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 (Mutector; 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

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Melanocytic lesions of the conjunctiva include nevus, racial melanosis, congenital melanosis, primary acquired melanosis, and melanoma.[1] The molecular events leading to the development of conjunctival melanoma are poorly understood. In a large series of 410 patients,[2] three 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.[3,4] 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.[2]

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,[5] conjunctival melanoma,[6,7] papillary thyroid carcinoma (PTC),[8] and to a lesser extent in other cancers.[9] 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.[5]

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.[6] 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-

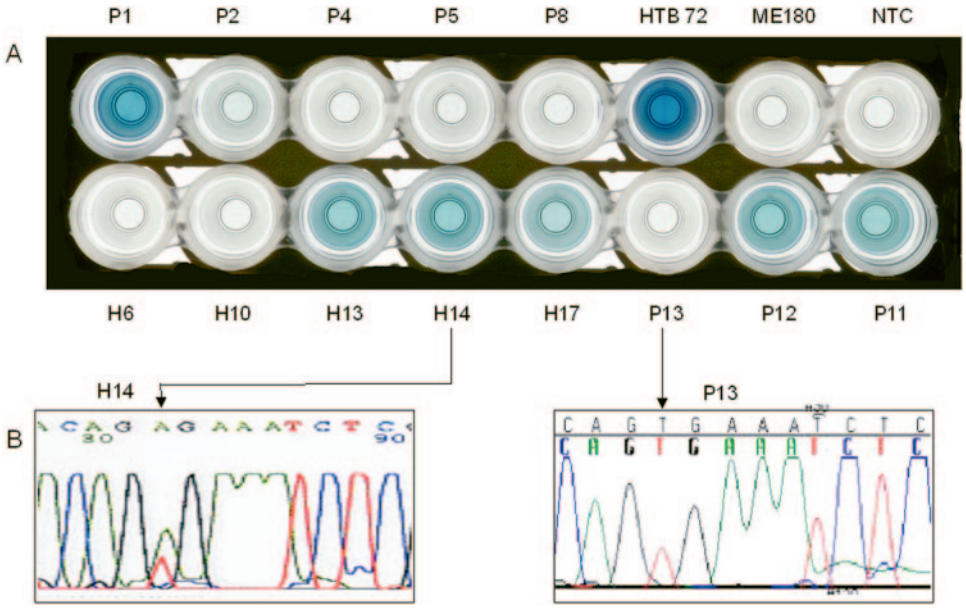

Figure 1. *BRAF* gene mutation analysis in conjunctival melanocytic lesions. (**A**) Mutector assay (*BRAF* codon 600 mutations detection kit; TrimGen, Sparks, MD) results. Wells with color reaction (*green*) represent samples positive for T1799A *BRAF* mutation. (NTC) no-template control, (ME180) cervical cancer cell line ME 180 served as the negative control, and (HTB 72) the melanoma cell line HTB 72 served as the positive control. The representative positive and negative samples were P1, P11, P12, H13, H14, H17, and P2, P4, P5, P8, P13, H6, H10, respectively. (**B**) The corresponding sequence chromatographs of samples H14 and P13 with *BRAF* T1799A mutation and wild-type *BRAF* T1799, respectively.

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.[13] 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.[14]

Detection of BRAF Mutations

Mutector Assay. To detect *BRAF* mutations at nucleotide position 1799, we used the Mutector assay (TrimGen, Sparks, MD).[15] 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.[15] 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 TGA 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 GTA AAA ATA GGT GA-3′ and 5′- CCA CAA AAT GGA TCC AGA CA-3′). PCR amplification was performed in a 30-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 $MgCl_2$, 16.6 mM $(NH_4)_2$ SO_4, 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 could be obtained from the other center.

T1799A *BRAF* mutations were identified in 14 (50%) of the 28 CN, in all age groups. Of the 13 CN samples with known 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.

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.[16,17] Activating mutations in the *BRAF* gene have been identified in human cancers, with the highest frequency of mutations found in cutaneous melanomas.[5] In contrast to cutaneous melanoma, *BRAF* mutations appear to be absent in uveal melanoma,[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 melanomas[23,24] may explain the lower frequency of *BRAF* mutations in conjunctival melanomas compared with cutaneous melanoma.

In accord with the high frequency reported in cutaneous nevi[12] 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 alarmingly 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[6] with a reported annual incidence of 0.5 per million[30] 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 (forniceal or palpebral) conjunctiva,[2] 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 observation[29] that *BRAF* mutations may be related to the formation of CN and that subsequent genetic changes, such as p53 mutation[29] lead to the development of melanoma.

References

1. Shields CL, Shields JA. Tumors of the conjunctiva and cornea. *Surv Ophthalmol.* 2004;49:3–24.
2. Shields CL, Fasiudden A, Mashayekhi A, Shields JA. Conjunctival nevi: clinical features and natural course in 410 consecutive patients. *Arch Ophthalmol.* 2004;122:167–175.
3. McDonnell JM, Carpenter JD, Jacobs P, Wan WL, Gilmore JE. Conjunctival melanocytic lesions in children. *Ophthalmology.* 1989;96:986–993.

4. Liesegang TJ, Campbell RJ. Mayo Clinic experience with conjunctival melanomas. *Arch Ophthalmol*. 1980;98:1385–1389.
5. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature*. 2002;417:949–954.
6. Gear H, Williams H, Kemp EG, Roberts F. BRAF Mutations in conjunctival melanoma. *Invest Ophthalmol Vis Sci*. 2004;45: 2484–2488.
7. Spendlove HE, Damato BE, Humphreys J, Barker KT, Hiscott PS, Houlston RS. BRAF mutations are detectable in conjunctival but not uveal melanomas. *Melanoma Res*. 2004;14:449–452.
8. Cohen Y, Xing M, Mambo E, et al. BRAF mutation in papillary thyroid carcinoma. *J Natl Cancer Inst*. 2003;95:625–627.
9. Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. *Nature*. 2002;418:934.
10. Cohen Y, Goldenberg-Cohen N, Parrella P, et al. Lack of BRAF mutation in primary uveal melanoma. *Invest Ophthalmol Vis Sci*. 2003;44:2876–2878.
11. Rimoldi D, Salvi S, Lienard D, et al. Lack of BRAF mutations in uveal melanoma. *Cancer Res*. 2003;63:5712–5715.
12. Pollock PM, Harper UL, Hansen KS, et al. High frequency of BRAF mutations in nevi. *Nat Genet*. 2003;33:19–20.
13. Merbs SL, Sidransky D. Analysis of p16 (CDKN2/MTS-1/INK4A) alterations in primary sporadic uveal melanoma. *Invest Ophthalmol Vis Sci*. 1999;40:779–783.
14. Parrella P, Sidransky D, Merbs SL. Allelotype of posterior uveal melanoma: implications for a bifurcated tumor progression pathway. *Cancer Res*. 1999;59:3032–3037.
15. TrimGen. Mutector on line. *TrimGen*: *Genetic Technology*. http://www.trimgen.com/mutector.htm; Accessed 2005.
16. Peyssonnaux C, Eychene A. The Raf/MEK/ERK pathway: new concepts of activation. *Biol Cell*. 2001;93:53–62.
17. Duesbery NS, Webb CP, Vande Woude GF. MEK wars, a new front in the battle against cancer. *Nat Med*. 1999;5:736–737.
18. Grin JM, Grant-Kels JM, Grin CM, Berke A, Kels BD. Ocular melanomas and melanocytic lesions of the eye. *J Am Acad Dermatol*. 1998;38:716–730.
19. Farber M, Schutzer P, Mihm MC Jr. Pigmented lesions of the conjunctiva. *J Am Acad Dermatol*. 1998;38:971–978.
20. Bataille V, Sasieni P, Cuzick J, Hungerford JL, Swerdlow A, Bishop JA. Risk of ocular melanoma in relation to cutaneous and iris naevi. *Int J Cancer*. 1995;60:622–626.
21. Folberg R, McLean IW, Zimmerman LE. Primary acquired melanosis of the conjunctiva. *Hum Pathol*. 1985;16:129–135.
22. Rodriguez-Reyes AA, Rios y Valles-Valles D, Corredor-Casas S, Gomez-Leal A. Advanced conjunctival melanoma. *Can J Ophthalmol*. 2004;39:453–460.
23. Folberg R, McLean IW, Zimmerman LE. Malignant melanoma of the conjunctiva. *Hum Pathol*. 1985;16:136–143.
24. Jakobiec FA, Folberg R, Iwamoto T. Clinicopathologic characteristics of premalignant and malignant melanocytic lesions of the conjunctiva. *Ophthalmology*. 1989;96:147–166.
25. Loewe R, Kittler H, Fischer G, Fae II, Wolff K, Petzelbauer P. BRAF kinase gene V599E mutation in growing melanocytic lesions. *J Invest Dermatol*. 2004;123:733–736.
26. Smalley KS, Herlyn M. Loitering with intent: new evidence for the role of BRAF mutations in the proliferation of melanocytic lesions. *J Invest Dermatol*. 2004;123:xvi-xvii.
27. Zamir E, Mechoulam H, Micera A, Levi-Schaffer F, Pe'er J. Inflamed juvenile conjunctival naevus: clinicopathological characterisation. *Br J Ophthalmol*. 2002;86:28–30.
28. Levi-Schaffer F, Micera A, Zamir E, et al. Nerve growth factor and eosinophils in inflamed juvenile conjunctival nevus. *Invest Ophthalmol Vis Sci*. 2002;43:1850–1856.
29. Patton EE, Widlund HR, Kutok JL, et al. BRAF mutations are sufficient to promote nevi formation and cooperate with p53 in the genesis of melanoma. *Curr Biol*. 2005;15:249–254.
30. Yu GP, Hu DN, McCormick S, Finger PT. Conjunctival melanoma: is it increasing in the United States? *Am J Ophthalmol*. 2003;135: 800–806.
31. Rodriguez-Sains RS. Pigmented conjunctival neoplasms. *Orbit*. 2002;21:231–238.
32. Whiteman DC, Whiteman CA, Green AC. Childhood sun exposure as a risk factor for melanoma: a systematic review of epidemiologic studies. *Cancer Causes Control*. 2001;12:69–82.
33. Bataille V. Genetic epidemiology of melanoma. *Eur J Cancer*. 2003;39:1341–1347.