norwich University Hospitals NHS Foundation Trust, Norwich NR4 7UY, UK; ⁵Norwich Medical School, University of East Anglia, Norwich NR4 7TJ, UK and ⁶Department of Dermatology, Norfolk and Norwich University Hospitals NHS Foundation Trust, Norwich NR4 7UY, UK

Background: Determining the BRAF mutation status of patients with advanced metastatic melanoma is essential in order to assess patients' eligibility for targeted BRAF inhibitor therapy. The aim of this study was to validate the utility of immunohistochemistry (IHC) to rapidly obtain the BRAF status in the UK cancer centre setting.

Methods: All samples sent for molecular testing for detection of the BRAF mutation over a 26-month period were prospectively tested using the VE1 monoclonal antibody IHC stain.

Results: Two-hundred and nineteen samples from 214 patients were identified. All patients were AJCC stage III/IV, except one. There was an overall 95.0% (208/219) concordance rate, with a sensitivity of 94.4% (84/89) and a specificity of 95.4% (124/130) when using genomic assays as the gold standard. Discordance resulted from the inability of the molecular technique to detect the V600E2 mutation and an inability of the IHC staining technique to detect non-V600E mutations. Molecular testing on smaller tumour deposits was also unreliable.

Conclusions: IHC staining has good sensitivity and excellent specificity for BRAF V600E mutations. BRAF IHC can be incorporated into a BRAF mutation testing algorithm for UK cancer centres to as a feasible first-line assay and identify a subset of cases that require subsequent genomic testing. It has the additional major advantages of reduced cost and rapid turnaround time.

The BRAF mutation in melanoma was first described in a landmark paper in 2002 (Davies *et al*, 2002). Mutations in BRAF are present in 41–56% of malignant melanomas (Lee *et al*, 2011;

Sosman *et al*, 2012). They are most prevalent in cutaneous lesions of superficial spreading and nodular types, whereas they occur at a lower frequency in acral and lentigo maligna melanomas (Saldanha

*Correspondence: Dr L Igali; E-mail: Laszlo.igali@nnuh.nhs.uk

⁷These authors contributed equally to this work.

⁸Former Skin Cancer Interface Fellow at Norfolk and Norwich University Hospital.

⁹Joint senior authors.

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et al, 2006; Lee *et al*, 2011). The mutations are almost exclusively missense and cluster at the kinase domain resulting in an increased kinase activity (Davies *et al*, 2002; Lee *et al*, 2011; COSMIC, 2014). The most common BRAF mutation, a single amino acid substitution of valine for glutamic acid at residue 600 (V600E), occurs in 90% of mutated cases. A lysine to valine substitution (V600K), accounts for a further 5–6%; while rarer BRAF mutations, such as V600E2, other V600 mutations, and non-V600 changes collectively comprise the remaining 4%.

Novel drugs, specifically aimed at inhibiting the overstimulation of the MAP-kinase cell-signalling pathway have revolutionised the treatment of unresectable or metastatic melanoma (Long *et al*, 2011). Over half of patients with BRAF V600-mutated metastatic melanoma had a clinical response to a BRAF inhibitor in a phase II clinical trial (Sosman *et al*, 2012), with further studies, including phase III trials, demonstrating an improved overall survival in patients treated with BRAF inhibitors compared with standard therapy (Flaherty *et al*, 2010; Chapman *et al*, 2011; Long *et al*, 2011; Hauschild *et al*, 2012; Sosman *et al*, 2012; McArthur *et al*, 2014). BRAF inhibitors are the current therapy of choice for patients with BRAF V600-mutated metastases following the publication of the UK NICE appraisal document in 2012 (National Institute for Health and Care Excellence, 2012).

Rapid BRAF mutation testing is therefore essential when considering the appropriate treatment pathway for these patients, particularly for those presenting with rapidly progressing, advanced and unresectable disease. Moreover, ascertaining BRAF status in metastatic melanoma facilitates patients' entry into clinical trials with BRAF inhibitors. There are several methods to detect BRAF mutations including immunohistochemistry (IHC), pyrosequencing, Sanger sequencing and real-time PCR (Colomba *et al*, 2013; Long *et al*, 2013; Just *et al*, 2014; Thiel *et al*, 2015). Table 1 highlights the abilities of these techniques to detect the various mutations. Before this study, all patients managed within the specialist skin multidisciplinary team (MDT) at our two centres required melanoma samples to be dispatched to a national molecular testing centre and tested using a real-time PCR test, the COBAS technique. The advantage of this system is that the diagnostic expertise is centralised to a limited number of high-volume centres, thereby maximising the quality assurance. The drawback is that it is relatively time-consuming and has a limited capacity. The VE1 antibody clone has been validated and shown to detect the V600E and the rarer V600E2-mutated variants with a sensitivity and specificity of 95–100% and 97–100%, respectively, (Long *et al*, 2011; Colomba *et al*, 2013; Just *et al*, 2014; Pearlstein *et al*, 2014; Thiel *et al*, 2015), although data from UK-based centres were lacking. IHC may be preferable for routine clinical use since it is quicker, cheaper and can be offered by more histopathology laboratories. However, since the antibody does not identify the less common V600K, V600R and non-V600 mutations, the overall sensitivity of IHC to detect BRAF mutations when compared with genomic methods is reported to be 76–89% (Long *et al*, 2011; Just *et al*, 2014; Thiel *et al*, 2015).

Given the potential advantages of using this in-house IHC technique over genomic methods, we embarked upon a prospective

audit in the two major cancer centres in our region to validate the diagnostic utility of the VE1 antibody in comparison with the external COBAS service, and in doing so determine the feasibility of establishing in-house BRAF IHC testing using the VE1 antibody for our practices with a large enough sample size to be able to potentially apply the findings to the UK in general.

MATERIALS AND METHODS

This study was designed as a prospectively planned audit on the basis that both cancers centres were investigating the feasibility of the previously validated IHC technique for the ongoing service provision of BRAF V600E mutation status testing. Accordingly, before commencing, the study was discussed and registered with the respective and appropriate audit departments in both participating centres at Addenbrooke's and Norfolk and Norwich University Hospitals (NNUH). All melanoma samples sent away for BRAF V600 mutation testing using the COBAS technique were identified from our respective electronic records databases held in the pathology departments. Cases with only BRAF IHC or genomic analysis were excluded. A single patient could be included more than once if both the IHC and genomic analysis were repeated on a subsequent sample from a different site.

IHC on paraffin-embedded samples using the VE1 monoclonal antibody (Spring Bioscience, Pleasanton, CA, USA) was undertaken prospectively by the in-house pathology services and reported by the local sub-specialty pathologists before the sample being sent for genomic analysis. Mutation testing was undertaken at a national molecular testing centre (Birmingham) using the COBAS technique. In three cases from Addenbrooke's hospital where IHC suggested the presence of the V600E mutation and the COBAS test was negative, pyrosequencing of BRAF was undertaken by the reference centre. The further genomic analysis was specifically requested in these cases by the local MDT to verify the reason for the false-negative COBAS test, since during the early part of the study a positive molecular result was required for the patient to receive treatment with a BRAF inhibitor. No further genomic analysis was undertaken in cases which were negative by IHC but positive on the COBAS test, since it is known that IHC only detects V600E mutations.

The results from IHC and genomic testing were cross-tabulated and the sensitivity, specificity, positive predictive value and negative predictive value determined.

RESULTS

Two-hundred and nineteen samples from 214 patients were identified from the Norfolk and Norwich and Addenbrooke's hospital databases. The reader is directed to Table 2 for the comprehensive description of the individual centres and overall cross-tabulated results comparing the IHC result with the COBAS technique. Furthermore, the table details concordance rates according to primary, locoregional and distant metastases.

Statistically, the data demonstrated consistent BRAF mutation rates between the two centres. The overall detected BRAF mutation rate was 40.6% (89/219 cases) for the COBAS technique and 41.1% (90/219 cases) for the IHC technique. Considering the COBAS technique (supplemented by sequencing in three cases as outlined in the methods) to be the reference for BRAF mutation status, IHC had an overall diagnostic accuracy of 95.0% (95% confidence interval (CI): 90.7–97.5%) indicating a misclassification rate of 5.0% (11/219 cases). The overall sensitivity for IHC was 94.4% (95% CI: 89.1–97.5%) indicating a false-negative rate of 5.6% (5/89 cases). The overall specificity for IHC was 95.4% (95% CI: 91.8–

Table 1. Ability of the different assays to test for the most common BRAF mutations. The frequency of their occurrence in melanomas is V600E >> V600K > non-V600 and other V600 mutations

Assay	V600E	V600E2	V600K	Other V600 Mutations	Non-V600
VE1 IHC	✓	✓	X	X	X
Cobas mutation test	✓	X	✓	✓/X	X
Sanger sequencing	✓	✓	✓	✓	✓

Abbreviation: IHC = immunohistochemistry.

Table 2. Comparison of IHC and COBAS techniques by centre and sample site

	PCR			Statistical summary
	Positive	Negative	Total	
Results by centre				
Cambridge				
IHC				
Positive	37 ^a	3	40	Sensitivity = 94.1% Specificity = 100%
Negative	1	49	50	
Total	38	52	90	Concordance = 96.9%
Norwich				
IHC				
Positive	47	3	50	Sensitivity = 92.2% Specificity = 94.9%
Negative	4	75	79	
Total	51	78	129	Concordance = 94.5%
Combined				
IHC				
Positive	84	6	90	Sensitivity = 94.4% Specificity = 95.4%
Negative	5	124	129	
Total	89	130	219	Concordance = 95.0%
Results by tumour sample site—combined centres				
Lymph-node metastases				
IHC				
Positive	48	0	48	Sensitivity = 94.1% Specificity = 100%
Negative	3	39	42	
Total	51	39	90	Concordance = 96.9%
In-transit metastases				
IHC				
Positive	11	1	12	Sensitivity = 84.6% Specificity = 96.9%
Negative	2	31	33	
Total	13	32	45	Concordance = 93.3%
Sentinel lymph nodes				
IHC				
Positive	3	4	7	Sensitivity = 100% Specificity = 71.4%
Negative	0	10	10	
Total	3	14	17	Concordance = 76.5%
All stage III				
IHC				
Positive	62	5	67	Sensitivity = 92.5% Specificity = 94.1%
Negative	5	80	85	
Total	67	85	152	Concordance = 93.4%
All primaries				
IHC				
Positive	13	1	14	Sensitivity = 100% Specificity = 96.4%
Negative	0	27	27	
Total	13	28	41	Concordance = 97.6%
All stage IV				
IHC				
Positive	9	0	9	Sensitivity = 100% Specificity = 100%
Negative	0	17	17	
Total	9	17	26	Concordance = 100%

Abbreviation: IHC = immunohistochemistry.

^aIn two cases, both from the same patient, initially mutation negative using the COBAS mutation test, direct sequencing demonstrated the presence of the V600E2 mutation, showing the IHC result to be correct.

97.5%) indicating a false-positive rate of 4.6% (6/130 cases). The positive predictive value for IHC was 93.3% (84/90 cases) and the negative predictive value was 96.1% (124/129 cases).

The cases with ‘false-positive’ IHC were reviewed to determine potential reasons for the discrepancies. In five cases, four sentinel lymph-node deposits and one in-transit deposit, the actual number

of tumour cells was very small and probably below the sensitivity threshold of the sequencing techniques, hence these cases most likely reflect true BRAF-mutated cases. On review of the sixth discordant case the BRAF IHC staining was patchy and non-specific in necrotic tumour with additional non-specific staining of the adjacent epithelium. Repeat BRAF IHC was undertaken and was negative, hence this case reflects an IHC false positive.

When looking at concordance by site, distant metastases (100%: 26/26 samples) followed by primary lesions (97.6%: 40/41 samples) proved to be the most reliable. The metastases tested from distant sites (*n* = 26) were derived from the following locations: brain (*n* = 6); bowel (*n* = 8); adrenal (*n* = 1); spleen (*n* = 1); lungs (*n* = 5); and liver (*n* = 5). In locoregional, stage III disease, the greatest concordance was found by testing macroscopic lymph-node deposits, derived from either core biopsy or completion lymphadenectomy specimens.

DISCUSSION

Our study is the largest reported comparison of BRAF IHC and genomic testing from the UK and reflects the performance of these tests in day-to-day clinical practice at two cancer centres using assays that are currently available to all UK pathology departments either in-house or via national reference centres. Our results are closely comparable to previous validation studies (Long *et al*, 2011; Colomba *et al*, 2013; Just *et al*, 2014; Pearlstein *et al*, 2014; Thiel *et al*, 2015).

Sensitivity. Five samples produced a negative result using the IHC stain when compared with the COBAS technique giving a false-negative rate of 5.6%. We suggest that the likely explanation is the inability of the IHC stain to detect non-V600E mutations, unlike the COBAS technique, which is non-specific, since it does not differentiate between V600E and V600K mutation subtypes (Long *et al*, 2013).

Specificity. Six samples were positive with the IHC stain but negative with the COBAS technique giving a false-positive rate of 4.6%. Five samples were small tumour deposits, four were sentinel lymph-node biopsies and one was a small in-transit deposit. As IHC staining can detect mutations at a single-cell level, very small tumour samples can yield interpretable data whereas, in comparison, the COBAS technique requires at least a 5% allele mutation level for detection by the COBAS test (Sosman *et al*, 2012). Furthermore, the COBAS technique is unable to detect the VE2 mutation, in contrast to the IHC technique (Table 1). It is important to note that strong macrophage infiltration can affect interpretation of the IHC stain. It is also recognised that the COBAS technique is relatively sensitive to a high concentration of melanin rendering the test difficult or impossible to interpret (Long *et al*, 2013; Thiel *et al*, 2015).

Sample site. The greatest concordance rates were seen with samples from distant metastatic sites followed by the primary. This most likely reflects the larger tumour volume and so reduces the false-negative rate from genomic assays. Accordingly, we would advocate IHC testing specimens from these locations in the first instance. Previous studies (Boursault *et al*, 2013; Kakavand *et al*, 2014; Eriksson *et al*, 2015) have demonstrated a very high paired concordance rate of BRAF status with the primary and distant metastasis, which allows clinicians to confidently treat stage IV disease on the basis of the BRAF result derived from the primary. Conversely, our data would suggest that caution is required when testing from sites of locoregional recurrence, particularly sentinel nodes and in-transit metastasis deposits where interpretation of a negative result for either test may not truly reflect the BRAF mutation status of the sample due to macrophage infiltration in the

case of the IHC test and high melanin concentration or diminutive tumour sample volume in the case of the COBAS technique (*vide supra*).

Feasibility. IHC can be performed quickly, with a turnaround time of less than 48 h, and since it does not require any additional equipment or expertise, it could be used in almost all pathology departments following optimisation of the IHC protocol. This would allow patients with advanced metastatic or unresectable melanoma (who would benefit from urgent treatment with BRAF inhibitors, particularly those with brain metastases) to start therapy if IHC is positive. In comparison, molecular testing for centres that do not have in-house genomic assays available, representing the majority of UK pathology departments, requires the blocks to be sent to a national reference centre which results in a delay of several working days.

On the basis of our results, one patient (0.5%) without a BRAF mutation could potentially have received a BRAF inhibitor due to false-positive IHC, although review in the MDT meeting prevented this. Safeguards can be introduced at multiple points to minimise the number of false-positive IHC results. Firstly, each BRAF IHC run should have both positive (genome analysis proven V600E mutation) and negative (genome analysis BRAF wild type) melanoma control cases analogous to HER2 testing in breast cancer. Training of pathologists reporting BRAF IHC should be undertaken to emphasise the expected BRAF staining pattern of cytoplasmic positivity within and restricted to the tumour cells. False-positive cases in the literature have been reported in tumours with a high degree of necrosis and large amounts of melanin pigment in tumour macrophages; therefore, extra care should be taken in these cases and a red chromogen could be considered (Thiel *et al*, 2015). We would recommend that all cases that are BRAF IHC positive should be reviewed as part of a MDT discussion, so that any errors in the interpretation of BRAF IHC can be identified before a decision to treat is made. Finally any equivocal cases should be sent for genomic BRAF V600 assessment.

Although the sensitivity and negative predictive value of BRAF IHC in our study was also high, 94.4% and 93.3%, respectively, it is known to miss patients with non-V600E BRAF mutations. These are predicted to account for 5/89 (5.6%) of BRAF mutations in our cohort, and are reported at a higher frequency the literature (Long *et al*, 2013; Just *et al*, 2014; McArthur *et al*, 2014; Thiel *et al*, 2015), resulting in lower BRAF IHC-negative predictive values of 88–91%. This supports the use of genomic analysis in cases with negative IHC to identify patients with other V600 mutations who would meet NICE criteria for BRAF inhibitors.

The dual use of BRAF IHC followed by the use of genomic assays in patients with negative BRAF IHC will expand the number of patients with BRAF mutations that are detected compared with either assay alone. BRAF IHC can detect BRAF mutations in cases with very few tumour cells for assessment which may be missed by genomic techniques due to the low allele frequency. The COBAS technique is also relatively sensitive to high concentrations of melanin rendering the test difficult or impossible to interpret in some cases (Long *et al*, 2013; Thiel *et al*, 2015). The range of potential mutations in BRAF also present challenges when deciding on the most appropriate assays to use. IHC has the narrowest range, only detecting V600E mutations; however, this does allow it to detect a two base pair substitution, the V600E2 mutation, which is missed by the COBAS test. The COBAS test predominantly detects V600E mutations but does detect other V600 mutations to a varying degree; however, it is less sensitive than other genomic methods such as next generation sequencing which is now offered in one of our centres.

In addition to expanding the proportion of patients with BRAF mutations that are identified, our proposed testing pathway of

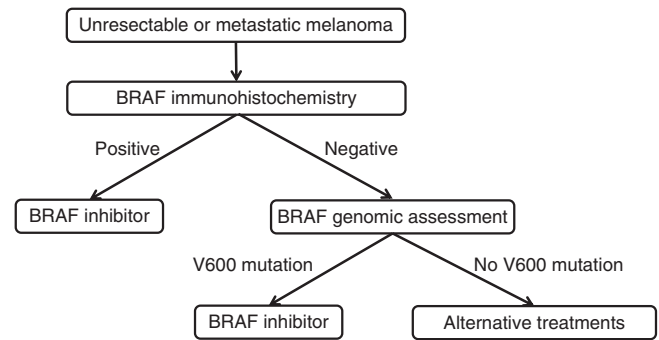


Figure 1. The proposed Anglian Cancer Network protocol for melanoma BRAF mutation testing and how this will be used to guide eligibility for BRAF therapy, if this is felt to be the most appropriate treatment option by the MDT.

undertaking BRAF IHC in all cases (estimated cost £40 per case) followed by a referral to a national reference centre or in-house genomic testing in IHC-negative cases (estimated cost £150–250 per case) would also offer an estimated cost-saving of at least 14% (£4740 for this study) compared with only undertaking genomic analysis; and at least a 33% cost-saving (£13 500 for this study) compared with undertaking IHC and genomic analysis in all patients.

CONCLUSION

We believe that our study has demonstrated that the IHC staining technique has excellent concordance and specificity rates to detect BRAF V600E mutation when compared with current accepted UK standard, namely molecular testing. IHC offers a rapid, sensitive and reliable method, which can be used by the majority of pathology departments to detect BRAF V600E mutations in patients with metastatic melanoma; however, genomic assays are still required in IHC-negative cases to detect other V600 mutations. We therefore propose the adoption of a BRAF mutation testing protocol as outlined in Figure 1.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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