**BRAF Mutations in Conjunctival Melanoma**

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**PURPOSE.** An activating mutation in exon 15 of the BRAF gene has been found in a high proportion of cutaneous melanomas and cutaneous nevi but not in uveal melanoma. Conjunctival melanoma shows greater clinical similarity to cutaneous melanoma than does uveal melanoma. The purpose of this study was to determine whether the T1799A BRAF mutation found in cutaneous melanoma is also present in conjunctival melanoma.

**METHODS.** DNA was extracted from paraffin sections obtained from glutaraldehyde or formalin-fixed, paraffin-embedded conjunctival melanomas. Forty-two specimens were identified from 25 patients. Seminested PCR was used to amplify exon 15 of the BRAF gene, and the resultant PCR product was purified and directly sequenced. Sequences from conjunctival melanomas were compared with the wild-type sequence of the reference.

**RESULTS.** The T1799A (V600E) mutation was detected by sequencing in melanomas from 5 of 22 patients as well as in the positive control, a cutaneous melanoma cell line. In this small series, no statistically significant associations between the presence of the BRAF mutation and clinicopathological characteristics were detected, although tumors with this mutation tended to have a larger diameter and greater depth of invasion and to contain epithelioid cells.

**CONCLUSIONS.** Others have demonstrated a BRAF T1799A-activating mutation in cutaneous but not uveal melanoma. In this study, this BRAF mutation was demonstrated in some conjunctival melanoma tissue samples, suggesting that some conjunctival melanomas may share biological features in common with cutaneous melanoma. (Invest Ophthalmol Vis Sci. 2004;45:2484–2488) DOI:10.1167/iovs.04-0093

Conjunctival melanoma accounts for 2% to 3% of all ocular melanomas, with a previously reported annual incidence of between 0.12 and 0.5 per million in white populations.1 There is evidence that the annual incidence of conjunctival melanoma is increasing, with one study reporting 0.8 cases per million in the year 2000.2 The etiology of conjunctival melanoma is uncertain. It has been reported in association with systemic conditions such as the dysplastic nevus syndrome,3 xeroderma pigmentosum,4 and neurofibromatosis.5 It has been suggested that exposure to ultraviolet light causes some conjunctival melanomas.6 This is supported by the increasing incidence of conjunctival melanoma in accordance with its cutaneous counterpart.2

The BRAF gene encodes a serine/threonine kinase in the mitogen-activated protein kinase (MAPK) pathway which is involved in signal transduction.7 Since the discovery of activating BRAF mutations in 66% of cutaneous melanomas,8 the search has been on to determine the presence and prevalence of BRAF mutations in other cancers. In cutaneous melanomas, most mutations involve a single point mutation in the activating segment of the kinase domain (exon 15; T1799A), leading to a V600E amino acid substitution and constitutive kinase activity.8 Several recent studies have failed to confirm the presence of the BRAF mutation in uveal melanomas including primary and metastatic choroidal and ciliary body melanomas.9–12 Clinically and histologically, conjunctival melanomas resemble cutaneous melanomas more closely than choroidal melanomas.13,14 The purpose of this study was therefore to determine whether the common BRAF mutation found in cutaneous melanoma is also present in conjunctival melanoma.

**MATERIALS AND METHODS**

**Samples**

Archival specimens of conjunctival malignant melanoma were obtained from the Western Infirmary Pathology files between 1980 and 2003. This included 42 specimens from 25 patients in whom melanoma was treated by local resection, enucleation, or exenteration. All tissues had been fixed in glutaraldehyde or formalin and embedded in paraffin wax. Samples were selected on the basis of there being sufficient remaining tumor tissue within the paraffin-embedded specimen with minimal surrounding non-tumor tissue to decrease the likelihood of contamination with non-tumor DNA. Full ethical approval in accordance with local policy was obtained for the use of these tissues, and the study protocol adhered to the tenets of the World Medical Association’s Declaration of Helsinki.

Clinical details, including age, sex, and site of tumor, were obtained from case notes and pathology reports. The original histologic sections were reviewed, and the size, depth of invasion, cell type, presence of necrosis, and presence of primary acquired melanosis (PAM) with atypia were recorded. Clinical outcomes measured included tumor recurrence and requirement for radical surgery (either enucleation or exenteration). These clinical and histologic features have been described to be of prognostic value in conjunctival melanoma.14–16

**DNA Extraction**

For each tumor sample, excess paraffin and non-tumor tissue was trimmed from a 25-μm section, which was then placed in a 1.5-ml microcentrifuge tube. For small tumors, two 25-μm sections were used. The sections were deparaffinized by the addition of 1 ml xylene. After 10 minutes, the samples were washed twice with ethanol and then allowed to air dry. To isolate genomic DNA, 100 μL digestion

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buffer (10 mM Tris [pH 8.5] and 1 mM EDTA) containing 100 μg proteinase K was added, and the samples were incubated overnight at 55°C. After centrifugation for 5 minutes, the supernatants were transferred to fresh tubes, and the enzyme was heat inactivated at 100°C for 5 minutes. Samples were stored at −20°C until used.

Polymerase Chain Reaction and Sequencing
The BRAF gene sequence was amplified with a seminested approach. Initially, primary polymerase chain reactions (PCRs) were performed in 25-μl reaction volumes containing 2× amplification buffer (Platinum Pfx; Invitrogen, Carlsbad, CA), 1 mM MgSO4, 0.3 mM dNTP mixture, 0.3 μM primer, and 0.625 U polymerase (Platinum Pfx; Invitrogen). One microliter of a 10-fold dilution of the supernatant containing extracted DNA was used as the template in the primary PCR reaction. Cycling conditions included an initial denaturation at 94°C for 5 minutes followed by 20 cycles of 94°C for 20 seconds, 50°C for 30 seconds, and 68°C for 30 seconds followed by a final extension cycle for 5 minutes at 68°C. Primer sequences for primary PCR were as follows: forward, 5'-TCATAATGCTGTGCTCTGATAGGA-3'; reverse, 5'-GGCCAAATTATTAATCTAGTGGA-3'. Two microliters of the primary PCR product was then used as a template in a seminested PCR reaction, increasing the cycle number to 25 and reaction volume to 50 μl. Primer sequences for seminested PCR were as follows: forward, 5'-GCTGATAGGAAAAATGAGATC-3'; reverse, 5'-GTTGAAAAATATAGCTCAATTT-3'.

PCR products were visualized by standard gel electrophoresis and were purified (GeneClean; Q Biogene, Carlsbad, CA). Purified PCR products were then bidirectionally sequenced by dye termination chemistry (BigDye Terminator chemistry; Applied Biosystems [ABI], Foster City, CA) and analyzed on a sequencer (MegaBACE; Amersham Biosciences, Amersham, UK). As a positive control, the cutaneous melanoma cell line SK-MEL-28 was used, which is known to contain the exon 15 T1799A (V600E) mutation. Sequences were compared to the melanoma cell line SK-MEL-28 was used, which is known to contain the exon 15 T1799A (V600E) mutation.8 Sequences were compared to the sequence (GenBank accession number: GI: 179532; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). Sequences were aligned and compared by eye. To confirm that the mutation was occurring only within the tumor cells, DNA was extracted from conjunctival mucosa adjacent to one melanoma containing the BRAF mutation. Semiautomated direct sequencing was the technique selected to detect this mutation, because it is considered the most reliable and accurate method of detection. Unlike other techniques such as detection of restriction fragment length polymorphisms it directly determines the precise sequence within the area of the gene under study and has the additional potential to identify as yet undiscovered mutations.

Statistical Analysis
The Fisher exact test was used to determine statistical correlations between the presence of the BRAF mutation and the clinical features, pathologic features, and outcomes measured.

RESULTS
Mutational Analysis
High-quality sequencing in both the 5' and 3' directions was obtained in 37 samples from 22 patients. Sequencing failed in five tumor samples, including two recurrent tumors, obtained from three patients. The T1799A (V600E) mutation in exon 15 was detected in the cutaneous melanoma cell line SK-MEL-28 and in melanomas from 5 of 22 patients. There were no other differences detected in the sequences obtained from the tumor DNA when compared with non-tumor DNA and with the known sequence of the wild-type human BRAF gene. In four of the five cases, similar to those previously described in the literature, the mutation appeared to be heterozygous. In one case, the mutation was homozygous. In seven patients samples from recurrent tumors were analyzed. A heterozygous mutation was found in two of these seven patients. In one case the mutation was present in the first excision but not in samples obtained 4 and 8 years later. In the second case the mutation was identified in the first recurrence, 6 years after the initial excision. The mutation was not present in the initial excision or in two subsequent recurrences 1 year later. The BRAF gene sequence was normal in conjunctival mucosa adjacent to one melanoma obtained from the sample with a homozygous mutation. Sequencing traces from non-tumor DNA and selected cases are shown in Figure 1.

Clinical and Pathologic Features
In the five patients (four women; one man) with the BRAF mutation, the mean age at initial diagnosis was 60 years (range, 30–77 years). The mean largest tumor dimension was 11.6 mm (range, 6–20 mm). In the 17 patients (11 women; 6 men) with no BRAF gene mutation the mean age at initial diagnosis was 65 years (range, 30–83 years). The mean largest tumor dimension was 8.2 mm (range, 2–18 mm). The tumor was located in a sun-exposed site in three of the 5 cases with the BRAF mutation and in 12 of the 17 cases with the wild-type BRAF gene. In our study four of the five melanomas containing the BRAF mutation were composed solely of epithelioid type cells and in one case contained a mixture of epithelioid and spindle cells. In contrast, the melanomas with a wild-type BRAF gene were composed solely of epithelioid cells in seven cases and in eight cases were composed of a mixture of cell types and in two of only spindle cells. In the melanomas containing the BRAF gene mutation the mean maximum depth of invasion was 6.1 mm (range, 0.5–11 mm) and areas of necrosis were present in two of the five cases. In the melanomas with a wild-type BRAF gene the mean maximum depth of invasion was 3.2 mm (range, 0.2–10 mm) and 0 of the 17 cases contained areas of necrosis. In five cases (one with and four without the BRAF gene...
mutation) the tumor extended to the deep resection margin. In these five cases the measurement to the deep resection margin was recorded as the maximum depth of invasion. PAM was present in 2 of the 5 melanomas with the BRAF mutation and in 12 of the 17 with a wild-type BRAF gene. In three of the five cases with the BRAF mutation, the melanoma had recurred and radical surgery (enucleation or exenteration) was necessary in two cases. In 12 of the 17 cases with the wild-type BRAF gene the melanoma had recurred, and radical surgery was necessary in 4 cases. According to the Fisher exact test, only the presence of necrosis reached a significance of $P < 0.05$. The clinical and pathologic features are summarized in Table 1.

**DISCUSSION**

The RAS-RAF-MEK-ERK-MAP kinase pathway mediates cellular responses to growth signals and is involved in a large number of physiological processes. This pathway has also been shown to play a role in cell transformation. In particular, activating mutations in the BRAF gene have been identified in human cancers, with the highest frequency of mutations found in cutaneous melanomas. The BRAF mutations identified are found predominantly in two small regions of the kinase domain of the BRAF molecule. The predominant mutation occurs in exon 15 of BRAF with a single T-to-A substitution, although some mutations have also been found in a region of exon 11. Mutations have also been detected in up to 82% of cutaneous melanocytic nevi. In contrast, this mutation appears to be absent from uveal melanomas, although it has been identified in choroidal melanoma cell lines. In this study, we identified the T1799A point mutation in 5 conjunctival melanomas from 22 patients.

In contrast to cutaneous and uveal melanoma, conjunctival melanoma is a rare neoplasm. The presence of this mutation in conjunctival melanomas may reflect its closer relationship with cutaneous melanoma than with uveal melanoma. Conjunctival melanoma is a tumor of melanocytes, which during the embryonic period originate in the neural crest and like their cutaneous counterpart migrate toward an epithelium. Uveal melanocytes also originate from the neural crest but in contrast migrate to the deeper mesodermal tissue of the uveal tract. Similarities between cutaneous and conjunctival melanoma are also evident clinically. Both of these melanoma classes tend to metastasize first to regional lymph nodes, as opposed to uveal melanoma, which tends to metastasize first to the liver. Conjunctival melanomas can appear histologically similar in many respects to cutaneous melanomas. The immunophenotypic expression of conjunctival melanoma has also been shown to be closer to cutaneous melanomas with epithelioid cells than to uveal melanomas. In particular, expression of S100 has been shown to be significantly higher in cutaneous and conjunctival melanomas than uveal melanomas. However, there are distinct differences between conjunctival and cutaneous melanomas. For example, there are no conjunctival counterparts of cutaneous lentigo maligna or superficial spreading melanoma. Conjunctival melanoma can arise de novo or in relation to a nevus or PAM with atypia. There is no cutaneous counterpart for PAM. In our study, the melanomas with the BRAF mutation tended to be composed solely of epithelioid cells in keeping with the proposed similarity with cutaneous melanoma. There was no obvious relationship with a history of PAM, which was present in 2 of the 5 cases with the BRAF mutation and in 12 of 17 cases with a wild-type BRAF gene.

In recent years, there is evidence that the incidence of conjunctival melanoma is increasing. This increasing incidence coincides with that of cutaneous melanoma, and a possible link to a sunlight-related etiology has been suggested. A similar etiology may therefore play a role in conjunctival melanomas occurring within the interpalpebral fissure, which may lead to common genetic mutations. However, sunlight is unlikely to contribute to the etiology of those melanomas that occur in the shielded areas of the fornices or palpebral conjunctiva. This may explain the lower incidence of BRAF mutations in conjunctival melanoma. Indeed, it has recently been shown that BRAF mutations are significantly more common in melanomas occurring on skin subject to intermittent sun ex-

### Table 1. Summary of the Clinical and Pathological Features and Outcomes of Patients with Conjunctival Melanomas, with or without a BRAF Gene Mutation

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Total Patients with Conjunctival Melanoma ($n = 22$)</th>
<th>Tumors with BRAF Mutation ($n = 5$)</th>
<th>Tumors with Wild-type BRAF Gene ($n = 17$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (y)</td>
<td>59.8 (range 30–77)</td>
<td>64.5 (range 30–85)</td>
<td></td>
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<tr>
<td>Mean maximum tumor dimension (mm)</td>
<td>11.6 (range 6–20)</td>
<td>8.2 (range 2–18)</td>
<td></td>
</tr>
<tr>
<td>Sun-exposed location, n (%)</td>
<td>3/5 (60)</td>
<td>13/17 (76)</td>
<td></td>
</tr>
<tr>
<td>Pathological features</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell type, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelioid only</td>
<td>4/5 (80)</td>
<td>7/17 (41)</td>
<td></td>
</tr>
<tr>
<td>Epithelioid and spindle</td>
<td>1/5 (20)</td>
<td>8/17 (47)</td>
<td></td>
</tr>
<tr>
<td>Spindle only</td>
<td>0/5 (0)</td>
<td>2/17 (12)</td>
<td></td>
</tr>
<tr>
<td>Presence of tumor necrosis, n (%)</td>
<td>2/5 (40)</td>
<td>0/17 (0)</td>
<td></td>
</tr>
<tr>
<td>Mean maximum depth of invasion (mm)</td>
<td>6.1 (range 0.5–11)</td>
<td>3.2 (range 0.2–10)</td>
<td></td>
</tr>
<tr>
<td>Presence of PAM, n (%)</td>
<td>2/5 (40)</td>
<td>12/17 (71)</td>
<td></td>
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<tr>
<td>Outcomes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Recurrence of melanoma, n (%)</td>
<td>3/5 (60)</td>
<td>12/17 (71)</td>
<td></td>
</tr>
<tr>
<td>Radical Surgerya</td>
<td>2/5 (40)</td>
<td>4/17 (24)</td>
<td></td>
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</table>

*aEnucleation or exenteration.*
posure than elsewhere.27 In contrast, \textit{BRAF} mutations in melanoma on chronically sun-damaged skin and melanomas occurring on the palms, soles, or subungual sites and mucosal membranes are rare.27 However, we did not identify any correlation between tumor site and presence of the \textit{BRAF} mutation.

A study by Shinozaki et al.29 has shown that the \textit{BRAF} mutation frequency is significantly higher in metastatic cutaneous melanoma than in primary melanoma. This implies that the \textit{BRAF} mutation may be acquired later in tumorigenesis and is associated with a more aggressive course. Conversely, Pollock et al.,19 have shown a high frequency of the \textit{BRAF} mutation in cutaneous nevi, which would suggest that this mutation was acquired early in melanocytic transformation. In this study, the conjunctival melanomas with the \textit{BRAF} mutation tended to be larger, with deeper invasion than those with the wild-type \textit{BRAF} gene, suggesting that they may be further advanced along the tumorigenesis pathway and pursuing a more aggressive course. However, in patients who had several recurrent samples analyzed, the \textit{BRAF} mutation was present in the first recurrence in one case and in the initial tumor in the second case and was not present in other samples. This may reflect selection of a particular tumor clone either at the time of biopsy or during DNA amplification. Therefore, in these cases with several samples no correlation can be drawn with tumor stage. Furthermore, in our study there were few cases with the \textit{BRAF} mutation, and as such it was not possible to obtain statistical significance with any of the clinical or pathologic parameters.

It is assumed that most mutations in tumors are heterozygous. The significance of a homozygous mutation is as yet unclear. It is interesting, however, that in this study the melanoma with a homozygous mutation recurred on several occasions and ultimately necessitated exenteration of the orbit, in keeping with an aggressive course. We were not able to perform mutational analysis on later samples from this patient. The same study by Shinozaki et al.29 also showed a higher frequency of the \textit{BRAF} mutation in younger patients (age, <60 years). Again, there were fewer cases in our study, but there appears to be no association with a younger age group.

In this study, the \textit{BRAF} mutation was present in only 5 of 22 patients with conjunctival melanoma. This suggests a lower mutation rate than is seen in cutaneous melanoma, which may reflect the previously described differences between these tumors or the techniques used in identification of the mutation. For example, we used direct sequencing to identify this mutation, and it is therefore possible that low levels of mutation could have been missed. Although every endeavor was made to remove all non-tumor tissue from the samples, it is possible that there was contamination from normal tissues in some cases. Direct sequencing is not as sensitive a technique as single-strand conformation polymorphism analysis or denaturing HPLC. Sequencing would not be able to detect mutant alleles present at a low frequency because of somatic mosaicism, but the presence of very low levels of mutant tumor cells is of doubtful significance. However, direct sequencing offers the advantages of determining the precise sequence within the area of the gene under study. It is also possible that there were mutations in other areas of the \textit{BRAF} gene; however, almost all previously reported mutations in melanoma have been concentrated in this region in exon 15 in the \textit{BRAF} kinase domain. We failed to obtain sequences in five samples. The cause for this is unclear. These tissue samples had been stored for up to 23 years (range, 6–23 years); however, sequences were successfully obtained from tissues of a similar age. The failure to obtain sequences may reflect the small amount of tumor tissue available in these cases.

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\textbf{References}