

SHORT REPORT

Absence of *BRAF* mutations in UV-protected mucosal melanomas

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Background: Mutations in *BRAF* have recently been identified in a significant percentage of primary and metastatic cutaneous malignant melanomas. As ultraviolet (UV) exposure may play a role in the development of cutaneous melanoma lesions with *BRAF* mutations, *BRAF* mutation frequency in melanomas arising in sites protected from sun exposure may be lower than those from sun-exposed areas. Thus, we determined the *BRAF* mutation frequency in a panel of 13 mucosal melanomas and compared those data with data from all currently published series of cutaneous melanomas.

Methods: *BRAF* exon 15 DNA from 13 archival primary mucosal melanomas (eight vulvar, four anorectal, and one laryngeal) was sequenced using intron-based primers. As archival DNA occasionally produces poor-quality template, results were confirmed with a *TspRI* restriction fragment length polymorphism (RFLP) that distinguishes wild-type *BRAF* from the common mutant form V599E. A binomial test was used to compare the mutation frequency in the mucosal melanomas with the published mutation frequency in cutaneous melanomas.

Results: None of the 13 mucosal melanomas in this series had an exon 15 *BRAF* mutation, as compared to 54/165 (33%) primary cutaneous melanomas with *BRAF* mutations in a compilation of all current published studies ($p=0.006$).

Discussion: These data suggest that UV exposure, plays a role in the genesis of *BRAF* mutations in cutaneous melanoma, despite the absence of the characteristic C>T or CC>TT mutation signature associated with UV exposure, and suggests mechanisms other than pyrimidine dimer formation are important in UV-induced mutagenesis.

Cutaneous malignant melanoma is a lethal form of skin cancer that is epidemiologically linked to solar UV exposure (reviewed in Gilchrest *et al*¹). Recently, oncogenic mutations in the DNA sequence encoding the kinase domain of *BRAF* have been identified in the majority of primary cell lines derived from cutaneous melanomas.^{2,3} The observation that about 90% of these mutations in melanomas are due to a recurrent single nucleotide substitution (T1796A; V599E)^{2,3} raises the possibility that a specific environmental exposure contributes to the genesis of this mutation. However, the common T1796A *BRAF* mutation is not a characteristic UV signature mutation. UV exposure causes DNA damage via two distinct mutational mechanisms. Cyclobutane pyrimidine dimers (CPD) and 6-4 photo-products are precursors to the C/T and CC/TT transitions that are the classic "UV signature" mutations.⁴ UV exposure also causes oxidative damage via generation of oxygen free

radicals, which in turn can lead to replicative errors and, ultimately, to base substitutions.^{4,5} In order to evaluate the role of UV exposure in the genesis of the T1796A *BRAF* mutation in cutaneous melanoma, we examined a series of melanomas arising in sites protected from sun exposure and compared our results with the published frequencies of *BRAF* mutations in cutaneous melanoma.

METHODS

Tumour specimens

Tissue blocks for 13 formalin-fixed paraffin-embedded mucosal melanomas (12 primary, one local recurrence), comprised of eight vulvar, four anorectal, and one laryngeal lesion, were retrieved from the Surgical Pathology archives of the Fox Chase Cancer Center and Pennsylvania Hospital under an IRB-approved protocol. The original histopathology data for each case were obtained and haematoxylin and eosin stained sections from all tumours were reviewed by a surgical pathologist (RHE).

DNA extraction

Tumour tissue was manually microdissected from surrounding normal tissue using 5×5 µm sections that were first stained using a modified H&E protocol. In sequential order, the steps for this protocol were incubation in: xylene×5 min×2, 100% ethanol×30 s×2, 95% ethanol×30 s, 80% ethanol×30 s, ddH₂O×30 s, Harris' modified haematoxylin×30 s, ddH₂O×30 s, ammonium hydroxide×30 s, 80% ethanol×30 s, 95% ethanol×30 s, eosin Y×30 s, 95% ethanol×30 s×2, 100% ethanol×30 s, and xylene×5 min. Slides were air-dried then placed in a vacuum dessicator for a minimum of 2 h to ensure complete dehydration of tissue. DNA extractions were performed using the QIAquick DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol with one modification: during microdissection of H&E stained sections, tissue was placed directly into Qiagen ATL buffer for proteinase K digestion.

Mutation detection

As archival material may yield poor quality DNA in some cases, mutation detection was performed by two independent methods. First, *BRAF* exon 15 was amplified by polymerase chain reaction (PCR) using previously published primers: *BRAF* exon 15F (forward): TCATAATGCTTGCTCTGATAGGA, *BRAF* exon 15R (reverse): GGCCAAAATTTAATCAGTGGA. The components of the PCR reaction mix were: 1× GeneAmp PCR Buffer II (Applied Biosystems, Foster City, CA), 2 mM MgCl₂, 250 µM dNTPs, 1 µM primers, 2.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA), 5%

Abbreviations: CPD, cyclobutane pyrimidine dimers; RFLP, restriction fragment length polymorphism; UV, ultraviolet

DMSO, and 3 μ l DNA template in a total reaction volume of 50 μ l. PCR cycling conditions were: 95°C \times 5 min, 33 cycles of denaturation at 95°C \times 1 min, annealing at 59°C \times 30 s, and extension at 72°C \times 2 min with a final extension at 72°C \times 10 min. PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and directly sequenced using *BRAF* exon 15F. Sequencing reactions were analysed on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using software supplied by the manufacturer.

Results from direct sequencing were confirmed independently using restriction fragment length polymorphism (RFLP) analysis by digestion with TspRI. For the restriction digest, DNAs from the mucosal melanomas were PCR-amplified using a hemi-nested PCR protocol. Primers for the first round of PCR were: *BRAF* exon 15N-F (forward): TTATTGACTCTAAGAGGAAAGATGAAG and *BRAF* exon 15N-R (reverse): TGATTTTTGTGAATACTGGGAAC. The second round of amplification was performed using the same forward primer (*BRAF* exon 15N-F) and the reverse sequencing primer (*BRAF* exon 15R). The reaction mix for the first round of PCR contained 1 \times PCR Reaction Buffer (Roche Applied Sciences, Indianapolis, IN), 2 mM MgCl₂, 250 μ M dNTPs, 1 μ M primers, 1 U Taq DNA Polymerase (Roche Applied Sciences, Indianapolis, IN), 5% DMSO, and 150 ng of DNA in a total reaction volume of 50 μ l. The second round of PCR utilised 2 μ l of PCR product from the first round of PCR as template in 2.5 mM MgCl₂. All other components were identical to those used in the first round. PCR cycling conditions were: 95°C \times 5 min, 32 cycles of denaturation at 95°C \times 1 min, annealing at 57°C \times 2 min and extension at 72°C \times 2 min with a final extension at 72°C \times 10 min. Second-round PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and digested with TspRI in NEB Buffer 4 (New England Biolabs, Beverly, MA) at 65°C \times 24 h. Digested and undigested products were analysed on a 12% polyacrylamide gel and visualised with ethidium bromide staining. Wild-type exon 15 *BRAF* DNA is completely digested by the enzyme, yielding three DNA fragments (214, 87, 12 bp), however, one of two TspRI sites present in the wild-type PCR product is eliminated by the common V599E mutation, as well as by the less common V599D, V599R, and V599K mutations. Thus a heterozygous mutant template produces four fragments (301, 214, 87, 12 bp) and a hemizygous mutant template produces two fragments (301, 12 bp).

Statistical analysis

The frequency of *BRAF* mutations in our series of mucosal melanomas was compared with the cumulative published frequency of *BRAF* mutations in primary cutaneous malignant melanomas. A binomial test was used to determine the likelihood that the mutation frequency we observed between these two series occurred by chance alone, under the null hypothesis that the mutation frequency in mucosal melanomas is the same as that estimated from published series of cutaneous melanomas.

RESULTS

Using a combination of direct sequencing (12/13 samples) and TspRI RFLP analysis (13/13 samples), we did not identify any exon 15 *BRAF* mutations in this series of mucosal melanomas (0/13). Representative results of the RFLP analysis are shown in figure 1. This result represents a highly significantly different *BRAF* exon 15 mutation prevalence in mucosal melanomas as compared to the cumulative exon 15 *BRAF* mutation frequency published for primary cutaneous melanoma (54/165; 33%; $p = 0.006$) (see table).

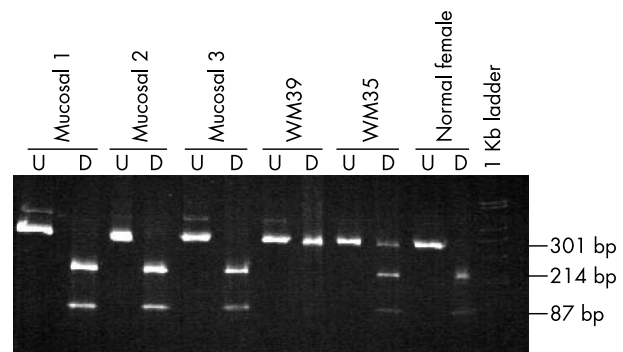


Figure 1 TspRI RFLP analysis of the V599E *BRAF* mutation. PCR-amplified DNA from three mucosal melanomas, the melanoma cell lines WM39 and WM35, and normal human female DNA were digested and analysed as described. None of the mucosal melanomas has a *BRAF* mutation; WM39 has one mutant *BRAF* allele and a subchromosomal deletion involving the other allele; WM35 has one mutant and one wild-type allele; the female DNA has two wild-type alleles. All PCR products are shown undigested (U) and digested (D). Restriction fragment sizes are as indicated.

DISCUSSION

In the present study, we did not identify any exon 15 *BRAF* mutations in 13 mucosal melanomas. This finding is in sharp contrast to the cumulative published rate of 33% in primary cutaneous malignant melanoma⁶⁻¹⁰ and thus represents a distinct genetic difference in melanomas arising from cutaneous v mucosal sites. Exon 11 was not evaluated in this study as mutations in this region account for a very small percentage of total *BRAF* mutations in both cutaneous melanoma and cancers arising from non-sun-exposed tissues.^{2,3} The lack of *BRAF* mutations identified in mucosal sites suggests that the prevalence of *BRAF* mutations in melanoma varies depending upon the anatomical origin of the tumour, possibly in direct relation to the extent of sun exposure for the tissue of origin. However, we cannot exclude the possibility that an inherent feature of the tissue type of origin is the determinant of *BRAF* mutagenesis in melanoma. Further studies of mutation prevalence in different subtypes of cutaneous melanoma (superficial spreading, nodular, acral lentiginous, and lentigo maligna melanoma) may be informative in this regard, as the extent and type of sun exposure varies between these subtypes.

While the mechanism of *BRAF* mutagenesis in melanoma is unknown, a role for UV exposure must be considered. The major UV-generated DNA lesions, cyclobutane pyrimidine dimers and 6-4 photoproducts, lead to C/T or CC/TT transitions, not the commonly described T/A transversion in *BRAF*. However, several additional minor photoproducts also are generated. The role of these compounds in UV mutagenesis is not well-studied, but they include thymine glycol, pyrimidine hydrate, 8,8-adenine dehydrate, and

Primary cutaneous melanomas analysed for *BRAF* mutations

Reference	Primary cutaneous melanomas studied	Number (%) with <i>BRAF</i> mutation
Dong <i>et al</i> ⁶	28	7 (25)
Uribe <i>et al</i> ⁷	25	13 (52)
Pollock <i>et al</i> ⁸	5	4 (80)
Yazdi <i>et al</i> ⁹	97	28 (29)
Cruz <i>et al</i> ¹⁰	10	2 (20)
Total	165	54 (33%)

thymidyl-(3'-5')-deoxyadenosine.¹¹ Of relevance to *BRAF* mutagenesis, production of thymidyl-(3'-5')-deoxyadenosine has been associated with T/A transversions.¹¹

In addition to generating DNA photoproducts, UV exposure can also lead to oxidative modification of DNA. To date, more than 30 different nucleotide modifications have been shown to be generated by oxidative damage (reviewed in Ohshima *et al*⁵). The most widely studied DNA oxidation product, 8-hydroxy-deoxyguanosine, mispairs with adenine, causing G/T transversions. Similarly, oxidative deamination of cytosine leads to mispairing with adenine, causing C/T transitions.⁵ Thus while a direct role for UV-induced oxidation in *BRAF* mutagenesis has not been demonstrated, it is possible that an as yet undetermined oxidative lesion serves as a precursor for the *BRAF* T/A transversion. Of note, oxidative damage also occurs in the context of inflammation, which is a component of UV-induced sunburn. The detection of *BRAF* mutations in several additional tumour types—potentially etiologically linked to inflammatory processes—papillary thyroid cancer with Hashimoto's thyroiditis,^{12–14} pancreatic cancer with chronic pancreatitis,^{15–17} and ovarian cancer¹⁸ with cyclic, ovulation-induced inflammation—raises the possibility that inflammation-associated oxidative changes play a role in the genesis of *BRAF* mutations.

Once a *BRAF* mutation is generated, UV exposure might also promote melanocytic tumour progression. *BRAF* is a component of the MAPK signalling pathway, with activation of *BRAF* leading to increased levels of phosphorylated ERK (reviewed in Smalley¹⁹). Depending upon the cellular context and level of activity, ERK activation may lead to cell proliferation, differentiation, or apoptosis.²⁰ In normal melanocytes, UV-induced activation of MAPK induces differentiation through α -MSH/MC1R-mediated increases in cAMP that directly or indirectly induce tyrosinase transcription.^{4, 19} In melanoma, however, constitutive ERK activation provides proliferative signals and inhibits differentiation. In support of this, dominant negative *RAS* mutations, *ERK* mutations, and MEK inhibitors lead to differentiation.¹⁹ Thus it is possible that in addition to inducing mutations, UV irradiation provokes a stronger proliferative response in melanocytes with an activating *BRAF* mutation than in neighbouring cells with wild-type *BRAF*, further promoting melanoma progression in UV-exposed sites.

In summary, these data demonstrate that exon 15 *BRAF* mutations are much less frequent in mucosal than cutaneous melanomas. Additional studies are needed to further assess the relationship between prevalence of this mutation in specific melanoma subtypes and ultraviolet exposure. Finally, the absence of *BRAF* mutations in mucosal melanoma suggests that mucosal lesions may not respond as well as cutaneous lesions in clinical trials of *BRAF*/MEK inhibitors.

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