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 54 (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 (MSS). MSI status (MSL, and MSH) 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 Ophthalmol 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 RBl locus and hypermethylation of tumor suppressors including RBl, 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: substitution DNA sequence 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 tumorigenesis, 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 tumorigenesis.7,18 However, the presence of genomic instability including MSI and chromosomal 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

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Nonmethylated alleles. In vitro SssI control for methylation and nonmethylation, respectively.

Lane M and lane U 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.

Microsatellite Instability in Retinoblastoma

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 cells.

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 the 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 Genethon 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). MSI was interpreted by computer (Genotyper; ABI).

Immunohistochemistry

Immunohistochemical staining on 5-μm-thick formalin-fixed, paraffin-embedded tumor tissue was performed as described previously. In
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/100 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 cell layer, INL, and ONL. The sections were counterstained with hematoxylin.

(Fig. 3) Immunohistochemical staining of MLH1 protein expression in typical human retinoblastoma sections. (A) Antibody reveals positive nuclear staining (brown) of MLH1 in normal human retinal cells, particularly in the retinal ganglion cell layer, INL, and ONL. The sections were counterstained with hematoxylin. (B) MSS tumor cells without MLH1 promoter methylation showing positive staining for MLH1 with intensity above normal adjacent retina cells. (C) Retinoblastoma cells stained positive in the tumor field with heterogeneous MLH1 protein expression. (D) Retinoblastoma sections from patient R15 with methylation of the MLH1 promoter showing negativity for the MLH1 protein. (E) A nonmethylated Y79 cell line showing positive nuclear staining for MLH1. (A-D) Formalin fixed, paraffin-embedded tissue sections; (E) sections prepared by cytopsin and fixed with 4% paraformaldehyde. Original magnification, ×400.
Null MLH1 protein expression was associated with MLH1 promoter hypermethylation ($P < 0.0005$). Treatment refers to prenucleation treatment with iodine plaque or chemo- or cyrotherapy. Scoring system for MLH1 protein detection is described in the Methods section. Bi, bilateral; Ui, unilateral; Di, well-differentiated; uni, poorly differentiated. For recurrence: R, local tumor recurrence; NR, no recurrence.

### 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 osteosarcoma 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 retinoblastoma genome (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.25

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.12 In colorectal cancer MSI was detected on 5q, 17p, and 18q and was associated with increased patient survival and tumor location in the colon.16 In this study, the absence of local tumor recurrence and optic nerve invasion among MSI-H 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.14,16 However, the number of cases in this study was too few to draw a statistical correlation between MSI and clinical features. Meanwhile, 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 colorectal14,16 and gastric cancer.26 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.20 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 retinoblastoma1 and the presence of genome instability in retinoblastoma.19 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 MSH1 protein production, as is known in gastric cancer.27 The presence of MSH1 methylation, irrespective of laterality and treatments, indicating that such epigenetic silencing was due neither to chemotherapy nor to cryotherapy before enucleation nor was it related to the hereditary or sporadic nature of the disease. The epigenetic silencing of the MLH1 gene could be an early event that occurs during tumor development. In addition, we identified the presence of MSI in a subset of retinoblastoma samples. The findings in this and our previous study7 indicated that epigenetic silencing of DNA repair genes MLH1 and MGMT may play a role in the development of retinoblastoma.

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**References**


