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Utility of BRAF V600E immunohistochemistry expression pattern as a surrogate of *BRAF* mutation status in 154 patients with advanced melanoma

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Summary

Successful BRAF inhibitor therapy depends on the accurate assessment of the mutation status of the *BRAF V600* residue in tissue samples. In melanoma, immunohistochemical (IHC) analysis with monoclonal anti-BRAF V600E has emerged as a sensitive and specific surrogate of *BRAF V600E* mutation, particularly when BRAF V600E protein expression is homogeneous and strong. A subset of melanomas exhibit heterogeneous labeling for BRAF V600E, but our understanding of the significance of heterogeneous BRAF V600E IHC expression is limited. We used next-generation sequencing (NGS) to compare BRAF V600E IHC staining patterns in 154 melanomas:

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79 *BRAF*^{WT} and 75 *BRAF* (including 53 *V600E*) mutants. Agreement among dermatopathologists on tumor morphology, IHC expression and intensity was excellent ($\rho=0.99$). A predominantly epithelioid cell phenotype significantly correlated with the *BRAF V600E* mutation ($P=.0085$). Tumors demonstrating either heterogeneous or homogeneous IHC expression were significantly associated with the *BRAF V600E* mutation ($P<.0001$) as was increased intensity of staining ($P<.0001$). The positive predictive value was 98% for homogenous IHC expression compared with 70% for heterogeneous labeling. Inclusion of both heterogeneous and homogeneous *BRAF V600E* IHC expression as a positive test significantly improved IHC test sensitivity from 85% to 98%. However, this reduced *BRAF V600E* IHC test specificity from 99% to 96%. Cautious evaluation of heterogeneous *BRAF V600E* IHC expression is warranted and comparison with sequencing results is critical, given its reduced test specificity and positive predictive value for detecting the *BRAF V600E* mutation.

Keywords

RAF V600E; melanoma; immunohistochemistry; next generation sequencing; sensitivity; specificity

1. Introduction

Investigations into the genomic landscape of cutaneous melanoma have identified mutations in the *BRAF* oncogene in 50–60% of patients as the most common oncogenic driver mutation [1, 2]. Among the activating *BRAF* mutations in melanomas, the majority (>99%) involves exon 15. The most frequent *BRAF* mutation type encountered (~75%-90%) is *BRAF V600E*, which involves a threonine to alanine (T to A) single DNA base substitution [1–3]. Compared with the kinase activity of the *BRAF*^{WT} protein, the *BRAF V600E* protein exhibits 480 times higher kinase activity; furthermore, patients with *BRAF V600E* mutant melanomas treated with selective small-molecule *BRAF* inhibitors (e.g., vemurafenib) had better clinical response, progression-free survival, and overall survival rates than did patients treated with standard chemotherapy [4, 5].

Various molecular testing platforms are available for determining *BRAF* mutations, including the Cobas® 4800 *BRAF V600* mutation test (Roche Molecular Diagnostics, Branchburg, NJ) as well as next-generation sequencing (NGS) platforms [6].

BRAF V600E can also be detected at the level of protein expression using immunohistochemical (IHC) methods with monoclonal anti-*BRAF V600E*. This antibody provides highly sensitive (97–100%) and specific (97–100%) insight into the *BRAF V600E* mutation status of patients with melanoma [7–12]. Homogeneous *BRAF V600E* IHC expression in melanoma highly correlates with the presence of *BRAF V600E* mutation when compared with sequencing methods as the gold standard. However, we and others have observed a subset of tumors exhibiting intratumoral or intertumoral heterogeneity for *BRAF V600E* IHC expression, and these tumors appear to harbor the *BRAF V600E* mutation at variable frequencies by molecular testing methods [9, 13]. Of note, however, Wilmott *et al.* [14] showed that the pattern of *BRAF V600E* protein expression did not predict response in patients receiving *BRAF* inhibitor therapy. However, knowledge of the clinical relevance of

heterogeneous tumor expression with anti-BRAF V600E immunostaining and correlation with *BRAF* mutation status is limited. Here, we compared the expression patterns from BRAF V600E IHC tests patients' mutation status, determined by concomitant NGS in 154 patients with metastatic melanoma. Our results confirmed the high sensitivity and specificity of the BRAF V600E IHC assay and further underscored the importance of recognizing and reporting heterogeneous labeling patterns.

2. Materials and methods

2.1. Patient selection and data collection

With the approval of the Institutional Review Board at The University of Texas MD Anderson Cancer Center, we retrospectively reviewed the results of clinical *BRAF* mutation testing performed over a 2-year period (January 1, 2011 to January 31, 2013) in patients with melanoma who were treated at MD Anderson and their tumor samples were analyzed by the Clinical Laboratory Improvement Amendments (CLIA)–certified Molecular Diagnostics Laboratory (MDL) of the Division of Pathology and Laboratory Medicine. Patients who had undergone BRAF IHC testing and concomitant *BRAF* molecular testing from the same tumor source for >99% of the cases were evaluated. NGS was performed on the majority of the cases (>99%), and in only one case the correlation with positive BRAF V600E IHC test and *BRAF* mutation status was achieved by pyrosequencing.

For all patients' tumor samples tested, we recorded the specific mutations detected, patient demographics (age and gender), tissue source (skin, lymph node, visceral), and primary tumor or metastasis.

2.2. *BRAF* mutation testing

2.2.1. BRAF V600E IHC test—IHC staining with anti-BRAF V600E (clone VE1) was performed on matched tumor samples submitted for molecular testing. Clone VE1 to *BRAF V600E* (Spring Bioscience), as previously described, [7] was used at a 1:50 dilution with an automated IHC staining instrument (Bond, Leica Biosystems, Buffalo Grove, IL; n=144 cases, or BenchMark XT, Ventana Medical Systems, Tucson, AZ; n=10 cases) and detected with a DAB (3,3'-diaminobenzidine) detection system.

IHC staining patterns with anti-BRAF V600E, along with corresponding hematoxylin and eosin (H&E) stains, were examined and scored by dermatopathologists (MTT and JLC) independent of NGS-based mutation status. BRAF V600E cytoplasmic protein expression was scored in three categories. *Positive heterogeneous pattern* was defined as any sub-population of tumor cells with negative staining and positive staining in < 95% tumor cells in the sample. *Positive homogeneous pattern* was defined as > 95% of tumor cells positive for anti-BRAF V600E, and *negative staining* was defined as absence of any cytoplasmic labeling in the tumor cells. Intensity of staining was also recorded: weak, moderate, and strong. Nuclear staining only was scored as negative, as previously reported [7]. The *predominant cell morphology*, defined as > 50% of tumor cells, was scored as spindle or epithelioid.

2.2.2. BRAF NGS test—*BRAF* mutation testing was performed on an Ion Torrent Personal Genome Machine (IT-PGM) 46/50 cancer-related gene NGS platform in the CLIA-certified MDL at our institution, as previously described [15].

2.3. Statistical methods

Fisher exact tests were used to examine associations between the BRAF V600E IHC test and other categorical variables. Spearman correlation determined the agreement between reviewers of the BRAF V600E IHC test. All statistical analyses were performed by using SAS 93 for Windows. *P* values <.05 were considered statistically significant.

3. Results

3.1. Clinical tumor characteristics and association with BRAF V600E IHC expression

A total of 154 patients (M:F = 100:54; median age, 61 years; range, 26 to 87 years) met the study criteria: 131 (85%) patients had a known anatomic site of their primary melanoma and 23 (15%) had melanoma of unknown primary. Table 1 shows the clinical tumor characteristics used for IHC and NGS testing.

There was no significant association with BRAF V600E IHC expression or intensity of staining with the anatomic site of primary melanomas or between the method of tissue procurement. However, there was some evidence that heterogeneous BRAF V600E expression occurred more commonly in metastatic tumor samples (10%, 10/105) than in primary tumor samples (0%, 0/49) (*P* = .05) (Table 1). However, this does not appear to be a function of the cutaneous location, since metastases to the skin exhibited this pattern.

3.2. BRAF V600E IHC expression and NGS molecular analysis

There was excellent agreement in determining BRAF V600E IHC expression pattern staining intensity and predominant tumor phenotype ($\rho = 0.99$). There was initial disagreement in the designation of heterogeneous and homogeneous BRAF V600E IHC expression pattern in four cases (3%), but the final consensus score by two dermatopathologists was heterogeneous expression for two samples and homogeneous for the other two. There was also some disagreement in the intensity (that differed by only one category) of BRAF V600E expression in 19 cases (12%), with differences between moderate and strong in 15 (10%) and between weak and moderate in 4 (3%).

Examination of the corresponding H&E-stained sections of tumor samples submitted for analysis with a 46/50 gene panel with NGS demonstrated that 92% (142/154) of the tumor samples contained an epithelioid cell phenotype as the predominant cell morphology (Fig.1), of which 32% (46/142) had homogeneous BRAF IHC expression, which significantly correlated with the probability of harboring the *BRAF V600E* mutation (*P* = .0085) (Table 2). The 12 tumors with spindle cells as the predominant cell morphology were negative for BRAF V600E IHC and none had a *BRAF V600E* mutation (Fig.1); only one tumor sample with spindle cell morphology had the *BRAF G469E* mutation.

Of the 154 patients tumors tested for BRAF V600E IHC expression, 98 (64%) were negative, whereas 46 (30%) had a homogenous IHC expression pattern and 10 (6%)

demonstrated heterogeneous labeling (Fig. 2). Patients whose tumors had positive (heterogeneous or homogeneous) BRAF V600E IHC expression were significantly associated with the presence of *BRAF V600E* mutation ($P < .0001$) (Table 2). The presence of nuclear restricted BRAF V600E IHC expression was seen in 7 tumor samples tested, and these were ultimately interpreted as negative [7]. Among the 56 positive BRAF V600E IHC tumor samples, 75% displayed strong IHC intensity that correlated significantly with the presence of the *BRAF V600E* mutation ($P < .0001$) (Table 2). All the *BRAF V600K* mutation were associated with epithelioid tumor morphology and ~94% had a negative BRAF V600E IHC test.

3.3. Comparison of BRAF V600E IHC test with *BRAF V600E* mutation status

Matched tumor blocks used for BRAF V600E IHC expression were also subjected to 46/50 gene panel mutation analysis by the NGS platform and identified 79 patients (51%) with wild-type *BRAF* (*BRAF^{WT}*) and 75 patients (49%) who harbored *BRAF* mutations. Among the 75 patients with *BRAF* mutations, 53 had *V600E*, 16 had *V600K*, and 6 had *BRAF non-V600* mutation types (*K601E*, *N581I*, *G596R*, *G469E*, *G469S*, *D595N*).

Compared with the *BRAF V600E* test by NGS, the sensitivity of homogeneous BRAF V600E IHC expression (when heterogeneous cases were considered negative) was 85%, specificity was 99%, positive predictive value (PPV) was 98%, and negative predictive value (NPV) was 93%. All 10 of the cases with BRAF V600E IHC labeling with Ventana automated stainer had concordant IHC and molecular results. One case demonstrated a strong and homogeneous BRAF V600E IHC expression pattern, but the molecular test by NGS demonstrated *BRAF^{WT}* (Table 3). However, due to the strong and diffuse BRAF V600E IHC expression pattern and the relatively small amount of tumor in the tissue selected for molecular testing, the same tissue sample was subjected to laser capture microdissection of a small focus of tumor, and subsequent pyrosequencing analysis confirmed the presence of *BRAF V600E* mutation [16].

Of the 10 patient tumor samples with heterogeneous labeling, the degree of heterogeneity for BRAF V600E IHC expression pattern ranged from 5% to 80% positive labeling of melanoma cells (Table 3). Seven tumors harbored the *BRAF V600E* mutation, whereas 3 did not and we were false-positive tests (2 had *BRAF* mutations [*V600K* and *D549N*] and 1 was *BRAF^{WT}*). In the 6 matched-tumor samples with positive heterogeneous BRAF V600E IHC that correlated with *BRAF V600E* by NGS (one positive heterogeneous sample did not have NGS data available), the percent of tumor cells labeling with anti-BRAF antibody ranged from 20–80% with 2 of the 6 samples showing < 55% of the tumor cells labeling (Fig. 2). Furthermore, when we correlated the IHC result with the relative *BRAF V600E* allele frequency, we found no significant difference in the percentage of *BRAF V600E* mutant alleles in the positive heterogeneous samples (range: 5%-55%) than in the homogenous BRAF IHC group (range: 2%-86%). The percentage of tumor cells labeling in the three false-positive BRAF IHC samples ranged from 5% to 25%. The sensitivity of heterogeneous BRAF IHC expression was 100%, but the specificity and PPV were only 70%.

When both heterogeneous and homogeneous BRAF V600E IHC labeling were classified as a positive IHC test, the sensitivity of the BRAF V600E IHC test was 98%, specificity was

96%, the PPV was 93%, and the NPV was 99% (Table 4). Incorporation of heterogeneous BRAF V600E IHC expression as a positive IHC test significantly improved IHC test sensitivity to 98% (95% CI, 94%-100%) compared with a sensitivity of 85% (95% CI, 75%-94%) when only homogeneous IHC expression was considered as positive ($P=.0403$). However, this caused a reduction in BRAF V600E IHC test specificity from 99% to 96%. Only one case that was scored negative by IHC harbored a *BRAF V600E* mutation by NGS. This sample was heavily pigmented, which obscured evaluation. As noted by Thiel *et al.* [17] this underscores an important pitfall in the interpretation of BRAF IHC (Fig. 3) and suggests the need for melanin bleaching in such samples, which reportedly does not affect BRAF V600E immunohistochemistry [11].

4. Discussion

Here, we report our experience with the largest single collection of melanomas assayed by both BRAF V600E IHC and NGS platform in a CLIA-certified environment. We showed that cumulative homogeneous and heterogeneous BRAF V600E IHC expression had a sensitivity of 98% and a specificity of 96%. Our findings are in agreement with prior studies showing high sensitivity (overall 97%, from 534/550 samples; range: 97%-100%) and specificity (overall 98%, from 723/738 samples; range: 97%-100%) for this assay from among more than 1250 samples of melanoma and underscore its utility as a reliable surrogate for *BRAF V600E* mutation status in patients with melanoma (Table 5)[8, 9, 12, 14, 17–21]. Heterogeneous BRAF V600E IHC expression was reported in ~15% of melanoma samples from 5 studies (including current study). Despite the heterogeneous pattern, there is a strong correlation with this pattern of IHC expression and the presence of mutant *BRAF V600E*. Of the 32 cases with heterogeneous BRAF V600E IHC, the overall sensitivity was 91% for the presence of the *BRAF V600E* mutation. In addition, Boursault *et al.* [22] reported ambiguous patterns of labeling (faint, equivocal brown staining) in 3 samples—1 of which harbored *BRAF V600E*.

Heterogeneous BRAF V600E IHC expression has been reported in 2.6% to 35% of melanoma tumor samples tested [9, 10, 14, 22, 23]. Here, we also confirm heterogeneous BRAF V600E IHC expression pattern in a subset of melanomas. Heterogeneous BRAF V600E immunoreactivity was observed in ~7% of the patient melanoma samples tested (10/154) and was detected only in metastatic tumor samples (10%, 10/105) and never in primary tumor samples (0/49) ($P=.05$). Furthermore, we show that 70% (7/10 samples tested) of tumor samples with heterogeneous BRAF V600E IHC expression actually harbored *BRAF V600E* mutation by the NGS platform.

A number of explanations for heterogeneous BRAF V600E IHC labeling are possible. Some cross reactivity among different BRAF epitopes is conceivable. In particular, other mutations occurring at V600 (including V600K) might occasionally sufficiently mimic the V600E epitope so as to variably cross react with the VE1 antibody; however, the vast majority of the V600K mutated tumor samples in our series were negative by BRAF V600E IHC. Prior studies have shown low-frequency cross-reactivity with BRAF V600E IHC with *non-V600E* mutations, including V600R [24, 25]. Alternatively, intratumoral molecular heterogeneity for the *BRAF V600E* mutation in melanoma has been described [26] and may

explain a subset of heterogeneous BRAF V600E IHC expression. Arguing against this is that there was no apparent difference in the allele frequencies observed in heterogeneous compared with homogeneous samples. Tumor heterogeneity for BRAF V600E IHC expression has also been reported in approximately 10% of thyroid and 1.5% of cases colorectal cancers cases [27–29]. Correlation with heterogeneous BRAF V600E IHC expression as a surrogate for the *BRAF V600E* mutation may be tumor dependent since there is a relative high rate of correlation with this pattern of labeling and the presence of *BRAF V600E* mutation in melanomas and thyroid carcinomas, but not in colorectal carcinomas [9, 27, 29].

Preanalytical variables in tissue preservation artifacts likely also contribute to heterogeneous BRAF V600E IHC staining. Tissue fixation studies with anti-BRAF V600E (VE1) antibody by Dvorak *et al.* [30] reported the optimal tissue fixation was with 10% neutral buffered formalin (NBF) for 12–24 hours within 2 hours of tissue collection. Fixation with NBF for fewer than 12 hours correlated with weaker staining, and fixation with zinc formalin, 95% ethanol, Z-5 (formalin, zinc, alcohol), or AFA (alcohol formalin acetic acid alcohol), in comparison to NBF, were suboptimal fixatives regardless of fixation time. Although the fixation time and type of fixative at our laboratory are generally standardized and validated for optimal conditions for ancillary studies (e.g., IHC and DNA analysis), one cannot exclude the possibility of uneven fixative penetration in a minority of tissue samples, thus contributing to heterogeneous BRAF V600E IHC staining.

A significant subset of tumors with heterogeneous BRAF V600E IHC expression harbor concomitant *BRAF V600E* mutation and inclusion of this staining pattern in our study as a positive test increased IHC test sensitivity but reduced IHC test specificity. Since patients with *BRAF non-V600* mutations treated with BRAF inhibitors may actually experience enhanced tumor growth, lowering test specificity for *BRAF V600E* mutation may have dramatic clinical implications [31]. Thus clinically, BRAF V600E IHC test specificity is more critical, and in our practice, we advise correlation with DNA sequencing analysis in tumor samples that demonstrate a heterogeneous (or negative) BRAF V600E IHC expression pattern.

A pitfall in the interpretation of BRAF V600E IHC expression may occur in tumor samples with notable melanin pigment because it complicates the assessment when a brown chromogen (3,3'-diaminobenzidine [DAB]) detection system is used. In our experience, examination of the corresponding H&E-stained slide together with BRAF V600E IHC stain often facilitates the distinction of true immunoreactivity from melanin. Some authors advocate pretreatment of tissue with a mild H₂O₂ and heat to remove endogenous melanin to help expose tumor that harbored the *BRAF V600E* mutation [11]. Alternatively, one may choose the use of red chromogen detection agent (3-amino-9-ethylcarbazole [AEC]) in cases where melanin obscures accurate interpretation.

In conclusion, we show that a subset of melanomas demonstrate heterogeneous labeling for BRAF V600E IHC. This occurred more frequently in metastases than in primary tumors. Although inclusion of heterogeneous labeling in the analysis significantly increased sensitivity of the BRAF V600E IHC test, this compromised specificity as well as PPV for

the detection of *BRAF V600E* mutation. Thus, reporting heterogeneous *BRAF V600E* IHC as well as lesions heavily pigmented with melanin necessitates correlation with concomitant mutational data before deciding to treat with *BRAF* inhibitors.

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Fig. 1. Melanoma cells with epithelioid cell morphology (A and C, H&E stain, $\times 40$ and $\times 400$, respectively) with homogeneous labeling with anti-BRAF V600E (B and D, immunohistochemical stain $\times 40$ and $\times 400$, respectively). Contrast, melanoma cells with spindle cell morphology (E, H&E stain, $\times 400$) negative for anti-BRAF V600E (F, immunohistochemical stain, $\times 400$).

Fig. 2.

Representative cases with areas of heterogeneous labeling (*) with anti-BRAF V600E (A and E, H&E stain $\times 100$, B and F immunohistochemical stain, $\times 100$). Higher magnification of scattered tumor cells positive (arrows) and negative (*) for anti-BRAF V600E (C and G immunohistochemical stain, $\times 400$). Corresponding molecular analysis by NGS detected *BRAF c.1799T>A p.V600E* (D) mutation and a *BRAF c.1780G>A p.D594N* (H) mutation. The bar at the top corresponds to the depth of sequencing at each nucleotide position. The bars corresponding to a mutated base shows the relative proportion of mutant and wild-type bases at each location in red and green, respectively. The pink and blue bands correspond to representative forward and reverse reads respectively where consensus wild-type sequences are hidden and only the mutant bases are shown in bold. The wild-type reference sequence and corresponding amino acids are shown at the bottom.

Fig. 3.

Case with heavily pigmented melanoma cells (A, H&E stain, $\times 400$) that obscures interpretation of BRAF V600E IHC (B, immunohistochemical stain $\times 400$). Corresponding molecular analysis by NGS detected *BRAF* c.1799T>A p.V600E (C) mutation. The bar at the top corresponds to the depth of sequencing at each nucleotide position. The bars corresponding to a mutated base shows the relative proportion of mutant and wild-type bases at each location in red and green, respectively. The pink and blue bands correspond to representative forward and reverse reads respectively where consensus wild-type sequences are hidden and only the mutant bases are shown in bold. The wild-type reference sequence and corresponding amino acids are shown at the bottom.

Table 1

Association with BRAF V600E IHC expression pattern and clinical tumor characteristics.

	N =154 (%)	BRAF IHC expression pattern N (%)		
		Negative	Heterogeneous	Homogeneous
Tissue type used for IHC test				
Primary melanoma	49 (32)	35 (23)	0	14 (9)
Metastatic melanoma	105 (68)	63 (41)	10 (7) ^a	32 (21)
Lymph node	54 (35)	32 (21)	6 (4)	16 (10)
Skin	25 (16)	12 (8)	2 (1)	11 (7)
Lung	6 (4)	4 (3)	0	2 (1)
Non-lung visceral	20 (13)	15 (10)	2 (1)	3 (2)
Tissue procurement method				
Endoscopic	4 (3)	4 (3)	0	0
Needle core	18 (12)	11 (7)	2 (1)	5 (3)
Shave/punch	31 (20)	21 (14)	0	10 (7)
Excision	101 (66)	62 (40)	8 (5)	31 (20)

Abbreviations: IHC, immunohistochemical

^a Statistically significant, $P = .05$.

Table 2

Results of BRAF V600E IHC expression and molecular tests.

Sample size (N)	Microscopic analysis	<i>BRAF</i> ^{WT} N = 79 (%)	Molecular analysis by 46/50 NGS gene panel		
	N=154 (%)		<i>BRAF</i> V600E N = 53 (%)	<i>BRAF</i> V600K N=16 (%)	<i>BRAF non-V600</i> N=6 (%)
Tumor cell morphology					
Epithelioid	142 (92)	68 (86)	53 (100)	16 (100)	5 (83)
Spindle	12 (8)	11 (14)	0	0	1 (17)
IHC expression pattern					
Negative	98 (64)	77 (98)	1 (2)	15 (94)	5 (83)
Heterogeneous	10 (6)	1 (1)	7 (13) ^b	1 (6)	1 (17)
Homogeneous	46 (30) ^a	1 (1)	45 (85) ^b	0	0
IHC intensity					
Negative	98 (64)	77 (97)	1 (2)	15 (94)	5 (83)
Weak	7 (5)	1 (1)	5 (26)	0	1 (17)
Moderate	7 (5)	0	6 (11)	1 (6)	0
Strong	42 (27) ^b	1 (1)	41 (77)	0	0

Abbreviations: IHC, immunohistochemical; NGS, next-generation sequencing; WT, wild-type for *BRAF*.

^aStatistically significant, *P* =.0085.

^bStatistically significant, *P* < .0001.

Table 3

List of patients with heterogeneous BRAF V600E IHC expression and melanoma samples with discordant IHC molecular results.

Age	Gender	Site of primary melanoma	Histologic type	Procurement method	Specimen type	Cell phenotype	BRAF V600E IHC expression (% of positive cells)	BRAF V600E IHC intensity	BRAF V600E IHC results (+ or −)	BRAF mutation type by NGS	BRAF V600E mutant alleles by NGS (%)	Other/co-existing mutation by NGS
60	M	Unknown primary	n/a	Excision	LN met	Epithelioid	Heterogeneous (80)	Moderate	+	V600E	54	0
35	F	Shoulder	SS	Core	Brain met	Epithelioid	Heterogeneous (70)	Strong	+	V600E	47	0
76	F	Foot	AL	Core	LN met	Epithelioid	Heterogeneous (70)	Strong	+	V600E	n/a ^b	CDKN2A
36	M	Back	Nodular	Excision	LN met	Epithelioid	Heterogeneous (80)	Moderate	+	V600E	4	0
57	M	Unknown	n/a	Excision	LN met	Epithelioid	Heterogeneous (70)	Strong	+	V600E	28	TP53
61	M	Back	Nodular	Excision	Brain met	Epithelioid	Heterogeneous (20)	Weak	+	V600E	55	0
60	F	Unknown	n/a	Core	Skin met	Epithelioid	Heterogeneous (55)	Moderate	+	V600E	6	0
Discordant BRAF V600E samples												
65	F	Unknown	n/a	Excision	Skin met	Epithelioid	Heterogeneous (15)	Weak	+	WT	0	KIT
60	M	Cheek	n/a	Excision	LN met	Epithelioid	Heterogeneous (25)	Moderate	+	V600K	0	CDKN2A, SMO
52	F	Unknown	n/a	Excision	LN met	Epithelioid	Heterogeneous (5)	Weak	+	D594N	0	None
44	M	Back	Nodular	Excision	LN met	Epithelioid	Homogeneous (95)	Strong	+	WT ^a	0 ^d	KDR
71	F	Foot	AL	Shave/punch	Primary	Epithelioid	Negative (0)	Negative	−	V600E	30	None

Abbreviations: AL, acral lentiginous; IHC, immunohistochemical; LN, lymph node; met, metastasis; +, positive; −, negative; n/a, not available; NGS, next-generation sequencing; SS, superficial spreading; WT, wild type.

^aLaser capture microdissection and pyrosequencing detected BRAF V600E mutation.

^bNot available, one heterogeneous case without matched tissue.

Table 4

Cumulative sensitivity and specificity of a positive (includes homogeneous and heterogeneous labeling) BRAF V600E IHC test with molecular analysis by NGS.

BRAF V600E			
IHC Test		NGS Test	
		Positive	Negative
Positive	56 ^a	52	4
Negative	98	1	97
Total	154	53	101
Sensitivity % (95% CI)		98 (90–100)	
Specificity % (95% CI)		96 (90–99)	
NPV % (95% CI)		99 (94–100)	
PPV % (95% CI)		93 (83–98)	

Abbreviations: CI, confidence interval; IHC, immunohistochemical; NGS, next-generation sequencing; NPV, negative predictive value; PPV, positive predictive value.

^a 46 tumor samples with homogeneous BRAF V600E IHC staining pattern and 10 tumor samples with heterogeneous BRAF V600E IHC staining pattern.

Table 5

Summary of BRAF V600E IHC expression and molecular tests in the literature.

Study (Ref)	Number of melanomas	Number of BRAF V600E melanomas	Method	Positive by BRAF V600E IHC	Antibody used	Number of non-V600E melanomas	Negative by BRAF V600E IHC	Tissues tested	Intratumoral Heterogeneity	
									% (N)	% (N) cases that harbor mutant BRAF V600E
Boursault [22]	230	112 ^a	HRM and Sanger	109 ^a	VE1 (Spring Biosciences)	118	121	Primary and metastases	Not identified ^c	Not reported
Busam [9]	51	25	Sequenome Mass ARRAY	25	VE1 Mab	26	26	Primary and metastases	32 (7/22)	100 (7)
Capper [7]	43	16	PCR-direct sequence	16	VE1 Mab	27	27	Brain metastases	Not identified	
Chen [11]	38	7	Pyrosequencing and allele specific PCR	7	VE1 (New East Bioscience)	29	27	Primary and metastases	Not reported	
Colomba [21]	89	40	Sanger, RT-PCR direct sequence, pyrosequence	40	VE1 (Mab)	49	49	TMA of metastases	Present (in minority)	Not reported
Feller [12]	35	6	Allele specific PCR and direct sequence	6	VE1 (New East Bioscience)	29	28	Primary	NA	
Ihle [25]	63	22	Multiple methods	22	VE1 (Spring Biosciences)	41	40	Melanomas (not specified)	Not identified	
Lade-Keller[19]	28	10	PCR-based methods (Cobas 4800, Sanger, CADMA, pyrosequencing, allele-specific PCR)+ Therascreen PCR	9	VE1 (Spring Biosciences)	18	15	Primary	Not reported	
Long [8]	97	38	HRM and PCR-mass spectrometry	37	VE1 (Mab)	59	58	Primary and metastases	Not identified	
Menzies [13]	64	30	HRM and PCR-mass spectrometry	30	VE1 (Spring Biosciences)	34	34	Primary and metastases	Not identified	
Pearlstein[23]	76	26	Pyrosequencing	22	VE1 (Spring Biosciences)	50	50	Primary and metastases	3 (2/76)	100 (2)
Routhier [18]	31	20 ^e	SNAPSHOT multiplex PCR	9	VE1 (Spring Biosciences)	42 ^e	20	Primary and metastases	Not reported	
				9	VE1 (New East Bioscience)		17			
Skorokhod [20]	75	35	Sanger and pyrosequence	29	VE1 Mab	40	40	Paired primary and metastases	Not identified	
Thiel [17]	127	52 ^b	Sanger and cMS	54 ^b	VE1 (Spring Biosciences)	75	73	Primary and metastases	Not reported	
Wilmott [14]	58	58	Somatic mutation testing (specific method not reported)	58	VE1 (Mab)	0	0	Primary and metastases	22 (13/58)	100 (13)
Tetzlaff (current)	154	53 ^d	NGS	52	VE1 (Spring Biosciences)	101	98	Primary and metastases	18 (10/56)	70 (7)
Totals	1259	550 ^f		534 ^f		738 ^g	723 ^g			

Abbreviations: CADMA, competitive amplification of different melting amplicons; cMS, cyclic minisequencing; HMR, high resolution melting; IHC, immunohistochemical; Mab, monoclonal antibody; PCR, polymerase chain reaction; TMA, tissue microarray.

^a 8 tumor samples *BRAF V600E2*.
^b 1 tumor sample *BRAF V600E2*.
^c 3 tumor samples of ambiguous pattern (faint pattern) mutation present in 1 of 3 tumor samples.
^d 1 tumor sample negative by NGS and positive by pyrosequencing on repeat analysis.
^e Samples counted twice since tested by two different VE1 antibody.
^f Sensitivity = 97% (534/550).
^g Specificity =98% (723/738).