Overexpression of DNA Methyltransferases 1, 3a, and 3b Significantly Correlates With Retinoblastoma Tumorigenesis

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Key Words: DNA methyltransferases; Retinoblastoma; Immunohistochemistry; Tumorigenesis; Biomarker

Abstract

DNA methyltransferases (DNMTs) 1, 3a, and 3b affect DNA promoter methylation; studies have suggested that they have important roles in the development of cancers. In this study, we analyzed the expression of DNMTs 1, 3a, and 3b; the MIB-1 labeling index; and their clinical significance in 6 normal retinas and 62 retinoblastomas using immunohistochemical analysis. We found that DNMT proteins were not expressed in normal retinas, whereas they were frequently expressed in retinoblastomas (DNMT1, 100%; DNMT3a, 98%; and DNMT3b, 92%). Compared with well-differentiated retinoblastomas, the expression of DNMTs 1 and 3a significantly increased in poorly differentiated retinoblastomas (P = .002 and P = .003, respectively); in addition, the frequency of their increased expression was high. DNMT1 expression was significantly higher in invasive retinoblastoma. Furthermore, the expression of DNMTs was positively correlated with the MIB-1 labeling index in retinoblastoma. Our findings suggest that the overexpression of DNMTs 1, 3a, and 3b may be related to retinoblastoma tumorigenesis and progression and may represent a novel approach for retinoblastoma therapy.

Retinoblastoma is a malignant neoplasm composed of embryonic tumor cells from retinoblasts of neuroepithelial origin. Retinoblastoma is the most common intraocular tumor in children, with a relative incidence of 3% of all pediatric tumors; approximately 1 in 20,000 children are affected by retinoblastoma worldwide.1 The development of retinoblastoma is associated with the inactivation of the retinoblastoma susceptibility gene, Rb1. The Rb1 gene codes for the retinoblastoma protein, RB, that functions as a tumor suppressor by controlling the cell cycle through complex interactions with multiple kinases and their inhibitors that form the RB pathway.2 Despite this landmark discovery, the precise pathogenesis of retinoblastoma is not completely understood. In a previous study, Mu et al3 observed that the overexpression of high-mobility groups A1 and A2 and the down-regulation of let-7 may be associated with retinoblastoma tumorigenesis and progression. Additional changes in the pattern of gene expression are necessary for the expression of the fully transformed malignant phenotype.4 Although retinoblastoma is not considered a deadly childhood cancer anymore and has become largely curable within the past 40 years, current treatment strategies are aimed at salvaging the eye and providing the best possible visual outcome.5 Molecular targeting therapy is emerging as a potential strategy for individualized therapy.

DNA hypermethylation of CpG islands is an epigenetic modification of eukaryotic genomes that is important for embryonic development, imprinting, and the inactivation of X chromosomes.6,7 DNA methylation is also one of the most common epigenetic changes observed in human cancers.8 Abnormal methylation of CpG islands can efficiently repress transcription of the associated gene and is a reversible process. Among the CpG methylation enzymes associated with gene
silencing, 3 functional DNA methyltransferases (DNMTs) have distinctive roles. DNMT1 is the primary enzyme responsible for copying methylation patterns after DNA replication because it localizes to replication foci and interacts with the proliferation cell nuclear antigen; DNMT3a and DNMT3b are responsible for de novo methylation. The overexpression of DNMT1, DNMT3a, and DNMT3b has been reported in various malignancies, including gastric, urothelial, and lung cancers, and may be related to tumorigenesis, tumor progression, and poor survival. Discovery of the aberrant epigenetic states of malignant cells that result in the silencing of tumor suppressor genes (TSGs) has led to an extensive search for new drugs that are capable of reactivating epigenetically silenced genes. In particular, drugs capable of reversing aberrant DNA methylation patterns by inhibiting DNMTs have been extensively explored in cancer therapy, and the specificities and efficacies of these drugs have been demonstrated in experiments and clinical trials.

Recent studies have suggested that the down-regulation of TSGs by promoter hypermethylation may have an important role in the tumorigenesis of retinoblastoma. Several TSGs and DNA repair genes, such as RB1, RASSF1A, NEUROG1, and MGMT, were inactivated by aberrant hypermethylation leading to transcriptional silencing and the development of retinoblastoma. However, to the best of our knowledge, no study has analyzed the roles of DNMT1, DNMT3a, and DNMT3b in retinoblastoma.

In this study, we examined the expression of DNMT1, DNMT3a, and DNMT3b using immunohistochemical staining in a large series of retinoblastomas. We analyzed the correlation between the expression of DNMT1, DNMT3a, and DNMT3b proteins and the clinicopathologic features and proliferative activity of retinoblastoma and explored the possible role of inhibiting DNMT expression in retinoblastoma therapy.

Materials and Methods

Patients and Retinoblastoma Samples

For the purpose of this study, 62 formalin-fixed, paraffin-embedded (FFPE) tumor samples from 62 patients with retinoblastoma, including 34 males and 28 females, and 6 FFPE normal retina samples were used. Among them, 5 normal retina and 9 tumor specimens were obtained from tissue microarrays (TMAs; Shanxi Chaoying Tissue MicroArray, Xi’an, China), and 1 normal retina was obtained from a pediatric donated eye. The TMA contained duplicate dots for each sample. The ages of the subjects ranged from 1 month to 15 years (median, 32 months). Among the patients, the retinoblastoma was unilateral in 48 (77%) and bilateral in 14 (23%); in patients with bilateral tumors, only 1 tumor was analyzed in this study.

Histopathologic analysis revealed that 17 tumors were poorly differentiated (PD; characterized by the absence of Flexner-Wintersteiner rosettes), and 45 were well differentiated (WD). Invasion of the choroid, optic nerve, and/or orbit was detected in 16 cases, while the tumors were localized without invasion in 46 cases (Table 1). Among the 16 invasive tumors, 5 showed diffuse choroidal invasion; 9, diffuse choroidal and optic nerve invasion; and 2, orbital invasion. This study was approved by the participating institutional review boards. Written informed consent for enucleation surgery and

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of Cases</th>
<th>DNMT1</th>
<th>DNMT3a</th>
<th>DNMT3b</th>
<th>MIB-1 LI</th>
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<td>Sex</td>
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<tr>
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<td>34</td>
<td>58.8 ± 26</td>
<td>57.9 ± 33</td>
<td>33.6 ± 21</td>
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<td>67.4 ± 26</td>
<td>65.1 ± 29</td>
<td>39.6 ± 26</td>
<td>46.9 ± 38</td>
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<tr>
<td>Location</td>
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<td>51.1 ± 33</td>
<td>35.4 ± 25</td>
<td>37.5 ± 34</td>
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<tr>
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<td>29.2 ± 29</td>
<td>38.9 ± 23</td>
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<tr>
<td>Bilateral</td>
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<td>59.4 ± 27</td>
<td>64.1 ± 29</td>
<td>39.2 ± 29</td>
<td>46.5 ± 38</td>
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<td>55.1 ± 31</td>
<td>35.4 ± 22</td>
<td>43.3 ± 35</td>
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<td>49.5 ± 32</td>
<td>35.2 ± 26</td>
<td>32.1 ± 30</td>
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<tr>
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<td>75.6 ± 29</td>
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<td>No</td>
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<td>58.3 ± 27</td>
<td>55.1 ± 33</td>
<td>33.6 ± 25</td>
<td>39.3 ± 35</td>
</tr>
<tr>
<td>Yes</td>
<td>16</td>
<td>75.1 ± 22</td>
<td>63.2 ± 25</td>
<td>44.3 ± 20</td>
<td>57.8 ± 35</td>
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</table>

LI, labeling index.

Table 1: Expression of DNMTs 1, 3a, and 3b and MIB-1 in 62 Retinoblastomas

Data are the mean ± SD for the percentage of DNMT1-, 3a-, and 3b-stained tumor cells and for the MIB-1 LI.

1 P < .05.
participation in this study were obtained from the guardians of all patients. All procedures conformed to the tenets of the Declaration of Helsinki.

**Immunohistochemical Staining**

The FFPE sections were analyzed for the presence of the DNMT1, DNMT3a, DNMT3b, and Ki-67 antigens by using the labeled streptavidin-biotin method. After deparaffinization, the antigens were detected by using the autoclave oven technique and blocking. The sections were incubated overnight with goat polyclonal anti-DNMT1 antibody (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal anti-DNMT3a antibody (dilution 1:150; Santa Cruz Biotechnology), goat polyclonal anti-DNMT3b antibody (dilution 1:100; Santa Cruz Biotechnology), or with MIB-1 mouse monoclonal antibody (dilution 1:100; DakoCytomation, Glostrup, Denmark) at 4°C. Antigen-antibody complexes were detected by using the cobalt-3,3'-diaminobenzidine reaction. The slides were lightly counterstained with hematoxylin and mounted for microscopic examination. Colon and breast carcinomas known to be positive for DNMT1, DNMT3a, and DNMT3b were used as positive control samples.18,19 Sections incubated in phosphate-buffered saline without the primary antibody served as negative control samples. The specificity of all reactions for DNMT1, DNMT3a, and DNMT3b was verified by replacing the primary antibody with normal serum and by preabsorbing each primary antibody with blocking peptide (Santa Cruz Biotechnology).

**Evaluation of Immunohistochemical Staining**

Each slide was examined by an observer (G.M.) blinded to the diagnosis and clinicopathologic data and then confirmed by a second blinded observer (Yan Wang). Nuclear staining was considered to represent a positive stain for DNMT1, DNMT3a, DNMT3b, and MIB-1. Images of several high-power fields (>400) were captured from regions with different staining densities, including high, moderate, low, and negative staining. In total, 1,000 cells were counted. In a few small samples, at least 500 cells were counted. In the TMA, all cells were counted in each sample. The results were expressed as the percentage of tumor cells with positive nuclear staining. The percentage of DNMT1-, DNMT3a-, and DNMT3b-stained tumor cells was then divided into the 2 following groups: low (less than the mean level) and high (more than the mean level) Table 2.

**Statistical Analysis**

To determine the significance of associations between the different variables, the data were statistically analyzed using the t test, Mann-Whitney U test, the Kruskal-Wallis test, the \( \chi^2 \) test, Pearson correlation coefficient, and Spearman correlation coefficient using StatView J-4.5 software (Abacus Concepts, Berkeley, CA). Probability values (\( P \)) less than .05 were considered statistically significant.

**Results**

Generally, DNMT1, DNMT3a, and DNMT3b were not expressed in normal retina samples Image 1A, Image 2A, and Image 2H. DNMT1 and DNMT3a were expressed in non-tumor retina samples located in the retinoblastoma marginal area Image 1B and Image 2B, while DNMT3b was not expressed in non-tumor retina samples Image 2F. In retinoblastoma tumor cells, DNMT1, DNMT3a, and DNMT3b proteins were observed in the nucleus Image 1C, Image 1D, Image 1E, Image 1F, Image 2C, Image 2D, Image 2G, and Image 2H.

DNMT1 was expressed in all 62 retinoblastoma samples (Table 1). Of the tumors, 32 (52%) showed high expression of DNMT1 (DNMT1+ cells ≥65%; Table 2). High expression of DNMT1 was more frequently detected in PD (15/17 [88%]) and invasive (12/16 [75%]) tumors than in WD (17/45 [38%]) and noninvasive (20/46 [43%]) tumors \( P = .0002 \) and \( P = .0268 \), respectively; Table 2). The level of DNMT1 expression in PD and invasive tumors was also significantly higher than that in WD and noninvasive tumors Figure 1A.

**Table 2**

**DNMT1, DNMT3a, and DNMT3b Expression in 62 Retinoblastomas and Pathologic Associations**

<table>
<thead>
<tr>
<th>Variable</th>
<th>DNMT1</th>
<th></th>
<th>DNMT3a</th>
<th></th>
<th>DNMT3b</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td>( P )</td>
<td>Low</td>
<td>High</td>
<td>( P )</td>
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<td>Histologic type</td>
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<td></td>
<td></td>
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<tr>
<td>WD (n = 45)</td>
<td>28 (62)</td>
<td>17 (38)</td>
<td>.0002</td>
<td>26 (58)</td>
<td>19 (42)</td>
<td>.0006</td>
</tr>
<tr>
<td>PD (n = 17)</td>
<td>2 (12)</td>
<td>15 (88)</td>
<td></td>
<td>2 (12)</td>
<td>15 (88)</td>
<td></td>
</tr>
<tr>
<td>Invasion</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noninvasive (n = 46)</td>
<td>26 (57)</td>
<td>20 (43)</td>
<td>.0268</td>
<td>24 (52)</td>
<td>22 (48)</td>
<td>.0547</td>
</tr>
<tr>
<td>Invasive (n = 16)</td>
<td>4 (25)</td>
<td>12 (75)</td>
<td></td>
<td>4 (25)</td>
<td>12 (75)</td>
<td></td>
</tr>
<tr>
<td>Total (n = 62)</td>
<td>30 (48)</td>
<td>32 (52)</td>
<td></td>
<td>28 (45)</td>
<td>34 (55)</td>
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</table>

PD, poorly differentiated; WD, well differentiated.

* Data are given as number (percentage). High expression was as follows: DNMT1, positive cells ≥65%; DNMT3a, positive cells ≥60%; and DNMT3b, positive cells ≥40%.
Image 11 DNMT1 immunostaining in normal retina, nontumor retina, and retinoblastoma. 

A, DNMT1 was not expressed in normal retina. B, DNMT1 expression was detected in nontumor retinal tissue. C and D, DNMT1 was highly expressed in poorly differentiated retinoblastoma. E and F, In well-differentiated retinoblastoma, with a typical rosette formation, DNMT1 was expressed at a low level (A-F, ×400).
Nuclear staining for DNMT3a was positive in 61 (98%) of 62 tumors (Images 2C and 2D and Table 1). A high level of DNMT3a expression (DNMT3a+ cells ≥60%) was detected in 34 tumors (55%; Table 2). High expression levels of DNMT3a were more frequently detected in PD (15/17 [88%]) than in WD (19/45 [42%]) tumors (P = .0006; Table 2). The level of DNMT3a expression in PD tumors was also significantly higher than that in WD tumors Figure 1B (P = .003; Table 1). The frequency of DNMT3a high expression and the level of DNMT3a expression in invasive tumors were potentially higher than those in noninvasive tumors, although they were not statistically significant Figure 1E (Table 2).

DNMT3b nuclear expression was detected in 57 (92%) of the 62 retinoblastomas (Images 2G and 2H and Table 1). High expression levels of DNMT3b (DNMT3b+ cells ≥40%) were detected in 25 tumors (40%; Table 2). The high expression levels of DNMT3b were not significantly related to the histopathologic classification or invasiveness of retinoblastoma Figure 1C and Figure 1F (Tables 1 and 2).

The MIB-1 labeling index was significantly correlated with tumor differentiation and invasion (Table 1). We detected a positive correlation between the MIB-1 labeling index and the protein expression levels of DNMTs in retinoblastoma Figure 1G, Figure 1H, and Figure 1I) (R2 = 0.554, P < .0001; R2 = 0.376, P < .0001; and R2 = .219, P < .0001; respectively).
In addition, there were no significant differences in the protein expression levels of DNMT1, DNMT3a, and DNMT3b with respect to patient age (data not shown), sex, tumor site, or tumor laterality (Table 1).

Discussion

The molecular signaling pathways in which retinoblastoma is involved are highly complex and regulated. Hereditary and sporadic retinoblastomas develop due to mutations in both alleles of the RB1 gene on chromosome 13q14. These mutations occur prezygotically in germ cells (the initial mutation in bilateral disease) or postzygotically (the mutation of the second allele in bilateral disease or mutations in both alleles in unilateral disease) in retinal cells during fetal development or early infancy. An astounding number of RB1 mutations (>900 mutations) have been reported; however, the precise pathogenesis of retinoblastoma remains unclear.

Epigenetic events such as DNA methylation of the CpG promoter are thought to be crucial in tumor initiation and progression. Although methylation of the RB1 promoter was detected, it is not a major mechanism for the development of retinoblastoma; additional genetic and epigenetic changes in other genes may be required for retinoblastoma tumorigenesis. Hypermethylation of TSG, RASSF1A, NEUROG1, and a DNA repair gene, MGMT, was detected in association with retinoblastoma. We used immunohistochemical assays to investigate the expression of the 3 functional DNMTs (1, 3a, and 3b) at the protein level in a...
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92%, respectively). DNMTs (1, 3a, and 3b) are overexpressed in other tumors, and their overexpression may be important for the deregulation of TSG expression, leading to tumor formation. Our findings suggest that DNMTs may contribute to the tumorigenicity of retinoblastoma. Further in vitro and in vivo investigations are necessary to clarify this issue.

Several previous studies have suggested that DNMT1 contributes to cancer development and progression through
alternative pathways. DNMT1 may have a significant role in the development of PD gastric cancers by inducing frequent DNA hypermethylation of multiple CpG islands.22 In addition, high levels of DNMT1 expression are significantly associated with poor overall survival of patients with non–small cell lung cancer, independent of tumor stage and grade; meanwhile, high levels of DNMT3b expression are significantly associated with poor prognosis only in young patients.13 The overexpression of DNMT1 may participate in multistage urothelial carcinogenesis, even at the precancerous stage, and in the development of nodular invasive carcinomas of the bladder.14 DNMT1 is the major DNMT in humans, although 2 other enzymes—DNMT3a and DNMT3b—have also been shown to possess DNMT activity.6 Genomic methylation patterns may be established through cooperation among these 3 DNMTs, even in cancer cells.

In this study, we found that DNMT1 and DNMT3a protein expression levels were significantly increased in PD retinoblastoma compared with WD retinoblastoma. In addition, the expression of DNMT1 was significantly higher in invasive retinoblastoma. Furthermore, we also found that the overexpression of DNMTs is related to tumor proliferation. This observation was confirmed by the positive correlation between the MIB-1 labeling index and the expression levels of DNMTs. Clinically, WD retinoblastoma is less malignant and has a better prognosis than PD retinoblastoma. In addition, tumor invasion is considered to be an important risk factor for metastatic retinoblastoma. Our data corroborate previous findings that high expression levels of DNMT1 and DNMT3a are associated with highly malignant phenotypes and are considered as poor prognostic indices.

Because retinoblastoma is largely curable, salvaging the eye and providing the best possible visual outcome represent the current treatment strategies for retinoblastoma. On the basis of advances in tumor genetics and tumor molecular signaling, molecular targeting therapy is emerging as a potential strategy for retinoblastoma. Recently, nutlin-3A, a small molecule inhibitor of the interaction between MDM2/MDMX and p53, and inhibitors of histone deacetylase were found to induce cell death in human retinoblastoma cell lines.26,27 Nutlin-3A, combined with topotecan, a chemotherapy drug, can kill human retinoblastoma cell lines.28 Hypermethylated gene promoters have the potential to be reactivated by nucleoside analogues, which are mechanism-based inhibitors of DNMTs.29 The US Food and Drug Administration has now approved the 2 DNMT-inhibiting nucleoside analogues 5-azacytidine and 5-aza-2′-deoxycytidine, which display anti-proliferative properties against cancer cells, for the treatment of myelodysplastic syndrome and acute myeloid leukemia.30 In addition, a number of non–nucleoside analogue DNMT inhibitors, such as hydralazine, have also been proposed as epigenetic cancer therapy drugs.31 Furthermore, clinical trials of the effectiveness of these DNMT inhibitors against solid tumors are in the early stages.30 According to the results of our study, we predict that DNMTs could be a good target for therapy in retinoblastomas that overexpress DNMT1, DNMT3a, and DNMT3b. It will be very important to identify the effectiveness of these drugs in retinoblastoma in vitro and in vivo. The clinical trial could be a big challenge.

These findings enhanced our understanding of the epigenetic changes that occur during retinoblastoma tumorigenesis. Patients whose tumors overexpress DNMTs seem to have more aggressive disease and a poor prognosis, possibly because many survival-associated TSGs are hypermethylated by DNMTs, which ultimately leads to their functional inactivation.32 Thus, our data imply that the protein expression levels of DNMTs may suggest a new mechanism of retinoblastoma development and can be a reliable predicting factor for the prognosis of this disease. Furthermore, our findings may open a new approach for retinoblastoma therapy.

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*Dr Qu and Mu contributed equally to this work.

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