Analysis of p16 (CDKN2/MTS-1/INK4A) Alterations in Primary Sporadic Uveal Melanoma
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INTRODUCTION. To define more clearly the role of the tumor suppressor gene p16 in uveal melanoma by determining the relative contribution of all known mechanisms of p16 inactivation in this tumor.

METHODS. A comprehensive genetic analysis of the p16 gene was performed in 33 primary sporadic ciliochoroidal and choroidal melanomas. Forty-one polyomavirus microsatellite markers surrounding the p16 locus on chromosome 9p21 were used for the microsatellite analysis. Sequence analysis of the p16 gene was performed on those tumors with 9p21 loss of heterozygosity. To investigate methylation as an alternative mechanism of inactivation of p16, methylation-specific polymerase chain reaction was performed on all tumor DNA samples.

RESULTS. Loss of heterozygosity (LOH) was found in 8 of 33 (24%) uveal melanomas. No evidence of a second region of LOH that did not include the p16 locus was found. Four cases had hemizygous losses including markers both distal and proximal to p16. Homozygous deletion of the p16 gene was detected in the 4 remaining cases by microsatellite analysis. Sequence analysis revealed no p16 mutations in the tumors with hemizygous loss of p16. Methylation of the 5' CpG island of p16 was found in one tumor with 9p21 LOH and in another without LOH.

CONCLUSIONS. p16 inactivation by HD or methylation occurs in 27% of uveal melanomas, representing the most common molecular genetic alteration identified thus far in uveal melanoma. (Invest Ophthalmol Vis Sci. 1999;40:779–783)

Uveal melanoma is the most common primary intraocular malignancy in adults and the second most common site of origin of malignant melanoma.¹ Although many of the clinical features and histopathologic indicators of uveal melanoma have been established, little is known about the genetic alterations that are involved in the development of uveal melanoma and the progression to metastatic melanoma. Malignant transformation during tumor progression is known to result from a series of genetic changes.² Determining the nature and timing of these changes in uveal melanoma will aid in understanding the biology of this tumor type and could lead to the development of new diagnostic and therapeutic strategies.

In human cancer, the frequency of inactivation of the tumor suppressor gene p16, located on chromosome 9p21, is reported to be second only to inactivation of p53.³ Germline mutations of p16 are found in about half of patients with familial cutaneous melanoma, and p16 mutations have been found in many cutaneous melanoma cell lines.⁴,⁵ Although loss of heterozygosity (LOH; usually due to deletion of one allele at a given locus) of 9p21 is one of the most frequent genetic alterations identified in human cancer, intractable mutations of p16 have been detected infrequently in many primary cancers with LOH at 9p21.⁶ Instead, very small homozygous deletions (HDs) of p16 have been detected in these cancers using mi-
rosatellite markers close to the p16 gene and chromosomal fluorescence in situ hybridization (FISH) analysis. \(^7\)

Inactivation of p16 also occurs by promoter hypermethylation.\(^8\) The methylation of 5' regulatory regions composed of CpG islands leads to repression of gene transcription.\(^9\) With the exception of X-inactivation and parental imprinting, CpG islands are not generally methylated in normal cells.\(^10,11\) However, methylation of specific regulatory genes appears to play a role in tumorigenesis. Methylation of the 5' CpG island of p16 was identified and associated with complete transcriptional block and p16 inactivation in many common human cancers.\(^12,13\) In some tumors, p16 promoter methylation and inactivation occurs quite frequently. For example, methylation occurs in approximately 21% of primary head and neck tumors and correlates with the absence of p16 protein by immunohistochemistry.\(^14\)

In contrast to familial cutaneous melanoma, no germline mutations of p16 have been found in patients with familial uveal melanoma.\(^15\) However, 9p21 loss has been demonstrated in some uveal melanomas by comparative genomic hybridization and microsatellite analysis.\(^16,17\) We have examined the p16 locus in a series of primary sporadic uveal melanomas for LOH of 9p21 and have determined the relative contribution of all known mechanisms of p16 inactivation in these tumors.

**Materials and Methods**

**Choroidal Melanomas and Constitutional DNA**

Thirty-three paraffin blocks containing specimens of histologically confirmed ciliochoroidal or choroidal melanoma were collected from the Eye Pathology Laboratory of the Wilmer Ophthalmological Institute. Eleven of 33 tumors involved the ciliary body. Maximum tumor dimension ranged from 6 mm to 19 mm (mean, 12 mm). All tumors were classified as mixed-cell type by the modified Callendar classification.\(^18\) Unstained paraffin sections of each block were microdissected into tumor and normal tissue. The normal ocular tissue was used as the source of normal control DNA for each patient.

Normal and tumor tissue was digested with sodium dodecyl sulfate/proteinase K and extracted with phenol/chloroform as previously described.\(^19\) Pigments, which coisolated with the DNA, tended to inhibit polymerase chain reactions (PCRs). Therefore, samples that contained visible pigments after phenol/chloroform extraction were addition of 10% dimethyl sulfoxide to the reaction mixture. Amplification was confirmed by agarose gel electrophoresis, and amplified products were purified by ethanol precipitation.

**PCR Amplification**

DNA from tumor and normal tissue was analyzed for LOH or HD by amplification of dinucleotide repeat-containing sequences using PCR and the conditions described previously.\(^19\) Oligonucleotide primers were obtained from Research Genetics (Huntsville, AL) or synthesized by Integrated DNA Technologies (Coralville, IA). In this study, the following 14 primers from the region 9p21 were used: D9S156, D9S157, IFNA, D9S1748, D9S1748, D9S171, D9S126, D9S265, D9S259, D9S169, D9S161, D9S270, D9S746, and D9S104. Primer sequences are available from the Genome Database (William H. Welch Medical Library, Johns Hopkins University, Baltimore, MD). Criteria for the detection of LOH and HDs were the same as previously described.\(^7,14\) For informative cases, allelic loss was scored if the intensity, as determined visually, of one allele was at least 30% reduced in the tumor DNA compared with the normal. Homozygous deletions were scored when one or more closely spaced markers demonstrated apparent retention when flanked by markers demonstrating clear loss of heterozygosity. The apparent retention results from amplification of a small amount of normal DNA present in the tumor DNA sample, without amplification of the homozygously deleted region in the tumor DNA, and correlates with HD by FISH analysis.\(^7,20\)

**Methylation-Specific PCR**

Bisulfite modification and methylation-specific PCR (MSP) were carried out on all 33 tumor DNA samples as previously described with the following modifications.\(^21\) Approximately 100 ng to 500 ng of tumor DNA without additional carrier DNA was modified by bisulfite treatment. DNA was obtained from unmethylated squamous carcinoma cell line 011 (Liggett WH Jr, unpublished observations) and methylated squamous carcinoma cell line 0122 as previously described.\(^21\) One microgram of DNA from each cell line was modified by bisulfite treatment and used as a positive control for the MSP.

The previously described primer sets p16-M and p16-U were synthesized by Life Technologies (Gaithersburg, MD). Approximately 50 ng of modified DNA in 1 X PCR buffer was used in the MSP.\(^21\) The reactions were hot-started at 95°C for 5 minutes before the addition of 2.5 U Taq polymerase (Life Technologies). The Taq polymerase was increased to 2.5 U because of inhibitory pigments present in some samples. Amplification was carried out for 40 cycles (30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C) followed by a final 4-minute extension at 72°C. Ten microliters of each PCR was loaded onto a 6% nondenaturing polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination.

**Sequence Analysis**

The four tumors showing LOH at 9p21 but without HD were analyzed for mutations of the p16 coding sequence by direct sequencing. p16 was amplified in two segments as described using primers 2F and 1108R for exon 1 and primers 42F and 551R for exon 2.\(^5\) The PCR amplification conditions were for 1 minute at 95°C, 1 minute at 53°C, and 1 minute at 72°C for 34 cycles. Primer sequences for exon 2 were 5'-TCCTGGACAGCTGCGAC-3' and 5'-TTCTGGACGCTGCGAC-3'. The amplified products were purified by ethanol precipitation.

Using \(5'\)-32P\)-labeled sequencing primers and the AmpliCycle sequencing kit (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ), the amplified products were sequenced according to manufacturers instructions with the addition of 10% dimethyl sulfoxide to the reaction mixture. Amplification was confirmed by agarose gel electrophoresis, and amplified products were purified by ethanol precipitation.
RESULTS

Microsatellite Analysis

Eight of 33 (24%) uveal melanomas showed LOH using 14 highly polymorphic markers surrounding the p16 locus (Fig. 1). Four of 11 (36%) ciliochoroidal tumors and 4 of 22 (18%) choroidal tumors showed 9p21 LOH, and this difference in tumor location was not statistically significant by the Fisher's exact test ($P = 0.39$). The mean maximum tumor dimension of those tumors with 9p21 loss (12 mm) was identical to the mean maximum tumor dimension of all tumors studied.

Homozygous deletion has been reported as the most common method of p16 inactivation in some tumor types. We screened for HDs with the microsatellite markers known to reside within approximately 100 kb of p16. Of the eight tumors with 9p21 LOH, four (numbers 20, 24, 28, and 32) displayed apparent retention of either microsatellite marker D9S1749 or D9S1748 or both (Figs. 1, 2). This pattern of apparent retention has been shown to represent HD of these markers by correlation with Southern blot and FISH analyses. Two other tumors with 9p21 LOH (numbers 10 and 29) were noninformative at one of these critical markers, thereby impairing our ability to detect small HDs in these tumors (Fig. 1).

Sequence Analysis

Independent deletion of one allele and point mutation of the other allele are thought to completely inactivate most tumor suppressor genes. However, point mutations of p16 have been relatively rare in many sporadic cancers including cutaneous melanoma. The four tumors showing 9p21 loss without HD were sequenced for a p16-inactivating mutation. We sequenced 97% (excluding four codons in exon 3) of the coding region, and no somatic p16 mutations were found (data not shown). Because we carefully microdissected all samples and excluded tumors with HD of the region containing p16, it is unlikely that predominantly normal DNA was amplified and sequenced.

Methylation Analysis

Methylation of the 5' CpG island of p16 has not been previously studied in uveal melanoma. Methylation-specific PCR allows the study of small DNA samples from paraffin-embedded tissues that could not previously be analyzed by Southern blot analysis. MSP is based on PCR recognition of a sequence difference, which is created by bisulfite modification of methylated and unmethylated cytosine bases. Unmethylated cytosine is chemically modified to uracil by bisulfite treatment, but methylated cytosine is resistant to modification and remains cytosine. MSP is more sensitive than Southern blot analysis,
allowing the detection of up to one methylated allele/1000 normal alleles at a given CpG island locus.

MSP was performed on all 33 samples of tumor DNA (Fig. 3). DNAs from an unmethylated cancer cell line (011) and a methylated cancer cell line (012) were used as positive controls for the unmethylated and methylated primer sets, respectively. Cell line 011 with only unmethylated alleles amplified with the unmethylated primer set; cell line 012 with only methylated alleles amplified with the methylated primer set, illustrating the specificity of the primer sets. The majority of tumors tested amplified only with unmethylated primers (Fig. 3, tumors 9, 14, and 29). One tumor with 9p21 LOH (number 10) and one tumor without 9p21 LOH (number 40) amplified with the methylation-specific primers, indicating detection of aberrantly methylated alleles in these tumors (Fig. 3). These tumors also amplified with the unmethylated primers, indicating the presence of unmethylated alleles contributed by the presence of a small amount of normal DNA in tumor samples. In previous studies, tumors found to be methylated at the p16 CpG island by Southern blot analysis were also found to be methylated by MSP. 

**FIGURE 2.** Examples of microsatellite analysis in uveal melanoma. Tumor numbers are noted above normal (N) and tumor (T) DNA lanes. Markers are labeled below each sample. Arrows indicate loss of alleles in tumor samples. Tumor 20 shows apparent retention of markers D9S1749 and D9S1748 flanked by LOH at markers IFNA and D9S171 indicating HD. Tumor 24 shows a similar pattern with retention of D9S1749 indicating HD.

**DISCUSSION**

The incidence of uveal melanoma is approximately 6 cases per one million people per year in the United States. Choroidal and ciliochoroidal melanomas pose a significant threat to life, with an overall mortality reported to be 35% in 5 years and 50% in 10 years. Although 8% to 12% of all cutaneous melanomas are familial, familial uveal melanoma (FUM) is rare, with fewer than 100 cases reported in the literature. No constitutional chromosomal abnormalities have been found in 14 patients with FUM. Sequence analysis of p16 in patients with FUM revealed no germline mutations. It appears that if we are to understand the genetic basis of uveal melanoma, we must also study somatic alterations in sporadically occurring tumors.

Inactivation of tumor suppressor genes is common in human cancers. Studies in colon cancer have confirmed the hypothesis that regions of allelic loss may inactivate tumor suppressor genes. In uveal melanoma, the most frequently detected chromosomal losses by comparative genomic hybridization have been monosomy 3 and loss of chromosomal arm 6q (each 45% of 11 tumors studied), followed by loss of chromosomal arm 9p in 3 of 11 tumors (27%). In fact, loss of chromosome arm 9p is one of the most common genetic changes in several tumor types. In an initial attempt to elucidate some of the molecular genetic events that lead to the transformation of a normal uveal melanocyte to metastatic melanoma, we have investigated the contribution of 9p21 LOH and p16 inactivation to the development of uveal melanoma.

We found 9p21 LOH by microsatellite analysis in 24% of tumors studied, consistent with the rate of 9p loss detected by comparative genomic hybridization. A previous report using several different microsatellite markers to study 17 uveal melanomas found LOH at 9p21-22 markers in 6 cases (32%). In contrast to this previous report, we found no evidence of additional regions of allelic loss that did not include the p16 locus (see below). All tumors with 9p21 LOH had loss of contiguous informative markers without interruption by retention of markers (with the exception of small retentions indicative of HD).

In our study, at least one half of uveal melanomas with 9p21 LOH had HD of microsatellite markers flanking the p16 locus. The observed frequency of HD of the approximately 200-kb region between the microsatellite markers D9S1749 and D9S1748 and surrounding p16 is likely to be an underestimate because microdeletions not extending to D9S1749 and D9S1748 would go undetected. In addition, two patients were noninformative at the critical locus D9S1749. Two tumors had
aberrantly methylated p16 promoter regions, which has previously been correlated with transcriptional silencing and absent protein production. 8

p16 is frequently inactivated by HD or by promoter hypermethylation, whereas inactivation by mutation of p16 is rare in most tumor types. Our failure to detect any inactivating point mutation in uveal melanomas with 9p21 LOH is certainly consistent with observations made in other tumor types including melanoma. Although it is possible that unusual mutations in the promoter or noncoding regions of this gene could be involved in inactivation of p16, this would be unlikely as the predominant mechanism of mutation and has not been previously demonstrated for p16.

It has been suggested that the absence of p16 mutations in uveal melanoma samples with 9p LOH suggests the presence of another tumor suppressor gene in the 9p21 region, which is involved in the pathogenesis of uveal melanoma. 29 The absence of point mutations of p16 in uveal melanoma with 9p21 LOH is not a sufficient argument against p16 as a critical target gene because HDs have been demonstrated in many primary tumors. Although the presence of a second suppressor cannot be ruled out, p16 appears to be at least the major target of inactivation on 9p21 in uveal melanoma; HD and methylation of p16 appear to account for the second inactivation event in the majority of uveal melanomas.

It is interesting to note that the rate of p16 LOH in uveal melanoma is roughly half that observed for sporadic cutaneous melanoma (46%-62%). 17,30 This suggests that uveal melanomas may arise from a series of genetic changes divergent from those contributing to the formation of cutaneous melanoma. Defining the genetic differences between uveal and cutaneous melanoma is important to develop novel diagnostic and treatment options for affected patients. New molecular diagnostic approaches and genetically based anticancer therapies provide the best hope to improve the treatment of this potentially deadly disease.

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References