The T1799A \textit{BRAF} Mutation Is Present in Iris Melanoma

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\textbf{PURPOSE.} An activating mutation in exon 15 of the \textit{BRAF} gene has been found in a high proportion of cutaneous pigmented lesions, but only in one case of uveal melanoma. Iris melanoma is the least common uveal melanoma and displays a less aggressive clinical course compared with posterior uveal melanoma. To date, no study has been conducted to investigate the T1799A mutation in iris melanoma. The purpose of this study was to determine whether the T1799A \textit{BRAF} mutation is present in iris melanoma.

\textbf{METHODS.} DNA was extracted from 19 archival, paraffin-embedded tissue sections of iris melanomas. Nested PCR was used to amplify exon 15 of the \textit{BRAF} gene, and the product was purified, cloned into a sequencing vector, and sequenced. The sequences obtained were compared with the wild-type sequence of the \textit{BRAF} gene. The presence or absence of the \textit{BRAF} mutation was also compared with the clinicopathological features.

\textbf{RESULTS.} The T1799A \textit{BRAF} mutation was identified by sequencing in 9 of 19 iris melanomas. Six of the 9 cases with the \textit{BRAF} mutation were recurrent tumors. All other tumors were resections for primary tumors. There was a statistically significant association between the \textit{BRAF} mutation and recurrent tumor \((P = 0.003)\). There was no association between the presence of the \textit{BRAF} mutation and other clinicopathological characteristics.

\textbf{CONCLUSIONS.} In this small study, the T1799A \textit{BRAF} mutation was identified in almost half of the iris melanoma tissues samples examined. This finding suggests that there may be genetic as well as clinical differences between iris and posterior uveal melanomas. (\textit{Invest Ophthalmol Vis Sci.} 2007;48: 4897–4900) DOI:10.1167/iovs.07-0440

Mutations in the \textit{BRAF} gene (a member of the Raf family that encodes a serine/threonine protein kinase) have been shown to occur in most cutaneous melanomas.\textsuperscript{1} In particular, a single point mutation in exon 15 (T1799A), which results in constitutive kinase activity and unregulated signal transduction, is involved in up to 80\% of cases.\textsuperscript{1} Despite its high incidence in cutaneous melanoma, there has been only one example of this mutation identified in posterior uveal melanoma.\textsuperscript{2} Several other studies have not found this mutation in uveal melanoma.\textsuperscript{3–8}

Uveal melanoma is the most common primary intraocular tumor in adults, comprising lesions of the choroid, ciliary body, and iris with an overall incidence of around per 1,000,000 per year.\textsuperscript{8,9} Melanomas of the iris are the least common form of uveal melanoma, accounting for between 3\% and 16\% of the total. Iris melanoma behaves differently from posterior uveal melanoma.\textsuperscript{10} In contrast with posterior uveal melanomas, iris melanomas tend to display a less-aggressive clinical course.\textsuperscript{10,11} They can grow in a locally aggressive manner but rarely metastasize. Occasionally they grow diffusely, extending posteriorly to involve the ciliary body and giving rise to the so-called ring melanoma.

In view of the clinical differences between posterior uveal melanoma and iris melanoma, we assessed samples from iris melanoma for the presence or absence of the T1799A point mutation in the \textit{BRAF} gene. The findings were compared with clinical and histopathologic features.

\textbf{MATERIALS AND METHODS}

\textbf{Case Selection}

Suitable cases of archival formalin- or glutaraldehyde-fixed, paraffin-embedded tissue sections were retrieved from the Eye Pathology Files, Western Infirmary Glasgow (1972–2004). Cases involving the ciliary body were excluded, to avoid the possibility of having a ciliary body tumor that extended into the iris. Very small samples that had been serially sectioned for earlier work were also excluded because of insufficient tissue remaining on the tissue block. Clinical details (such as age, sex, site, and whether a primary or recurrent tumor) were obtained from the pathology report. The original histology slides were reviewed and tumor cell type, invasion of the trabecular meshwork, and number of mitoses per 40 high-power fields were recorded. This project received the full approval of the West Ethics Committee, North Glasgow Hospitals, and adhered to the tenets of The Declaration of Helsinki.

\textbf{Laser Capture Microdissection}

Laser capture microdissection (LCM) was performed with a commercial system (PixCell II; Arcturus, Mountain View, CA) to isolate 10 to 100 tumor cells from each section. In brief, the H&E-stained section was placed on the microscope stage under a thermoplastic film (Capsure HS; Arcturus) and the area of interest was observed with a charge coupled device (CCD) video camera and displayed on a computer monitor. The laser microbeam was directed through the cap onto single cells, and each cell was picked up and collected on the thermoplastic film of the cap. Several different areas were microdissected from each tumor. Adjacent normal tissue was taken from selected cases for comparison.

\textbf{Genomic DNA Isolation}

Genomic DNA was isolated by proteinase K digestion in a Tris-EDTA buffer. The caps containing the cells were placed on an alignment tray and 100 \(\mu\)g proteinase K was added to the thermoplastic film. The caps were covered by a 0.5-ml microcentrifuge tube, incubated in a preheated incubation block, and placed in an oven at 65°C for 20 hours. The contents of each cap were collected by centrifugation at 4000g for 1 minute, and the proteinase K was inactivated by incubation at 95°C


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for 10 minutes. The isolated DNA was stored at 4°C until further use in PCR. Genomic DNA was also isolated from the in vitro cell line SK-MEL-28, as a positive control for PCR and sequencing.

Oligonucleotide Primer Design

Sequences of primers used in PCR were designed in exon 15 of the BRAF gene sequence (GenBank Accession number NM_004333; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

Polymerase Chain Reaction

All PCR amplification reactions were performed in a 25-μL reaction (ReddyMix; Aligene, Epsom, UK). 25 picomoles of forward (5′-TCATAATGCTTGCTGTAGGAA-3′) and reverse (5′-GGCCAAAAAATTATCAGTGGAGA-3′) primer, and 5 μL of genomic (g)DNA template isolated from tumor cells. This mix contains a recombinant Taq polymerase with a low error rate. With the use of a programmable thermal cycler (Peltier PTF-100; Bio-Rad, Hemel Hempstead, UK), reactions consisted of an initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing for 45 seconds at 55°C, and extension at 72°C for 1 minute. A final extension was performed at 72°C for 10 minutes. Nested PCR was performed in the same manner, with forward (5′-GCTCTGATAGGAAAATGAGATC-3′) and reverse (5′-GTGGAAAAATAGCCTCAATTC-3′) primer with 5 μL of template from the primary PCR reaction. All PCR reactions were performed in duplicate.

A-U Cloning of PCR-Amplified Products and Sequencing

The PCR reactions were analyzed on a 2% agarose electrophoresis gel (Fig. 1), and the DNA fragments were excised and purified with a kit (Gel Extraction MinElute Kit; Qiagen, Crawley, UK) according to the manufacturer’s instruction. The PCR amplified products were ligated into the pDRIVE vector with a PCR cloning kit (Qiagen). Three micro-liters of the ligation reaction was used to transform DH5α by the heat-shock method of Cohen et al.13 White colonies were selected and grown overnight in Luria Bertani (LB) broth at 37°C with shaking at 225 rpm. Plasmids were purified from cultures by using a kit (Miniprep Kit; Qiagen), according to the manufacturer’s instruction. Automated sequencing in both forward and reverse direction overlapping the mutation site was performed on two to three cloned products (Gene Codes, Cambridge, UK) and sequences were analyzed (Sequencher; Gene Codes, Ann Arbor, MI).

Statistical Analysis

The association between the tumor type (primary or recurrent) and presence of the T1799A BRAF mutation was assessed by the Fisher exact test.

RESULTS

BRAF Mutations

A total of 22 samples were identified but 3 were subsequently excluded, as repeated PCRs of extracted DNA were unsuccessful. Of the 19 remaining samples, there were 4 sector iridectomies, 9 iridocyclectomies, and 7 enucleations. The T1799A point mutation was identified in 9 of these 19 iris melanomas (Fig. 2C). The mutation was not identified in the normal tissue.

Clinical Features

BRAF-Positive Tumors. Of the nine tumors with the T1799A point mutations, seven were from women and two were from men. The patients’ average age was 51.2 years (range, 44 – 71). The location of the tumor was interior (six cases), superior (one case), or diffuse (one case), and in one case the location was not stated. Surgery was for removal of a primary tumor in four cases and for recurrent tumor after primary excision in five cases. Six cases were treated by enucleation, two by iridectomy, and one by iridocyclectomy. The intraocular pressure was raised in three cases all treated by enucleation. There was a statistically significant association between the BRAF mutation and recurrent tumor (P = 0.003).

BRAF-Negative Tumors. Of the 10 tumors without the T1799A point mutation, four were from women and six were from men. The patients’ average age was 49.6 years (range, 28 – 67). The location of the tumor was inferior (four cases), superior (two cases), or interpapillary (one case); in three cases the location was not stated. Surgery was for removal of a primary tumor in all cases. One case with raised intraocular pressure was treated by enucleation, eight by iridocyclectomy, and one by iridectomy.

Histologic Features

BRAF-Positive Tumors. Six of the nine tumors consisted of spindle cells, two were mixed tumors, and was one composed purely of epithelioid cells. In six cases, no mitotic figures were identified, in two cases there were between one and five mitotic figures, and in one case there were more than five mitotic figures. Tumor cells were identified within the trabecular meshwork in six cases (Figs. 2A, 2B).

BRAF-Negative Tumors. Nine of the 10 tumors consisted of spindle cells with 1 mixed tumor. In three cases there were between one and five mitotic figures, but no mitotic figures were identified in the remaining seven cases. Tumor cells were identified in the trabecular meshwork in five cases.

DISCUSSION

Activating mutations in the BRAF gene have been identified in many human cancers, with the highest frequency of mutations found in cutaneous melanomas. In melanoma, these BRAF mutations are found in two small regions of the kinase domain of the BRAF molecule. The predominant mutation occurs in exon 15 of the BRAF gene with a single T-to-A substitution, although a smaller number of mutations have been found in a region of exon 11. The mutation has also been reported in 22% to 40% of conjunctival melanomas. However, despite several studies, in uveal melanoma including primary and metastatic choroidal and ciliary body melanomas, the BRAF mutation has been identified in only one case. In this study we identified the BRAF mutation in 9 (48%) of 19 samples of the least common uveal melanoma, iris melanoma. As the cases that were positive for the BRAF mutation were amplified by PCR and sequenced in duplicate, it is highly unlikely that the mutations identified were due to PCR error.

Iris melanoma tends to pursue a less aggressive course than tumors of the posterior uveal tract, which may in part reflect earlier diagnosis. However, there may be different etiological factors that in part account for the differences in BRAF gene mutations. For example, exposure to sunlight has been sug-

FIGURE 1. A 2% agarose electrophoresis gel showing bands of approximately 200 bp from five different samples and a negative control (–).
gested as a risk factor for iris melanoma. Indeed, the iris receives a greater amount of UV light than does the ciliary body or choroid because of specific filtering effects of the lens, retinal pigment epithelium, and choroid. This is further supported by the fact that most iris melanomas occur inferiorly in the part of the eye exposed to most sunlight. In cutaneous melanoma, it has been suggested that exposure to ultraviolet light is a key factor in melanomas with the T1799A point mutation. Previous research has shown that the BRAF mutation frequency is lower in melanomas arising in sites protected from sun exposure compared with those from sun-exposed areas. It is recognized that the T1799A point mutation is not a UV-signature mutation, but it has been suggested that it may occur as a result of error-prone reduplication of UV-damaged DNA. However, although exposure to sunlight may explain the presence of the BRAF mutation in iris melanoma compared with posterior uveal melanoma there was no significant difference in location for BRAF-positive compared with BRAF-negative tumors of the iris.

Tumors of the iris are less common than those of the ciliary body and choroid. Their rarity is reflected in the relatively small number of suitable cases identified for this study. In addition, there is an inevitable element of selection bias in the tumors studied since many iris melanomas are simply observed and may never come to surgery. Sector iridectomy specimens may yield only small amounts of tumor, and all of it may be needed for diagnostic purposes. By contrast, the tumors that are treated surgically are by implication faster growing and generally larger. Nonetheless, there was one interesting and statistically significant association with the clinical features recorded. Specifically, 6 of the 9 BRAF-positive tumors were recurrent tumors treated by enucleation compared with the 10 primary tumors that were BRAF-negative and required only local excision (P = 0.003). One possible explanation is that the BRAF mutation is responsible for the more aggressive phenotypes and that these melanomas are more likely to recur. It has been reported that recurrent melanoma often assumes a higher grade of epithelioid morphology than primary iris melanoma that frequently displays low-grade cytology. However, in this study, there were no morphologic differences between the primary and recurrent tumors, with or without the BRAF mutation. An alternative explanation may be that the BRAF mutation is more likely to occur in tumors as they progress. To answer this question we attempted to identify the primary tumor for sequencing in the cases of recurrent melanoma. In one case, it was the patient’s only seeing eye, and the primary tumor had been treated by cryotherapy; in another two cases, the primary excision was not performed at our hospital, and in the final two cases, we were unable to obtain sufficiently high-quality DNA for sequencing, in part due to the small amount of identifiable residual tumor present in the tissue block. There were no differences between BRAF-positive and BRAF-negative tumors for other clinical or histologic features.

In summary, this study has shown that the T1799A point mutation in the BRAF gene is present in a significant number of iris melanomas. This finding suggests that there may be cytogenetic as well as clinical differences between iris and posterior uveal melanomas where the mutation has rarely been identified. In this study it was identified predominantly in recurrent tumors, suggesting that it may indicate a more aggressive tumor phenotype.

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References


Erratum in: “Flt-1 Intraceptor Induces the Unfolded Protein Response, Apoptotic Factors, and Regression of Murine Injury-Induced Corneal Neovascularization” by Singh et al. (\textit{Invest Ophthalmo Vis Sci}. 2006;47:4787–4793.)

The first sentence of the Figure 2 legend should read: “\textit{Lane 1}: PBS; \textit{lane 2}: empty pCMV; \textit{lane 3}: pCMV.Fl24K.”

The first sentence of the Figure 3 legend should read: “\textit{Lane A}: mouse corneas injected with empty pCMV vector; \textit{lane B}: pCMV.Fl24K.”

The first sentence of the Figure 4 legend should read: “\textit{Lane 1}: HMECs in medium; \textit{lane 2}: cells transfected with empty pCMV; \textit{lane 3}: cells transfected with pCMV.Fl24K.”

In Figure 5, the labels “\textit{β-Actin}” and “\textit{Caspase-3}” are reversed. “\textit{Caspase-3}” should be the top label, while “\textit{β-Actin}” should be the bottom label.