Absence of BRAF mutations in UV-protected mucosal melanomas


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 al3). 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.4 5 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 photoproducts are precursors to the C/T and CC/TT transitions that are the classic “UV signature” mutations.6 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.6 7 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, 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 desiccator 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): GGCAAAATTAATCAGTGGGA. 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%
DMSO, and 3 μl DNA template in a total reaction volume of 50 μl. PCR cycling conditions were: 95°C×5 min, 33 cycles of denaturation at 95°C×1 min, annealing at 59°C×30 s, and extension at 72°C×2 min with a final extension at 72°C×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 semi-nested PCR protocol. Primers for the first round of PCR were: BRAF exon 15N-F (forward): TTATTGACTCTAAGGAGAAAGATGAAG and BRAF exon 15N-R (reverse): TGATTITTTGGAATCTGGGAAAC. 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× PCR Reaction Buffer (Roche Applied Sciences, Indianapolis, IN), 2 mM MgCl₂, 250 μM dNTPs, 1 μM 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 μl. The second round of PCR utilised 2 μl 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×5 min, 32 cycles of denaturation at 95°C×1 min, annealing at 57°C×2 min and extension at 72°C×2 min with a final extension at 72°C×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×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).

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 melanoma and thus represents a distinct genetic difference in melanomas arising from cutaneous v mucosal sites. In the present study, we did not identify any exon 15 BRAF mutations in this series of mucosal melanomas. 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.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Primary cutaneous melanomas studied</th>
<th>Number (%) with BRAF mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dong et al</td>
<td>28</td>
<td>7 (25)</td>
</tr>
<tr>
<td>Urbe et al</td>
<td>25</td>
<td>13 (52)</td>
</tr>
<tr>
<td>Pollock et al</td>
<td>5</td>
<td>4 (80)</td>
</tr>
<tr>
<td>Yazidi et al</td>
<td>97</td>
<td>28 (29)</td>
</tr>
<tr>
<td>Cruz et al</td>
<td>10</td>
<td>2 (20)</td>
</tr>
<tr>
<td>Total</td>
<td>165</td>
<td>54 (33%)</td>
</tr>
</tbody>
</table>
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 al13). 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 transversions. 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,13-16 pancreatic cancer with chronic pancreatitis,17-19 and ovarian cancer20 with cyclical, 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 Smalley13). 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.21 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.22 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.

Authors’ affiliations
R H Edwards, M R Ward, C A Medina, M S Brose, P Volpe, B L Weber, Abramson Family Cancer Research Institute, University of Pennsylvania Medical Center, Philadelphia, PA, USA
H Wu, S R Lessin, Fox Chase Cancer Center, Philadelphia, PA, USA
S Nussen-Lee, H M Houp, A M Martin, Pennsylvania Hospital, Philadelphia, PA, USA
M Herlyn, The Wistar Institute, Philadelphia, PA, USA

Research support was received from the National Cancer Institute, grant numbers 1K08CA93748 (to RHE) and 5POCA093372 (to MH), the National Institute of Arthritis and Musculoskeletal and Skin Diseases, grant number K24 AR02102 (to SRL), and from the Abramson Family Cancer Research Institute (to BLW).

Conflict of interest: none declared.

Correspondence to: B L Weber, MD, Abramson Family Cancer Research Institute, University of Pennsylvania Medical Center, 513 BRB III/II, 421 Curie Boulevard, Philadelphia, PA 19104, USA; weberbl@mail.med.upenn.edu

Revised version received 12 December 2003
Accepted for publication 12 December 2003

REFERENCES
3 Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero E, Hinrich MC, Alence R, Nars SRL, Drasnina G. ERK mutations 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.21 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.22 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.

Authors’ affiliations
R H Edwards, M R Ward, C A Medina, M S Brose, P Volpe, B L Weber, Abramson Family Cancer Research Institute, University of Pennsylvania Medical Center, Philadelphia, PA, USA
H Wu, S R Lessin, Fox Chase Cancer Center, Philadelphia, PA, USA
S Nussen-Lee, H M Houp, A M Martin, Pennsylvania Hospital, Philadelphia, PA, USA
M Herlyn, The Wistar Institute, Philadelphia, PA, USA

Research support was received from the National Cancer Institute, grant numbers 1K08CA93748 (to RHE) and 5POCA093372 (to MH), the National Institute of Arthritis and Musculoskeletal and Skin Diseases, grant number K24 AR02102 (to SRL), and from the Abramson Family Cancer Research Institute (to BLW).

Conflict of interest: none declared.

Correspondence to: B L Weber, MD, Abramson Family Cancer Research Institute, University of Pennsylvania Medical Center, 513 BRB III/II, 421 Curie Boulevard, Philadelphia, PA 19104, USA; weberbl@mail.med.upenn.edu

Revised version received 12 December 2003
Accepted for publication 12 December 2003

REFERENCES
3 Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero E, Hinrich MC, Alence R, Nars SRL, Drasnina G. ERK mutations 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.21 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.22 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.

Authors’ affiliations
R H Edwards, M R Ward, C A Medina, M S Brose, P Volpe, B L Weber, Abramson Family Cancer Research Institute, University of Pennsylvania Medical Center, Philadelphia, PA, USA
H Wu, S R Lessin, Fox Chase Cancer Center, Philadelphia, PA, USA
S Nussen-Lee, H M Houp, A M Martin, Pennsylvania Hospital, Philadelphia, PA, USA
M Herlyn, The Wistar Institute, Philadelphia, PA, USA

Research support was received from the National Cancer Institute, grant numbers 1K08CA93748 (to RHE) and 5POCA093372 (to MH), the National Institute of Arthritis and Musculoskeletal and Skin Diseases, grant number K24 AR02102 (to SRL), and from the Abramson Family Cancer Research Institute (to BLW).
Absence of *BRAF* mutations in UV-protected mucosal melanomas


*J Med Genet* 2004 41: 270-272
doi: 10.1136/jmg.2003.016667

Updated information and services can be found at:
http://jmg.bmj.com/content/41/4/270

*These include:*

**References**
This article cites 20 articles, 8 of which you can access for free at:
http://jmg.bmj.com/content/41/4/270#BIBL

**Email alerting service**
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

**Topic Collections**
Articles on similar topics can be found in the following collections
- Cancer: dermatological (43)
- Dermatology (240)
- Molecular genetics (1254)

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/