Exon 15 *BRAF* Mutations Are Uncommon in Melanomas Arising in Nonsun-Exposed Sites

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ABSTRACT

**Purpose:** An activating point mutation of the *BRAF* oncogene has been identified in a high proportion of cutaneous nevi and cutaneous melanomas, but its frequency in melanomas arising from the mucosa of head and neck is unknown.

**Experimental Design:** We tested 17 malignant mucosal melanomas of the head and neck for the thymine (T)—adenine (A) missense mutation at nucleotide 1796 in the *BRAF* gene using direct sequencing and a newly developed assay that uses a novel primer extension method (Mutector assay). We also tested 21 cutaneous melanomas, including 13 arising from sun-exposed sites and 8 from a nonsun-exposed site, the vulvar skin.

**Results:** The 1796T→A mutation was detected in only 1 (6%) of the sinonasal melanomas. As for cutaneous melanomas, a *BRAF* mutation was detected in 8 (62%) of the tumors arising in sun-exposed sites but in none (0%) of vulvar melanomas.

**Conclusions:** In contrast to cutaneous melanomas arising in sun-exposed sites, mucosal melanomas of the head and neck do not frequently harbor an activating mutation of *BRAF*. This finding additionally supports the view that the various subtypes of melanoma are not equivalent and that distinct genetic alterations may underlie well recognized differences in risk factors and behavioral patterns. Accordingly, patients with melanomas should not be collectively regarded as a uniform group as new strategies are developed that target specific genetic alterations.

INTRODUCTION

Melanocytes are normally present in the mucosa of the upper aerodigestive tract (1, 2), and just like their cutaneous counterparts, these mucosal melanocytes can undergo malignant transformation. Although cutaneous and mucosal melanomas arise from the same cell type—the neural crest derived melanocyte—these subgroups of melanoma are distinct. Mucosal melanomas of the head and neck are not associated with UV exposure, an important risk factor in the development of cutaneous melanomas (3). Mucosal melanomas tend to arise in an older age group compared with cutaneous melanomas. The onset of mucosal melanoma is typically two decades later than that of cutaneous melanoma (4). Finally, mucosal melanomas are more aggressive than their cutaneous counterparts. They are characterized by advanced local growth, frequent local recurrences and distant dissemination, and exceedingly high fatality rates even in the setting of apparently complete tumor resection (4–6). Such divergence in clinical patterns points to key differences in genetic pathways driving tumorigenesis, but insufficient insight into the pathogenesis of mucosal melanomas has frustrated attempts to compare genetic profiles. The frequency of even the most common genetic alterations in cutaneous melanomas remains conspicuously undefined for mucosal melanomas of the head and neck.

*BRAF* encodes a serine-threonine kinase that acts in the mitogen-activated protein kinase pathway (7). Activating *BRAF* mutations induce constitutive activation of the signal transduction pathway, providing a potent promitogenic force that drives malignant transformation (8, 9). The role of *BRAF* mutational activation in the development of cutaneous melanocytic tumorigenesis appears to be substantial. *BRAF* mutations have been identified in up to 82% of cutaneous melanocytic nevi (10) and 66% of primary melanomas (8). Little is known about the role of the mitogen-activated protein kinase pathway activation in mucosal melanomas, but a conjunctive effort to explore the frequency of *BRAF* mutations in melanomas from cutaneous and noncutaneous sites could provide new and practical insights by helping to: (a) explain divergence in clinical patterns; (b) link specific genetic targets to specific exposure profiles (e.g., UV exposure); and (c) determine whether patients with mucosal melanomas of the head and neck, a uniformly fatal tumor that is resistant to conventional modes of therapy, could potentially benefit from novel anti-*BRAF* therapy.

PATIENTS AND METHODS

Sample Selection and DNA Isolation. Seventeen surgically excised malignant mucosal melanomas of the head and neck, and 21 patients with cutaneous malignant melanomas were identified from a search of archival surgical pathology files of The Johns Hopkins Hospital. The patients with cutaneous melanomas included 13 patients with melanomas arising from sun-exposed sites and 8 patients with melanomas arising from the vulvar skin. After initial patient identification, all original histological slides were reviewed to confirm the diagnosis, and an appropriate block or, when available, fresh frozen tissue was
retrieved for DNA extraction. For all of the cutaneous melanomas, samples were taken from the vertical component of tumor growth. The tissues were sectioned, and sections were micro-dissected to obtain >80% neoplastic cells. DNA was extracted using standard protocols as previously published (11).

Detection of BRAF Mutations. All tumor samples and controls were analyzed for the thymine (T)→adenine (A) missense mutation at nucleotide 1796 in the BRAF gene. This hot spot was chosen because the reported BRAF-activating mutations in cutaneous nevi and cutaneous melanoma occur almost exclusively at this position (8, 10).

PCR primer sequences were designed to amplify a 102-bp fragment of exon 15 (5'-GAAGACCTCACCTAAATAG-GTGA-3' and 5'-CCACAAAAATGGATCCAGACA-3'). PCR amplification was performed using 100 ng of tumor sample DNA as template. The PCR reactions were carried out in a 96-well thermocycler. Cycling conditions were as follows: a denaturation step at 95°C for 5 min was followed by 2 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, primer extension at 72°C for 1 min, 2 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, primer extension at 72°C for 1 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, primer extension at 72°C for 1 min, and one final extension at 72°C for 5 min. Amplified fragments were separated on an agarose gel and visualized by ethidium bromide staining.

Analysis of the PCR products for a BRAF mutation at nucleotide position 1796 was performed using direct automated sequencing and the Mutector assay (TrimGen, Sparks, MD). The Mutector assay is designed for the detection of any type of known DNA mutation. In brief, a detection primer is designed that does not permit primer extension when the target base is wild-type. As a result, primer extension does not occur, labeled nucleotides are not incorporated, and a color reaction is not observed. When the target base is mutated (e.g., T→A transversion at BRAF T1796), primer extension continues, and a strong color reaction is observed. We used as a template 10 μl of PCR products of the 102-bp fragment of exon 15. The assay was preformed according to the manufacturer instructions. When we previously compared BRAF detection for a large number of human tumors, we found a 100% correlation between the Mutector assay and direct sequencing (unpublished results). The results of the Mutector assay were confirmed by direct sequencing of exon 15 for all of the non-sun-exposed melanomas.

As a positive control for the BRAF T1796A mutation, we tested the cutaneous melanoma cell line HTB 72. The cervical cell line ME180 served as a negative control.

RESULTS

Of the 17 patients with malignant mucosal melanomas of the head and neck, 11 were females and 6 were males. Nine tumors arose from the nasal cavity, five from the ethmoid sinus, two from the maxillary sinus, and one from the nasopharynx. Of the patients with cutaneous melanomas, 13 were females and 8 were males. Eight tumors arose from the vulvar skin, six from the facial skin, five from the skin of the back, and two from the skin of the arm.

BRAF mutations were also detected in 8 of the 13 (62%) cutaneous melanomas arising from sun-exposed sites, in 1 of the 17 (6%) mucosal melanomas, and in 0 of the 8 (0%) vulvar melanomas. Thus, BRAF mutations were more frequent in melanomas arising from sun-exposed sites compared with nonsun-exposed sites (62 versus 4%, P = 0.0002, Fisher’s exact two-tailed test). Mutations were detected by a strong color reaction with the Mutector assay as determined visually and confirmed by a high relative absorbance reading. A BRAF mutation was noted in the HTB72 positive control, but a mutation was not detected in the ME180 negative control (Fig. 1). For all of the non-sun-exposed melanomas, direct sequencing of exon 15 confirmed the Mutector assay results for the hot spot thymine (T)→adenine (A) missense (T1796A) mutation at nucleotide 1796 (i.e., V599E mutation), and it did not uncover the presence of any non-V599E oncogenic mutations that occasionally cluster in or near the V599 site (e.g., GT1795-96AG, GT1795-96AA, and C1786G accounting for L596V).

DISCUSSION

Melanocytes are not confined in their distribution to the skin; instead, they are dispersed throughout the mucosa lining the upper aerodigestive tract, female genital tract, and anorectum and urinary tracts. Although the melanocyte (or its progenitor) can undergo malignant transformation irrespective of its precise anatomical location, unmistakable differences in incidence rates, exposure profiles, and clinical behavior suggest that cutaneous and mucosal melanomas may not necessarily share identical pathways of tumorigenesis (4, 5, 12). The notion that melanocytic tumorigenesis may target different genes in a sitespecific manner has found support in a limited number of studies. Van Dijk et al. (13), for example, recently found that sinonasal mucosal melanomas display a consistent pattern of chromosomal abnormalities that are distinct from the pattern seen in cutaneous melanomas. Even so, mucosal melanomas have not received the scientific scrutiny of their cutaneous counterparts. Overshadowed by cutaneous melanoma in terms of sheer cancer incidence, mucosal melanomas remain to be characterized even for those genetic alterations known to be important in melanocyte tumorigenesis. Consequently, opportunities to compare genetic profiles and gain new insights into the complex interactions between genetic targets, exposure profiles, and behavioral patterns have been limited.

The most frequently targeted gene in melanoma is BRAF. Activating mutations of the BRAF oncogene have been detected in a large majority of cutaneous melanoma (8, 14), and their common presence in cutaneous nevi suggests that BRAF may be targeted at the initiation of melanocytic tumorigenesis (10). The high frequency of BRAF mutations in cutaneous melanomas was confirmed in the present study where a mutation was detected in 62% of those cutaneous melanomas arising from sun-exposed sites. The relative importance of BRAF mutational activation in tumorigenesis is clearly not the same across the various subtypes of melanoma. Two previous studies were unable to detect
even a single \textit{BRAF} mutation in the total 77 uveal melanomas evaluated (15, 16). In the present study, we detected a \textit{BRAF} mutation in only 1 (6%) of 17 mucosal melanomas of the sinonasal tract. Despite sharing a common progenitor cell, the melanoma subtypes appear to use different genetic pathways of tumorigenesis.

The genetic divergence observed in melanoma subtypes may underlie epidemiological variations such as differences in exposure profiles. Sun exposure has not been shown to directly induce the \textit{1796T$\rightarrow$A} transition at codon 15, but the large discrepancy in mutation rates across the various subtypes of melanoma circumstantially implicates sun exposure as a possible contributing factor. \textit{BRAF} mutations occur at high frequency in those melanomas that are strongly linked to sun exposure, but their frequency in melanomas unassociated with sun exposure is much lower. With this in mind, we also evaluated eight genital melanomas arising from the vulvar skin. Despite their cutaneous origin, these melanomas arising from nonsun-exposed sites did not harbor a \textit{BRAF} mutation. \textit{BRAF} is not the only member of the mitogen-activated protein kinase pathway to be implicated as a target of UV-induced mutation. N\textit{-RAS} mutations occur in $\sim$15% of cutaneous melanoma, but they rarely occur in melanomas arising in nonsun-exposed sites (17, 18). Thus, their presence has been strongly correlated with chronic sun exposure (19). More work is needed to better understand the interaction of UV exposure and \textit{BRAF} mutations because this relationship does not appear to be direct. The \textit{BRAF} gene does not harbor the C$\rightarrow$T or CC$\rightarrow$TT transitions that signature UV-induced genetic damage, and \textit{BRAF} mutations are sometimes encountered in nevi that develop prior to any sun exposure (i.e., congenital nevi; Ref. 10). Furthermore, \textit{BRAF} mutations are sometimes encountered in tumors arising from internal organs entirely unassociated with UV exposure (8, 20, 21).

\begin{figure}[h]
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\caption{BRAF mutation analysis of two malignant mucosal melanomas. Using the Mutector assay, the presence of a BRAF mutation is indicated by a colorimetric change from clear to green (case 7). In the absence of a mutation, a color signal is not visible (case 6). Direct sequencing confirmed the presence of a T$\rightarrow$A transversion at BRAF T1796 (arrow) in case 7 and the absence of a mutation for case 6. The controls used include the ME180 cervical cell line (wild-type for BRAF), the HTB 72 melanoma cell line (homozygous for the T$\rightarrow$A transversion at T1796), and a water sample without a DNA template.}
\end{figure}
BRAF has been hailed as an ideal target for the development of anticancer agents that target key components of the mitogen-activated protein kinase pathway: BRAF gene is commonly mutated in melanoma, and there are no existing therapies that are highly effective for advanced melanoma (22). Indeed, drugs targeting Raf proteins have already entered clinical trials (23, 24). The call for novel therapeutic strategies is particularly compelling for mucosal melanomas of the head and neck because the conventional therapeutic armament of surgery, post-operative irradiation, and systemic chemotherapy is ineffective in this subgroup of melanomas (4). Despite the promise of anti-BRAF therapy, our findings indicate that only a small percentage of mucosal melanomas of the head and neck actually harbor BRAF mutations and, in turn, that few patients with these tumors may likely benefit from these anticancer agents. On the basis of the fundamental differences in the genetic pathways that underlie melanocytic tumorigenesis, it is clear that the melanoma subtypes need to be viewed individually, not collectively, as novel strategies are designed that target critical genetic alterations.

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