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Concordant *BRAF*^{V600E} mutation status in primary melanomas and associated naevi: implications for mutation testing of primary melanomas

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Summary

There is concern that *BRAF* mutant naevus cells admixed with melanoma cells could cause false positive mutation tests in *BRAF* wild-type melanomas. We sought to assess the frequency of *BRAF*^{V600E} mutations in primary melanomas arising with/without associated naevi and determine *BRAF*^{V600E} concordance between melanomas and associated naevi. Formalin fixed, paraffin embedded (FFPE) tissue from 57 patients with primary melanomas with/without associated naevi was immunohistochemically stained to detect *BRAF*^{V600E} mutation. In a subset of patients ($n=29$), molecular mutation testing was also carried out using a panel of 238 known genetic variants. Of the primary melanomas with an associated naevus ($n=29$), 55% were *BRAF*^{V600E} mutant with 100% concordance between the melanoma and associated naevus. In contrast, only 21% of the primary melanomas unassociated with naevi were *BRAF*^{V600E} mutant ($p=0.009$). Our results suggest that melanomas with associated naevi have a higher frequency of *BRAF*^{V600E} mutations than melanomas unassociated with naevi. Furthermore, melanomas and their associated naevi were concordant in *BRAF*^{V600E} status, which suggests that false positive mutation tests occurring as a consequence of admixed *BRAF* mutant naevus cells in *BRAF* wild-type primary melanomas are unlikely to be a problem in clinical practice. The findings have important implications for adjuvant clinical trials of targeted therapies.

Key words: BRAF, diagnosis, immunohistochemistry, melanoma, mutation testing, naevus, pathology, targeted therapy, treatment.

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INTRODUCTION

The constitutive activation of the mitogen-activated protein kinase (MAPK) pathway driven by mutant BRAF signalling leads to proliferation and cell cycle dysfunction in many solid tumours, including melanocytic tumours.[1] In melanoma, the most common *BRAF* mutation causes a valine to glutamic acid substitution at position 600 (*BRAF*^{V600E}) and occurs in 70–95% of patients.[1–6] *BRAF* mutations are an early event in the development of melanocytic lesions with reported rates of up to 82% in benign naevi (including congenital, intra-dermal, compound and dysplastic naevi)[7] and rates of approximately 40% in metastatic melanomas.[8–10] Approximately 20–30% of melanomas arise in association with a naevus, although reported rates can vary from less than 20% to 70% depending on case selection and pathological interpretation of samples.[11,12]

Selective BRAF inhibitors are a standard of care for treating *BRAF*^{V600} mutant metastatic melanoma, with high response rates and a prolonged progression-free and overall survival compared with chemotherapy.[13,14] These inhibitors are only effective in patients with *BRAF* mutant melanoma and are potentially detrimental to patients with *BRAF* wild-type melanoma,[15–17] so the presence of a *BRAF* mutation in the melanoma must be verified prior to initiation of therapy. Mutation testing is routinely undertaken on archival formalin fixed, paraffin embedded (FFPE) tumour tissue.

The current conventional methods for mutation testing have a variety of limitations and costs.[18,19] The development of a monoclonal antibody (VE1) targeted to bind specifically to the mutant protein has provided a new method for detecting *BRAF*^{V600E} mutations[20,21] and in one study was reported to be more accurate than molecular mutation testing for detecting *BRAF*^{V600E} mutations in melanomas.[18] Immunohistochemistry (IHC) has the added advantage of allowing visualisation of individual antigen-bearing tumour cells, e.g., single metastatic tumour cells in lymph nodes or melanoma cells admixed with naevus cells. Furthermore, immunohistochemical detection of *BRAF*^{V600E} is an ideal method for mutation testing small biopsy samples and is a readily available tool in nearly all pathology laboratories. Therefore, it can efficiently expedite the determination of the *BRAF*^{V600E} mutation status, decrease the amount of tissue required and provide useful translational information to assess potential correlations between response, heterogeneity and intensity of staining.[18,19]

There is concern that if standard approved molecular genetic mutation testing is performed using a specimen with *BRAF* wild-type primary melanoma cells admixed with *BRAF* mutant naevus cells, the latter would lead to a false positive mutation result with subsequent adverse effects on the patient if they are

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treated with a BRAF inhibitor. In this study, we performed BRAF IHC on primary melanomas arising in association with compound/dermal naevi to determine the rates of *BRAF*^{V600E} mutations in primary melanoma with an associated naevus as well as the concordance in mutation status between the melanoma and naevus. Furthermore, we also compared the *BRAF*^{V600E} mutation rates in melanomas occurring with and without associated naevi.

METHODS

Patient selection

Patients presenting with primary melanoma associated with naevi between May 2000 and May 2013 were identified from the Melanoma Institute Australia Melanoma Research Database. Available archival FFPE tissue samples were retrieved from the archival files of the Department of Tissue Pathology and Diagnostic Oncology at the Royal Prince Alfred Hospital, Sydney, Australia, and outside institutions (in cases where the pathology of the specimen had been originally reported elsewhere). Twenty-nine patients were identified. The FFPE tissue blocks of an unselected cohort of 28 primary melanomas without an associated naevus diagnosed and treated during the same time period were also retrieved. This study was conducted with Human Ethics Review Committee approval.

Immunohistochemistry

Two 4-μm thick sections were cut and a haematoxylin and eosin (H&E) slide was analysed in each case to confirm the presence of a primary melanoma and the presence or absence of an associated naevus. IHC was performed using an automated IHC system (Ventana BenchMark Ultra; Ventana Medical Systems, USA) and OptiView DAB IHC Detection Kit (Ventana). Following deparaffinisation of FFPE sections, heat-induced epitope retrieval (HIER) was applied using CC1 for 64 min. The sections were incubated with anti-BRAF mouse antiserum VE1 (1:50 dilution; Spring Bioscience, USA) for 1 h followed by incubation with haematoxylin II counterstain for 4 min and then with bluing reagent for 4 min

Histological assessment

All histology slides were evaluated independently by three observers (HK, OC, and RAS) blinded to all clinical information. Melanoma and naevus cells were scored separately in each case. The staining was scored as positive when the melanoma and naevus cells showed definitive cytoplasmic staining and negative when there was no staining or focal faint staining. Positive cases were scored using a semi-quantitative scale (0–3), with 0 for absent staining, 1 for low/weak staining, 2 for moderate staining, and 3 for high/strong staining.

Molecular testing

BRAF mutation testing was performed on sections from archival FFPE tissue blocks of 29 patients at the Royal Prince Alfred Hospital (RPAH), Department of Tissue Pathology and Diagnostic Oncology (Sydney, Australia). From the FFPE sections, the melanoma was carefully macro-dissected using an H&E section as a guide. The DNA was extracted and amplified for 238 variant targets in a 24 multiplex polymerase chain reaction (PCR) using the OncoCarta Panel v1.0 Kit and analysed based on the matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF) technology on the MassArray platform (Sequenom, USA).

Statistical analysis

Categorical comparisons between patient cohorts were tested with the Pearson's chi-square. Continuous variables were tested with the Mann–Whitney U test for difference across two groups. SPSS statistic v21.0 (IBM, USA) was used to run all the statistical analyses and a p value less than 0.05 was considered statistically significant.

RESULTS

Immunostain for *BRAF*^{V600E} mutation in primary melanomas with/without an associated naevus

Sixteen of the 29 (55%) primary melanomas arising in association with a naevus had a *BRAF*^{V600E} mutation in both the melanoma and the naevus. In contrast, six of 28 (21%) melanomas unassociated with naevi had a *BRAF*^{V600E} mutation. Melanomas associated with naevi were more likely to have the *BRAF*^{V600E} mutation than those without an associated naevus ($p = 0.009$). There were no statistically significant differences in clinical and pathological characteristics (i.e., age, gender, Breslow thickness, ulceration, melanoma subtype and anatomical site) in the patients with primary melanoma arising in association with a naevus and primary melanomas unassociated with a naevus (Table 1). As expected, patients with a *BRAF*^{V600E} mutation were younger than *BRAF* wild-type patients ($p = 0.007$).

Concordance in *BRAF*^{V600E} mutation status between primary melanomas and the associated naevus

In the 29 primary melanomas with an associated naevus, there was 100% concordance in the *BRAF*^{V600E} mutation status between the primary melanoma component and the associated dermal/compound remnant naevus component of the lesion (Fig. 1 and 2).

Differential intensity of BRAF^{V600E} protein expression between primary melanomas and their associated naevus

Immunostaining for BRAF^{V600E} was strongly and diffusely positive (3+) in 82% of the positive primary melanomas (18/22) and moderately positive (2+) in 18% (4/22). The three independent observers were concordant in the assessment of the BRAF^{V600E} staining intensity for all cases. The intensity of the staining for BRAF^{V600E} was weaker (1+ to 2+) in a proportion (5/16) of associated naevi compared to the strong diffuse staining (3+) in the primary melanoma (Table 2; Fig. 3). However, there was no intratumoural heterogeneity in the intensity of staining; in the *BRAF*^{V600E} mutant melanomas, all melanoma cells showed strong BRAF^{V600E} protein expression.

Molecular mutation testing

In a subset of patients (29/57) that were tested using the BRAF^{V600E} immunohistochemistry in our study, molecular mutation testing of the primary melanoma was also undertaken. *BRAF*^{V600E} mutations were detected in 24% (7/29) of the cases. There was 100% concordance between the *BRAF* molecular mutation testing results and the BRAF^{V600E} immunohistochemistry results. One of the 29 cases that had molecular mutations testing performed had an associated naevus, with the *BRAF*^{V600E} mutation being the only one positive in the panel. The molecular mutation testing results also showed that there were 17% (5/29) non-V600E *BRAF* mutations [G469R, L597Q, V600M, V600R, and K601N), 34% (10/29) *NRAS* mutations (G12D, G13R, Q61H, Q61K, Q61L(X2), and Q61R(X4)], 3.4% (1/29) *HRAS* (G12D) and 3.4% (1/29) *cKIT* (L576P). As expected, all of these cases were negative with BRAF^{V600E} immunohistochemistry. No *BRAF*^{V600K} mutations were detected in this patient cohort.

DISCUSSION

The current literature on *BRAF* mutation status in primary melanomas arising in association with naevi is very limited, with these reports only including small numbers of cases and mostly using mutation analysis techniques that do not allow visualisation of the mutation status of individual cells

Table 1 Clinicopathological characteristics of the patient cohorts

	Primary melanoma with associated naevus (1)			Primary melanoma without associated naevus (2)			Significance (cohort 1 vs 2)	
		BRAF V600E protein expression			BRAF V600E protein expression			
Patient cohort	All patients ($n=29$)	Negative ($n=13$)	Positive ($n=16$)	All Patients ($n=28$)	Negative ($n=22$)	Positive ($n=6$)	Mann–Whitney U	Chi-square
Age, median years	65	70	56	71	72	61	NS	
Gender								
Male, *n* (%)	59 (17)	9	8	75 (21)	17	4		NS
Female, *n* (%)	41 (12)	4	8	25 (7)	5	2		NS
Breslow thickness, median mm	2.6	2.8	2.4	3.15	3.25	2.15	NS	
Ulcerated primary melanoma	10	5	5	9	8	1		NS
Associated naevus								
Dermal	20	10	10	N/A	N/A	N/A		
Compound	9	3	6	N/A	N/A	N/A		
Melanoma subtype								
NM	15	8	7	14	11	3		NS
SSM	8	2	6	9	7	2		NS
Naevoid	2	2	0	0	0	0		NS
Mixed	3	1	2	2	2	0		NS
LMM	0	0	0	2	2	0		NS
Desmoplastic	0	0	0	1	0	1		NS
AL	1	0	1	0	0	0		NS
Anatomical site of tumour								
Head and neck	9	4	5	10	8	2		NS
Trunk	11	5	7	10	8	2		NS
Extremities	8	4	4	8	6	2		NS

AL, acral lentiginous; LMM, lentigo maligna melanoma; NM, nodular melanoma; NS, not significant ($p>0.05$); SSM, superficial spreading melanoma.

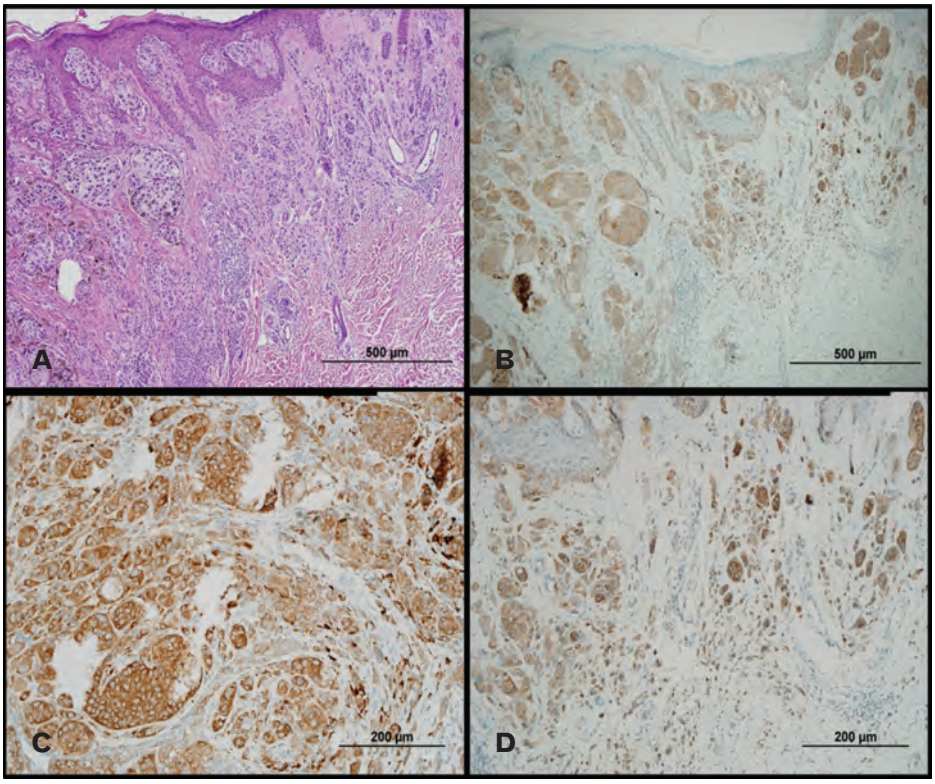

Fig. 1 Concordant positive anti-BRAF^{V600E} antibody (VE1) staining in a primary melanoma with an associated naevus. (A) H&E staining of the lesion at low power magnification. (B) BRAF^{V600E} protein expression in a primary melanoma and associated naevus, both staining strongly (3+) positive. (C) High power magnification demonstrating BRAF^{V600E} protein expression in the primary melanoma; and (D) high power magnification demonstrating BRAF^{V600E} protein expression in the associated naevus.

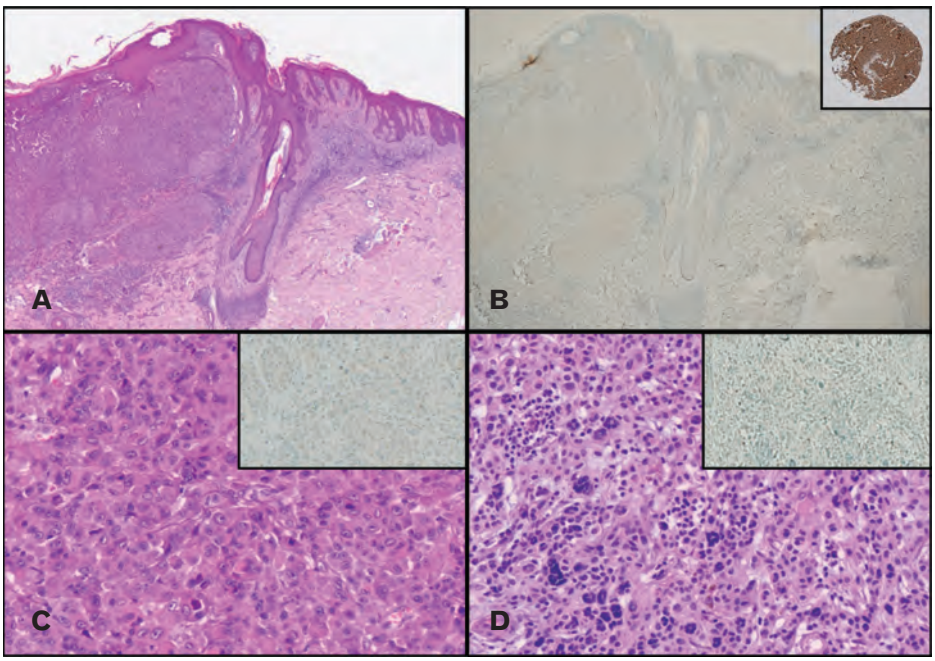

Fig. 2 Concordant negative anti-BRAF^{V600E} antibody (VE1) in a primary melanoma with an associated naevus. (A) H&E staining of the lesion at low power magnification. (B) BRAF^{V600E} protein expression in a primary melanoma and associated naevus both staining negative (inset showing positive control). (C) High power magnification of the primary melanoma (inset showing negative BRAF^{V600E} protein expression); and (D) high power magnification of the associated naevus (inset showing negative BRAF^{V600E} protein expression).

(and hence cast doubt regarding the reliability of the findings).[22,23] Recently, Tschandl and colleagues reported a concordance rate of only 80% for *BRAF* status between melanomas and associated naevi in a study of 46 cases using IHC and Sanger-sequencing.[23] The proposed explanation for the discordance was that there was a possibility the melanoma arose independently of the pre-existing naevus. Another study of 14 cases utilising laser capture micro-dissection followed by PCR amplification and sequencing, reported a *BRAF* mutation rate of 29% (4/14) in the primary melanomas and 21% (3/14) in the associated naevi.[24] The results of these studies with discordant BRAF cases are in contrast to our 100% concordance rate, and might be explained by the technical difficulty of reliably micro-dissecting out admixed naevus/melanoma cells and obtaining sufficient quantities and purity of DNA for BRAF mutation testing from both cell types. Nevertheless, it is also possible that occasional melanomas collocated with naevi arise independently of the naevus and their spatial association is coincidental.

Table 2 BRAF^{V600E} staining intensity score in primary melanomas and associated naevi

Sample no.	Naevus BRAF	Primary BRAF	Δ naevus to primary
Case 1	2	2	0
Case 2	3	3	0
Case 3	3	3	0
Case 4	3	3	0
Case 5	2	2	0
Case 6	3	3	0
Case 7	2	2	0
Case 8	3	3	0
Case 9	3	3	0
Case 10	2	2	0
Case 11	3	3	0
Case 12	2	3	1
Case 13	2	3	1
Case 14	2	3	1
Case 15	1	2	1
Case 16	1	3	2

Previous studies assessing the development of primary melanomas arising in association with naevi concluded that the melanomas arose from precursor naevus cells and that the chance of a primary melanoma developing *de novo* coincidently at the same site as a pre-existing naevus is low.[11] It was argued that the reported rates of 20–30% of primary melanomas with an underlying or adjacent naevus supported a causal association as opposed to a coincidental one.[11,12,25] In all 29 melanomas arising in association with naevi in this study, there was 100% concordance in *BRAF*^{V600E} mutation status between the primary melanoma and the associated melanocytic naevus (Fig. 1 and 2), further strengthening the case that the primary melanoma arose from the naevus.

In our study, melanomas arising in association with a naevus were more likely to be *BRAF* mutant than melanomas unassociated with naevi (55% versus 21%; $p = 0.009$). This suggests that the *BRAF* mutation rate in common acquired naevi may be lower than the initially reported rate of 82%.[7] More recent studies of small numbers of naevi have also reported lower *BRAF* mutation rates.[26–28] The intensity of the IHC staining for BRAF^{V600E} protein expression in a proportion (5/16) of the associated naevi was lower than in the primary melanoma (Table 2; Fig. 3). A similar finding was also previously highlighted in a subset of primary melanomas with associated naevi.[23,29] The higher intensity of VE1 IHC in melanomas compared to naevi is likely due to increased expression of the protein as a consequence of genetic/epigenetic alterations that amplify the reliance on the MAPK pathway.

There are a number of limitations to traditional mutation testing techniques that are overcome by the use of IHC. Most traditional mutation testing techniques provide limited

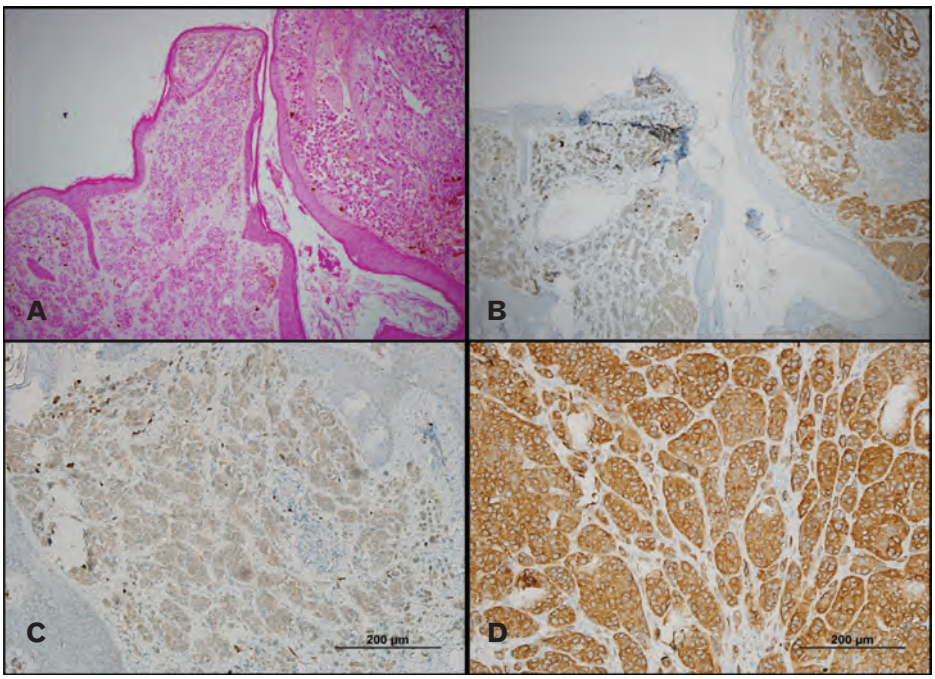

Fig. 3 Differential intensity of anti-BRAF^{V600E} antibody (VE1) staining between a primary melanoma and the associated naevus. (A) H&E staining of the lesion at low power magnification. (B) BRAF^{V600E} protein expression in a primary melanoma with an associated naevus. (C) High magnification showing strong staining intensity (3+) BRAF^{V600E} protein expression in the primary melanoma. (D) High magnification showing moderate intensity staining intensity (2+) BRAF^{V600E} protein expression in naevus.

technical sensitivity (the minimum percentage of mutant tumour cells that can be detected as a positive test) which can be problematic for specimens with a low percentage of tumour cells such as in some primary melanomas or sentinel lymph node metastases. In contrast, IHC allows detection of even single *BRAF*^{V600E} mutant tumour cells and verification that the positive cells are in fact tumour cells and not caused by contamination by naevus or other cells. However, like some traditional methods for mutation testing (such as allele-specific PCR and mass array techniques), IHC with the VE1 antibody will not detect non-V600E *BRAF* mutations, which occur in up to 20% of *BRAF* mutated melanomas.[10] Whilst massively parallel sequencing (so-called next generation sequencing) offers great promise as a technique, both highly sensitive and comprehensive, it is still reliant on the tumour purity of the tissue being tested and hence pathological verification and careful dissection of tumour tissue remains a necessity for accurate results.

In conclusion, this is the first study using IHC to demonstrate that primary melanoma associated with a naevus within the same patient are concordant for *BRAF*^{V600E} mutation status. The clinical implication of the concordance is that the theoretical possibility of a false positive *BRAF* mutation result, occurring when a *BRAF* wild-type primary melanoma is admixed with *BRAF* mutant naevus cells and tested by traditional molecular methods, is unlikely to occur. Adjuvant clinical trials (www.clinicaltrials.gov) are well advanced testing drugs targeting the MAP kinase pathway in stage IIC and III mutant *BRAF* melanoma patients. In these patients, as well as in some patients with stage IV disease, tissue for testing may be extremely limited and important clinical decisions may rest on the results of testing of the primary tumour. Strong concordance has been demonstrated between the primary tumour and metastases.[30] This, together now with the fact that contaminating mutant BRAF naevus cells are unlikely to provide false positive mutant *BRAF* status, gives increased clinical confidence for decisions dependent on *BRAF* testing of the primary tumour.

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References

1. Davies H, Bignell GR, Cox C, *et al*. Mutations of the BRAF gene in human cancer. *Nature* 2002; 417: 949–54.
2. Arkenau H, Kefford R, Long G. Targeting BRAF for patients with melanoma. *Br J Cancer* 2010; 104: 392–8.
3. Bauer J, Büttner P, Murali R, *et al*. BRAF mutations in cutaneous melanoma are independently associated with age, anatomic site of the primary tumor, and the degree of solar elastosis at the primary tumor site. *Pigment Cell Melanoma Res* 2011; 24: 345–51.

4. Broekaert S, Roy R, Okamoto I, *et al*. Genetic and morphologic features for melanoma classification. *Pigment Cell Melanoma Res* 2010; 23: 763–70.
5. Scolyer RA, Long GV, Thompson JF. Evolving concepts in melanoma classification and their relevance to multidisciplinary melanoma patient care. *Mol Oncol* 2011; 5: 124–36.
6. Dutton-Regester K, Irwin D, Hunt P, *et al*. A high-throughput panel for identifying clinically relevant mutation profiles in melanoma. *Mol Cancer Ther* 2012; 11: 888–97.
7. Pollock PM, Harper UL, Hansen KS, *et al*. High frequency of BRAF mutations in nevi. *Nat Genet* 2002; 33: 19–20.
8. Mann GJ, Pupo GM, Campain AE, *et al*. BRAF mutation, NRAS mutation, and the absence of an immune-related expressed gene profile predict poor outcome in patients with stage III melanoma. *J Invest Dermatol* 2013; 133: 509–17.
9. Long GV, Menzies AM, Nagrial AM, *et al*. Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. *J Clin Oncol* 2011; 29: 1239–46.
10. Menzies AM, Haydu LE, Visintin L, *et al*. Distinguishing clinicopathologic features of patients with V600E and V600K BRAF-mutant metastatic melanoma. *Clin Cancer Res* 2012; 18: 3242–9.
11. Bevona C, Goggins W, Quinn T, Fullerton J, Tsao H. Cutaneous melanomas associated with nevi. *Arch Dermatol* 2003; 139: 1620.
12. Massi D, Carli P, Franchi A, Santucci M. Naevus-associated melanomas: cause or chance? *Melanoma Res* 1999; 9: 85–91.
13. Chapman PB, Hauschild A, Robert C, *et al*. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 2011; 364: 2507–16.
14. Hauschild A, Grob J-J, Demidov LV, *et al*. Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. *The Lancet* 2012; 380: 358–65.
15. Halaban R, Zhang W, Bacchiocchi A, *et al*. PLX4032, a selective BRAFV600E kinase inhibitor, activates the ERK pathway and enhances cell migration and proliferation of BRAFWT melanoma cells. *Pigment Cell Melanoma Res* 2010; 23: 190–200.
16. Poulikakos PI, Zhang C, Bollag G, Shokat KM, Rosen N. RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature* 2010; 464: 427–30.
17. Hatzivassiliou G, Song K, Yen I, *et al*. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature* 2010; 464: 431–5.
18. Long GV, Wilmott JS, Capper D, *et al*. Immunohistochemistry is highly sensitive and specific for the detection of V600E BRAF mutation in melanoma. *Am J Surg Pathol* 2013; 37: 61–5.
19. Wilmott JS, Scolyer RA, Long GV, Hersey P. Combined targeted therapy and immunotherapy in the treatment of advanced melanoma. *Oncoimmunology* 2012; 1: 997–9.
20. Capper D, Berghoff AS, Magerle M, *et al*. Immunohistochemical testing of BRAF V600E status in 1, 120 tumor tissue samples of patients with brain metastases. *Acta Neuropathol* 2012; 123: 1–11.
21. Capper D, Preusser M, Habel A, *et al*. Assessment of BRAF V600E mutation status by immunohistochemistry with a mutation-specific monoclonal antibody. *Acta Neuropathol* 2011; 122: 11–9.
22. Dadzie O, Yang S, Emley A, Keady M, Bhawan J, Mahalingam M. RAS and RAF mutations in banal melanocytic aggregates contiguous with primary cutaneous melanoma: clues to melanomagenesis. *Br J Dermatol* 2009; 160: 368–75.
23. Tschandl P, Berghoff AS, Preusser M, *et al*. NRAS and BRAF mutations in melanoma-associated nevi and uninvolved nevi. *PloS One* 2013; 8: e69639.
24. Yazdi AS, Palmedo G, Flaig MJ, *et al*. Mutations of the BRAF gene in benign and malignant melanocytic lesions. *J Invest Dermatol* 2003; 121: 1160–2.
25. Urso C, Giannotti V, Reali U, Giannotti B, Bondi R. Spatial association of melanocytic naevus and melanoma. *Melanoma Res* 1991; 1: 245–50.
26. Poynter JN, Elder JT, Fullen DR, *et al*. BRAF and NRAS mutations in melanoma and melanocytic nevi. *Melanoma Res* 2006; 16: 267–73.
27. Bauer J, Curtin JA, Pinkel D, Bastian BC. Congenital melanocytic nevi frequently harbor NRAS mutations but no BRAF mutations. *J Invest Dermatol* 2006; 127: 179–82.
28. Uribe P, Wistuba II, González S. BRAF mutation: a frequent event in benign, atypical, and malignant melanocytic lesions of the skin. *Am J Dermatopathol* 2003; 25: 365–70.
29. Busam KJ, Hedvat C, Pulitzer M, von Deimling A, Jungbluth AA. Immunohistochemical analysis of BRAFV600E expression of primary and metastatic melanoma and comparison with mutation status and melanocyte differentiation antigens of metastatic lesions. *Am J Surg Pathol* 2013; 37: 413–20.
30. Colombino M, Capone M, Lissia A, *et al*. BRAF/NRAS mutation frequencies among primary tumors and metastases in patients with melanoma. *J Clin Oncol* 2012; 30: 2522–9.