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Immunohistochemistry with a mutation-specific monoclonal antibody as a screening tool for the *BRAFV600E* mutational status in primary cutaneous malignant melanoma

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The *V600E* mutation of *BRAF* has emerged as both an effective biomarker and therapeutic target for select benign and malignant cutaneous and non-cutaneous human tumors and is typically determined using DNA-based techniques that include allele-specific PCR and direct DNA sequencing. Recently however, the development of new antibodies directed against the V600E protein has opened the door for an easier and more efficient strategy for identifying this mutation. Our present aim was to determine the efficacy of one such antibody, anti-B-Raf (V600E), a mouse monoclonal antibody in which the immunogen is a synthetic peptide derived from the internal region of *BRAFV600E*. A total of 35 cases of primary cutaneous melanoma were evaluated using a combination of DNA-based techniques that included allele-specific PCR and/or direct DNA sequencing and immunohistochemistry. Cases of papillary thyroid carcinomas ($n = 5$) and colorectal carcinomas ($n = 5$), known to harbor the *BRAFV600E* mutation, served as positive controls for the study. DNA analyses revealed that 6 of 35 (17%) cases of the primary cutaneous malignant melanoma possessed the *BRAFV600E* mutation. For immunohistochemical analyses, cytoplasmic positivity with anti-B-Raf was noted in 7 of 35 (20%) cases of primary melanoma and in all 10 positive controls. Statistical analyses of the data demonstrated that the sensitivity of the immunohistochemistry was 100% and specificity was 97%. Findings from the current study support the potential use of immunohistochemistry as an ancillary screening tool to assess the *BRAFV600E* mutation status in primary cutaneous melanoma.

Activating *BRAF* mutations are found at a high frequency in benign and malignant, cutaneous and non-cutaneous human tumors. Over 90% of these mutations are characterized by a glutamic acid to valine substitution at position 600 (*V600E*) resulting in a substantial increase in protein kinase activity.[1–3] The *BRAFV600E* mutation is seen at particularly high rates in primary and metastatic melanoma (20–70%), papillary thyroid carcinoma (40–70%), colorectal carcinoma (5–10%), and in select benign tumors such as melanocytic nevi.[2,4–10]

Given its prevalence and role in increasing tumor cell proliferation and metastases, *BRAFV600E* has emerged as an important biomarker for clinicians. In early melanomas, the *BRAF* mutation status has not been shown to affect overall survival; however, in metastatic melanoma, it has been associated with a poorer survival rate.[11,12] With regard to papillary thyroid carcinomas, evidence suggests that molecular testing for *BRAFV600E* in thyroid fine-needle aspirations can be a useful tool to separate this malignancy from other thyroid nodules.[13] The mutation status also serves as a prognostic marker as its presence is associated with higher rates of tumor recurrence and mortality.[14] In the case of colorectal carcinomas, *BRAFV600E* mutation positive patients have significantly shorter progression-free survival

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when compared with patients with tumors with *BRAF* wild-type and its presence is shown to impair the therapeutic effect of drugs such as cetuximab and panitumumab.[15] The recognition of *BRAFV600E* mutation and its prognostic value has led to accelerated attempts to utilize it as a target for cancer therapy. Briefly, recent drugs targeted against mutated *BRAF* (particularly in melanomas) have shown great promise in early clinical trials.[10]

As it stands today, *BRAFV600E* mutation status is typically determined by means of DNA-based techniques such as direct DNA Sanger sequencing and allele-specific PCR. However, both of these strategies are expensive and time consuming. Additional confounding issues include the variable quality of DNA extracted from formalin-fixed paraffin-embedded tissue and the obscuring effects of melanin pigment.[16] These issues underscore the need for establishing faster and more efficient methods to screen for this mutation. Of particular interest, the development of new antibodies directed against the V600E protein has opened the door for the usage of immunohistochemistry to identify the *BRAFV600E* mutation. The primary aim of the current study was to determine the efficacy of one such antibody in cases of primary cutaneous melanoma and correlate the results obtained from immunohistochemistry with that evidenced by DNA analyses.

Materials and methods

Sample Selection

This study was approved by Boston University School of Medicine institutional review board (IRB docket #H-31715). Archival tissue with a diagnosis of primary cutaneous malignant melanoma ($n = 35$) were retrieved from the pathology files of the Skin Pathology Laboratory, Boston University School of Medicine, Boston, MA, USA. Inclusion criteria were randomly selected cases of primary cutaneous melanoma from both chronically and intermittently sun-exposed skin with adequate tumoral tissue in the block to allow for genetic and immunohistochemical analyses. Control tissue included cases of thyroid papillary carcinomas ($n = 5$; cases C1–4, 7) and colorectal carcinomas ($n = 5$; cases C5, 6, 8–10) previously determined to harbor the *BRAFV600E* mutation. Histologic sections of all cases were reviewed by two board-certified dermatopathologists (initial sign-out on all by a dermatopathologist; cases were then re-reviewed, and the diagnoses were confirmed by the senior author). All patient data were de-identified. Demographics of the patients included in the study are detailed in Table 1.

DNA Analyses

DNA was extracted from the sections of formalin-fixed paraffin-embedded primary cutaneous melanoma, papillary thyroid carcinoma and colorectal carcinoma tissues by a simple proteinase K digestion and boiling method without

Table 1 Patient demographics

Sample	Age	Sex	Biopsy site
M1	70	M	Left infraorbital
M2	60	M	Right lateral hairline
M3	74	F	Posterior lateral right arm
M4	75	M	Back
M5	74	M	Right lower abdomen
M6	83	M	Right upper back
M7	69	M	Left ear
M8	82	F	Left tibia
M9	43	M	Right occipital
M10	50	M	Right neck
M11	27	F	Left lower leg
M12	81	M	Left upper lateral earlobe
M13	29	M	Mid back inferior
M14	57	F	Right upper arm
M15	72	M	Dorsolateral mid left forearm
M16	84	F	Back
M17	63	M	Right mid back
M18	79	M	Right lower back
M19	50	M	Right upper back
M20	66	F	Right shoulder
M21	38	F	Left wrist
M22	58	M	Left back
M23	51	M	Right scalp
M24	74	F	Right distal shin
M25	75	F	Left back superior
M26	83	F	Left mid lateral most calf
M27	77	M	Left back
M28	33	M	Left scapular back
M29	50	F	Left upper arm
M30	87	M	Arm
M31	63	M	Left hip
M32	57	M	Right upper back
M33	67	M	Left lateral upper thigh
M34	62	F	Right breast
M35	41	F	Left buttock

further purification. *BRAF* mutations were first assayed by allele-specific PCR (codon *V600E*) by previously described methods.[17–19] Two sets of allele-specific primers (forward and reverse gene coding strand) were designed to duplex with a single pair of GAPDH primers as DNA quality internal control to amplify the mutated allele. Following allele-specific PCR, direct DNA Sanger sequencing was used to confirm the mutation positive cases and a few allele-specific PCR failed cases (as indicated by negative GAPDH PCR). Positive, negative and no-DNA controls were included in each batch of analysis.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue of primary cutaneous malignant melanoma ($n = 35$) and controls were baked at 75 °C for 30 min. Sections were deparaffinized with xylene and rehydrated in a series of decreasing concentrations of ethanol solutions. Heat-induced antigen retrieval was carried out in a DAKO retrieval solution (DAKO, Carpinteria, CA, USA) with a pH of 9.0 in a 98 °C water bath for 12 min. The slides were treated with dual endogenous enzyme block (DAKO) and then incubated with the anti-B-Raf (V600E) mouse monoclonal antibody (NewEast Bioscience, Malvern, PA, USA) at a dilution of

1:100 for 2 h at room temperature. The remaining steps were carried out in a DAKO Autostainer Plus (DAKO). Color development and contrast were achieved using DAB and hematoxylin, respectively.

Cases were marked as positive if they exhibited cytoplasmic staining that was similar in intensity to the positive control in each batch. Any isolated nuclear staining was determined to be negative. All stained slides were reviewed by two observers (first author KF and the senior author MM) in a blinded fashion with respect to genotype.

Results

DNA Analyses

The combined results of allele-specific PCR and direct DNA sequencing revealed that 6 of 35 (17%) of the primary cutaneous melanoma cases (case numbers 9, 10, 11, 20, 22, 33) were positive for the *BRAFV600E* mutation (Table 2). Of the 29 cases that were negative, 27 of them were shown to have wild-type DNA and the remaining two (cases 2 and 8) possessed mutations other than *V600E* (case 2 had a glutamic acid to lysine substitution; case 8 had a glutamic acid to methionine substitution). All papillary thyroid carcinoma and colorectal carcinoma cases were positive for *BRAFV600E* mutation by allele-specific PCR.

Immunohistochemical Analyses

The results of immunohistochemical analyses showed that 7 of 35 (20%) of the primary cutaneous melanoma cases (cases 9, 10, 11, 20, 22, 26, 33) demonstrated positive cytoplasmic staining for the V600E mutated protein (Figure 1; Table 2). In addition to strong cytoplasmic staining, case 20 also possessed nuclear staining. All papillary thyroid carcinoma and colorectal carcinoma cases demonstrated positive cytoplasmic staining for the mutated protein (Figure 1).

Statistical Analyses

Data analyses revealed that when it came to detecting the *BRAFV600E* mutational status as determined by direct DNA Sanger sequencing and allele-specific PCR, the anti-B-Raf (V600E) antibody had a sensitivity of 100% and specificity of 97%. All six cases that were positive by DNA analyses were also positive by immunohistochemistry. One case (case 26) was positive only by immunohistochemical analysis.

Discussion

The concept of 'oncogene addiction' emphasizes that cancer cells, in contrast to normal cells, rely more heavily on hyperactivated pathways and, therefore, on the oncogenes that drive those pathways for the maintenance of the malignant phenotype.[20] A high

Table 2 Results of DNA and immunohistochemical analyses

Sample	*BRAFV600E* status	*Immunohistochemical analyses*
M1	−	−
M2[a]	−	−
	(GTG to AAG)	
M3	−	−
M4	−	−
M5	−	−
M6	−	−
M7	−	−
M8[a]	−	−
	(GTG to ATG)	
M9	+	+
M10	+	+
M11	+	+
M12	−	−
M13	−	−
M14	−	−
M15	−	−
M16	−	−
M17	−	−
M18	−	−
M19	−	−
M20[b]	+	+
M21	−	−
M22	+	+
M23	−	−
M24	−	−
M25	−	−
M26	−	+
M27	−	−
M28	−	−
M29	−	−
M30	−	−
M31	−	−
M32	−	−
M33	+	+
M34	−	−
M35	−	−
C1	+	+
C2	+	+
C3	+	+
C4	+	+
C5	+	+
C6	+	+
C7	+	+
C8	+	+
C9	+	+
C10	+	+

For *BRAFV600E* mutation status, cases were marked as positive/negative using a combination of direct DNA Sanger sequencing and allele-specific PCR.
[a]Cases with a mutation in *BRAF* other than *V600E*.
[b]Case presented with both nuclear and cytoplasmic staining.

frequency of *BRAF* mutations has been previously demonstrated in rapidly changing melanocytic lesions and an increase in frequency of these mutations has also been observed in primary cutaneous malignant melanoma progressing from the radial to the vertical growth phase.[21,22] In light of this, it is not surprising that *BRAF* has emerged as the 'Achilles' heel' and is a validated therapeutic target in melanoma as *BRAFV600E* substantially increases protein kinase activity resulting in constitutive *BRAF/MEK/ERK* signaling.[20] To date,
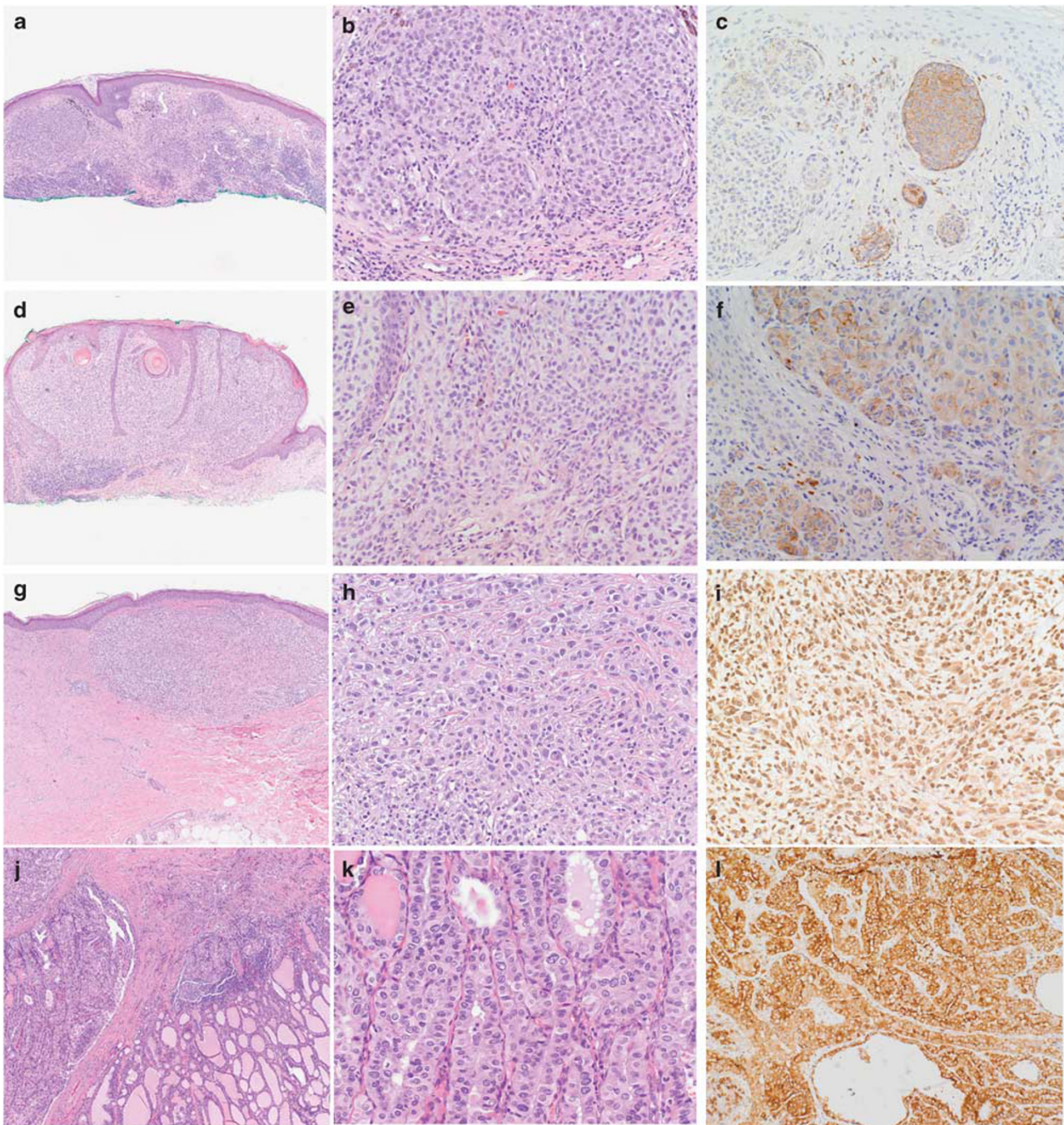
Figure 1 Immunohistochemical results for select *BRAFV600E* mutation positive cases by DNA analyses (case 9 = **a–c**; case 10 = **d–f**; case 20 = **g–i**; papillary thyroid carcinoma control = **j–l**). (**a/d/g/j**): Scanning magnification, H&E. (**b/e/h/k**): High power, H&E. (**c/f/i/l**): Immunohistochemical stain with the anti-B-Raf (V600E) antibody.

select *RAF* kinase inhibitors are in different phases of several clinical trials, making screening for this mutation of clinical relevance.[10]

The gold standard for the detection of *BRAFV600E* is direct DNA sequencing.[3] This method takes several days, is expensive and has a relatively low sensitivity in that tumoral population must constitute at least 20% of the tissue sample.[23] An additional confounding issue is the suboptimal quality of DNA extracted from formalin-fixed paraffin-embedded tissue, a feature which can necessitate additional rounds of extraction further adding to the delay in turn-round-time. Furthermore, the known interference of melanin in PCR-based techniques as a consequence of its interaction with DNA polymerase can compromise the sensitivity of the procedure.[16] Allele-specific PCR circumvents several of the problems associated with direct DNA sequencing in that it has been shown to have the ability to detect roughly 5% of the mutant
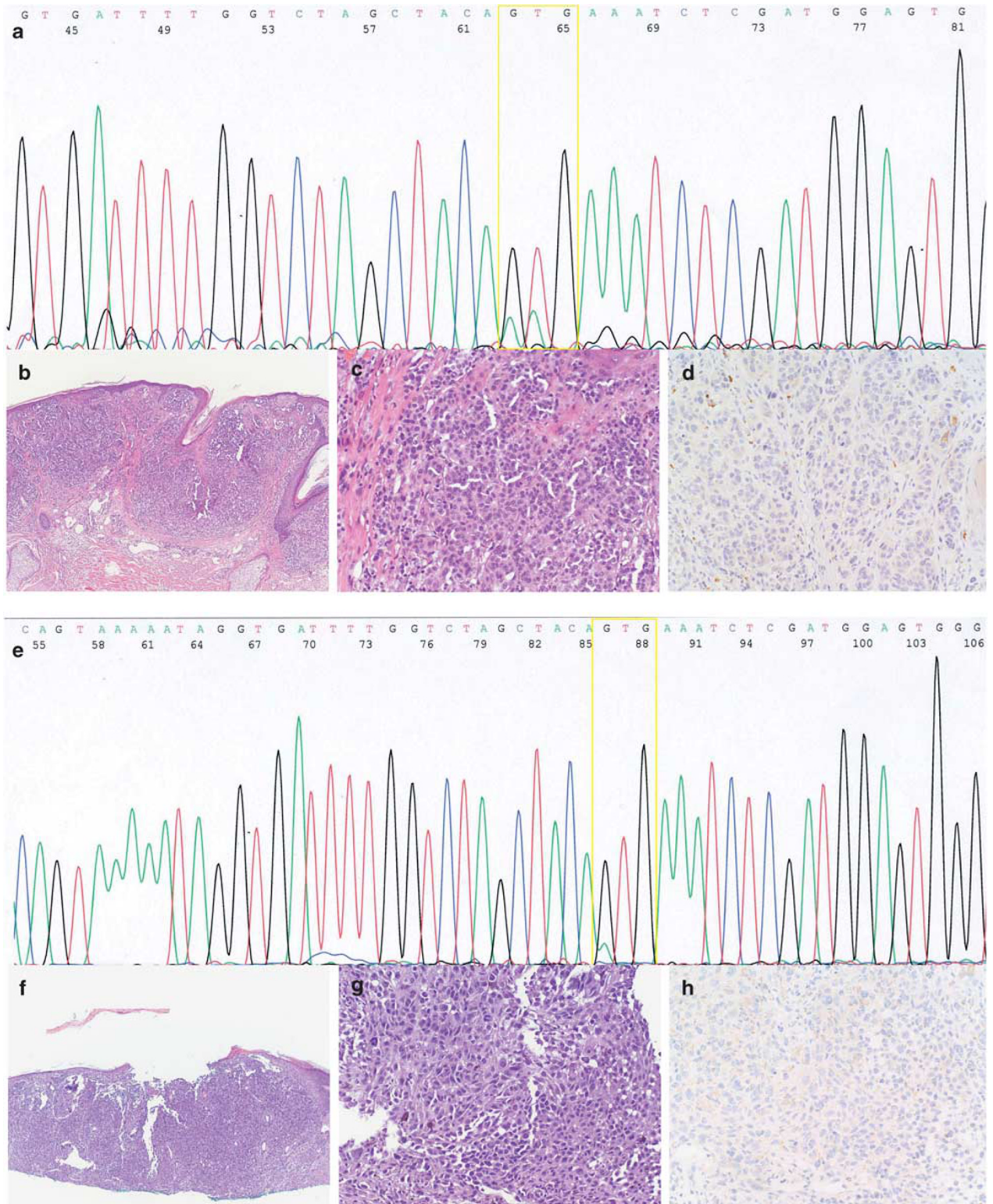
Figure 2 Cases with *BRAF* mutation other than *V600E* (case 2 = **a–d**; case 8 = **e–h**). (**a**) Direct DNA sequencing demonstrating the glutamic acid to lysine substitution (GTG to AAG). (**b/f**) Scanning magnification, H&E. (**c/g**) High power, H&E. (**d/h**) Immunohisto-chemical stain with the anti-B-Raf (V600E) antibody (brown staining seen is melanin). (**e**) Direct DNA sequencing demonstrating the glutamic acid to methionine substitution (GTG to ATG).

BRAF in a pool of wild-type, making it particularly attractive in skin samples.[24] However, it still does not address the issues of time, cost-effectiveness and DNA extraction.

The advent of monoclonal antibodies on formalin-fixed paraffin-embedded tissue has greatly facilitated the use of immunohistochemistry as a histological adjunct.[25] This technique, which is easy to perform and interpret, is also considered to be highly sensitive and specific because the antibodies used are typically directed against the antigen of interest. In other entities associated with genetic abnormalities such as Muir-Torre syndrome, we have previously presented data to support the use of immunohisto-chemistry as the initial screening test as it has been effective in identifying the loss of one or more mismatch repair proteins in sporadic sebaceous neoplasms.[26] An additional minor, yet practically relevant factor relates to the ease of the methodology, which translates to a rapid turn-round-time. Concerning the detection of *BRAFV600E*, three recent publications highlight the utility of immunohistochemistry using a mutation-specific monoclonal antibody. In the first, Capper *et al*[3] evaluated a total of 47 intracerebral melanoma meta-stases and 21 primary papillary thyroid carcinomas by techniques that included direct DNA sequencing and immunohistochemistry, albeit with a different antibody (clone VE1), and found a complete corre-lation between both methodologies. In the second study, using the same antibody Capper *et al* extended their previous sample size to a larger, more comprehensive cohort of patients with brain metasta-ses and found a correlation between immunohisto-chemistry and *BRAFV600E* sequencing in 68/70 cases (~97%).[27] In the third, the same group found a correlation between expression of the protein and the *BRAFV600E* mutation in 32 cases of hairy cell leukemia.[28] However, in each of the latter two studies the authors noted that two cases were posi-tive by immunohistochemistry but negative by sequ-encing of exon 15 of *BRAF* and suggested a more sensitive technique to clarify the genetic status. Using a different antibody from that of Capper *et al* ie anti-B-Raf (V600E), in which the immunogen is a synthetic peptide derived from the internal region of *BRAFV600E*, we noted that the sensitivity of imm-unohistochemistry was 100% and specificity was 97%. Of note, only one (case 26) was positive by immunohistochemistry but negative by DNA analyses. As expected, cytoplasmic staining was the pattern observed in all cases. In only one, case 20, additional non-specific nuclear staining was demonstrated. However, given the uniform cytoplasmic staining also observed in this case, it was scored as a positive.

The focus of the current study was not on evaluating the frequency of *BRAFV600E* mutations in primary cutaneous melanoma, but on ascertain-ing the utility of a mutation-specific monoclonal antibody. Therefore, our cases were not selected specifically to verify mutation rates. However, it should be noted that our reported incidence of *BRAFV600E* in primary cutaneous melanoma is relatively low, albeit not unprecedented. Studies on the prevalence of *BRAF* mutations in melanoma indicate that they are most commonly seen in melanomas from skin sites subject to sun exposure, with a relatively higher prevalence being noted in those that are intermittently sun-exposed *vs* con-tinually sun-exposed.[7] This might account for the slightly lower frequency of *BRAFV600E* in the current cohort as a proportion of our cases were from continually sun-exposed sites (cases 1, 2, 7, 10–12, 15, 21, 23, 26, 30). Furthermore, it has been previously shown using the same technique that the frequency of *BRAFV600E* mutations in primary cutaneous melanoma can vary greatly with inci-dences as low as 30% being reported.[11] Our own previous experience has shown that the frequency could be even lower.[8]

In spite of the low incidence rate, findings from this preliminary study shed light on the potential use of immunohistochemistry with the anti-B-Raf (V600E) antibody as an ancillary screening tool to assess the *BRAFV600E* mutation status in primary cutaneous melanoma. Further, in support of the specificity of this antibody, two cases in our cohort possessed mutations other than *V600E* (case 2 had a glutamic acid to lysine substitution while case 8 had a glutamic acid to methionine substitution) and were negative by immunohistochemistry (Figure 2). Ours is not the first study to document *V600* mutations other than *V600E*. In one previous study, the same glutamic acid to lysine substitution was noted and found in a higher proportion (7%) of cases.[29] While the precise significance of these other mutations is unclear, it further validates the specificity of the antibody for the *BRAFV600E* mutation.

While we observed the staining intensity to be somewhat variable, a consistent feature of all seven positive cases was that the staining was confined to lesional melanocytes. In contrast to DNA-based techniques, this allows for the distinction to be made between populations harboring the mutant allele *vs* those harboring wild-type. From a scientific perspec-tive, this feature facilitates insights into the biology of nevic lesions particularly those in which the mela-noma arises in the setting of a banal or congenital nevus. Additional studies on a larger cohort are required to validate our findings and for immuno-histochemistry to supplement or replace DNA-based techniques in identifying *BRAFV600E* in primary cutaneous melanoma. Given the similar results seen in both the thyroid papillary carcinoma and color-ectal carcinoma controls, we are encouraged about the potential of this mutation-specific antibody.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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