Microsatellite Instability and MLH1 Promoter Methylation in Human Retinoblastoma

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PURPOSE. To investigate the link between microsatellite instability and epigenetic silencing of the MLH1 gene in the human retinoblastoma genome.

METHODS. Methylation at the 5’ region of MLH1 was studied, along with its protein expression level by using immunohistochemical staining in 51 retinoblastoma tumors and 2 retinoblastoma cell lines. Also assessed was the genomic stability of 26 retinoblastoma DNAs from microdissected tumor tissue and matched normal retina tissue obtained from the same patient by microsatellite instability (MSI) analysis. The National Cancer Institute–designed reference panel, and 85 markers on chromosomes 1, 6, 9, and 13 were used.

RESULTS. Hypermethylation of the MLH1 promoter was detected in the WERI-Rb1 cell line and in 34 (67%) of the 51 tumors, but not in cell line Y79 and the other 17 tumors. MLH1 hypermethylation was associated with null MLH1 protein expression (P < 0.0005) and with well-differentiated histology (P < 0.05). MSI at three markers (D2S123, D6S470, and D13S265) was frequently identified among 26 retinoblastoma specimens with matched normal DNA. Among these 26 retinoblastomas, high-frequency MSI (MSI-H) tumors were detected in 19% (5/26) and low-frequency MSI (MSI-L) in another 19% (5/26). The remaining 62% (15/26) were genetically stable (MS). MSI status (MS, MS-L, and MS-H) was not associated with MLH1 promoter hypermethylation (P = 0.088; Kruskal-Wallis test).

CONCLUSIONS. Epigenetic silencing of the DNA repair gene MLH1 by promoter hypermethylation is a frequent event in retinoblastoma. The results showed that somatic genetic changes involving MSI occur in a subset of retinoblastoma and implicated the presence of a defective DNA mismatch repair pathway resulting in MSI in retinoblastoma. (Invest Ophtalmol Vis Sci. 2004;45:3404–3409) DOI:10.1167/iovs.03-1273

The development of cancer is a multistep process involving somatic activation of proto-oncogenes, inactivation of tumor suppressor genes, and epigenetic alterations such as DNA methylation.1,2 Genetic and epigenetic alterations that have been determined in retinoblastoma include loss of heterozygosity (LOH) at the RB1 locus and hypermethylation of tumor suppressors including RBB1, RASSF1A,3–6 and MGMT7, a DNA repair gene. Another cardinal feature of cancer cells is genomic instability. In human tumors, genetic alterations can be divided into at least four major categories: single-nucleotide polymorphism, copy number changes, such as microsatellite instability (MSI); chromosomal instability; chromosomal translocation; and gene amplification or deletion. All except MSI have been described in retinoblastoma. MSI may be a molecular marker of tumorogenesis, and the MSI assay may be useful for assessment of genetic aberrations in specific human tumor types and stages. Analysis of microsatellite markers at a specific locus not only helps to identify tumor-related genes, but also contributes to prognostic indications.3–8–9

MSI is a genome-wide alteration characterized by a global instability phenomenon affecting repetitive microsatellite sequences. First discovered in colorectal cancers and subsequently in many other cancers,10,11 it is caused by a failure of the DNA replication error repair system to repair errors during DNA replication.12,13 The extent of MSI varies considerably in different tumors. In MSI, there is frequent accelerated accumulation of single-nucleotide alterations in the length of repetitive microsatellite sequences that occurs ubiquitously throughout the genome. Tumors displaying MSI occur as a result of germ-line mutations in certain genes, such as MLH1, MSH2, MSH6, and MLH3, that compose the DNA mismatch repair system, whereas in sporadic cancers, methylation of the MLH1 promoter appears to be the dominant mechanism leading to MSI.14,15 MSI has also been implicated in tumor development and clinical prognosis.16

In retinoblastoma, inactivation of RB1 occurs in both familial and sporadic cases. Heritable susceptibility to retinoblastoma is transmitted in the autosomal-dominant mode, with 80% to 90% penetrance.17 In vitro studies and epigenetic investigations have shown that RB1 gene alteration is a prerequisite for tumorogenesis.7,18 However, the presence of genomic instability including MSI and chromosome instability (CIN) in the retinoblastoma genome19,20 indicate that such genetic instability may have the putative oncogenic effects necessary for retinoblastoma formation. We sought to determine whether MSI in tumor cells is associated with development of retinoblastoma and to explore the link between MSI and MLH1 gene methylation in retinoblastoma. Methylation status of the CpG island of the MLH1 promoter on retinoblastoma tissues was studied. We performed microsatellite analysis using the 10 reference panel markers of the National Cancer Institute (NCI), together with 85 markers on chromosomes 1, 6, 9, and 13, where prevalent abnormalities had been reported.8–9,20
In vitro control for methylation and nonmethylation, respectively. Treated DNA from normal lymphocytes were used as the positive controls. The PCR products in lane M indicate the presence of methylated alleles and in lane U of nonmethylated alleles. In vitro Ssd methyltransferase-treated and untreated DNA from normal lymphocytes were used as the positive control for methylation and nonmethylation, respectively.

**Materials and Methods**

**Specimen Preparation**

We retrieved 51 unrelated formalin-fixed, paraffin-embedded retinoblastoma specimens archived in the retinoblastoma clinic between 1990 and 2002 at the Hong Kong Eye Hospital (n = 23) and New York Presbyterian Hospital (n = 28). Informed consent and institutional review board approval was obtained from the participating institutions, and the protocol adhered to the provisions of the Declaration of Helsinki.

**Laser Captured Microdissection**

We obtained more than 90% tumor cells from the retinoblastoma tissue samples by using a laser capture microdissection (LCM) system (PALM, Bernried, Germany) to select cancerous tissue cells on slides, according to the manufacturers’ protocol. Briefly, the stained and dehydrated tissue section was overlaid with a thermoplastic film mounted on an optically transparent cap. The visually selected areas of tumor cells were bound to the membrane by short, low-energy laser pulses, resulting in focal melting of the polymer. On average, approximately 20,000 tumor cells were yielded by LCM shots. They were incubated in 100 μL buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 400 μg/mL proteinase K, and 1% Tween 20 for digestion at 55 °C overnight. DNA was extracted with a DNA extraction kit (Qiagen, Hilden, Germany). Genomic DNA was also extracted from the corresponding normal eye tissue of the same individual.

**Methylation-Specific PCR Assay**

The methylation status in the CpG island 5′ to the transcription start site of the MLH1 gene was examined by methylation-specific PCR (MSP) analysis, based on the sequence differences between methylated and nonmethylated DNA after bisulfite modification (CpGenome DNA Modification kit; Intergen, Purchase, NY). Nonmethylated cytosine was converted to uracil, which is recognized as thymine by Taq polymerase. Methylated cytosine was not affected (Fig. 1). Subsequent PCR using primers specific for methylation or nonmethylation sequences discriminated methylated from nonmethylated DNA. The PCR program: 95 °C for 12 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension for 7 minutes at 72 °C.

**Microsatellite DNA Markers and MSI Analysis**

DNA extracted from the microdissected tumor cells and matched normal DNA from the same person were used for MSP analysis using fluorescence-labeled primers on 10 microsatellite markers recommended by NCI (D2S123, D5S346, D7S501, D10S197, D17S250, D18S58, BAT25, BAT26, BAT40, and D3S1611) to distinguish the form of genomic instability in the RB genome. We also analyzed 85 polymorphic microsatellite markers on chromosomes 1, 6, 9, and 13, with an average spacing of 10 cM and heterozygosity of 0.79. These were fluorescence labeled (Prism Linkage Mapping Set-MD10; Applied Biosystems, Inc. [ABI], Foster City, CA). All 95 markers were selected from the Génétique linkage map on the basis of chromosomal location and heterozygosity. PCR products were electrophoresed on a sequencer (377 Prism; ABI), and fluorescent signals from the different sized alleles were analyzed (Genotyper ver. 2.1 and Genescan, ver. 3.1 software; ABI). A given informative marker was considered to display MSI, if one or both alleles in the tumor DNA exhibited size variation due to expansion or contraction of the repeat sequences in comparison with matched normal DNA from the same individual (Fig. 2). Tumors with high-frequency MSI (MSI-H) have instability in three or more markers, whereas tumors with low-frequency MSI (MSI-L) have instability in less than three markers. Tumor specimens showing no apparent instability were designated microsatellite stable (MSS).

**Immunohistochemistry**

Immunohistochemical staining on 5-μm-thick formalin-fixed, paraffin-embedded tumor tissue was performed as described previously.

**Figure 1.** MSP analysis of the MLH1 gene in retinoblastoma tissues. This is a typical example of MSP analysis in retinoblastoma samples of R4, R9, R15, R40, and R38, which are microsatellite unstable, and of R2, R13, and R50, which are microsatellite stable. The PCR products in lane M indicate the presence of methylated alleles and in lane U of nonmethylated alleles. In vitro Ssd methyltransferase-treated and untreated DNA from normal lymphocytes were used as the positive control for methylation and nonmethylation, respectively.

**Figure 2.** MSI and LOH analyses in paired normal (N, top) and tumor (T, bottom) tissue obtained by microdissection of a tissue specimen from the same individual. Electrophoregram of the dinucleotide repeat marker D2S123 from a homozygous individual. Appearance of extra alleles at lower molecular weights in tumor sample R4 indicates the presence of genomic instability (MSI).
brief, the epitope site was retrieved by heating slides twice for 5 minutes in a microwave. After the endogenous peroxidase was blocked with 0.6% hydrogen peroxide, the tissue sections were incubated overnight at 4°C with mouse monoclonal antibody MLH1 (BD-PharMingen, San Diego, CA) at a 1:100 dilution containing 5% normal sheep serum. Sections were rinsed with PBS (0.1 M phosphate buffer NaCl), incubated with biotinylated anti-mouse IgG (Dako, Carpinteria, CA) according to the instructions in the avidin-biotin complex staining kit (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA), and finally counterstained with hematoxylin. Immunoreactive cells were identified by their brown nuclei, whereas negative-staining cells had blue nuclei from hematoxylin counterstaining. Negative controls were generated by omitting the primary antibody in each case. Two of the authors (KWC, KFC) examined the entire tumor area under the microscope for consensus (at least five/1000 fields). We scored tumors positive for MLH1 protein expression when immunohistochemical staining revealed the presence of MLH1 protein in more than 80% of the cells in the tumors investigated: (+ +) More than 80% of the tumor cells were positive and the staining intensity was the same or above normal adjacent retina cells; (+) positive staining in two thirds of the tumor field and cells with equal intensity compared with normal adjacent cells; and (−) tumor cells are completely negative for immunohistochemical stain.

Statistics Analysis
The χ² test, Kruskal-Wallis test, and Kaplan-Meier curves were used to analyze the MSI in relationship to the methylation status of the MLH1 gene or the expression of the gene to any clinical significance. P < 0.05 was considered statistically significant.

RESULTS
MLH1 Promoter Methylation and Expression
Our MSP analysis (Fig. 1) detected hypermethylation of the MLH1 promoter in 34 (67%) of the 51 tumors and hemimethylation in the WERI-Rb1 cell line. No MLH1 gene promoter methylation was detected in the Y79 retinoblastoma cell line or the remaining 17 (33%) of the 51 retinoblastoma specimens. Immunohistochemical staining for MLH1 protein expression showed that in normal retina, MLH1 was predominately present in the nuclei of retinal ganglion cells and inner nuclear layer (INL). Fainter nuclear expression was found in some cells of the outer nuclear layer (ONL; Fig. 3A). In retinoblastoma, the intensity of nuclear MLH1 immunostaining varied from positive, reduced to negative (Figs. 3B–D). For the 15 nonmethyl-
Table 1. Clinical and Pathologic Features of the 26 Retinoblastoma Patients with Matched Normal and Cancerous Retinal Tissue Cells in MSI Analysis

<table>
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<tr>
<th>Patient Number</th>
<th>Sex</th>
<th>Age at Diagnosis (y)</th>
<th>Eye Survival (mo)</th>
<th>MSI Status</th>
<th>MLH1 Methylation</th>
<th>MLH1 Protein</th>
<th>Optic Nerve</th>
<th>Recurrence</th>
<th>Laterality</th>
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Null MLH1 protein expression was associated with MLH1 promoter hypermethylation (P < 0.0005). Treatment refers to pretreatment with iodine plaque or chemo- or cryotherapy. Scoring system for MLH1 protein detection is described in the Methods section. Bi, bilateral; Uni, unilateral; Di, well-differentiated; uni, poorly differentiated. For recurrence: R, local tumor recurrence; NR, no recurrence.

Microsatellite Instability

From the 51 retinoblastoma specimens, normal tissue cells were successfully dissected and removed from cancerous cells. Genomic DNA was extracted separately from the cancer cells of all 51 specimens but from normal cells in only 26 specimens. MSI was detected in tumors at both differentiated and undifferentiated stages of malignancy but not in constitutional normal DNA. MSI was detected in 29 of the 51 retinoblastoma specimens with MLH1 promoter hypermethylation, and the rest (5/54) showed presence of focally positive cells and impaired MLH1 expression (data not shown). Null MLH1 protein expression was significantly associated with hypermethylation of the MLH1 promoter (P < 0.0005; Table 1).

Association between MSI and Clinical Features

There was no significant association between MSI status and the clinical and pathologic features of retinoblastoma in age of diagnosis, histologic differentiation, optic nerve invasion beyond the lamina cribrosa, and treatment before enucleation (Table 1). Two of the 26 patients had optic nerve invasion and another two had a recurrence; all in MSS or MSI-L tumors (Table 1). Although none of the MSI-H patients had a recurrence or optic nerve invasion, the number of samples in this study is too small to suggest MSI status to be of prognostic value.

Association between Methylation Status and Clinical Features

The 51 retinoblastoma cases in this study had different clinical and pathologic features (Table 2). All patients were diagnosed under the age of 5 years and followed up (minimum 2 months, median 32 months, maximum 8 years). All were still surviving at the time of this report except one patient (R2), who had osteosacroma develop in the right maxilla and died at 12 years of age. There was no association between MLH1 promoter methylation and all the clinical or pathologic features, except that it was significantly associated with retinoblastoma with
well-differentiated histology ($P < 0.05$; Table 2). Although the median eye survival of patients with methylation was shorter than that of those without methylation, 12 against 17 months for patients with and without methylation, respectively, the difference was not statistically significant, based on the Kaplan-Meier curves.

**DISCUSSION**

Human retinoblastoma contains genomic alterations. Some of these genetic defects may contribute to the development and progression of retinoblastoma or even resistance to therapy. In this study, we investigated *MLH1* gene hypermethylation, which implicates a defect in mismatch repair and MSI in the retinoblastoma genome and their possible links to tumorigenesis. We detected MSI-H at a moderate frequency (19%) in the 26 available cases (Table 1). This was much lower than MSI-H in hereditary nonpolyposis colorectal cancer (75%–100%) but was comparable to ovarian cancer (17%) and sporadic colorectal carcinoma (15%). In human cancers, particularly hereditary nonpolyposis colorectal cancer, most of the MSI association was associated with genetic mutations or epigenetic alterations in the mismatch repair genes *MSH2* and *MLH1*, which was located in the D2S123 marker region of the Rb1 gene. In contrast, somatic instability is observed either as a substantial change in repeat length or LOH at particular loci. We found that impaired expression of *MGMT* was associated with promoter methylation in retinoblastoma and the presence of genome instability in retinoblastoma. In this study, the identification of epigenetic silencing of *MLH1* in a high proportion of retinoblastoma cases (67%, 34/51) shows *MLH1* promoter hypermethylation to be a frequent event in retinoblastoma, providing additional evidence of involvement of a DNA repair defect in the development of retinoblastoma. There is also an association between *MLH1* hypermethylation and impairment of *MLH1* protein production, as is known in gastric cancer.

We found CpG methylation of the *MLH1* promoter in retinoblastoma samples with different MSI status (Table 1). Although retinoblastoma samples with MSI-H had higher frequency than the MSI-L and MSS samples, statistical comparison is not meaningful due to small sample size. Because only 26 of 51 specimens were available for the MSI analysis, we cannot exclude a potential source of bias in our results between MSI status and *MLH1* promoter hypermethylation. Although our results may indicate the possibility of DNA methylation at the *MLH1* promoter to be involved with the generation of an MSI-H phenotype in retinoblastoma, as has been reported in colorectal and gastric cancers, confirmation by a large number of samples is required. In addition, two of the four MSI-H retinoblastoma specimens showed MSI in the D2S123 locus, which is located within the *MSH2* gene (Fig. 2). In contrast to the other retinoblastoma specimens, there was no *MSH2* protein expression in these two cases (data not shown). Therefore, we suggest that the MSI observed in these two retinoblastoma cases may also be associated with lesion in the *MSH2* gene, as has been reported in colorectal carcinoma.

MSI varies considerably in different tumors and the degree of MSI reflects the frequency of strand slippage events during replication and the efficiency of subsequent mismatch repair. In colorectal cancer MSI was detected on 5q, 17p, and 18q and was associated with increased patient survival and tumor location in the colon. In this study, the absence of local tumor recurrence and optic nerve invasion among MSI-H retinoblastoma patients may indicate survival advantages among the MSI-H retinoblastoma patients, as has been documented in patients with hereditary colorectal cancer of the MSI-H genotype. However, the number of cases in this study was too few to draw a statistical correlation between MSI and clinical features. Moreover, using a panel of 85 microsatellite markers on chromosomes 1, 6, 9, and 13, together with the 10 markers recommended by the NCI for MSI analysis, we frequently found the presence of MSI in 3 markers (D2S123, D6S470, and D13S265), particularly among our MSI-H retinoblastoma cases. Only D2S123 was commonly identified in other cancers, such as colorectal and gastric cancer. Our results suggest that a different panel of microsatellite markers should be used to assess MSI in retinoblastoma. It is notable that van der Wal et al. detected no MSI in retinoblastoma by using the five reference panel markers from the NCI.

Genomic instability at the retinoblastoma genome is mostly reported at the nucleotide level, such as base substitutions, small deletions or insertions in the *Rb1* gene. In contrast, somatic instability is observed either as a substantial change in repeat length or LOH at particular loci. We found that impaired expression of *MGMT* was associated with promoter methylation in retinoblastoma and the presence of genome instability in retinoblastoma. In this study, the identification of epigenetic silencing of *MLH1* in a high proportion of retinoblastoma cases (67%, 34/51) shows *MLH1* promoter hypermethylation to be a frequent event in retinoblastoma, providing additional evidence of involvement of a DNA repair defect in the development of retinoblastoma. There is also an association between *MLH1* hypermethylation and impairment of *MLH1* protein production, as is known in gastric cancer.

We found CpG methylation of the *MLH1* promoter in retinoblastoma samples with different MSI status (Table 1). Although retinoblastoma samples with MSI-H had higher frequency than the MSI-L and MSS samples, statistical comparison is not meaningful due to small sample size. Because only 26 of 51 specimens were available for the MSI analysis, we cannot exclude a potential source of bias in our results between MSI status and *MLH1* promoter hypermethylation. Although our results may indicate the possibility of DNA methylation at the *MLH1* promoter to be involved with the generation of an MSI-H phenotype in retinoblastoma, as has been reported in colorectal and gastric cancers, confirmation by a large number of samples is required. In addition, two of the four MSI-H retinoblastoma specimens showed MSI in the D2S123 locus, which is located within the *MSH2* gene (Fig. 2). In contrast to the other retinoblastoma specimens, there was no *MSH2* protein expression in these two cases (data not shown). Therefore, we suggest that the MSI observed in these two retinoblastoma cases may also be associated with lesion in the *MSH2* gene, as has been reported in colorectal carcinoma.

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**References**


