Status of the NF1 Tumor Suppressor Locus in Uveal Melanoma

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Background: A clinical association has been observed between uveal melanoma and neurofibromatosis type 1 (NF1). This study aims to determine whether the NF1 tumor suppressor gene is mutated in uveal melanoma.

Methods: Thirty-eight uveal melanomas, as well as normal uveal melanocytes, were examined for NF1 deletions by dual-color fluorescence in situ hybridization, and for expression of the NF1 protein (neurofibromin) by immunohistochemistry and Western blot analysis.

Results: Normal uveal melanocytes strongly express neurofibromin. Eighteen (47%) of uveal melanomas demonstrated weak expression of neurofibromin. One large tumor contained a deletion of the NF1 locus and lacked neurofibromin expression. Two other tumors contained additional copies of the NF1 chromosomal region.

Conclusion: Mutations of the NF1 gene may occasionally play a role in the pathogenesis of uveal melanoma.

Clinical Relevance: A search for biallelic NF1 mutations in uveal melanomas from patients with neurofibromatosis will be of interest to determine whether germline NF1 mutations may predispose to uveal melanoma.

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UVEAL MELANOMA is the most common primary malignancy of the eye, but the genetic abnormalities that lead to this cancer remain obscure. Linkage analysis is not possible in most patients with uveal melanoma because of the rarity of familial cases. Cytogenetic studies have identified frequently altered regions on chromosomes 3, 6, and 8, but these changes have not been linked to specific cancer genes.1 Tumor suppressors such as p53, Rb, and p16 are rarely mutated in this cancer.2 Another approach to identifying cancer genes in sporadic tumors is to analyze genes known to be mutated in hereditary cancer syndromes.3 The cancer syndrome that has been most commonly associated with uveal melanoma is neurofibromatosis type 1.4-14 Several other lines of evidence suggest that the neurofibromatosis type 1 (NF1) gene could potentially be involved in the pathogenesis of uveal melanoma. The NF1 gene encodes the protein neurofibromin, which regulates cell growth by inhibiting the Ras oncprotein in neural crest-derived cells such as uveal melanocytes.15 The NF1 gene is mutated in some cutaneous melanomas,16 and hamartomatous uveal melanocytic proliferations of the iris, called Lisch nodules, are commonly found in patients with NF1. Finally, spectral karyotyping has recently shown abnormalities involving the NF1 locus on chromosome 17q11 in some uveal melanomas.17 To test whether mutations involving the NF1 tumor suppressor locus may occur in uveal melanoma, we analyzed the structure and expression of the NF1 gene in 38 primary uveal melanomas and in normal uveal melanocytes by means of dual-color fluorescence in situ hybridization (FISH), immunohistochemistry, and Western blot analysis.
cluded inadequate tissue or extensive necrosis (no available samples were excluded). Formalin-fixed, paraffin-embedded blocks were obtained from all cases, and diagnoses were confirmed. Tumors were classified as predominantly spindle, mixed, or epithelioid according to the modified Callander classification. Clinical data (age, sex, eye, largest basal dimension, ultrasound thickness, location, and pathological findings) were recorded from patient charts. Anterior tumors were those predominantly anterior to the equator. None of the patients had a clinical diagnosis of neurofibromatosis. Survival data were not included, since the follow-up interval for most patients was insufficient for analysis.

DUAL-COLOR FISH

Dual-color FISH was performed on 38 tissue blocks as previously described.18 Briefly, paraffin-embedded tissue sections were deparaffinized with a deparaffinization solution (Histo-Clear; National Diagnostics, Atlanta, Ga), dehydrated in 100% ethanol, subjected to target retrieval by steam heating in citrate buffer (pH 6.0) for 20 minutes, digested in pepsin solution (4 mg/mL in 0.9% sodium chloride) for 30 minutes at 37°C, rinsed in 2X SSC (300mM sodium chloride and 30mM sodium citrate) at room temperature for 5 minutes, and air dried. A commercial SpectrumGreen-labeled chromosome 17 centromeric DNA probe (CEP17; Vysis Inc, Downers Grove, Ill) was paired with a rhodamine-labeled P1 artificial chromosome-derived DNA probe containing the exon 28 to 3’ end of the NF1 locus at 17q11.2 (PAC-13). Probes were diluted 1:50 in DNA hybridization buffer (DenHlyb; Insitut Biotechnologies, Albuququerque, NM). Hybridization mix was applied to sections, followed by denaturation in a 90°C oven for 13 minutes. Hybridization was performed overnight at 37°C in a humidified chamber. Slides were then washed in 50% formamide/1X SSC and then 2X SSC for 5 minutes each at room temperature. Slides were allowed to air dry, then nuclei were counterstained with 4,6-diamidino-2-phenylindole (Insitut Biotechnologies). Sections were visualized on a fluorescent microscope (Olympus BX60, Olympus America Inc, Melville, NY). At least 100 nuclei were analyzed for each tumor.

To adjust for the truncation artifacts associated with cutting through nuclei in thin paraffin sections, the mean plus 2 SDs of the number of nuclei with 1 NF1 signal in normal control brain sections was used to establish the threshold for deletion of the NF1 locus. This threshold was determined to be 47% nuclei with a single NF1 signal.

IMMUNOHISTOCHEMISTRY

Sections 4 µm thick were obtained from 38 paraffin-embedded tissue blocks, deparaffinized by immersion in xylene, and rehydrated in water. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide for 5 minutes followed by antigen retrieval with 0.4% pepsin in 0.01N hydrochloric acid for 30 minutes at 37°C. Sections were then blocked in 1% bovine serum albumin for 1 hour at room temperature. Neurofibromin-specific affinity purified rabbit polyclonal antibodies (NF1-GRD; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) was used at a 1:200 dilution overnight at 4°C. Then, slides were developed by the streptavidin-biotin method with the use of a kit (Vectastain; Vector Laboratories Inc, Burlingame, Calif). Staining was performed with peroxidase substitute (Vector SG; blue), and counterstained with nuclear fast red. Use of blue substrate readily allowed pigmented tumors to be analyzed, and no tumor samples had to be excluded because of excessive pigmentation. Sections were then dehydrated and mounted for visualization by light microscopy. At least 8 high-power fields were analyzed for each specimen, and overall immunostaining of the entire section available for analysis was scored as weak (+), moderate (++), or strong (+++), compared with normal human brain sections (external positive control) and retinal photoreceptor outer segments (internal positive control). Negative control was secondary antibody alone. Clinicopathologic and immunohistochemical data were analyzed for correlation by Pearson correlation coefficients.

WESTERN BLOT ANALYSIS

In 3 cases (cases 7, 9, and 10), fresh tissue was obtained from the tumor and normal uveal tissue at the time of enucleation. Informed consent was obtained according to a protocol approved by the institutional Human Studies Committee. The normal and neoplastic tissues were immediately placed in Dulbecco modified Eagle medium containing 15% fetal calf serum and gentamicin to eliminate contaminating fibroblasts. The tissues were fragmented in the culture hood maintained in a 37°C incubator at 4% oxygen. Within 3 to 7 days, pure colonies of adherent normal melanocytes or melanoma cells were identified and confirmed to be melanocytic on the basis of morphologic features, intracytoplasmic pigmentation, and HMB-45 staining (data not shown). Mel-290 uveal melanoma cells were obtained from Bruce Ksander, PhD (Harvard University, Boston, Mass).

After 1 to 3 passages, cells were collected and lysates were prepared and subjected to electrophoresis on a 6% polyacrylamide gel, transferred overnight to a nitrocellulose membrane (Nitropure; Osmonics Inc, Westborough, Mass), and probed overnight by means of an NF1 antibody (NF1-GRD; Santa Cruz Biotechnology) at a dilution of 1:300. The blot was then probed with an antirabbit secondary antibody (Santa Cruz Biotechnology) at room temperature for 1 hour and imaged with a reagent kit (Chemiluminescence Reagent Plus kit; NEN Life Science Products, Inc, Boston). An astrocytoma lacking neurofibromin expression was used as negative control.

RESULTS

DUAL-COLOR FISH

Results of FISH analysis are summarized in the Table. In case 23, which was the thickest tumor in the study, 92% of tumor nuclei contained only 1 NF1 signal, consistent with deletion of the NF1 locus (Figure 1B). The presence of 2 signals in the majority of adjacent nonneoplastic retinal cells suggested that this was a somatic, rather than a germline, deletion (data not shown). In case 34, 27% of tumor nuclei contained 3 NF1 signals, and 28% contained 4 or more signals. Additional copies of the centromeric probe for chromosome 17 (CEP17) were also present in some nuclei (18% with 3 copies and 9% with ≥4 copies), suggesting that most or all of chromosome 17 may be overrepresented in this tumor. Case 32 displayed areas in which tumor nuclei contained a gain of the NF1 region relative to the centromere. In the remaining 35 cases, the majority of tumor nuclei displayed 2 copies of NF1 (Figure 1A).

IMMUNOHISTOCHEMISTRY

Clinicopathologic features and results of immunohistochemical analysis are summarized in the Table. Strong immunostaining was observed in normal cone and rod retinal photoreceptor outer segments (Figure 1C). This
staining was used as an internal positive control. Variable cytoplasmic immunostaining for neurofibromin was detected in the 38 tumors (Figure 1A). Eighteen cases (47%) demonstrated weak expression, 11 (29%) moderate expression, and 8 (21%) strong expression. One case (3%) had minimal immunostaining, and this was the same case with NF1 deletion by FISH (Figure 1B). No other significant correlations were observed between the intensity of immunostaining and the clinicopathologic features that were measured.

WESTERN BLOT ANALYSIS

Four uveal melanoma cell cultures (cases 7, 9, and 10, and Mel-290 cells) and 3 cultures of normal uveal melanocytes were available for Western blot analysis to confirm the neurofibromin expression observed by immunohistochemistry. All of the melanoma cells and uveal melanocytes expressed similar levels of a 250-kDa protein detected by the neurofibromin antibody (Figure 2).

Abbreviations: A, anterior; CEP17, centromeric probe for chromosome 17; FISH, fluorescence in situ hybridization; L, left; LBD, largest basal tumor dimension; NA, not available; NF1, neurofibromatosis type 1; P, posterior; R, right; −, absent; +, weak; ++, moderate; ++++, strong.

* Gain of NF1 alleles.
The NF1 gene is extremely large, preventing routine screening of the entire DNA sequence. However, most NF1 gene mutations in NF1-related tumors and sporadic tumors involve gene deletions or other mutations that can usually be detected by the techniques used in this study. Using dual-color FISH, we found that the NF1 locus was deleted in 1 tumor (case 23). This deletion does not appear to be a nonspecific loss of the entire chromosome 17, since the centromeric probe was not deleted in this case. Furthermore, the lack of deletion in adjacent retinal cells and normal choroidal melanocytes rules out the possibility of a germline deletion in a patient with unrecognized NF1. In addition, case 32 displayed some evidence of a germline deletion in a patient with untreated neurofibromatosis. In 50% of cases, moderate to strong neurofibromin expression was observed at intensities similar to that in surrounding normal photoreceptors and choroidal melanocytes. Western blot analysis in 3 of these cases showed normal protein size and expression of neurofibromin compared with normal uveal melanocytes, suggesting an absence of NF1 mutations. No focal areas of reduced expression were identified in any of these cases. In 47% of cases, weak immunostaining was observed. It is unclear from these initial studies whether the reduced expression in these cases may represent mutational inactivation of the NF1 gene, or simply an artifact of immunohistochemistry. In case 23, deletion of NF1 on FISH was accompanied by minimal neurofibromin expression on immunohistochemistry. This specimen was the thickest tumor in the study, suggesting that NF1 deletion may either occur later in tumor progression or confer a distinct growth advantage over tumors without this alteration. No other correlations between neurofibromin immunostaining and clinicopathologic features were identified. Similar to our findings in uveal melanoma, loss of heterozygosity at the NF1 locus has been observed in only 3% of typical cutaneous melanomas, but loss of heterozygosity was noted in 67% of desmoplastic neurotropic melanomas, which share morphologic characteristics with nerve sheath tumors. Further studies with larger numbers of cases will be needed to determine whether these findings can be extrapolated to all posterior uveal melanomas, and functional studies in uveal melanoma cells will be required to determine whether alterations in NF1 are pathogenetically relevant.

In summary, the NF1 tumor suppressor locus is occasionally the target of mutations in uveal melanoma among patients without neurofibromatosis. A search for biallelic loss of the NF1 locus in uveal melanomas from patients with neurofibromatosis will be of interest to determine whether germline NF1 mutations may predispose to uveal melanoma.

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REFERENCES

