T1799A BRAF Mutations in Conjunctival Melanocytic Lesions

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PURPOSE. To gain a better understanding of the molecular events leading to the development of conjunctival melanocytic lesions and conjunctival melanoma, this study was conducted to investigate the presence of T1799A BRAF oncogenic mutation in these lesions.

METHODS. Forty-eight surgically excised conjunctival melanocytic lesions from 48 patients were examined for the presence of the BRAF T1799A mutation. Twenty-eight lesions were conjunctival nevi, of which 20 were excised from children younger than 18 years. Fifteen lesions were conjunctival primary acquired melanosis (PAM; 11 without atypia and 4 with atypia) and five were conjunctival melanomas. To detect the BRAF T1799A mutation, both a newly developed kit (Mutec-tor; TrimGen, Sparks, MD) and direct DNA sequence analysis of exon 15 after PCR amplification were used.

RESULTS. The T1799A BRAF mutation was identified in 14 of 28 (50%) conjunctival nevi analyzed, but in none of the 15 conjunctival PAMs, with and without atypia. The T1799A BRAF mutation was identified in two of the five (40%) conjunctival melanomas. There was no difference in the BRAF mutation detected in conjunctival nevi in children or adults, as the BRAF mutation was detected in 50%.

CONCLUSIONS. The results showed that conjunctival nevi, similar to skin nevi, have a high frequency of oncogenic BRAF mutations. Furthermore, the results suggest that the oncogenic event leading to BRAF mutations affect only conjunctival nevi and not conjunctival PAM. The clinical significance of these observations remains to be determined. (Invest Ophthalmol Vis Sci. 2005;46:3027–3030) DOI:10.1167/iovs.04-1449

Melanocytic lesions of the conjunctiva include nevus, racial melanosis, congenital melanosis, primary acquired melanosis, and melanoma. The molecular events leading to the development of conjunctival melanoma are poorly understood. In a large series of 410 patients, two patients had conjunctival malignant melanoma that developed from a preexisting compound nevus (two cases) or a blue nevus (one case) during a mean interval of 7 years. Clinicopathologic review suggested that primary acquired melanosis of the conjunctiva (conjunctival PAM) with atypia and conjunctival nevi (CN) might be associated with higher risk of conjunctival melanoma. Clinically, melanocytic conjunctival lesions that have prominent blood vessels, rapid growth, or changes in color are suspicious, and therefore excision of these lesions is recommended.

The RAF serine/threonine kinases are regulated by Ras and mediate cellular responses to growth signals. Activating mutations in one of the RAF genes, BRAF, has been recently reported in a significant proportion of cutaneous melanoma,7 conjunctival melanoma,3,8 papillary thyroid carcinoma (PTC),6 and to a lesser extent in other cancers.5 In contrast to the high incidence reported in cutaneous melanoma,5 uveal melanoma, which is the most common melanocytic ocular malignancy, does not harbor BRAF mutations.10,11 Of the reported BRAF mutations, the T1799A point mutation in exon 15 is the most common mutation in cutaneous melanoma and PTC as well as in other tumor types.5,8 The resultant valine-to-glutamic acid substitution in BRAF codon 600 (V600E) has been shown to cause constant kinase activation independent of Ras activation and to induce NIH3T3 cell transformation.3

A recent observation,12 that BRAF-activating mutations are present in high frequency in nevi, suggests that this mutation represents an initial step in the process of melanocytic transformation.

Recently, The T1799A (V600E) mutation was detected in conjunctival melanomas from 5 of 22 patients.5 No statistically significant associations were detected between the presence of the BRAF mutation and clinicopathologic characteristics.6

To better understand the molecular events leading to the development of conjunctival melanoma, we investigated the presence of a T1799A BRAF oncogenic mutation in various benign and premalignant melanocytic lesions of the conjunctiva as well as in conjunctival melanoma.

MATERIALS AND METHODS

Patients and Sample

In all, 48 patients with conjunctival melanocytic lesions were identified between 1993 and 2004 from a search of archival surgical pathology files of the ophthalmic pathology laboratory of the Hadassah-Hebrew University Medical Center (Jerusalem, Israel) and from Rabin Medical Center (Petach Tikva, Israel) with institutional review board approval. After initial identification of patients, their charts were retrieved and reviewed for gender, age at diagnosis, location of the lesion, and clinical indications for excision. All patients included in this study underwent excision of melanocytic lesion of the conjunctiva due to one or more of the following indications: suspicious clinical appear-

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ance, accelerated growth, or color change of the lesions. All original histologic slides were reviewed by an experienced ocular pathologist. The lesions were classified as CN (compound or junctional), PAM with or without atypia, and conjunctival melanoma. In this study, we included five conjunctival melanomas, 28 CN, of which 27 were categorized as compound nevi and one was categorized as junctional nevus, and 15 conjunctival PAM (11 without atypia and 4 with atypia). Of the 28 CN analyzed, 20 CN were excised from children under 18 years of age.

**DNA Isolation**

Archival formalin-fixed, paraffin-embedded tissues were used for DNA isolation, as previously described. In brief, hematoxylin-eosin stained thin sections were first reviewed by a pathologist and melanocytic pigmentary lesions containing at least 75% melanocytic cells were outlined. Five consecutive 10-μm unstained paraffin sections of each block were carefully microdissected using a no. 11 surgical blade. DNA was then digested, extracted, and precipitated with ethanol using standard protocols, as previously published.

**Detection of BRAF Mutations**

**Mutector Assay.** To detect BRAF mutations at nucleotide position 1799, we used the Mutector assay (TrimGen, Sparks, MD). The assay was preformed according to the manufacturer’s instructions, using 10 μL of PCR products (102-bp fragment). In brief, the Mutector assay is designed to detect point mutations of known DNA sequence variation. A detection primer is designed not to 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 point mutation at BRAF, T1799), primer extension continues, and a strong color reaction is observed (Fig. 1A). The Mutector assay is highly sensitive and can detect as little as 1% of mutant DNA from a mixed sample. As positive controls for the BRAF T1799A mutation, we used the melanoma cell lines HTB71 (T1799A heterozygous) and HTB72 (A1799 homozygous); for negative controls, we used the cell lines ME180 (cervical cancer) and HCT116 (colorectal carcinoma). PCR primer sequences were designed to amplify a 224-bp fragment of exon 15 (5’-TCA TAA TGC TTG CTC TAG GA-3’ and 5’-GGC CAA AAA TTT AAT CAG TGG A-3’); and a 102-bp fragment of exon 15 (5’-GAA GAC CTC ACA GTGA AAA ATA GGT GA-3’ and 5’-CCA CAA AAT GGA TGC AGA CA-3’). PCR amplification was performed in a 50-μL reaction volume containing 50 to 100 ng of sample DNA as a template. The amplification was performed in a buffer containing 1.5 mM MgCl2, 16.6 mM (NH4)2 SO4, 6.25% dimethylsulfoxide (DMSO), 200 μM dNTPs, 250 nM of each forward and reverse primers, and 2.5 units of polymerase (Platinum Taq, Invitrogen, Carlsbad, CA). Cycling conditions were as follows: a denaturation step at 95°C for 5 minutes was followed by two cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, primer extension at 72°C for 1 minute; two cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute, primer extension at 72°C for 1 minute; 35 cycles of denaturation at 95°C for 1 minute, annealing at 56°C for 1 minute, primer extension at 72°C for 1 minute; and a final extension at 72°C for 5 minutes. Amplified fragments were separated on 2% agarose gel and visualized by ethidium bromide staining.

**Direct Sequencing of PCR Products.** PCR amplification of exon 15 (224 bp fragment) was followed by direct sequencing of PCR products using dye terminator chemistry (Big Dye Terminator Cycle Sequencing reagents; Applied Biosystems, Inc., Foster City, CA). Every mutation detected by the Mutector assay was confirmed by direct-sequence analysis and there were no discrepancies.

**RESULTS**

All the samples analyzed in this study were excised due to one of the indications mentioned in the methods sections and were localized in the sun-exposed bulbar conjunctiva. The results are presented below according to the histologic subgroup.

**Conjunctival Nevi**

In this study we analyzed 28 patients with CN, of whom 20 were younger than 18 years. The median age of the juvenile group was 12.5 years (mean, 12.2 ± 3) and the patients’ ages ranged from 7 to 17 years. Four of 13 CN from the ophthalmic disease laboratory of the Hadassah University Hospital (Jerusalem, Israel) were inflamed juvenile CN (IJCN). No data regarding nevi inflammatory status, 1 (25%) of the 4 inflamed CN samples harbored the BRAF mutation, as did 5 (55.5%) of the 9 noninflamed CN.
Conjunctival PAM

The median age of the conjunctival PAM group of patients was 59 years (mean 52.2 ± 22), and the patients’ age ranged from 25 to 77. We analyzed 11 samples of PAM without atypia. None of the 11 conjunctival PAM samples harbored the T1799A BRAF mutation. We also analyzed four samples of PAM with atypia. None of the PAM with atypia samples harbored the BRAF mutation.

Conjunctival Melanoma

Because of the rarity of conjunctival melanoma, we analyzed five conjunctival melanoma samples. The T1799A BRAF mutation was found in two (40%) of five conjunctival melanomas analyzed.

DISCUSSION

The recent hypothesis that oncogenic mutations in the BRAF gene are the principal genetic event in the development of malignant melanoma prompted us to evaluate the presence of BRAF mutations in conjunctival melanocytic lesions. In this study, we analyzed benign melanocytic lesions of the conjunctiva (CN and PAM without atypia), premalignant lesions (conjunctival PAM with atypia), and conjunctival melanoma for T1799A BRAF mutation.

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The RAS-RAF-MEK-ERK-MAP kinase pathway mediates cellular responses to growth signals and is involved in a large number of physiological processes as well as in cancer.6,16-17 Activating mutations in the BRAF gene have been identified in human cancers, with the highest frequency of mutations found in cutaneous melanomas.6 In contrast to cutaneous melanoma, BRAF mutations appear to be absent in uveal melanoma,6,10,11 supporting the known differences between ocular and cutaneous melanomas.18

The similarities between conjunctival and skin melanocytic lesions and melanomas has been described.19,20 BRAF mutations were also identified in conjunctival melanomas, a tumor closely resembling cutaneous melanomas.6 In accordance with the frequency found by Gear et al.6 BRAF mutations were detected in two of five conjunctival melanomas. Spendlove et al.7 studied both conjunctival and uveal melanomas for BRAF mutations. Similar to previous studies,10,11 no BRAF mutations were detected in uveal melanomas, whereas 3 of the 21 CN examined harbored the mutation.7 These data further support the similarities between conjunctival and skin nevi and melanomas, but not uveal melanoma.

Although conjunctival melanoma resembles that of the skin, the higher frequency of BRAF mutations in cutaneous melanoma may indicate different biological origin. Conjunctival melanoma can arise de novo or in relation to preexisting nevi or conjunctival PAM with atypia.2,21-22 Although conjunctival PAM with atypia is highly associated with the development of conjunctival melanoma,23,24 we did not find BRAF mutations in conjunctival PAM (with or without atypia). The lack of BRAF mutations in conjunctival PAM with atypia which constitutes most of the conjunctival melanomas25,24 may explain the lower frequency of BRAF mutations in conjunctival melanomas compared with cutaneous melanoma.

In accord with the high frequency reported in cutaneous nevi12 we found BRAF mutations in 50% of the CN analyzed. Given the fact that CN constitutes approximately one third of conjunctival melanomas we speculate that most of the conjunctival melanomas harboring BRAF mutations originate from CN.

Although CN are common, malignant transformation is infrequent. It is not clear whether the BRAF activating mutation is an early step in melanoma tumorigenesis. Loewe et al.25 reported that growing nevi had a strikingly high incidence of the BRAF mutation compared with nevi without growth. The correlation of melanocytic lesion growth and the presence of BRAF V600E mutations support the assumption made in vitro that this mutation induces cell proliferation.26 The question of whether melanocytic lesions harboring BRAF V600E mutation represent lesions at risk of developing into melanoma should be investigated further.

IJCN are often erroneously suspected to be malignant because of rapid growth. Zamir et al.27 reported that IJCN is associated with allergic conjunctivitis, and despite periods of alarming rapid growth, it is histologically benign. Alternatively, this process may represent a direct immune response induced by the nevus itself.28 In this study, the BRAF mutation was found in one (25%) of four IJCN, suggesting a lower frequency in this group. Because the IJCN study group was small, further investigation is needed.

In an unexpected finding, there was not only increased but also early occurrence of BRAF mutations in CN. Similar to the frequency found in nevi excised from adults, half of the nevi excised from children younger than 18 years were positive for the BRAF activating mutation suggesting that BRAF mutations can occur at any age. Because conjunctival melanoma is very rare in childhood and most often occurs in adults, the finding of BRAF mutations in children’s CN argues that these mutations may not be directly related to the development of melanoma. Although the BRAF mutation may simply be associated with certain melanocytic lesions, it is more likely that additional molecular events are necessary for the development of malignant melanoma.

In a recent study, it was found that the BRAF mutation is essential to the development of nevi, and with the presence of defective p53 function, leads to the development of melanoma in a zebrafish model.29

 Conjunctival melanoma is a relatively rare tumor, accounting for 2% to 3% of all ocular melanomas with a reported annual incidence of 0.5 per million whereas CN are much more common.31 Recently, evidence has shown an increase in the annual incidence of conjunctival melanoma.6,30 The changing incidence patterns coincide with those in cutaneous melanoma, suggesting a possible link to a sunlight-related etiology.

Sunlight exposure is a well-known risk factor for melanoma and intensive exposure to sunlight in childhood is a strong determinant of melanoma risk later in life.32,33 Because conjunctival melanoma usually arises in the UV-exposed bulbar conjunctiva and less often in the unexposed ( fornical or palpbral) conjunctiva, a possible etiological relationship between sunlight exposure and BRAF mutations should be explored.

Our data suggest that the molecular events leading to the development of conjunctival PAM and nevi are different. However, the premalignant subgroup of PAM with atypia is too small for a conclusion to be drawn. Conjunctival melanoma containing BRAF mutations may originate from CN harboring the same mutation. Our data support the recent observation29 that BRAF mutations may be related to the formation of CN and that subsequent genetic changes, such as p53 mutation lead to the development of melanoma.

References


