Method of Mutation Analysis May Contribute to Discrepancies in Reports of ^{V599E}BRAF Mutation Frequencies in Melanocytic Neoplasms

To the Editor:

Since the initial discovery that BRAF mutations occur in >60% of primary melanomas (Davies *et al*, 2002), multiple studies have reported widely discrepant BRAF mutation frequencies in melanocytic neoplasms of all stages of progression (Dong *et al*, 2003; Gorden *et al*, 2003; Kumar *et al*, 2003; Lang *et al*, 2003; Maldonado *et al*, 2003; Pollock *et al*, 2003; Uribe *et al*, 2003; Yazdi *et al*, 2003; Kumar *et al*, 2004; Tsao *et al*, 2004). Resolving these discrepancies has critical implications for clarifying the role of BRAF mutations in melanocytic tumorigenesis and for determining the relevance of BRAF as a potential target for therapy. We noted that the lowest frequencies of BRAF mutation were reported in studies that used direct fluorescent sequencing as the sole method of mutational analysis (Dong *et al*, 2003; Gorden *et al*, 2003; Lang *et al*, 2003; Yazdi *et al*, 2003). This finding prompted us to compare the sensitivities of bidirectional direct fluorescent sequencing and allele-specific polymerase chain reaction (AS-PCR) to detect the ^{V599E}BRAF mutation and to evaluate whether method of mutation analysis could be one factor contributing to variations in mutation frequencies.

Initially, titration assays were performed, demonstrating that AS-PCR detects the ^{V599E}BRAF mutation with greater sensitivity compared to direct fluorescent sequencing.

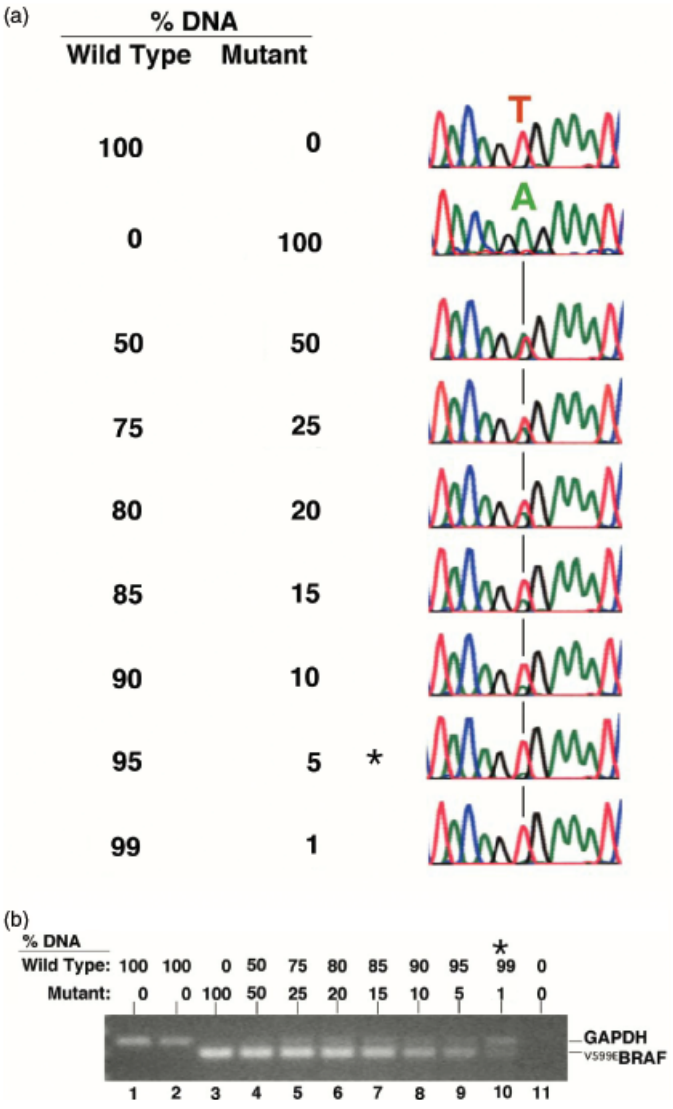

Figure 1
Results of titration assay assessing the sensitivity of direct fluorescent sequencing *versus* allele-specific polymerase chain reaction (AS-PCR) to detect the ^{V599E}BRAF mutation. (*a*) Results of titration assay assessing the sensitivity of direct fluorescent sequencing to detect the ^{V599E}BRAF mutation. DNA sequencing profiles showing the results of direct fluorescent sequencing of template DNA containing decreasing percentages of mutant ^{V599E}BRAF. The 100% wild-type BRAF (T) sequence contained template DNA that was obtained from normal human fibroblast cells in which only the wild-type BRAF gene was present. The 100% mutant ^{V599E}BRAF (A) sequence contained template DNA that was obtained from the melanoma cell line UACC 903 in which 100% of the cells were mutant. The remaining sequencing profiles represent varying concentrations of template genomic DNA from the melanoma cell line UACC 903 diluted with DNA from normal human diploid fibroblasts to give the indicated concentration of wild-type to mutant ^{V599E}BRAF. Asterisk indicates the minimum concentration at which mutant ^{V599E}BRAF DNA must be present to be able to discern the presence of a mutant spike from background. (*b*) Results of titration assay assessing the sensitivity of AS-PCR to detect the ^{V599E}BRAF mutation. Ethidium bromide-stained gel showing the results of the AS-PCR analysis of template DNA containing decreasing percentages of mutant ^{V599E}BRAF. Lanes 1 and 2 serve as negative controls since template DNA contained only the wild-type BRAF gene from human lymphoblast and fibroblast cells, respectively. Lane 3 represents results from template DNA obtained from the melanoma cell line UACC 903 in which 100% of the cells contain mutant ^{V599E}BRAF. Lanes 4–10 contain template genomic DNA from the melanoma cell line UACC 903 diluted with DNA from normal human diploid fibroblasts to give the indicated concentration of wild-type to mutant ^{V599E}BRAF. Lane 11 is a water only negative control. Asterisk indicates the minimum concentration at which mutant ^{V599E}BRAF DNA must be present to be detected by AS-PCR. The GAPDH control bands lack uniform intensity due to preferential PCR amplification in multiplex reactions, but does not impact the interpretation of our results (Markoulatos *et al*, 2002).

Abbreviation: AS-PCR, allele-specific polymerase chain reaction

Table I. Comparison of frequencies of ^{V599E}BRAF mutation by direct fluorescent sequencing and AS-PCR

Melanocytic lesion	Direct sequencing	AS-PCR
Atypical nevi	22% (2/9)	44% (4/9)
Primary melanomas	50% (17/34)	59% (20/34)
Metastatic melanomas	71% (17/24)	79% (19/24)
Combined frequency	54% (36/67)	64% (43/67)*

*$p = 0.0082$, McNemar's test comparing detection rates of direct fluorescent sequencing and AS-PCR.
AS-PCR, allele-specific polymerase chain reaction.

UACC 903 genomic DNA, a melanoma cell line containing the ^{V599E}BRAF mutation, was mixed with increasing amounts of normal human diploid fibroblast genomic DNA. Direct fluorescent sequencing and AS-PCR were performed at each stage of the dilution. Direct fluorescent sequencing required $\geq$ 5% of mutant DNA in the total mixture in order to detect the ^{V599E}BRAF mutation. (Fig 1*a*). Below the 5% threshold, the presence of a mutant spike could not reliably be discerned from background on the electropherogram and was determined to be wild-type. By comparison, AS-PCR reliably detected the ^{V599E}BRAF mutant DNA if it comprised 1% or more of the mixture of mutant and wild-type DNA. At a 1% concentration of ^{V599E}BRAF mutant DNA, a mutant band was clearly visible on the gel of the AS-PCR products (Fig 1*b*).

Next, both direct fluorescent sequencing and AS-PCR were performed to screen for the ^{V599E}BRAF mutation on DNA extracted from macrodissected human tissue samples obtained from conventional paraffin-embedded blocks archived at the Penn State Milton S. Hershey Medical Center. The institutional review board of the Penn State College of Medicine/Penn State Milton S. Hershey Medical Center approved the study, which was conducted according to the Declaration of Helsinki Principles. Conditions for direct fluorescent sequencing and AS-PCR have been previously described (Davies *et al*, 2002; Pollock *et al*, 2003). Both methods of mutation analysis were successful in 67 samples, including nine atypical nevi, 34 primary melanomas, and 24 metastatic melanomas. PCR amplification of exon 15 of the BRAF gene followed by direct fluorescent sequencing detected the ^{V599E}BRAF mutation in 22% (two of nine) of atypical nevi, 50% (17 of 34) primary melanomas, and 71% (17 of 24) metastatic melanomas. By comparison, AS-PCR detected the mutation in seven additional samples, including 44% (four of nine) of atypical nevi, 59% (20 of 34) of primary melanomas, and 79% (19 of 24) of metastatic melanomas (Table I). Consistent with the results of the titration assay, AS-PCR detected the mutation with higher frequency than direct fluorescent sequencing in each of the three subgroups (atypical nevi, $p = 0.16$; primary melanomas, $p = 0.083$; and metastatic melanomas, $p = 0.16$ [McNemar's test for each group]). The overall mutation frequency was significantly higher using AS-PCR (64% [43 of 67]) compared to direct sequencing (54% [36 of 67]) ($p = 0.0082$, McNemar's test). To ensure that mutations detected by AS-PCR did not represent false positives, normal DNA derived from human diploid fibroblasts and lymphoblasts was used as negative controls (Fig 2). As a further measure to preclude contamination, genomic DNA was isolated from additional sections from the tissue blocks of three of seven samples in which the mutation was detected by AS-PCR but not by direct fluorescent sequencing. In all three samples, the presence of the mutation was reproduced on subsequent AS-PCR. The mutant band was then cut from the AS-PCR gel, and direct fluorescent sequencing of the isolated DNA confirmed the mutant sequence in each of the three samples. In our study, therefore, both titration assays and direct comparison of mutation analysis of human tissues demonstrated that AS-PCR detects the ^{V599E}BRAF mutation with greater sensitivity than direct fluorescent sequencing. Similar to our results, another study which used both AS-PCR and direct sequencing for detection of ^{V599E}BRAF mutations had samples in which AS-PCR, but not direct sequencing, detected the mutant allele (Pollock *et al*, 2003). Since we analyzed only two methods of mutation analysis, we cannot comment on the sensitivities of other methods of mutation analysis, such as PCR-single-strand conformation polymorphism/heteroduplex analysis,

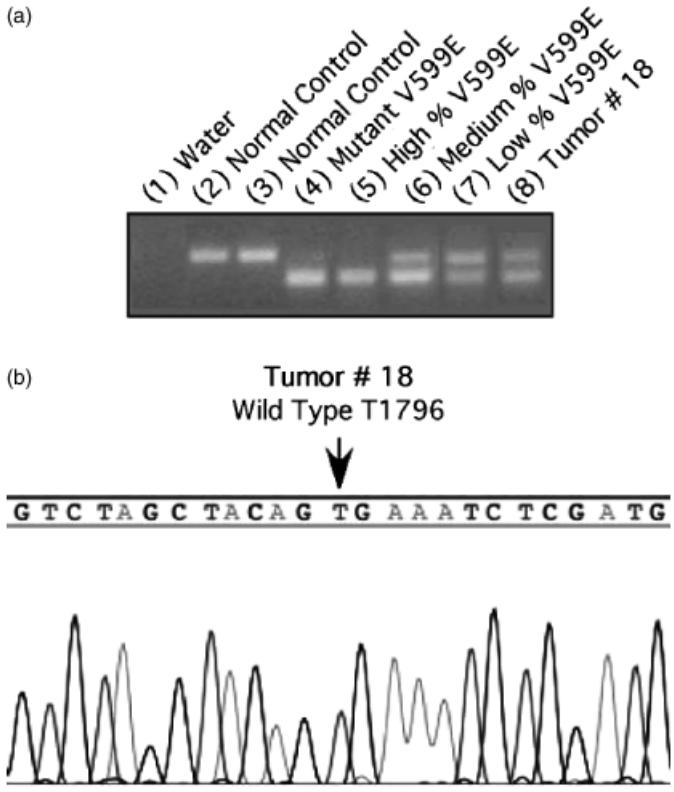

Figure 2
Results of allele-specific polymerase chain reaction (AS-PCR) analysis and direct fluorescent sequencing for the ^{V599E}BRAF mutation in human tissue. (*a*) Ethidium bromide-stained AS-PCR gel showing the absence of the ^{V599E}BRAF mutant band in the first three control lanes representing water, fibroblast genomic DNA and lymphoblast genomic DNA, respectively. A mutant band is clearly visible in lane 4, representing the positive control SK-Mel-111 cell line known to contain the ^{V599E}BRAF mutation. Lanes 5, 6, and –7 show a clear mutant band from tumor samples in which direct fluorescent sequencing also detected a mutant allele with high, medium, and low levels of intensity, respectively. The final column, representing tumor #18, shows a clear mutant band that was undetectable by direct fluorescent sequencing. (*b*) Direct fluorescent sequencing of DNA from tumor #18, which shows a wild-type sequence despite detection of the mutation by AS-PCR.

which has been used by some authors to assess BRAF mutation frequencies (Kumar *et al*, 2003, 2004; Tsao *et al*, 2004). Nevertheless, our findings indicate that differences in the sensitivities of methods of mutation analysis may be one factor contributing to discrepancies in reports of mutation frequency. Other factors may also contribute to discrepancies in mutation frequency, such as selection of melanomas from skin with intermittent sun exposure *versus* from chronically sun-damaged and relatively unexposed skin (Maldonado *et al*, 2003).

Resolving discrepancies in reported mutation frequencies will help to clarify current conflicts about the role of BRAF mutations in melanocytic tumor progression. Due to detection of high rates of BRAF mutation in subsets of benign and atypical nevi, most authors conclude that mutation of BRAF is a critical step in the initiation of many melanocytic neoplasms (Pollock *et al*, 2003; Uribe *et al*, 2003; Yazdi *et al*, 2003; Kumar *et al*, 2004). Moreover, most authors agree that BRAF mutation alone appears insufficient to cause a melanoma (Pollock *et al*, 2003; Uribe *et al*, 2003; Yazdi *et al*, 2003), as evidenced by the fact that the majority of nevi do not progress to melanoma (Bevona *et al*, 2003). By contrast, Dong *et al* (2003) conclude that BRAF mutations are not involved in the initiation of the great majority of melanomas, but instead reflect a progression event in melanoma tumorigenesis. This conflicting conclusion is based on their finding that BRAF mutation was present in only 10% of radial growth phase compared to 63% of vertical growth phase and 62% of metastatic melanomas [pooled statistical difference, $p < 0.01$] (Dong *et al*, 2003). Our results suggest that the low rate of mutation detected in radial growth phase melanomas may have resulted in part from the lack of sensitivity of direct sequencing, which was the sole method of mutational analysis used in the study by Dong *et al*.

Resolving discrepancies in BRAF mutation frequencies also has important therapeutic implications. Increasing evidence suggests that suppression of BRAF and the MAP kinase pathway may be a therapeutic option against melanomas with BRAF mutation (Sebolt-Leopold, 2000; Collisson *et al*, 2003; Hingorani *et al*, 2003; Tuveson *et al*, 2003; Sharkey *et al*, 2004). Whereas several studies, including our own, report a BRAF mutation rate of $>60\%$ in metastatic melanomas (Dong *et al*, 2003; Kumar *et al*, 2003; Pollock *et al*, 2003), others studies, which used direct fluorescent sequencing as the sole method of mutation analysis, report mutation rates as low as 21%–40% in metastatic melanomas (Gorden *et al*, 2003; Lang *et al*, 2003; Yazdi *et al*, 2003). These low rates of mutation suggest that the development of therapies that inhibit BRAF activity may be relevant to fewer melanomas than initially suspected.

In conclusion, using both titration assays and mutation analysis in human tissue samples, our study demonstrates that AS-PCR detects the ^{V599E}BRAF mutation with greater sensitivity than direct fluorescent sequencing. Our results suggest that method of mutation analysis may be one factor contributing to widely discrepant reports of BRAF mutation frequency. Accurate methods of mutation analysis are necessary to clarify the role of BRAF mutation in melanocytic tumorigenesis and to determine the percentage of melanomas for which BRAF inhibitors will be relevant.

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