Microsatellite Instability and Alterations of Mismatch Repair Protein Expression in Choroidal Melanomas

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**Objectives:** To examine choroidal melanomas for genomic instability, manifested by microsatellite instability (MSI) and mismatch repair (MMR) protein alterations, and to determine the association of these alterations with selected clinicopathological features of the tumors.

**Methods:** Polymerase chain reaction–based microsatellite assays were applied to analyze 57 cases of choroidal melanomas using 11 microsatellite markers at 5 chromosomal regions: 1p, 2p, 4q, 9p, and 17p. Immunoperoxidase staining methods and mouse monoclonal antibodies were used to investigate the expression patterns of MMR proteins.

**Results:** Microsatellite instability was found at the 1p, 9p, and 17p regions in these lesions with an overall prevalence of 35% (20/57). The frequency of MSI ranged from 9% (1/11) to 27% (3/11), ie, low-level MSI (MSI-L). The instability was most commonly found at the 1p region (D1S2734, 55%; D1S2832, 40%; and D1S233, 20%). Two MSI banding patterns, band shifts and the appearance of additional bands, were found in 10% and 90% of the unstable lesions, respectively. The average percentages of hMLH1 and hMSH2 positively stained cells were significantly reduced in the unstable lesions (81.7 ± 9.3 and 76.7 ± 16.7) as compared with the stable lesions (84.1 ± 15.5 and 78.6 ± 19.6; P = .62 and 0.74 for hMLH1 and hMSH2, respectively). There was no significant difference in survival between the 2 groups; however, relative to the stable subset, the unstable tumors showed a trend (P < .10) toward occurring at a younger age and having tumor cells in vascular lakes.

**Conclusions:** The presence of MSI-L in some choroidal melanomas defines a novel genetic subset of these tumors and suggests that MSI (genomic instability) may play a role in their molecular pathogenesis. Elucidation of the underlying mechanisms for MSI will require further investigation.

**Clinical Relevance:** Detection of the MSI-L pattern might prove to be useful as an adjunct to the conventional diagnosis of choroidal melanomas. Larger series are needed to determine whether any of the correlative trends noted in this study will achieve statistical significance. To the best of our knowledge, this study is the first to define both the MSI and MMR protein expression features of choroidal melanomas.

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formalin-fixed, paraffin-embedded tissues of 57 choroidal melanomas were obtained from the archival files of the Collaborative Ocular Melanoma Study, 1988 to 2000. The clinical data and the tumor characteristics are shown in Table 1. Histopathological classification and staging were performed according to Armed Forces Institute of Pathology (Washington, DC) modification of the Callender classification.1 Sections were stained with hematoxylin and eosin to identify the areas of interest, and stereomicroscopy was used to microdissect additional sections, 5 µm thick, by the Turbett method.7 These sections, from matched tumorous and nontumorous tissues, were stained with eosin. The nontumorous tissues included adjacent retina, choroid, and corneal epithelium. We extracted DNA from these tissues using the DNeasy Tissue Kit (Qiagen, Valencia, Calif) as described by the manufacturer.

### METHODS

**TISSUES SPECIMENS AND DNA EXTRACTION**

Our group and others have reported the presence of MSI-L, reduced MMR protein expression, and MMR gene mutations in the cutaneous melanocytic lesions.8,9 These studies support a role for these genetic changes in the pathogenesis of the melanocytic lesions. Notably, similar reports regarding the ocular counterparts are thus far absent. We hypothesized that choroidal melanoma tumorigenesis involves comparable alterations of the microsatellite repeats and MMR proteins. To test our hypothesis, we used polymerase chain reaction–based microsatellite assays and immunoperoxidase staining methods to analyze 57 cases of choroidal melanomas. We addressed 4 questions: is there MSI in choroidal melanomas, and if so, what is the pattern? Are there any alterations in MMR protein expression in these lesions? Is there a relationship between MSI and MMR expression? Is there a relationship between MSI and the clinicopathological features of choroidal melanomas?

### SELECTION OF THE CHROMOSOMAL REGIONS

The 5 chromosomal regions investigated in this study were chosen for several reasons. First, choroidal melanomas have reasonably consistent karyotypic abnormalities at the 1p36-32 and 9p22 regions.10-14 Second, the 17p and 9p regions harbor tumor suppressor genes with their protein products (p16, p15, and apoptosis-related factor) being deregulated in these lesions.15-17 Third, our previous studies established the high sensitivity of these loci at these regions in the detection of MSI in the melanocytic lesions.4,6,8,13,14 Finally, although genes at these regions are rarely mutated in choroidal melanomas, the primary purpose of studying these loci was not for the genes located there but simply to establish the microsatellite status, ie, whether they are microsatellite stable, MSI-L, or MSI-H.

### SELECTION OF MICROSATELLITE MARKERS

The 11 markers investigated in this study were chosen for the following reasons. First, the markers at 1p36-32 and 9p22 regions (D1S2740, D1S2734, D1S233, D1S2832, D1S513, and MYCL1) were tested previously and proved to be reasonably sensitive in the detection of MSI in the melanocytic lesions.4,6,8,13,14 Finally, although genes at these regions are rarely mutated in choroidal melanomas, the main purpose of studying these loci was not for the genes located there but simply to establish the microsatellite status, ie, whether they are microsatellite stable, MSI-L, or MSI-H.

### POLYMERASE CHAIN REACTION–BASED MICROSATellite ASSAYS

Polymerase chain reaction was performed in a 10-µL volume containing 1× polymerase chain reaction buffer (10 mmol/L Tris, 50 mmol/L KCl [pH 8.3], 0.02% Tween 20); 0.2 mmol/L each of unlabeled and labeled ATP primers (γ-32P; ICN Biomedicals, Costa Mesa, Calif); 1 µL of DNA supernatant; 0.2 U
of Taq DNA polymerase (PGC Sciences, Frederick, Md); and 125 mmol/L each of dNTPs and 1.5 mmol/L MgCl₂. Polymerase chain reaction was performed using a “touch-down” approach: 3 minutes at 94°C; 36 to 38 cycles of 1 minute at 94°C, 1 minute at 60°C to 56°C and 1 minute at 72°C; and 9 minutes at 72°C for final elongation. Hot-start was also employed by adding Taq DNA polymerase when the temperature in the initial ramp was greater than 80°C. Polymerase chain reaction products were resolved on a 6% Long Ranger sequencing gel (FMC, Rockland, Md) and exposed to BioMax MR film (Kodak, Rochester, NY) for 24 to 72 hours. All polymerase chain reaction amplifications and gel loadings were repeated at least twice, and both purchased from Oncogene Science, Cambridge, Mass, at a dilution of 1:500) for 30 minutes at room temperature. After brief rinsing in phosphate buffer solution, a catalyzed signal amplification system (K1300 from DAKO Corp, Carpinteria, Calif) was used according to the manufacturer’s instructions. Sections were next treated with peroxidase-labeled streptavidin for 30 minutes at room temperature and incubated with 1,4-diaminobenzidine and 0.06% hydrogen peroxide for 5 minutes. They were counterstained with hematoxylin, dehydrated, cleared, and mounted under coverslips.

**DEFINITION OF MSI**

Microsatellite instability was defined as variations in the banding pattern between tumor and matching normal DNA. Lesions with MSI at 1 or more loci were scored as MSI positive. Instability at more than 30% of the tested loci was used as a cutoff between MSI-L and MSI-H. Lesions without variations in the banding pattern were labeled as microsatellite stable.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Immunostaining was carried out as described in previous studies. Briefly, sections mounted on glass slides were deparaffinized and rehydrated through graded alcohols to water. Endogenous peroxidase activity was blocked with 0.6% hydrogen peroxide in methanol. Sections were then immersed in the retrieval solution (10 mmol/L sodium citrate buffer, pH 6.0) and subjected to heat-induced antigen retrieval for 20 minutes. The slides, in plastic Coplin jars containing retrieval solution, were microwaved at high (~750 W) for 4 cycles of 5 minutes’ duration each. Nonspecific protein binding was blocked with 10-minute exposure to 10% normal goat serum. Sections were then incubated with mouse monoclonal antibodies as primary antibodies (clone G168-15 for hMLH1 and clone FE11 for hMSH2, both purchased from Oncogene Science, Cambridge, Mass, at a dilution of 1:500) for 30 minutes at room temperature. After brief rinsing in phosphate buffer solution, a catalyzed signal amplification system (K1300 from DAKO Corp, Carpinteria, Calif) was used according to the manufacturer’s instructions. Sections were next treated with peroxidase-labeled streptavidin for 30 minutes at room temperature and incubated with 1,4-diaminobenzidine and 0.06% hydrogen peroxide for 5 minutes. They were counterstained with hematoxylin, dehydrated, cleared, and mounted under coverslips.

**EVALUATION OF hMLH1, hMSH2, AND hMSH6 STAINING**

Staining intensity, percentage of positive tumor cells (PP), and immunoreactivity score were evaluated as described in previous studies. Briefly, staining intensity was scored according to the expression levels in the positive controls as strong, moderate, weak, or negative. The PP was assessed by counting at least 5 different areas at a magnification of 400. The resulting immunoreactivity score for tumors was determined by multiplying staining intensity by PP; results were scored as negative (0-1), weak (2-3), moderate (4-6), or strong (8-12).

**CONTROLS**

As previously described, additional sections of the samples were stained in parallel but with omission of the primary antibody. The retinal cells (the inner and outer nuclear layers) and corneal epithelium served as positive controls.

**STATISTICAL ANALYSIS**

Statistical analysis was done using the Fisher exact test (Statistix for Windows; Analytical Software, Tallahassee, Fla), analysis of variance, and Spearman correlation coefficient tests. Differences were considered statistically significant at P<.05.

### Table 2. Characteristics of the Microsatellite Markers

<table>
<thead>
<tr>
<th>Source</th>
<th>Marker</th>
<th>Location</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Repeat</th>
<th>Tm</th>
<th>Size, Base Pairs</th>
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<tbody>
<tr>
<td>Hussein et al⁵</td>
<td>D1S2740</td>
<td>1p36.23</td>
<td>AGCGTCTTTGACCTTCTAGCTTG</td>
<td>Di</td>
<td>55</td>
<td>20-104</td>
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<tr>
<td></td>
<td>D1S2734</td>
<td>1p35.1</td>
<td>GGTTCAAGGGATCTCCTGT</td>
<td>Di</td>
<td>55</td>
<td>108-134</td>
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<tr>
<td></td>
<td>D1S513</td>
<td>1p34.3</td>
<td>AGCCCTCAGAGCTGCTGG</td>
<td>Di</td>
<td>55</td>
<td>179-197</td>
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<tr>
<td></td>
<td>D1S233</td>
<td>1p34.3</td>
<td>GGCTGCGGCAAGAGST</td>
<td>Di</td>
<td>55</td>
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<td></td>
<td>D12832</td>
<td>1p34.2</td>
<td>GCGAAGACCTCCTGCTAAAGA</td>
<td>Di</td>
<td>55</td>
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<td></td>
<td>MYCL1</td>
<td>1p34.1</td>
<td>TGCGGAGACTCTCAATCAAG</td>
<td>Tetra</td>
<td>55</td>
<td>140-209</td>
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<tr>
<td>Liu et al¹⁸</td>
<td>BAT40</td>
<td>1p13.1</td>
<td>ATTAACCTCTACGACACACAA</td>
<td>Mono</td>
<td>55</td>
<td>80-100</td>
</tr>
<tr>
<td>Hussein et al⁵</td>
<td>BAT26</td>
<td>2p16</td>
<td>TGACTCTTTTGACTCTGACACG</td>
<td>Mono</td>
<td>55</td>
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<tr>
<td></td>
<td>BAT25</td>
<td>4q16</td>
<td>AACTTCAGACTTACATTTAACCC</td>
<td>Mono</td>
<td>55</td>
<td>90</td>
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<td></td>
<td>Interferon α</td>
<td>9p22</td>
<td>TGGCGTTAATGAATTGTTGT</td>
<td>Di</td>
<td>55</td>
<td>138-150</td>
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<td>TP53</td>
<td>17p</td>
<td>AGAGACAGATGCAATCCAAC</td>
<td>Di</td>
<td>55</td>
<td>259-274</td>
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Abbreviations: Di, dinucleotide repeats; Mono, mononucleotide repeats; Tetra, tetranucleotide repeats; Tm, annealing temperature.
RESULTS

Focal MSI (alterations at some but not all the examined loci) was found in these lesions. The overall prevalence (ie, number of tumors with MSI/total number of the lesions) was 35% (20/57). The highest MSI rates were found at the 1p region, followed by the 17p and 9p regions, respectively (90% [18/20] vs 15% [3/20] vs 10% [2/20]). These results, however, could be related to the use of more markers at the 1p region (Table 2 and Table 3).

The frequency of MSI (number of unstable markers/total number of markers) ranged from 9% (1/11 markers) to 27% (3/11 markers); ie, there was MSI-L. No instabilities were found at the 2p and 4q chromosomal regions. The level of MSI among different loci ranged from no change (BAT25, BAT26, and BAT40) to frequent alterations (D1S2734 and D1S2832). The highest level was observed with D1S2734 and was highest for markers with dinucleotide repeats, lower with tetranucleotides (MYCL1), and absent with mononucleotides (BAT25, BAT26, and BAT40) (Figure 1).

The appearance of additional bands (MSI-1 pattern) and band shifting (MSI-2 pattern) were found in 75% and 25% of tumors, respectively, as compared with autologous normal tissue (Figure 2). The retinal cells (the inner and outer nuclear layers) and corneal epithelium demonstrated strong nuclear staining for the repair proteins (Figure 3). As shown in Table 4, the average values of PP, staining intensity, and immunoreactivity score were insignificantly reduced in the unstable tumors when compared with the stable ones (for example, PP showed P = .62 and P = .74 for hMLH1 and hMSH2, respectively).

The association between MSI and selected clinicopathological features of choroidal melanomas was examined and tested for statistical significance. There was a trend (P < .10) toward earlier age at onset and tumor cells in vascular lakes among the MSI-L subgroup. Mitotic activity; ciliary body involvement; invasion of the blood vessels, long ciliary nerve, vortex veins, and scleral lamellae; and extraocular extension were insignificantly higher among the unstable tumors than the stable ones. There was no significant difference in survival between patients with MSI-L and microsatellite-stable tumors (Table 1 and Figure 4).

COMMENT

To date, little has been determined about MSI and MMR protein status in choroidal melanomas. Moreover, little

<table>
<thead>
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<th>Table 3. Microsatellite Instability in Choroidal Melanomas</th>
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<tr>
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</table>

Figure 1. Proportion of microsatellite instability (MSI)–positive cases with MSI at specific microsatellite loci.

Figure 2. Microsatellite instability in choroidal melanomas. Arrowheads indicate the microsatellite instability bands. N indicates normal; T, tumor.

Figure 3. Mismatch repair protein expression. A, Absence of hMLH1 expression in the tumor cells (negative control). B, Uniform hMLH1 nuclear expression in the cells of the inner and outer nuclear layers of the retina (positive control). C and D, hMSH2 protein expression in the nuclei of the tumor cells.
is known about the association of these alterations with the clinicopathological characteristics of these tumors. We carried out the present investigation to gain insights into these issues. Our data clearly demonstrate that the MSI-L pattern defines a subset of choroidal melanomas. In contrast, using a panel of microsatellite markers roughly half the size of ours, one prior study reported MSI in only 1 of 52 uveal melanomas.22

**MSI-L AT THE 1P, 9P, AND 17P REGIONS**

The observation of MSI-L in choroidal melanomas agrees with other studies in their cutaneous counterpart1,6 and the notion that tumors outside human nonpolyposis colonic carcinoma have MSI-L rather than MSI-H patterns.5,6 It also suggests the involvement of MSI in choroidal melanoma tumorigenesis. Four different possibilities may explain the MSI-L pattern in the choroidal melanomas. First, the variable expression of MMR genes with weakly penetrant mutations and attenuated phenotype may be manifested as MSI-L. A similar finding has been observed in yeast, where inactivation of hMSH3 results in a less severe phenotype than inactivation of hMSH2.23 Second, there may be inherent intrinsic instability of these loci. This is an unlikely alternative because none of the tumors derived from other tissues, such as colorectal adenomas and hepatocellular carcinomas,24,25 demonstrated MSI at any of these loci. Third, there may be inactivation of non-MMR genes or additional MMR genes other than those encountered in human nonpolyposis colonic carcinoma, such as the hMSH6 gene.26 By far, the most important loci involved in choroidal melanoma map to chromosomes 3, 6, and 8. Therefore, future studies are mandated to examine their microsatellite status. Fourth, it is still possible that MSI also reflects an increased rate of spontaneous mutations in these lesions.

**TWO MSI BANDING PATTERNS FOUND IN THE CHOROIDAL MELANOMAS**

The 2 MSI banding patterns observed in our study are similar to those described in human nonpolyposis colonic carcinoma, familial ovarian carcinomas, and melanocytic skin tumors.5,6 The MSI-1 pattern, entailing the
presence of additional bands, may be due to division of
the sequence within 1 of the original alleles.27,28 The band-
shifting pattern (MSI-2) may be due to the migration of
CA-repeat bands as a result of instability of those re-
peats at 1 or both alleles.27 To the best of our knowl-
edge, the present investigation is the first to character-
ize the banding pattern in the choroidal melanomas.

MMR PROTEIN EXPRESSION IN CHOROIDAL
MELANOMAS WITH MSI

To investigate the underlying reasons for this MSI-L pat-
tern, we further examined the MMR protein expression
and its correlation with MSI status in choroidal melan-
omas. The presence of strong nuclear MMR protein ex-
pression, particularly in the retina and corneal epithe-
lum, suggests transcriptional and translational control
analogous to that of other proteins involved in DNA re-
lication and compartmentalization of the MMR proteins
and the presence of a nuclear localization signal. It is also
consistent with the MMR protein biochemical function
in DNA repair.29,30

In agreement with similar findings in their cutane-
ous counterparts, MMR protein expression was less in
choroidal melanomas with MSI-L as compared with the
microsatellite-stable lesions. However, this reduction was
not statistically significant and suggests (1) the majority
of the tumor cells in these lesions may carry functional
MMR genes, (2) these tumors may harbor missense mu-
tations in the MMR genes that result in dysfunctional but
still detectable proteins, or (3) these lesions could have
a defect in other unknown MMR or non-MMR genes or
epigenetic mechanisms.26,31 As MMR genes fit into the
Knudsen 2-hit theory of tumor suppressor genes, a ger-
miline mutation in 1 allele cannot abolish the repair func-
tion. An additional somatic mutation in the second al-
lele would be required for complete loss of expression
and for the development of MSI.18,32

DIAGNOSTIC AND PROGNOSTIC RAMIFICATIONS
OF MSI IN CHOROIDAL MELANOMAS

From the diagnostic point of view, the presence of MSI-L
has been used as an adjunctive diagnostic tool in other tu-
mors.33 Our data, along with extended screening and fol-
low-up of a larger sample size in future studies, may help
establish a consensus panel of markers for adjunctive di-
agnostic use in choroidal melanomas. The finding of MSI-L
in choroidal melanomas might also have some prognostic
value as it has in some other types of tumors.34 Al-
though not statistically significant in the present series of
cases, some of the clinicopathological associations we ex-
amined did exhibit a trend (P<.10) toward statistical sig-
nificance. They might prove to be of prognostic utility if
substantiated by larger studies in the future.

In our series, there was no significant difference in sur-
vival between the patients with stable and MSI-L cho-
roidal melanomas. This finding contrasts with the find-
ings in breast cancer, where MSI was associated with a
worse prognosis,35 and with the findings in human non-
polyposis colon carcinoma, where MSI is associated with
better prognosis.29,36,37 These differences could be ex-
plained by 2 possibilities: the association between MSI
and prognosis has organ specificity and MSI may target
different genes in different organs with different impact
on the prognostic outcomes.

CONCLUSIONS

We report here the analysis of both MSI alterations and
MMR protein expression in choroidal melanomas. Our
findings suggest that MSI may be involved in the devel-
opment of at least a subset of these tumors. The funda-
mental causes and functional effects of MSI alterations
in choroidal melanomas are yet to be determined. To the
best of our knowledge, this is the first report investigat-
ing choroidal melanomas for MSI and the expression of
MMR proteins.

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From the Archives of the ARCHIVES

In March 1902, after an absence of about 6 weeks, he returned with exclusion of the right pupil, “bombé” iris, and “crater” pupil. The iris had not begun to bulge when the eye was examined in Chicago a few days previously. Dr Webster performed an iridectomy through fluid.