Impaired Expression and Promotor Hypermethylation of O6-Methylguanine-DNA Methyltransferase in Retinoblastoma Tissues

Kwong Wai Choy,[1] *Chi Pui Pang,*[1] *Ka Fai To,*[2] *Christopher B. O. Yu,*[1] *Joan S. K. Ng,*[1] *and Dennis S. C. Lam*[1]

Purpose. To investigate the role of epigenetic changes in the promoter region of tumor-suppressor genes in the retinoblastoma genome and to study the disruption of expression of O6-methylguanine-DNA Methyltransferase (*MGMT*) due to aberrant methylation and its association with retinoblastoma.

Methods. A series of 23 retinoblastoma tissue specimens and 2 retinoblastoma cell lines (Y79 and WERI-Rb1) were subjected to methylation-specific PCR (MSP) analysis of hypermethylated genes identified in human cancers, including $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$, *VHL*, and *MGMT*. Further, the expression of MGMT was studied by immunohistochemistry and, when fresh tissue was available, by Western blot analysis and RT-PCR.

Results. Aberrant methylation of at least one *MGMT* locus was detected in 8 of the 23 tumors (35%), all of which (100%) had impaired or absent expression of MGMT . The remaining 15 tumor specimens were nonmethylated, and, among them, 7 (43%) showed defective expression. No methylation of tumor DNA was found on the $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$, and *VHL* genes. Hypermethylation in the *MGMT* promoter was found to be prominently present in retinoblastoma with poor tissue differentiation, and was more frequently detected among patients with bilateral disease. Production of MGMT was consistent with expression of mRNA. No methylation of *MGMT* promoter was detected in the two retinoblastoma cell lines (Y79, WERI-Rb1).

Conclusions. The data show a clear association between impaired production of MGMT and hypermethylation of the *MGMT* promoter, which appeared to relate to early onset and poor differentiation, suggesting that epigenetic silencing of *MGMT* by methylation of the promoter and reduced expression of MGMT may play an important role in the development and progression of retinoblastoma. (*Invest Ophthalmol Vis Sci.* 2002;43:1344–1349)

Retinoblastoma is one of the most common causes of cancer in children in Hong Kong and poses a serious health problem. Etiologic studies have shown 80% to 90% of retinoblastoma to be due to the loss of function of the *Rb1* gene.[1,2] Inactivation of *Rb1* occurs in both familial and sporadic retinoblastoma.[3,4] The trait is transmitted in an autosomal-dominant manner with 80% to 90% penetrance.[1,5] *Rb1* is important in cell cycle regulation in most cells and is inactivated or mutated in many human cancers. Epigenetic control of mRNA expression by methylation of discrete regions of the CpG island has been reported in neoplasia of many human tissues, but not in retinoblastoma, except methylation in *Rb1*. The Association of methylation of DNA with loss of gene function is well documented.[6] Recent studies have demonstrated that hypermethylation of the promoter region is one of the major mechanisms by which cancer-related genes are inactivated, including $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$, *VHL*, *Rb1*, *hMLH*, *HIC*, *MGMT*, *RAR-β2*, and *DAP* kinase.[6–12] Searching for hypermethylated CpG islands has been shown to be an efficient approach for identification of disease-associated genes in human cancer.[11–13]

Tumorigenesis is most likely a multistage process involving somatic activation of proto-oncogene(s), inactivation of tumor-suppressor gene(s), and epigenetic alterations such as methylation of DNA.[1] Some of these genetic alterations have been described in human retinoblastoma, including loss of heterozygosity (LOH) at the *Rb1* locus at chromosome 13 and retention of heterozygosity on chromosome 17.[14,15] The identification of hypermethylated CpG islands provides an alternative pathway to isolate related genes involved in the development and progression of retinoblastoma. Methylation of *Rb1* and other genes associated with tumor suppression plays a definite role in the development of retinoblastoma and other cancers. In particular, the properties and silencing by promoter hypermethylation of *MGMT* have been revealed in various human tumors, such as glioma. Animal studies have also indicated a relationship between elevated activity of O6-methylguanine-DNA methyltransferase (MGMT) and the formation of tumors.[16,17] However, the expression of MGMT in retinoblastoma has received relatively little attention. It remains to be clarified whether the DNA repair gene(s), such as *MGMT*, is involved in the oncogenesis of human retinoblastoma. In this study, we investigated the patterns of methylation of DNA in genes that are hypermethylated in other cancers ($p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$, *VHL*, and *MGMT*) and determined the disruption of MGMT's expression due to aberrant methylation in retinoblastoma tissue. We report herein that in our study MGMT was frequently absent in human retinoblastoma tissue. This event was associated with methylation of the promoter region and was due to inability of tumor cells to synthesize the protein.

Materials and Methods

Cell Lines and DNA Isolation

Two human retinoblastoma cell lines (WERI-Rb1, Y79) and a breast cancer cell line (MCF-7) obtained from the American Type Culture Collection (ATCC; Manassas, VA) were cultured in the recommended

From the Departments of [1]Ophthalmology and Visual Sciences and [2]Anatomic and Cellular Pathology, The Chinese University of Hong Kong, Hong Kong, China.

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Corresponding author: Chi Pui Pang, Department of Ophthalmology & Visual Sciences, The Chinese University of Hong Kong, Hong Kong Eye Hospital, 3/F, 147K Argyle Street, Kowloon, Hong Kong; cppang@cuhk.edu.hk.

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conditions. Genomic DNA from 23 retinoblastoma tissues was extracted from frozen or archived retinoblastoma samples and from cell lines by the proteinase K digestion procedure, according to the manufacturer's instructions.

Methylation-Specific PCR Assay

We examined the status of methylation of the 23 retinoblastoma specimens in the reported density of CpG immediately 5′ to the transcription start site of the $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$, *VHL*, and *MGMT* genes by MSP analysis.[18–20] The assay is based on the DNA sequence differences between methylated and nonmethylated DNA after bisulfite modification by a DNA modification kit (CpGenome; Intergen, Purchase, NY). The bisulfite reaction converts all nonmethylated cytosine to uracil, which is recognized as thymine by *Taq* polymerase, but does not affect methylated cytosine. Subsequent PCR with primers specific for discriminating between methylated and nonmethylated DNA was performed with *Taq* polymerase (Ampli *Taq* Gold; PE-Applied Biosystems, Foster City, CA): 95°C for 12 minutes followed by 35 amplification cycles (denaturation for 30 seconds at 94°C, annealing for 30 seconds at optimal temperature, and extension for 30 seconds at 72°C) and final extension for 7 minutes at 72°C. PCR products were analyzed on 3% agarose gel.

Immunohistochemical Analysis

Consecutive sections of retinoblastoma tissue cut at 3-μm thickness were subjected to double immunostaining. Tissue samples of nasopharyngeal carcinoma (NPC) from two unrelated patients were used as the control. Primary antibodies including antibody to MGMT (3B8, which recognizes MGMT at codons 30-60; a gift from Benjamin F. Li, Institute of Molecular and Cell Biology, National University of Singapore), and to p53 (CM1; rabbit polyclonal IgG; Novocastra, Newcastle-upon-Tyne, UK) were used at optimum dilution. Sections were incubated overnight at 4°C with primary antibodies, washed with PBS containing 0.1% Triton X-100, and further incubated for 90 minutes in the dark at room temperature with the secondary antibodies (1:50 dilution): sheep anti-mouse Ig–conjugated with fluorescein isothiocyanate (FITC; Roche Molecular Biochemicals, Mannheim, Germany) and sheep anti-rabbit IgG conjugated with Cy3 (Sigma, St. Louis, MO). DNA was stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (Roche). Immunoreactive cells were counted at ×200 magnification in at least 30 neoplastic fields under a fluorescence microscope (Lieca, Heerbrugg, Switzerland). Nonimmune sheep serum was used as a negative control for all primary antibodies.

Semiquantitative Reverse Transcription–Polymerase Chain Reaction

Total mRNA was extracted from the six frozen retinoblastoma sections with a kit (RNeasy; Qiagen, Hilden, Germany). PCR was performed with cDNA synthesized from total mRNA with reverse transcriptase (Superscript II; Gibco BRL, Bethesda, MD), with reported sense and antisense primers,[20] in a 30-cycle amplification process: denaturation at 94°C for 30 seconds, annealing at 57°C for 40 seconds, and extension at 72°C for 30 seconds. β-Microglobulin–specific PCR with 30 cycles served as a positive control for mRNA preparation and for semiquantitative analysis of *MGMT* mRNA expression. Three 2× serial diluted cDNA samples and eight 2× serially diluted standard (PCR product from an earlier experiment) were used to ensure the cycle number to be at the logarithmic phase. The band volume of *MGMT* and β-microglobulin were measured by computer with quantitation software (Quantity One; Bio-Rad, Hercules, CA).

Determination of Specific Protein Expression

Total protein extracts from each frozen retinoblastoma sample and cell lines were prepared with lysis buffer (50 mM Tris-HCl, 0.3 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). Equal amounts (100 μg) of cell extracts were separated on SDS-polyacrylamide gels and subsequently transferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK) in trans-buffer (25 mM Tris; 129 mM glycine; 10% methanol; 0.05% SDS). These filters were blocked overnight with ovalbumin (Sigma)-saturated TBS (50 mM Tris [pH 8.0] and 150 mM NaCl). Primary antibodies at 5 μg/mL in TBST (50 mM Tris, 150 mM NaCl, and 0.1% Tween-20) were incubated for 1 hour at 37°C. These are monoclonal antibodies to p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA), MGMT (3B8), and β-actin (C4; Roche Molecular Biochemicals). The filters were then incubated with secondary antibodies (anti-mouse anti-rabbit horseradish peroxidase; Amersham) in TBST at room temperature for 1 hour and treated with an enhanced chemiluminescence (ECL) sensitization kit (Amersham), according to the manufacturer's protocol. The band volume was quantified by computer, using the quantitation software (Quantity One; Bio-Rad).

Results

Promoter Hypermethylation in Retinoblastoma Genome

Genomic DNA from normal lymphocytes, with or without in vitro treatment by *SssI* methyltransferase (New England BioLabs, Beverly, MA), was used as positive control for methylation and nonmethylation, respectively. DNA from 8 of the 23 primary tumors was methylated at the *MGMT* CpG island promoter region (Table 1). Among them, six samples had amplification with both the methylated primer set and the nonmethylated primer set and were therefore partially methylated. The remaining two samples, Yu44 and Yu45, showed complete methylation at the promoter region of the *MGMT* gene, because they had amplification with the methylated primers only (Fig. 1). Nonmethylated alleles were found in the remainder of the 15 samples and the WERI-Rb1 and Y79 cell

Table 1. Assessment of the Positivity of *MGMT* and p53 in Retinoblastoma

	MGMT Gene Status			
	Methylated	**Partial Methylated**	**Nonmethylated**	**RB Cases (*n*)**
p53 positivity				
Negative (<2%)	0	1	5	6
Focally positive (10–20%)	1	1	5	7
Strong (>40%)	1	4	5	10
MGMT positivity				
Negative (<2%)	2	4	1	7
Focally positive (10–20%)	0	2	6	8
Strong (>40%)	0	0	8	8

[a] The staining intensity was expressed in three categories: negative, focally positive, and intensively stained. Tumors that were positive for less than 2% of cells were considered negative. RB, retinoblastoma.

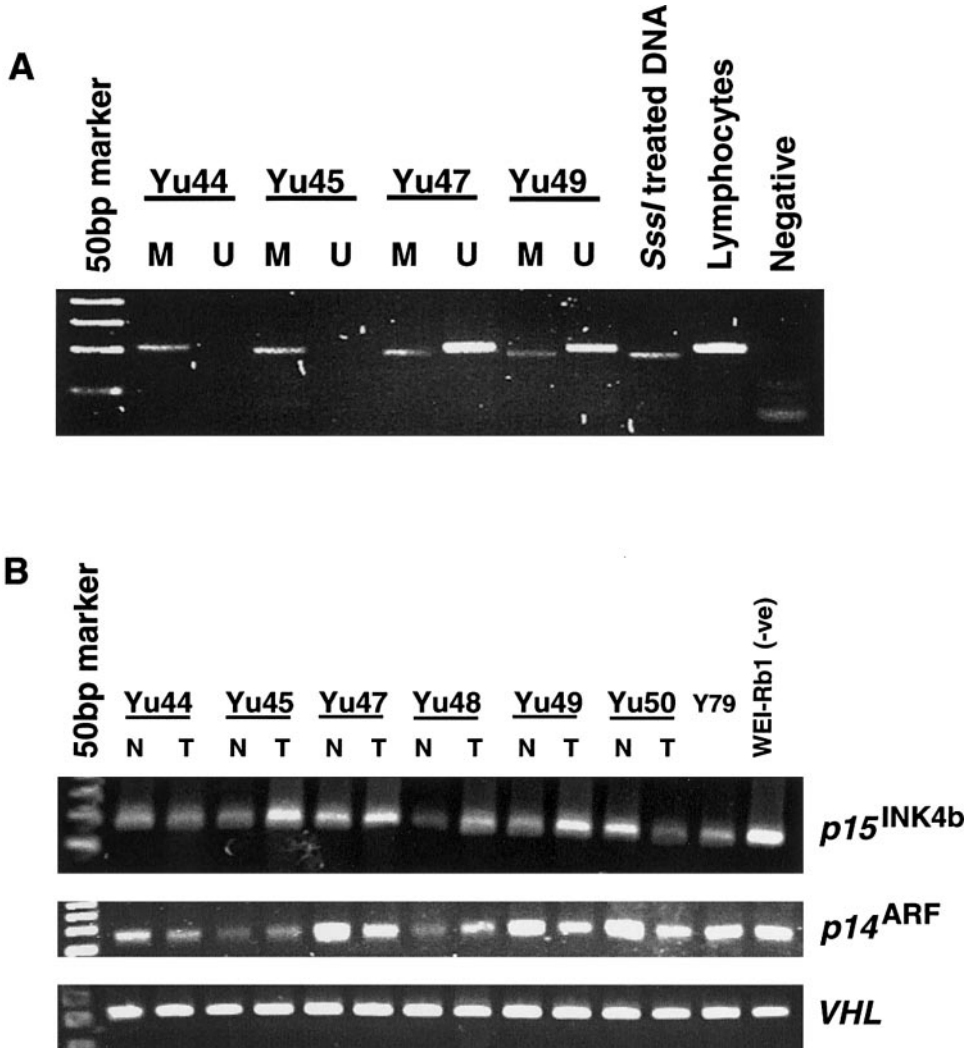

FIGURE 1. MSP analysis of DNA of retinoblastoma tissues. Existence of PCR products in *lane M* indicates the presence of methylation. MSP product in *lane U* indicates the presence of nonmethylation alleles. In vitro *SssI* methyltransferase–treated and –untreated DNA from normal lymphocytes were used as the positive controls for methylation and nonmethylation, respectively. (**A**) MSP analysis of *MGMT* in retinoblastoma tissues and cancer cell lines. (**B**) Selected retinoblastoma tumor sample DNA demonstrated the presence of nonmethylated but no methylated $p14^{ARF}$, $p15^{INK4b}$, and *VHL* genes in primary retinoblastomas. N, normal; T, tumor.

lines. As a control, DNA extracted from two pieces of healthy retinal tissue was used for MSP analysis. The tissues were histologically normal, and the *MGMT* promoter was nonmethylated (data not shown), further indicating hypermethylation to be linked to tumorigenesis in the retinoblastoma tissue.

Immunohistochemistry of Retinoblastoma

To determine whether the DNA repair gene *MGMT* is transcriptionally silenced by hypermethylation of the promoter, we examined MGMT's expression in 23 enucleated retinoblastoma samples by immunofluorescence staining. Intensive nuclear staining with the monoclonal antibody to MGMT was detected in the nuclei of nasopharyngeal epithelial cells of both NPC samples that were included as a positive control (data not shown). Double-immunofluorescence analysis of the retinoblastoma samples for expression of MGMT and p53 showed three clear categories of specimens, according to the immunofluorescence score: negative expression (<2% positively stained cells in one field), focally positive (2%–40% positive cells, but all our specimens in this category had 10%–20% positive cells), and strong expression (>40% positive cells). In 8 of 23 (35%) retinoblastoma tissues, strong expression of MGMT was detected. No MGMT protein was detected in seven (30%) samples, and the remaining eight (35%) showed presence of focally positive cells, indicating an intermediate level of expression (Table 1). Deposition of MGMT was found predominantly in the cell nuclei (Fig. 2). The immunofluorescence score on staining with MGMT alone was consistent in different tissue sections of the same patient, thus confirming the presence of MGMT in retinoblastoma cells. The p53 protein was present predominately in the cell nuclei of the samples (Fig. 2). No association was found between the level of MGMT and expression of p53 in the tumor tissues.

Semiquantitative Analysis of Levels of *MGMT* mRNA

Presence of *MGMT* transcripts in the six cases available with frozen retinoblastoma tissues (Yu44, Yu45, Yu47, Yu48, Yu49, and Yu50) and in the two nonmethylated retinoblastoma cell lines (WERI-Rb1, Y79) was detected by RT-PCR. β-Microglobulin amplification was performed to ensure uniformity in mRNA preparation and in the first-strand cDNA synthesis by reverse transcriptase (Fig. 3). To account for the variability in the starting cDNA concentration, the amounts of amplified *MGMT* transcripts were normalized to expression of β-microglobulin. The relative expression of *MGMT* mRNA in fibroblasts was assigned with an arbitrary value of 1 U (Table 2). Although β-microglobulin signal was detected in all samples, no *MGMT* mRNA was detected in two retinoblastoma tissue samples (Yu44 and Yu45) with extensively methylated *MGMT* alleles. Samples with the partially methylated *MGMT* gene expressed relatively low levels (0.7–3 U) of *MGMT* transcript, whereas

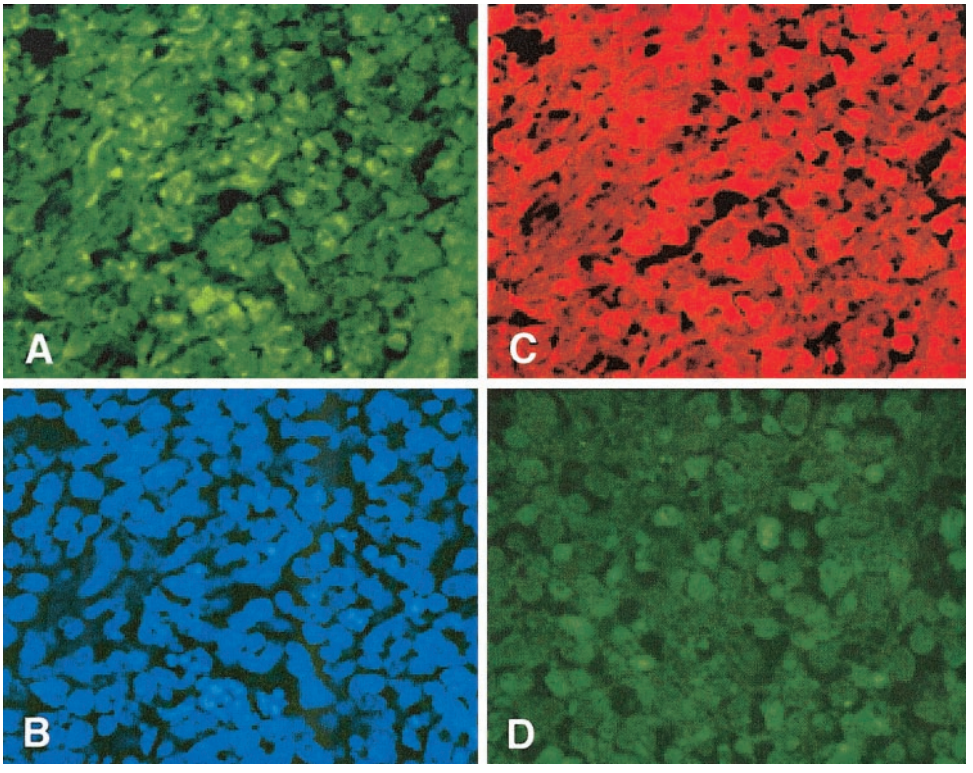

FIGURE 2. Immuohistochemical analysis of selected tumorous tissues. A typical retinoblastoma sample double-immunofluorescence–stained for (**A**) immunoreactive MGMT (3B8; 1.5 μg/mL in *green*) showed strong fluorescence in the nuclei cell; (**B**) 4′,6′-diamino-2-phenylindole (DAPI; (1.5 μg/mL in *blue* for nuclear DNA), and (**C**) p53 (CM1; 2 μg/mL in *red*). (**D**) An example of retinoblastoma tissue with focally positive and heterogeneous expression of MGMT. Enhanced chemiluminescence (2 seconds for DAPI, 30 seconds for MGMT and p53). Magnification, ×200.

high levels (9–25 U) were found in nonmethylated retinoblastoma cell lines (Table 2).

Expression of MGMT Protein in Frozen Retinoblastoma Extracts

We examined MGMT protein expression by Western blot analysis to confirm the association between its promoter methylation and its impaired synthesis by tumor cells. MGMT migrated as a single 21-kDa protein in extracts derived from retinoblastoma tissues, and from retinoblastoma (WERI-Rb1) and breast cancer (MCF-7) cell lines. Figure 4 shows that methylated RB cells (Yu44 and Yu45) were without expression of MGMT. To quantify the relative expression of MGMT, the levels in fibroblasts were taken as the basal level. A low level of MGMT was observed in partially methylated tissues, but there was markedly more (2.5–4 times higher) in nonmethylated cell lines (Table 2). Although these results confirmed the presence of MGMT protein in retinoblastoma cells, they also showed that hypermethylation of the CpG islands located in the promoter region of the *MGMT* gene is associated with loss of *MGMT* mRNA and expression of protein in retinoblastoma tissues.

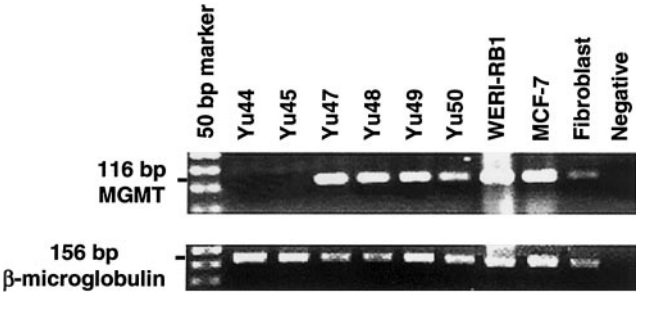

FIGURE 3. Amplification products of *MGMT* (116 bp) and β-microglobulin (165 bp) cDNA extracted from frozen retinoblastomas and cancer cell lines on 2% agarose gel. The relative expression level of *MGMT* mRNA was calculated by comparing the relative ratio of *MGMT* to β-microglobulin mRNAs with the corresponding ratio in human fibroblasts.

Clinical and Pathologic Features of Retinoblastoma Associated with Methylated MGMT

A striking common feature of the eight patients with methylated promoter in at least one *MGMT* locus was the disease's onset before the age of two. Seven of the patients had bilateral disease with poorly differentiated or undifferentiated retinoblastoma (Table 3). The 15 nonmethylated samples, however,

TABLE 2. Levels of *MGMT* Expression Detected by Western and RT-PCR Techniques in Retinoblastoma Tissues

Case Identity	Relative Expression in RB Tumor: *MGMT* Protein (U)*	Relative Expression in RB Tumor: *MGMT* mRNA (U)†	Methylation Status
Yu 44	ND	ND	Methylated
Yu 45	ND	ND	Methylated
Yu 47	0.5	0.7	Partially methylated
Yu 48	1	3.0	Partially methylated
Yu 49	1.3	1.5	Partially methylated
Yu 50	1.5	0.9	Partially methylated
WEI-Rb1	2.5	9	Nonmethylated
Y79	4	25	Nonmethylated
Fibroblast	1	1	Nonmethylated

ND, not detected.

* The protein band volume was normalized with fibroblast as the basal level (1 U).

† Amount of *MGMT* mRNA was normalized to the fibroblast, which was assigned an arbitrary value of 1 U *MGMT* mRNA.

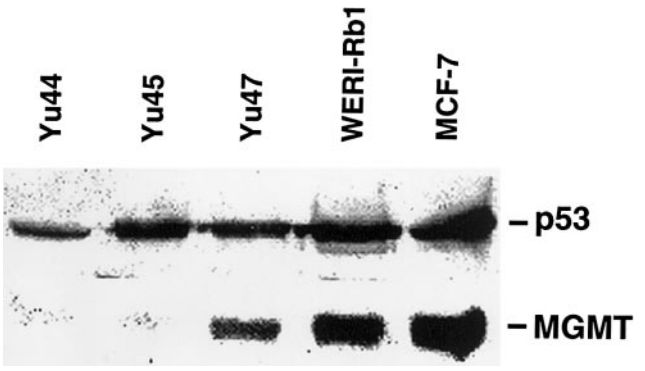

FIGURE 4. Western blot analysis of MGMT expression in retinoblastoma tissues and in a retinoblastoma cell line (WER1-Rb-1). Equal amounts (100 μg) of protein were loaded into each lane. A representative result showing a good coincidence of *MGMT* transcript detection by RT-PCR analysis and by Western blot analysis was demonstrated by retinoblastoma samples Yu 44 (*lane 1*), Yu45 (*lane 2*), and Yu47 (*lane 3*). The breast cancer cell line (MCF-7) was used as a positive control for MGMT. The blot was reprobed with anti-β-actin antibody as a positive loading control.

had even proportions of differentiated and undifferentiated phenotypes. Optic nerve involvement was observed only in nonmethylated tumors (Table 3). However, the number of cases in this study was insufficient to draw a statistical correlation between methylation of *MGMT* and prognostic implication.

DISCUSSION

It is increasingly apparent that in cancer, loss of gene function may be mediated as often by epigenetic as by genetic alterations.[21,22] In this study, we showed for the first time the occurrence of epigenetic lesion in *MGMT* by hypermethylation of the promoter in retinoblastoma. We demonstrated that MGMT deficiency was consistent with reduced or absent mRNA transcription. Heterogeneous variation in expression of MGMT was also observed at the transcription level through normalization with the recovered mRNA between human fibroblasts and retinoblastoma tissue (Table 2). Previous studies have shown that absence of MGMT is rarely due to deletion, rearrangement, or mutation of the *MGMT* gene.[23,24] An epigenetic control of mRNA expression (i.e., hypermethylation of discrete regions of the *MGMT* CpG island) has been reported in human neoplasia and in glioma cell lines.[20,25] In addition, three of the eight methylated DNA samples were found to have a G-to-A transition in the *Rb1* gene. This finding is consistent with the suggestion that methylation and subsequent inactivation of MGMT may generate G-to-A mutations in cancer.[26] Results of our investigation directly linked the absence of impairment of expression of MGMT to a mechanism involving cytosine methylation in the *MGMT* promoter sequence in retinoblastoma. Meanwhile, seven of eight patients with methylated MGMT had bilateral disease (Table 3). Genetic testing showed that they all had a germline *Rb1* mutation, but the patient with unilateral disease did not (data not shown). Such findings are consistent with the younger age of presentation (Table 3), indicating that increased methylation at the MGMT promoter may be associated with the inherited disease genotype. A further study with a large sample size should affirm whether such association may be statistically significant. Furthermore, in normal retina tissue, there was no *MGMT* promoter methylation detected, suggesting that methylation of *MGMT* promoter may arise during tumor progression in retinoblastoma. We further speculate that alteration of expression of MGMT may be a predisposing factor during retinoblastoma carcinogenesis.

It has been shown that hypermethylation of the promoter can disrupt the function of tumor-suppressor genes such as $p15^{INK4b}$, *VHL*, and $p16^{INK4a}$, and the frequency differs between tumor types. The $p15^{INK4b}$ and $p16^{INK4a}$ genes encode cyclin-dependent kinase inhibitors, and inactivation of these genes leads to transformation and immortalization.[27,28] Loss of function by hypermethylation of the 5′ CpG island of $p15^{INK4b}$ and $p16^{INK4a}$ is often found in malignant tumors.[29] Similarly, $p14^{ARF}$, whose physical association with MDM2 regulates the metabolism of p53, was hypermethylated in colonic polyps.[18] Our results provide evidence that extensive methylation of $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$, and *VHL* is not common in retinoblastoma. Accordingly, in retinoblastoma, initial oncogenic mutation in the *Rb1* gene can bypass early mortality checkpoints critical to the onset of cellular immortality, as has been demonstrated by experiments in the transgenic mouse.[30]

We found that some retinoblastoma tissues, like those of other tumors, did not appear completely methylated or completely nonmethylated in this promoter region. These results suggest that fresh human tumor samples may contain both normal and cancerous tissues because of the heterogeneous expression of MGMT by individual cells, as was observed in our immunofluorescence analysis. Selective analysis of cancerous and normal cells obtained by laser-captured microdissection should clarify this situation. The MSP protocol used in this study is highly sensitive and allows detection of aberrantly methylated alleles. Our results therefore clearly indicate that absence of expression of MGMT is associated with hypermethylation in the *MGMT* promoter region. We are following up the clinical course of our patients to associate their MGMT properties with responses to chemotherapeutic agents.

Recognition of methylation of DNA and inactivation of the *MGMT* gene in retinoblastoma has important clinical implications. The DNA repair activity of MGMT is a major factor that protects cells from mutagenic and cytotoxic O6-alkylguanine lesions caused by carcinogens. It also limits the chemotherapeutic efficacy of nitrosoureas. A positive correlation has been observed between high MGMT activity in tumors and poor initial response to postoperative combination chemotherapy with cyclophosphamide (CTX) in patients with ovarian cancer[31] and lung tumor xenografts.[32] Our results show that impaired MGMT expression due to hypermethylated promoter is a frequent event in human retinoblastoma. The absence of or

TABLE 3. Prevalence of *MGMT* Methylation by Age and Gender, Phenotype, and Differentiation of Tumor

	***MGMT* Methylation Status**		
	Full	**Partial**	**Nonmethylated**
Tumors (*n*)	2	6	15
Age at onset (y)			
≤2	2	6	9
>2	0	0	4
Sex			
Female	2	2	6
Male	0	4	9
Laterality			
Unilateral	0	1	7
Bilateral	2	5	8
Histologic classification			
Differentiated	0	1	3
Poor differentiated	2	4	5
Undifferentiated	0	1	7
Presence of recurrence	0	1	2
Optic nerve involvement	0	0	2

intermediate expression of MGMT in 15 of 23 of our cases indicates that at least a subset of patients with retinoblastoma may benefit from alkylating-based chemotherapy. Indeed, methylation of MGMT is correlated with increased overall and disease-free survival and improved response to the alkylating agent in patients with glioma.[33] Another clinical application for identification of the *MGMT* methylated allele in tumor cells is to provide a genetic marker for the occurrence of cancer and for prognostic indication. Our results show that such application can be achieved with the use of a small amount of DNA. Finally, epigenetic changes are potentially reversible. The recognition of hypermethylation of the promoter in gene suppression has spurred a new therapeutic target for cancer.

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References

1. Vogel F. Genetics of retinoblastoma. *Hum Genet.* 1979;52:1–54.
2. Bookstein R, Lee WH. Molecular genetics of the retinoblastoma suppressor gene. *Crit Rev Oncog.* 1991;2:211–227.
3. Blanquet V, Turleau C, Gross-Morand MS, Senamaud-Beaufort C, Doz F, Besmond C. Spectrum of germline mutations in the RB1 gene: a study of 232 patients with hereditary and non hereditary retinoblastoma. *Hum Mol Genet.* 1995;4:383–388.
4. Hagstrom SA, Dryja TP. Mitotic recombination map of 13cen-13q14 derived from an investigation of loss of heterozygosity in retinoblastomas. *Proc Natl Acad Sci USA.* 1999;96:2952–2957.
5. Naumova A, Sapienza C. The genetics of retinoblastoma, revisited. *Am J Hum Genet.* 1994;54:264–273.
6. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res.* 1998;72:141–196.
7. Mancini D, Singh S, Ainsworth P, Rodenhiser D. Constitutively methylated CpG dinucleotides as mutation hot spots in the retinoblastoma gene (RB1). *Am J Hum Genet.* 1997;61:80–87.
8. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA.* 1999;96:8681–8686.
9. Merlo A, Herman JG, Mao L, et al. 5′CpG island methylation is associated with transcriptional silencing of the tumor suppressor *p16/CDKN2/MTS1* in human cancers. *Nat Med.* 1995;1:686–692.
10. Widschwendter M, Berger J, Herman M, et al. Methylation and silencing of the retinoic acid receptor-β2 gene in breast cancer. *J Natl Cancer Inst.* 2000;92:826–832.
11. Huang THM, Laux DE, Hamlin BC, Tran P, Tran H, Lubahn DB. Identification of DNA methylation markers for human breast carcinoma using the methylation-sensitive restriction fingerprinting technique. *Cancer Res.* 1997;57:1030–1034.
12. Gonzalgo ML, Liang G, Spruck CH III, Zingg JM, Rideout WM III, Jones PA. Identification and characterization of differentially methylated regions of genomic DNA by methylation-sensitive arbitrarily primed PCR. *Cancer Res.* 1997;57:594–599.
13. Liang G, Salem CE, Yu MC, et al. DNA methylation differences associated with tumor tissues identified by genome scanning analysis. *Genomics.* 1998;53:260–268.
14. Kato MV, Shimizu T, Ishizaki K, et al. Loss of heterozygosity on chromosome 17 and mutation of the p53 gene in retinoblastoma. *Cancer Lett.* 1996;106:75–82.
15. Munier FL, Thonney F, Balmer A, et al. Prognostic factors associated with loss of heterozygosity at the RB1 locus in retinoblastoma. *Ophthalmic Genet.* 1997;18:7–12.
16. Dumenco LL, Allay E, Norton K, Gerson SL. The prevention of thymic lymphomas in transgenic mice by human O6-alkylguanine-DNA alkyltransferase. *Science* 1993;259:219–222.
17. Becker K, Dosch J, Gregel CM, Martin BA, Kaina B. Targeted expression of human O(6)-methylguanine-DNA methyltransferase (MGMT) in transgenic mice protects against tumor initiation in two-stage skin carcinogenesis. *Cancer Res* 1996;56:3244–3249.
18. Esteller M, Tortola S, Toyota M, et al. Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. *Cancer Res.* 2000;60:129–133.
19. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA.* 1996;93:9821–9826.
20. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res.* 1999;59:793–797.
21. Baylin SB, Herman JG. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet.* 2000;16:168–174.
22. Jones PA. The DNA methylation paradox. *Trends Genet.* 1999;15:34–37.
23. Skorpen F, Krokan HE. The methylation status of the gene for O6-methylguanine-DNA methyltransferase in human Mer+ and Mer− cells. *Carcinogenesis* 1995;16:1857–1863.
24. Wang L, Zhu D, Zhang C, et al. Mutations of O6-methylguanine-DNA methyltransferase gene in esophageal cancer tissues from Northern China. *Int J Cancer.* 1997;71:719–723.
25. Costello JF, Futscher BW, Tano K, Graunke DM, Pieper RO. Graded methylation in the promoter and body of the O6-methylguanine DNA methyltransferase (MGMT) gene correlates with MGMT expression in human glioma cells. *J Biol Chem.* 1994;269:17228–17237.
26. Esteller M, Toyota M, Sanchez-Cespedes M, et al. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. *Cancer Res.* 2000;60:2368–2371.
27. Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell.* 1995;81:323–330.
28. Haber DA. Splicing into senescence: the curious case of p16 and p19ARF. *Cell.* 1997;91:555–558.
29. Herman JG, Civin CI, Issa JP, Collector MI, Sharkis SJ, Baylin SB. Distinct patterns of inactivation of p15INK4B and p16INK4A characterize the major types of hematological malignancies. *Cancer Res.* 1997;57:837–841.
30. Windle JJ, Albert DM, O'Brien JM, et al. Retinoblastoma in transgenic mice. *Nature* 1990;343:665–669.
31. Chen SS, Citron M, Spiegel G, Yaros, D. O6-methylguanine-DNA methyltransferase in ovarian malignancy and its correlation with postoperative response to chemotherapy. *Gynecol Oncol.* 1994;52:172–174.
32. Mattern J, Eichhorn U, Kaina B, Volm M. O6-methylguanine-DNA methyltransferase activity and sensitivity to cyclophosphamide and cisplatin in human lung tumor xenografts. *Int J Cancer.* 1998;77:919–922.
33. Esteller M, Garcia-Foncillas J, Andion E, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med.* 2000;343:1350–1354.