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Clinical Implications of Promoter Hypermethylation in *RASSF1A* and *MGMT* in Retinoblastoma[1]

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Abstract

We investigated the epigenetic silencing and genetic changes of the RAS-associated domain family 1A (*RASSF1A*) gene and the O^6-methylguanine-DNA methyltransferase (*MGMT*) gene in retinoblastoma. We extracted DNA from microdissected tumor and normal retina tissues of the same patient in 68 retinoblastoma cases. Promoter methylation in *RASSF1A* and *MGMT* was analyzed by methylation-specific PCR, *RASSF1A* sequence alterations in all coding exons by direct DNA sequencing, and *RASSF1A* expression by RT-PCR. Cell cycle staging was analyzed by flow cytometry. We detected *RASSF1A* promoter hypermethylation in 82% of retinoblastoma, in tumor tissues only but not in adjacent normal retinal tissue cells. There was no expression of *RASSF1A* transcripts in all hypermethylated samples, but *RASSF1A* transcripts were restored after 5-aza-2′-deoxycytidine treatment with no changes in cell cycle or apoptosis. No mutation in the *RASSF1A* sequence was found. *MGMT* hypermethylation was present in 15% of theretinoblastoma samples, and the absence of *MGMT* hypermethylation was associated (*P* = .002) with retinoblastoma at advanced Reese-Ellsworth tumor stage. Our results revealed a high *RASSF1A* hypermethylation frequency in retinoblastoma. The correlation of *MGMT* inactivation by promoter hypermethylation with lower-stage diseases indicated that *MGMT* hypermethylation provides useful prognostic information. Epigenetic mechanism plays an important role in the progression of retinoblastoma.

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Introduction

Alteration in genomic DNA methylation is an important mechanism in the pathogenesis of human cancers and is associated with the transcriptional repression of cancer-associated genes [1]. Promoter hypermethylation and subsequent transcription silencing of tumor-suppressor genes such as *p16*, *RB1*, *BRCA1*, and *VHL* promote tumorigenesis in many types of cancers [2–5]. Identifying hypermethylation at the CpG islands is an efficient alternative approach for detection of disease-associated genes in ovarian cancer, renal carcinoma, colorectal cancer, and breast cancer [2,5–10]. In retinoblastoma, several tumor-suppressor genes and DNA repair genes have been shown to be frequently inactivated due to aberrant methylation at the 5′ promoter regions including *RB1* (14%) [11], *RASSF1A* (59%) [12], and *MGMT* (35%) [13]. These studies provide evidence that genes associated with tumorigenesis acquire hypermethylation changes during retinoblastoma development. The gene for the RAS association domain family protein, *RASSF1A*, which is located at 3p21.3, encodes an M_r of 39,000 predicted peptide with a Ras association domain. There is a predicted NH_2 terminal diacylglycerol binding domain. Rarely inactivated by mutations, it has been suggested to be a tumor-suppressor gene on the basis of its frequent inactivation through promoter hypermethylation in human cancers [14,15]. The epigenetic silencing of the *RASSF1A* gene, brought forth by promoter hypermethylation, commonly occurred in (40–72%) lung cancer [14,15], (66.7%) nasopharyngeal carcinoma [16], (62%) primary mammary carcinoma [17], (59%) retinoblastoma [12], and (45%) adenocarcinoma of the uterine cervix [18]. *RASSF1A* is likely involved in cell cycle control through the inhibition of cyclin D1 accumulation [19]. Recently, *RASSF1A* has been identified as a microtubule binding protein that may play a role in the control of microtubule stability [20], and regulates the stability of mitotic cyclins and mitotic progression by inhibiting APC–Cdc20 activity [21]. *In vitro* expression of *RASSF1A* in lung

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cancer cells resulted in suppression of colony formation and anchorage-independent soft agar growth. It also suppressed the malignant phenotype [14,15]. Thus, *RASSF1A* likely serves as a tumor suppressor during cancer development [14,15,22,23].

Loss of heterozygosity (LOH) on chromosome 3p is a common and early event in the pathogenesis of lung cancer, nasopharyngeal carcinoma, and other tumors. However, LOH at 3p or 3p21.3 is not common in retinoblastoma [23]. Loss of chromosome 3 has so far been reported only in six retinoblastoma cases among a total of 101 cases reported in 16 studies [24–29]. Recently, a high frequency (59%, 10/17) of promoter hypermethylation in *RASSF1A* has been detected in retinoblastoma by Harada et al. [12]. They suggested that epigenetic silencing in *RASSF1* might be one of the genetic targets involved in the development of retinoblastoma. However, their number of study subjects (n = 17) was small and there was a lack of assessment on clinical correlations. It was unclear how epigenetic silencing in *RASSF1A* correlated with the development of retinoblastoma and whether epigenetic silencing of *RASSF1A* could be an independent prognostic factor. Meanwhile, in a previous study, we have shown that promoter hypermethylation in *MGMT* in retinoblastoma DNA was associated with impaired or absent *MGMT* expression at both mRNA and protein levels [13]. In this study, we investigated the presence of 5′ promoter methylation and sequence changes at the coding regions of *RASSF1A* on 40 bilateral and 28 unilateral retinoblastoma cases and characterized their clinical significance. Also studied was the relationship between promoter hypermethylation of *RASSF1A* and *MGMT*, as well as whether demethylation and reactivation of the *RASSF1A* gene by 5-aza-2′-deoxycytidine (5-AzaCdR) affect cell proliferation in retinoblastoma cell lines.

Materials and Methods

Retinoblastoma Samples

Human retinoblastoma cell lines (WERI-Rb1, Y79) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according to the recommended conditions. Retinoblastoma tumor tissues were collected from 68 retinoblastoma patients treated at the Hong Kong Eye Hospital (n = 36) and the New York Presbyterian Hospital (n = 32). Among the patients, 40 (59%) were bilateral and 28 (41%) were unilateral. We obtained normal retinal tissues from four unrelated donor cadaver eyes as controls. Informed consent and institutional IRB have been obtained for this study.

Reese-Ellsworth (RE) Classification for Retinoblastoma

Intraocular retinoblastoma was classified according to the RE classification for retinoblastoma: group I [a) solitary tumor, smaller than four disc diameters in size, at or behind the equator; or b) multiple tumors, none larger than four disc diameters in size, all at or behind the equator]; group II [a) solitary tumor, 4 to 10 disc diameters in size, at or behind the equator; or b) multiple tumors, 4 to 10 disc diameters in size, behind the equator]; group III [a) any lesion anterior to the equator; b) solitary tumor, larger than 10 disc diameters in size, behind the equator]; group IV [a) multiple tumors, some larger than 10 disc diameters in size; or b) any lesion extending anterior to the ora serrata]; and group V [a) massive tumors involving more than one half the retina; or b) vitreous seeding].

Laser Capture Microdissection (LCM)

We obtained over 90% tumor cells from the retinoblastoma tissue samples by using the LCM system (PALM, Bernried, Germany) to select cancerous tissue cells on slides according to the manufacturer's protocols. The procedure has been described previously [13]. On average, about 20,000 tumor cells from one specimen were yielded by LCM shots. DNA was extracted by DNA extraction kit (Qiagen, Hilden, Germany). Genomic DNA was also extracted from the corresponding normal eye tissue cells.

Methylation-Specific Polymerase Chain Reaction (PCR)

For all retinoblastoma samples, normal control samples, and the two retinoblastoma cell lines (WERI-Rb1 and Y79), the 5′ promoter DNA methylation status of *RASSF1A* and *MGMT* was investigated by methylation-specific PCR (MSP) assay as described previously [12,13]. Genomic DNA of the microdissected specimens were modified by bisulfite treatment, prior to MSP amplification [30]. Bisulfite-modified DNA was also obtained from the retinoblastoma cell lines and eight microdissected normal retinal tissues for MSP analysis. Negative controls without DNA were included in each set of assays. The methylation status was determined as a reflection of the presence of the methylated fraction of the examined genes. MSP analysis for each sample was repeated three times. PCR products were analysed by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV illumination.

DNA Sequencing

DNA was extracted from all 68 retinoblastoma samples for direct sequencing to identify sequence alterations in all *RASSF1A* coding exons and splicing regions using 10 PCR amplicons [16]. The samples were sequenced by using the dRhodamine Dye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA) on an automated ABI PRISM 377 Sequencer (Perkin Elmer).

Bisulfite Sequencing

DNA from the WERI-Rb1 and Y79 cell lines and four normal control retinal tissues were subjected to bisulfite sequencing to detect the methylation status of the promoter region of *RASSF1A*. Genomic DNA were modified by bisulfite treatment and purified using the CpGenome DNA Modification Kit (Intergen, Purchase, NY) according to the manufacturer's recommendations. Seminested PCR amplification was performed on 100 ng of bisulfite-modified genomic DNA as described by Dammann et al. [14]. The amplified

fragments were subcloned. Ten clones of each sample were selected for sequencing.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total mRNA was prepared from the tumor tissue sections and normal adjacent retinal tissues obtained after microdissection of the retinoblastoma specimens by an RNeasy kit (Qiagen). PCR was carried out with cDNA synthesised from total mRNA using Superscript II reverse transcriptase (Gibco BRL, Bethesda, MD) with 1 mM sense and antisense primers as previously reported [16]. The RNA samples were also amplified by the primers of the *GAPDH* gene as control.

5-AzaCdR Treatment and RT-PCR

To determine whether *RASSF1A* expression could be restored by application of a demethylating agent, the WERI-Rb1 and Y79 cell lines were subjected to 5-AzaCdR treatment. Cells were grown in suspension culture and incubated for 2 and 4 days with 3 or 5 μM 5-AzaCdR (Sigma Chemical Co., St. Louis, MO). The medium and 5-AzaCdR were replaced every 24 hours. Expression of the *RASSF1A* transcript in the cell lines was examined by RT-PCR [16].

Cell Cycle Analysis

For flow cytometry analysis, cells ($\sim 5 \times 10^6$) were harvested and washed with Hank's balanced salt solution (HBSS; Sigma-Aldrich, St. Louis, MO) and fixed in 70% ethanol at 4°C for 2 hours. After passing through a 40-μm cell strainer (Falcon; Becton Dickinson, Franklin Lakes, NJ), cells were adjusted to 1×10^6 ml^{-1} and stained with propidium iodide (50 μg/ml). The cellular DNA content was assessed by the System II software in a Coulter EPICS XL MCL flow cytometer (Coulter Corporation, Miami, FL) and analyzed with the Multicycle software (Phoenix Flow Systems, San Diego, CA).

Statistical Analysis

Fisher's exact test or chi-square analysis was conducted to estimate the relationships between hypermethylation and the pathologic and clinical features of retinoblastoma.

Results

Methylation Status of the RASSF1A Promoter in Retinoblastoma Samples

Of the 68 retinoblastoma carcinoma samples, 56 (82%) showed hypermethylation at the CpG sites of the *RASSF1A* promoter (Figure 1*A*). The retinoblastoma cell lines WERI-Rb1 and Y79 were completely methylated (Figure 2*A*). No *RASSF1A* promoter hypermethylation was detected among the four normal control retinal specimens and the microdissected normal retinal tissue sections adjacent to the tumor tissues of the retinoblastoma specimens. Direct sequencing further confirmed the 16 CpG sites of the *RASSF1A* promoter from each of the two retinoblastoma cell lines to be extensively methylated. There was no CpG island methylation among the four normal retina tissues examined (Figure 1*B*). These findings suggest that promoter hypermethylation of the *RASSF1A* gene is a common epigenetic event in human retinoblastoma cells.

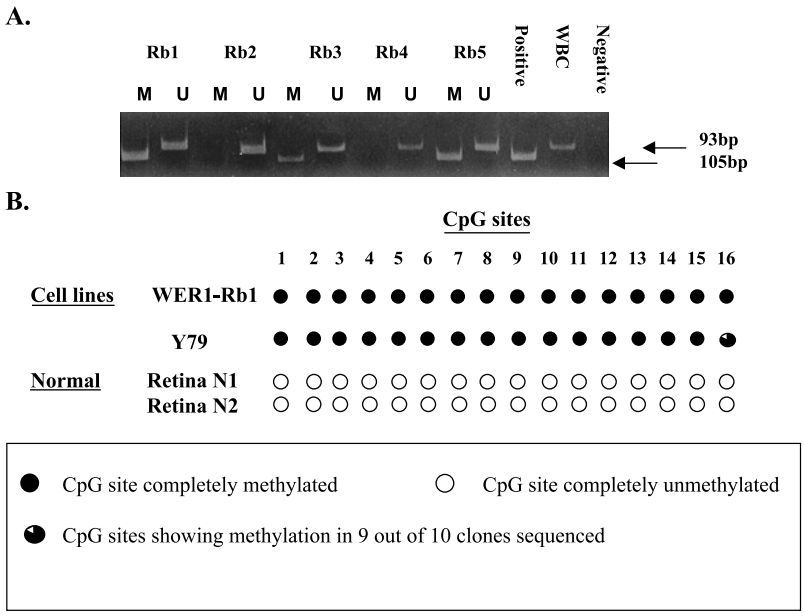

Figure 1. *Methylation analysis of RASSF1A in retinoblastoma. (A) Representative MSP results of RASSF1A for retinoblastoma cancers. The PCR products in lane U showed the presence of unmethylated templates, whereas, in lane M, they indicated the presence of methylated templates. In vitro SssI methyltransferease-treated (positive) and untreated (WBC) DNA from normal lymphocytes were used as positive control for hypermethylation and unmethylation, respectively. (B) Summary of 5-methylcytosine levels of the RASSF1A promoter detected by bisulfite sequencing. Two cell lines (WERI-Rb1 and Y79) and four normal and nonmalignant retina tissues were examined. N1 and N2 are representative samples from normal retina.*

Mutation Screening of the RASSF1A Gene

Direct DNA sequencing after PCR of the *RASSF1A* coding exons and splicing regions confirmed the presence of polymorphisms at codons 53 (CGC to CGT), 56 (CCC to CCT), and 57 (GCG to GCA) in our study samples [16]. No somatic *RASSF1A* mutation has been detected in any of the 68 retinoblastoma tumor samples.

Expression of RASSF1A in Retinoblastoma Cell Lines and Tissues

The WERI-Rb1 and Y79 cell lines and tumor specimens with *RASSF1A* promoter hypermethylation did not express the *RASSF1A* transcript (Figure 2*B*). However, expression of *RASSF1A* mRNA was detected in all four control normal retinal tissue samples and in all unmethylated retinoblastoma tumors. Restoration of the gene expression was observed in the retinoblastoma cell lines WERI-Rb1 and Y79 after 2 days of treatment with 5 μM 5-AzaCdR (Figure 2*B*). Furthermore, demethylation of the promoter region was detected (Figure 2*A*). These results confirmed that loss of expression in the retinoblastoma cell lines was associated with promoter hypermethylation (Figure 2*A*). Flow cytometry examination of the cells after partial reactivation of *RASSF1A* by 5-AzaCdR treatment showed that WERI-Rb1 had a slight (less than 7 ± 3%) increase in G2/M phase cells on day 4 after treatment. No change in cell cycle was observed for Y79 (Figure 2*C*). In both cells, no significant increase of apoptosis was detected.

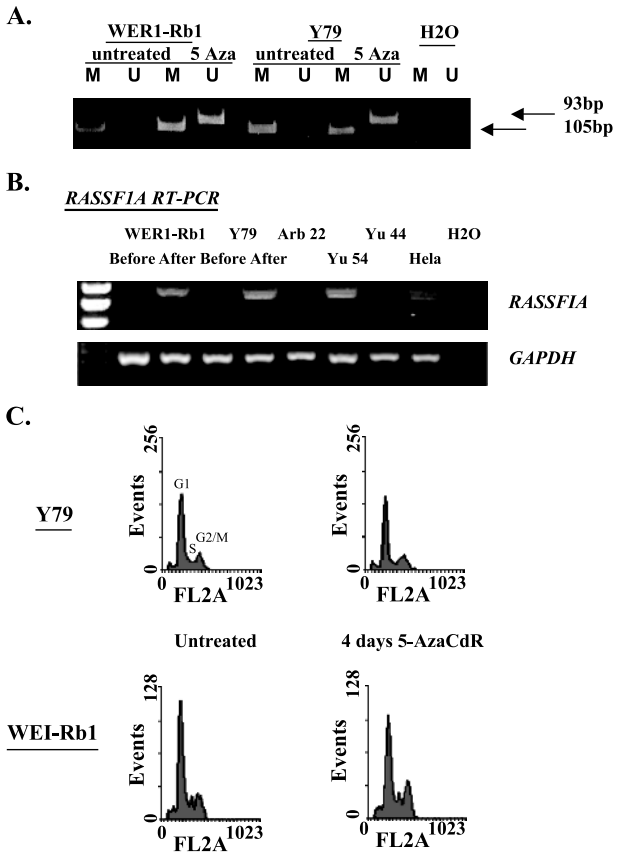

Figure 2. *(A) Methylation analysis of RASSF1A in RB cell lines showing demethylation by 5-AzaCdR resumed the unmethylated sequence (U). (B) RT-PCR analysis of RASSF1A expression in retinoblastoma. Cell lines (WERI-Rb1 and Y79) expressing RASSF1A transcripts after 2 days treatment with 5 μM 5-AzaCdR. Examples of completely methylated retinoblastoma tumors (Arb22 and Yu44) showing the absence of RASSF1A mRNA expression. Presence of RASSF1A transcript in unmethylaed retinoblastoma (Yu54). HeLa cells were used as positive control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), housekeeping gene, was used as a control for RNA integrity. (C). FACS cell cycle profiles of RB cell lines treated with 5 μM 5-AzaCdR for 4 days. The x-axis measures the DNA content of the cell (FL2A). The y-axis represents the number of cell counts (events). A slight increase of G2/M-phase fraction was observed in WERI-Rb1. However, there was no significant difference in the G2/M fraction between the two cell lines after treatment.*

Relationship between RASSF1A and MGMT Methylation Status in Retinoblastoma

Although promoter hypermethylation was detected in 56 (82%) of 68 retinoblastoma samples for *RASSF1A*, hypermethylation of the *MGMT* promoter was detected in 10 of 68 retinoblastoma samples (15%). There was no *MGMT* hypermethylation detected in the retinoblastoma cell lines WERI-Rb1 and Y79. There were no significant difference in the ages at presentation, family history, sex, tumor staging, or laterality between *RASSF1A*-methylated and unmethylated cases. Among the 56 retinoblastoma specimens with promoter hypermethylation at *RASSF1A*, nine of them also had *MGMT* hypermethylation. However, when the frequencies of *MGMT* hypermethylation in retinoblastoma with and without *RASSF1A* hypermethylation were compared (9/56 *vs* 1/12), they were statistically insignificant. Clinical information on tumor staging by RE classification was available for 47 retinoblastoma tumors. Patient characteristics were summarized in Table 1. Most of them were at RE stage V (74%; 35/47) and RE stage IV (17%; 8/47); only a few were at stage III (4%; 2/47) and stage II (3%; 2/47). Due to the small number of retinoblastoma cases at individual RE stages II and III, we grouped them together and compared with retinoblastoma tumors at RE stages IV and V. *MGMT* or *RASSF1A* hypermethylation was found in all tumor stages (RE II–V). *MGMT* hypermethylation was frequently detected in RE stages II to III (50%; 2/2) or stage IV (50%; 6/12), but significantly less among 6% (2/35) RE stage V tumors ($P = .002$; Fisher's exact test). Meanwhile, *RASSF1A* hypermethylation was commonly detected (63–100%) among tumors at all different RE stages (Table 1).

Discussion

In this study, we detected promoter hypermethylation of *RASSF1A* in 56 of 68 (82%) human retinoblastoma tumors. The frequency is higher than the 59% reported in a previous study, which involved 17 retinoblastoma samples and provided no information on *RASSF1A* expression [12]. We showed for the first time that there was a complete concordance between promoter hypermethylation and expression of *RASSF1A* in retinoblastoma cells. Our finding showed that *RASSF1A* was not hypermethylated in normal

Table 1. Clinicopathologic Characteristics of Retinoblastoma Tumor as a Function of *RASSF1A* and *MGMT* Promoter Methylation Status.

	RASSF1A Gene Status		*P*	*MGMT* Gene Status		*P*
	Methylated	Unmethylated		Methylated	Unmethylated	
Laterality						
Bilateral	32	8	.543	6	34	.935
Unilateral	24	4		4	24	
Histology						
Differentiated	21	5	.297	4	32	.730
Undifferentiated	29	6		6	22	
N/A	6	1		0	4	
RE classification						
II to III	4	0	.180	2	2	.002*
IV	5	3		4	4	
V	31	4		2	33	
N/A	16	5		2	19	
Optic nerve invasion	8	1	.567	1	8	.826

MGMT hypermethylation is significantly less among 6% (2/35) RE stage V tumors ($P = .002$; Fisher's exact test) compared to RE stage II, III, or IV.
N/A = not available.
*All *P* values <.05 are considered statistically significant.

and nonmalignant retinal tissues adjacent to the tumor tissues in retinoblastoma specimens, clearly indicating that promoter hypermethylation of *RASSF1A* occurs specifically in cancer. Our results thus implicated that alteration in activity of the RAS signal transduction pathways (*RASSF1A*) may play an important role during the pathogenesis of retinoblastoma. There is mounting evidence that *RASSF1A* functions as a tumor-suppressor gene. Transfection and expression of *RASSF1A* in lung and renal carcinoma cells have been shown to suppress colony formation, anchorage-independent soft agar growth, and nude mouse tumorigenicity [14,20,22,23]. *RASSF1A* is frequently inactivated by hypermethylation in a variety of tumors, including those in which 3p21 allele loss is common (lung, breast, and nasopharyngeal carcinomas) or rare (papillary renal cell carcinoma) [14–16,31]. Its occurrence is different in specific types of tumors. In uterine cervix cancer, it occurred at a high frequency in adenocarcinoma, but was not found in squamous cell carcinoma [18]. Alternated DNA methylation at the CpG promoter region could be a potentially promising molecular marker for making early cancer diagnosis, predicting cancer risk, and monitoring prognosis [32]. Methylated *RASSF1A* was associated with impaired patient survival and poor prognosis in non small cell lung cancer [15]. However, we found no such association in retinoblastoma. The tumor-suppressive properties of *RASSF1A* may therefore be variable in different cancer types.

Previously, Shivakumar et al. [19] have demonstrated that *RASSF1A* induced G0/G1 cell cycle arrest in H1299 lung carcinoma cells by decreasing cyclin D1 accumulation. In this study, we found that both WERI-Rb1 and Y79 retinoblastoma cell lines carried a completely methylated *RASSF1A* promoter and did not express *RASSF1A*. But the *RASSF1A* gene was reactivated and was able to express *RASSF1A* transcript after treatment with 5-AzadCdR, which inhibited DNA methylation (Figure 2, *A* and *B*). No significant alterations in cell cycle or apoptosis that were associated with the reactivation of *RASSF1A* were found. Only a slight increase in the G2/M-phase fraction was observed in the WERI-Rb1 cell line, in which *RASSF1A* was reactivated after 4 days of 5-AzadCdR treatment (Figure 2*C*). This suggested that the G2/M cell phase changes identified in the WERI-Rb1 cell line might not be a specific tumor-suppressing effect caused by *RASSF1A* reactivation. However, the lack of effects on cell growth or cell cycle may be due to the limited amount of *RASSF1A* transcript that was reexpressed. Our results indicated that 5-AzadCdR could not completely restore *RASSF1A* because there was still presence of methylated alleles (Figure 2*A*). To understand the functional role of *RASSF1A* in retinoblastoma development, it is important to study the effects of the exogenous expression of *RASSF1A* on retinoblastoma tumor cell phenotype. Whether loss of *RASSF1A* may affect spindle function and hence mitotic aberrations in retinoblastoma should be investigated.

MGMT promoter hypermethylation was associated with a significant increase in overall survival in diffuse large B-cell lymphomas [33] and gliomas [34]. The pathogenesis of retinoblastoma is known to involve inactivation of both copies of the *RB1* gene and other genetic or epigenetic alterations in independent molecular pathways. In this study, the presence of *MGMT* methylation was significantly less among RE stage V tumors (Table 1). Consistent with our previous report, which indicated that *MGMT* hypermethylation might be related to poor differentiation [13], in this study, hypermethylation of *MGMT* promoter was more frequently identified among undifferentiated cases (60%) than differentiated tumor samples (40%). When *MGMT* methylation status was correlated with clinical staging, there was greater frequency of hypermethylation in lower-stage diseases (Table 1). However, the relatively low frequency of cases (10 of 68, 15%) with *MGMT* hypermethylation precluded us from making statistical inference, although it is known to be significantly associated with longer progression-free survival time in diffused large B-cell lymphomas [33]. The levels of *MGMT* activities and protein levels vary widely

in tumors, are abundant in B-cell lymphomas, and are undetectable in gliomas [33,34]. *MGMT* deficiency increased the sensitivity of brain tumor to alkylating agents [34]. In retinoblastoma, alkylating agents such as the platinum compounds carboplatin or cisplatin have been commonly used for treatment [34,35]. Therefore, *MGMT* inactivation may render the retinoblastoma cells more prone to genotoxic effects of alkylating agents, as has been proposed in glioma [34]. Besides, the presence of *MGMT* may confer increased resistance on the elimination of transformed retina cells to methylating and chloroethylating agents. Effects of *MGMT* hypermethylation on response to chemotherapy should be investigated in retinoblastoma.

In contrast, we found the presence of *RASSF1A* promoter hypermethylation in a very high proportion (82%) of retinoblastoma cases regardless of age at presentation, sex, laterality, histology, family history, tumor staging, or optic nerve involvement (Table 1). Our data showed that loss of *RASSF1A* expression by promoter hypermethylation is the most common epigenetic change that is known in retinoblastoma. Such epigenetic silencing was not detected among normal or nonmalignant retina tissues, further suggesting the contribution of inactivation of *RASSF1A* to the progression of retinoblastoma.

In conclusion, the large proportion of retinoblastoma cases with *RASSF1A* and *MGMT* promoter hypermethylation as well as the absence of mutations in the *RASSIFIA* sequences that cause retinoblastoma clearly show the important role of epigenetic mechanism on retinoblastoma progression.

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References

[1] Baylin SB, Herman JG, Graff JR, Vertino PM, and Issa JP (1998). Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* **72,** 141–196.

[2] Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB, and Sidransky D (1995). 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor *p16*/CDKN2/MTS1 in human cancers. *Nat Med* **1,** 686–692.

[3] Mancini D, Singh S, Ainsworth P, and Rodenhiser D (1997). Constitutively methylated CpG dinucleotides as mutation hot spots in the retinoblastoma gene (*RB1*). *Am J Hum Genet* **61,** 80–87.

[4] Chan KY, Ozcelik H, Cheung AN, Ngan HY, and Khoo US (2002). Epigenetic factors controlling the *BRCA1* and *BRCA2* genes in sporadic ovarian cancer. *Cancer Res* **62,** 4151–4156.

[5] Herman JG, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, Samid D, Duan DS, Gnarra JR, Linehan WM, et al. (1994). Silencing of the *VHL* tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci U S A* **91,** 9700–9704.

[6] Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, and Issa JP (1999). CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A* **96,** 8681–8686.

[7] Widschwendter M, Berger J, Hermann M, Muller HM, Amberger A, Zeschnigk M, Widschwendter A, Abendstein B, Zeimet AG, Daxenbichler G, et al. (2000). Methylation and silencing of the retinoic acid receptor-beta2 gene in breast cancer. *J Natl Cancer Inst* **92,** 826–832.

[8] Huang TH, Laux DE, Hamlin BC, Tran P, Tran H, and Lubahn DB (1997). Identification of DNA methylation markers for human breast carcinomas using the methylation-sensitive restriction fingerprinting technique. *Cancer Res* **57,** 1030–1034.

[9] Gonzalgo ML, Liang G, Spruck CH III, Zingg JM, Rideout WM III, and Jones PA (1997). Identification and characterization of differentially methylated regions of genomic DNA by methylation-sensitive arbitrarily primed PCR. *Cancer Res* **57,** 594–599.

[10] Liang G, Salem CE, Yu MC, Nguyen HD, Gonzales FA, Nguyen TT, Nichols PW, and Jones PA (1998). DNA methylation differences associated with tumor tissues identified by genome scanning analysis. *Genomics* **53,** 260–268.

[11] Greger V, Debus N, Lohmann D, Hopping W, Passarge E, and Horsthemke B (1994). Frequency and parental origin of hypermethylated *RB1* alleles in retinoblastoma. *Hum Genet* **94,** 491–496.

[12] Harada K, Toyooka S, Maitra A, Maruyama R, Toyooka KO, Timmons CF, Tomlinson GE, Mastrangelo D, Hay RJ, Minna JD, et al. (2002). Aberrant promoter methylation and silencing of the *RASSF1A* gene in pediatric tumors and cell lines. *Oncogene* **21,** 4345–4349.

[13] Choy KW, Pang CP, To KF, Yu CB, Ng JS, and Lam DS (2002). Impaired expression and promotor hypermethylation of O^6-methylguanine-DNA methyltransferase in retinoblastoma tissues. *Invest Ophthalmol Vis Sci* **43,** 1344–1349.

[14] Dammann R, Li C, Yoon JH, Chin PL, Bates S, and Pfeifer GP (2000). Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* **25,** 315–319.

[15] Burbee DG, Forgacs E, Zochbauer-Muller S, Shivakumar L, Fong K, Gao B, Randle D, Kondo M, Virmani A, Bader S, et al. (2001). Epigenetic inactivation of *RASSF1A* in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst* **93,** 691–699.

[16] Lo KW, Kwong J, Hui AB, Chan SY, To KF, Chan AS, Chow LS, Teo PM, Johnson PJ, and Huang DP (2001). High frequency of promoter hypermethylation of *RASSF1A* in nasopharyngeal carcinoma. *Cancer Res* **61,** 3877–3881.

[17] Dammann R, Yang G, and Pfeifer GP (2001). Hypermethylation of the CpG island of Ras association domain family 1A (*RASSF1A*), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. *Cancer Res* **61,** 3105–3109.

[18] Cohen Y, Singer G, Lavie O, Dong SM, Beller U, and Sidransky D (2003). The *RASSF1A* tumor suppressor gene is commonly inactivated in adenocarcinoma of the uterine cervix. *Clin Cancer Res* **9,** 2981–2984.

[19] Shivakumar L, Minna J, Sakamaki T, Pestell R, and White MA (2002). The *RASSF1A* tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. *Mol Cell Biol* **22,** 4309–4318.

[20] Liu L, Tommasi S, Lee DH, Dammann R, and Pfeifer GP (2003). Control of microtubule stability by the *RASSF1A* tumor suppressor. *Oncogene* **22,** 8125–8136.

[21] Song MS, Song SJ, Ayad NG, Chang JS, Lee JH, Hong HK, Lee H, Choi N, Kim J, Kim H, et al. (2004). The tumour suppressor *RASSF1A* regulates mitosis by inhibiting the APC–Cdc20 complex. *Nat Cell Biol* **6,** 129–137.

[22] Yoon JH, Dammann R, and Pfeifer GP (2001). Hypermethylation of the CpG island of the *RASSF1A* gene in ovarian and renal cell carcinomas. *Int J Cancer* **94,** 212–217.

[23] Dreijerink K, Braga E, Kuzmin I, Geil L, Duh FM, Angeloni D, Zbar B, Lerman MI, Stanbridge EJ, Minna JD, et al. (2001). The candidate tumor suppressor gene, *RASSF1A*, from human chromosome 3p21.3 is involved in kidney tumorigenesis. *Proc Natl Acad Sci U S A* **98,** 7504–7509.

[24] van der Wal JE, Hermsen MA, Gille HJ, Schouten-Van Meeteren NY, Moll AC, Imhof SM, Meijer GA, Baak JP, and van der Valk P (2003). Comparative genomic hybridisation divides retinoblastomas into a high and a low level chromosomal instability group. *J Clin Pathol* **56,** 26–30.

[25] Chaum E, Ellsworth RM, Abramson DH, Haik BG, Kitchin FD, and Chaganti RS (1984). Cytogenetic analysis of retinoblastoma: evidence for multifocal origin and *in vivo* gene amplification. *Cytogenet Cell Genet* **38,** 82–91.

[26] Kusnetsova LE, Prigogina EL, Pogosianz HE, and Belkina BM (1982). Similar chromosomal abnormalities in several retinoblastomas. *Hum Genet* **61,** 201–204.

[27] Benedict WF, Banerjee A, Mark C, and Murphree AL (1983). Nonrandom chromosomal changes in untreated retinoblastomas. *Cancer Genet Cytogenet* **10,** 311–333.

[28] Kusnetsova LE, Pogosianz HE, Muratova TT, and Koslovskaya GM (1985). Nonrandom changes of the karyotype on human retinoblastomas. *Genetika (Moskva)* **21,** 321–326.

[29] Mitelman Database of Chromosome Aberrations in Cancer from the

Cancer Genome Anatomy Project (http://cgap.nci.nih.gov/Chromosomes/Mitelman).

[30] Herman JG, Graff JR, Myohanen S, Nelkin BD, and Baylin SB (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* **93,** 9821–9826.

[31] Morita R, Ishikawa J, Tsutsumi M, Hikiji K, Tsukada Y, Kamidono S, Maeda S, and Nakamura Y (1991). Allelotype of renal cell carcinoma. *Cancer Res* **51,** 820–823.

[32] Esteller M, Corn PG, Baylin SB, and Herman JG (2001). A gene hypermethylation profile of human cancer. *Cancer Res* **61,** 3225–3229.

[33] Esteller M, Gaidano G, Goodman SN, Zagonel V, Capello D, Botto B, Rossi D, Gloghini A, Vitolo U, Carbone A, et al. (2002). Hypermethylation of the DNA repair gene *O*(6)-methylguanine DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma. *J Natl Cancer Inst* **94,** 26–32.

[34] Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, Baylin SB, and Herman JG (2000). Inactivation of the DNA-repair gene *MGMT* and the clinical response of gliomas to alkylating agents. *N Engl J Med* **343,** 1350–1354.

[35] Demirci H, Eagle RC Jr, Shields CL, and Shields JA (2003). Histopathologic findings in eyes with retinoblastoma treated only with chemoreduction. *Arch Ophthalmol* **121,** 1125–1131.