High levels of global genome methylation in patients with retinoblastoma

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Abstract. Retinoblastoma is a tumor of the embryonic neural retina in young children. The DNA methyltransferase 1 (DNMT1) gene has been demonstrated to be transcriptionally activated in cells lacking retinoblastoma 1 (RB1). Thus, there is a direct interaction between DNMT1 and RB1. The present study hypothesized that uncontrolled DNMT1, DNMT2 and DNMT3 expression may lead to a high level of global genome methylation causing a second hit or where both alleles are altered, in RB1 and/or inactivation of other genes in retinal cells. To test this, the global genome methylation levels were analyzed in 69 patients with retinoblastoma, as well as 26 healthy siblings and 18 healthy unrelated children as the control groups. Peripheral blood and tumor tissue samples were obtained from 32 patients. The expression levels of DNMT genes were also determined in cell lines. Based on the median levels of global genome methylation in patients, higher genome-wide methylation levels in peripheral blood were associated with a 3.33-fold increased risk for retinoblastoma in patients compared with all healthy controls (95% confidence interval, 0.98-11.35; P<0.0001). The level of global genome methylation and the expression of DNMT genes were increased in the WERI-RB-1 cell line, which has a mutated RB1 gene, compared with a wild-type RB1-expressing cell line. These results supported the hypothesis that epigenetic alterations, as well as mutations in RB1, may be associated with the oncogenesis and inheritance of retinoblastoma. The repression of genes that interact with RB1, such as the DNMT gene family, may be important in patients with retinoblastoma with alterations in RB1, and may serve a role in the treatment and regression of retinoblastoma.

Introduction

Retinoblastoma is a childhood cancer (1). The majority of the clinical phenotypes can be explained by the double mutational inactivation of the tumor suppressor gene retinoblastoma 1 (RB1) (2,3). However, additional mutations or epigenetic changes in other signaling pathways may also be involved in tumor development in retinoblastoma (4).

Previous studies have demonstrated that although abnormal epigenetic gene silencing can occur at any time during tumor progression, it most frequently occurs during the early stages of the neoplastic process, such as in the precancerous stages of tumor development (5,6). Epigenetics studies have revealed that gene expression can be modified with no total structural changes in the genomic DNA by the environment alone, or epigenetic alterations may occur early in tumor development, which may lead to genetic changes (4,7-9). The importance of epigenetic abnormalities in tumor initiation and maintenance has been demonstrated in studies of genes that were identified through random screening of cancer-cell genomes (10,11). Cancer cells consistently have DNA methylation on their genome. Although global DNA hypomethylation is a very common alteration in tumor tissue, also the regional methylation which is known as promoter methylation frequently occurs in the tumor cells. However, it is not known how global hypomethylation and regional promoter methylation occur together and how they affect each other, but it is known that the enzymes responsible for these conditions are DNMT enzymes and the enzymes are expressed at higher levels in tumor cells. Global hypomethylation is a highly effective mechanism in carcinogenesis as it causes the formation of aberrant gene expression, chromosomal instability, reactivation of retrotransposons, and loss of imprinting. Furthermore, promoter methylation
is known to cause the silencing of tumor suppressor genes. It will be understood that both global hypomethylation mechanisms and promoter methylations are thought to be involved in tumor formation together or separately. Therefore, Methylation mechanisms that can be recycled can be used for disease prevention or treatment. Indeed, there are agents such as decitabine and 5-azacytidine that are capable of reversing methylation changes and are being successfully applied, particularly in the treatment of leukemia now.

In addition, the epigenetically modified DNA landscape is stable and can be transmitted from one generation to the next. Thus, the study of these epigenetic modifications is important (10).

McCabe et al and Kooi et al (12,13) demonstrated that DNA methyltransferase 1 (DNMT1) is transcriptionally activated in cells lacking RB1 (RB1Δ73), suggesting that DNMT1 is under the control of retinoblastoma protein (pRb) and retinoblastoma-associated protein (E2F) transcription factors. Overexpression of DNMT1 is associated with tumor suppressor gene hypermethylation, which contributes to tumorigenesis (14). Alterations in both alleles of RB1 are involved in the development of retinoblastoma (4). Therefore, epigenetic changes in the genome of patients with retinoblastoma may stimulate or be stimulated by RB1 and/or DNMT1 gene alterations to generate the first and second hits or both for cancer in early life. However, Qu et al (15) have reported that DNMT proteins were not expressed in normal retinas, whereas they were frequently expressed in tumor tissue of patients with retinoblastoma. The expression levels of DNMT1 and DNMT3a proteins were increased in poorly differentiated retinoblastoma compared with well-differentiated retinoblastoma (15). Patients with tumors exhibiting upregulated expression of DNMTs appeared to have aggressive disease and poor prognosis (15). The expression level of DNMT1 was also identified to be increased in cases with invasive retinoblastoma (15). However, it is unclear whether patients with retinoblastoma carry RB1 mutations. Therefore, the association between RB1 mutation and the level of DNMT1 expression warrants further investigation. Germ line mutations in RB1 account for 15 -35% of patients with retinoblastoma (15). Patients with tumors exhibiting upregulated expression of DNMTs appeared to have aggressive disease and poor prognosis (15). It will be understood that both global hypomethylation mechanisms and promoter methylations are thought to be involved in tumor formation together or separately. Therefore, Methylation mechanisms that can be recycled can be used for disease prevention or treatment. Indeed, there are agents such as decitabine and 5-azacytidine that are capable of reversing methylation changes and are being successfully applied, particularly in the treatment of leukemia now.

The present study aimed to investigate the level of global genome methylation in peripheral blood samples of patients with retinoblastoma, their healthy siblings and unrelated controls to improve the understanding of the importance of global genome methylation in retinoblastoma. In addition, the levels of genome-wide methylation in tumor tissue and in the peripheral blood samples of patients with retinoblastoma were investigated to better understand whether genome-wide methylation serves a role in the oncogenesis of retinoblastoma. Finally, the association between the expression levels of DNMT genes (DNMT1, DNMT2, DNMT3a and DNMT3b), global genome methylation and RB1 mutation was investigated in the commercially available WER1-RB-1 cell line, established from the tumor tissue of a patient with retinoblastoma who had a mutation in RB1, and the RWPE-2 cell line with wild-type RB1.

Materials and methods

Samples. The study samples comprised 145 blood and/or tumor specimens from 69 retinoblastoma cases (46 unilateral and 23 bilateral), 26 siblings matched to the cases and 18 unrelated controls. Both peripheral blood and tumor tissues were obtained from 32 patients; 30 patients, 26 unaffected siblings and 18 unrelated healthy controls provided peripheral blood samples only and 7 patients provided tumor tissue only. The study was approved by the ethics committee of the Istanbul Medical Faculty, Istanbul University (Istanbul, Turkey). All patients and their siblings were selected from individuals who were diagnosed and treated between January 1991 and December 1996 at the Oncology Institute in Istanbul University and their relatives. Fresh tumor tissue and peripheral venous blood samples of the patients were collected during surgery, transferred to the laboratory and immediately processed. Informed consent was signed prior to collecting 10 ml peripheral blood samples from the patient and healthy controls. The lymphocytes were separated from whole blood using the Ficoll-1077 (Sigma-Aldrich; Merck KGaA) gradient centrifugation method at 700 x g for 30 min at room temperature. The white blood cells were harvested using sterile pipette tips from the surface of the Ficoll solution followed by centrifugation. The lymphocytes were washed with PBS twice and centrifuged at 700 x g for 10 min at room temperature. Subsequently, the cell pellets were resuspended with PBS and divided into 2 cryo tubes and centrifuged at 700 x g for 20 min at room temperature. The supernatant was discarded and the cell pellets were frozen at - 80°C immediately. Then they were stored as cell pellets in liquid nitrogen until further use.

Fresh tissues were dissected into 2-3 mm3 pieces and incubated into The RNALater solution at 4°C for at least 24 h. The following day, the tissues were removed from the RNALater stabilization solution (cat. no. AM7020; Invitrogen; Thermo Fisher Scientific, Inc.) and placed in cryotubes for storage in liquid nitrogen. Unrelated healthy controls were selected from healthy children attending Istanbul Medical Faculty, Department of Pediatrics at Istanbul University, for routine health assessment, with no history of cancer in first, second and third-degree relatives and were age- and ethnicity matched with the patients. A total of 5 ml of peripheral blood was obtained from each healthy control and also for healthy...
siblings of the patients. Family histories were obtained from the patients, and pedigrees were drawn. The mean (±SD) and median ages of the patients with retinoblastoma were 18.46±15.61 and 14 months (range, 1.32-84.29 months), respectively. The mean (±SD) and median ages of the siblings were 93.10±60.59 and 84.96 months (range, 12.48-204.10 months), respectively. The mean (±SD) and median ages of the unrelated healthy controls were 19.96±16.16 and 15 months (range, 2.27-92.44 months), respectively. 47% of cases, including the patients, siblings and healthy controls participated in the study, were female and 53% were male. The DNA was isolated by the phenol/chloroform (1:1) extraction method following overnight incubation with proteinase K at 37°C as described previously published article (20). The DNA concentration was measured using a Pico Green ds DNA Assay kit (Thermo Fisher Scientific, Inc.) at a wavelength of 485/535 nm in visible/UV spectrometer.

**Mutation analysis of the RB1 gene in patients with retinoblastoma.** A total of 27 patients in the cohort were tested for the presence of small indel mutations and large genomic rearrangements (LGRs). The peripheral blood of patients with diagnosed retinoblastoma was used for sequencing for the full exons of the RB1 gene by Sanger sequencing using CEQ8000 and GXL dye terminator cycle sequencing (Beckman Coulter, Inc.) systems, and LGRs were screened using multiplex ligation-dependent probe amplification (MLPA) analysis. All coding exons and adjacent intronic splice junction regions of the RB1 gene were screened for mutations in the fragments between 150 and 823 bp for Sanger sequencing. The reference sequence ENST00000267163/NM_000321.2 (21) was used for the RB1 gene. All DNA sequencing results were read according to the hg19 genomic sequence.

LGRs in the RB1 gene were evaluated using MLPA analysis in the study cohort. MLPA analysis was performed using MRC-Holland probe set for the RB1 gene (MLPA R1: P047; MRC-Holland) according to the manufacturer’s instructions. At least one negative and three normal controls were included in each experimental batch, as well as DNA molecular weight markers. Amplified DNA was run on CEQ8000 and GXL Beckman Coulter DNA sequencer (Beckman Coulter, Inc.) for fragment analysis. Raw data of fragment analyses were analyzed and peak areas were calculated using Coffalyser analysis software (22,23).

**Methyl acceptance assay.** Genome-wide DNA methylation was determined by the methyl acceptance assay using the protocol described by Balaghi and Wagner (24). A mixture of 100-250 ng genomic DNA, 2 µCi S-adenosyl-L-(methyl 3H) methionine (SAM; GE Healthcare Life Sciences) and 1X enzyme buffer (New England BioLabs, Inc.) were incubated at 37°C for 80 min. All samples were run in duplicate and the reactions were performed in a 30 µl reaction volume with stopping on ice. The enzymatically methylated DNA was isolated from the reaction mixture by filtering 15 µl of the reaction solution on Whatman DE81 ion exchange filters (Whatman, Inc.) and washing three times with 5 ml 0.5 M phosphate buffer (pH 7.0) and twice with 2 ml 70 and 100% ethanol in a sampling manifold unit (EMD Millipore). The dried filters were placed into vials containing 5 ml scintillation liquid (Ultima Gold; Perkin Elmer, Inc.), and counted in a Packard-Tricarb 2100TR liquid scintillation analyzer (Perkin Elmer, Inc.). The mean disintegrations per minute (DPM) values were divided by the amount of DNA used for evaluating the global genome methylation levels. Duplicate incubations without the Sss1 methylase enzyme were used as the blank control. Commercial methylated DNA (Chemicon International; Thermo Fisher Scientific, Inc. cat. no. S7821), DNA from a random lymphoblastoid cell line (required from the sample registry of Oncology Institute, Istanbul University), whole genome amplified DNA and commercial unmethylated DNA (Chemicon International; Thermo Fisher Scientific, Inc. cat. no. S7822) were assayed with each batch to determine reaction precision, integrity and variability (data not shown).

The cost-effective and fast protocol developed by Balaghi and Wagner (24) was used for measuring the level of global genome methylation in the present study. The unmethylated CpGs that were present throughout the genome became methylated. Methylation levels were determined by measuring DNA radioactivity; high amounts of radioactive methyl groups binding to DNA in the assay suggested that the DNA exhibited low initial methylation levels, whereas low amounts of methyl binding to the DNA indicated that the DNA was highly methylated initially.

**Expression levels of DNMT genes in retinoblastoma and normal cell lines.** Expression levels of the DNMT genes (DNMT1, DNMT2, DNMT3a and DNMT3b) were investigated in the commercially available WERI-RB1-1 cell line, which was established from the tumor tissue of a retinoblastoma patient with an RB1 mutation, and a prostatic epithelial cell line RWPE-2 (both American Type Culture Collection; ATCC), which carried an intact RB1 gene. The extent to which RB1 gene mutations affect the expression of DNMT genes in different cell lines was investigated in these experiments, and the experimental design has been based on the genetic status of the cells. Therefore, two different cell lines with either RB1 gene mutations or the wild-type gene were selected. The only cell line from ATCC known to have the RB1 gene intact is the RWPE-2 cell line. The two cell lines were cultured in appropriate media (RPMI-1640 media for WERI-RB1, Keratinocyte Serum Free Medium for RWPE-2; Invitrogen; Thermo Fisher Scientific, Inc.) according to the ATCC protocols and were harvested to extract RNA and DNA using a commercial DNA/RNA isolation kit and protocols given by the firm (AllPrep DNA/RNA mini kit; Qiagen, Inc.). RNA was converted to cDNA using Invitrogen Superscript III kit with random primers and oligo(dT)20, and reverse transcriptase enzyme (SuperScript IV) at 50°C for 30 min (Invitrogen; Thermo Fisher Scientific, Inc.) in a thermal cycler (Biorad T100; Bio-Rad Laboratories Inc.). Expression levels of DNMT genes were measured using SyberGreen (Thermo Fisher Scientific, Inc.) by quantitative PCR using ABI 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.) and normalized to GAPDH expression levels using primers described by Saito et al (25): DNMT1 forward, 5'-CCCTGAGCCCTACC GAA-T-3' and reverse, 5'-CTCGTGGAGTGGACCTTG-3', 142 bp; DNMT2 forward, 5'-AAGCTGTAGCCAGCCTAC TATA-3' and reverse, 5'-TCAGCAGTGAACAGAACCCTAC
ATG-3', 148 bp; DNMT3a forward, 5'-TCTACCCGCCTC
CTGCAAGATGTCCTCT
TGCACTA-3', 113 bp; DNMT3b forward, 5'-GAATTACTC
ACGGCCCAAGGA-3' and reverse, 5'-ACCGTGAGATGT
CCTCTTGTTC-3', 101 bp; GAPDH forward, 5'-GAAGGT
GAAAGTGCTGGAGTCT-3' and reverse, 5'-GAAGATGGGAT
GGGATTC-3', 226 bp. Gene expression was calculated using
the following formula: 2^(-∆∆Cq) (26). According to this
method, the results were determined based on the Cq value,
which was the first significant increase in the PCR product
amount. The ∆Cq value, which determined the difference
between the target gene and the reference gene were calculated
separately for both the mutated cell line and the cell line
containing the intact RB1 gene. The magnitude of increase or decrease
in DNMTs gene expression of both cell lines in reference to
GADPH gene expression was determined using the 2^(-∆∆Cq)
method (26).

Statistical analysis. The levels of global methylation data
are presented as median ± interquartile range (IQR). The
Mann-Whitney U test was used to compare the differences in
median global methylation levels between tumor tissues and
peripheral blood samples of the patients and between cases
and controls. In addition, the Spearman's Rank-Order correla-
tions test was used to determine the correlation between the
global methylation level in peripheral blood and tissue. Sex-
and age-adjusted logistic regression was used to calculate the
odds ratio; the cut-off value was the median methylation level
in peripheral blood among patients. A Bonferroni-corrected
P<0.025 was considered to indicate a statistically significant
difference. All analyses were performed with SAS software 9.4
(SAS Institute).

Results

Levels of global genome methylation in peripheral blood
samples in patients with retinoblastoma and healthy control.
Global genome methylation levels were significantly higher in
the peripheral blood (P<0.0001) and tumor tissues (P<0.004)
of patients with retinoblastoma compared with those in the
peripheral blood of the healthy controls (Table I). In the subset
of patients (n=26) whose healthy siblings provided peripheral
blood, the global genome methylation levels in the peripheral
blood of patients were higher compared with their healthy
siblings (P<0.02; Table II). Global genome methylation levels
in the peripheral blood were significantly higher in patients
with bilateral (P<0.0004) and unilateral retinoblastoma
(P<0.007) compared with all healthy controls (Table III). The
levels of global genome methylation in the peripheral blood
of patients with retinoblastoma were 2.6-fold higher compared
with the levels in the peripheral blood of siblings and unrelated
healthy controls. Based on the median levels of global genome
methylation in patients with retinoblastoma, high global
genome methylation levels in peripheral blood samples were
identified to be associated with a 3.33-fold increased risk for
retinoblastoma compared with all healthy controls (95% CI, 0.98-11.35; P<0.0001; Table IV).

Levels of global genome methylation in tumor tissue and
peripheral blood of patients with retinoblastoma. The levels
of global genome methylation were 1.5-fold higher in the
DNA isolated from the tumor tissue compared with that from
the peripheral blood in patients with retinoblastoma. There
was a correlation between global genome methylation levels
in pairs of tumor tissues and peripheral blood samples from
patients with retinoblastoma (P<0.046; R=0.366; Table I). No
significant association was observed between global genome
methylation levels and family history of other types of cancer
(P>0.27).

Levels of DNMT1, DNMT2, DNMT3a and DNMT3b gene
expression and global genome methylation in the WERI-RB-1
and RWPE-2 cell lines. No appropriate RNA samples were
available at Istanbul University-Oncology institute biopspec-
imen bank to measure the expression levels of DNMTs in
the patients and healthy controls. However, the expression levels of
DNMT genes that are responsible for methylation were investi-
gated in vitro as higher levels of global genome methylation
levels were demonstrated in the peripheral blood samples of
the patients in this study. The expression levels of DNMT1,
DNMT2, DNMT3a and DNMT3b were investigated in a human
retinoblastoma tumor cell line (WERI-RB-1), which is RB1+,
and a cell line that has an intact RB1 (RWPE-2). The expres-
sion levels of all DNMT genes were higher in WERI-RB-1
cells compared with those in RWPE-2 cells. The expression
levels were 6.2-fold higher for DNMT1, 90.3-fold higher for
DNMT2, 57.2-fold higher for DNMT3a and 5.8-fold higher for
DNMT3b in the WERI-RB-1 cell line compared with RWPE-2
cells. The values of DPM/µg were 75.598 and 432.951 in the
WERI-RB-1 cell line and the RWPE-2 cell line, respectively.
The level of global genome methylation in the WERI-RB-1
cell line with a RB1 mutation was 5.72-fold higher compared
with the levels in the RWPE-2 cell line with wild-type RB1
gene. No significance differences were observed in the values
of DPM/µg between the WERI-RB-1 cell line and tumor
tissues from patients with retinoblastoma, as well as between
the RWPE-2 cell line and the peripheral blood of controls
(P>0.05).

Mutation frequency of RB1 gene in patients with retinoblas-
toma. A subset of 27 patients (39% of the patient cohort) in
the study was screened for RB1 mutations. The total mutation
frequency in patients with retinoblastoma was 37% (10/27);
among patients with mutations, the frequency of LGRs was
30% (3/10), whereas the frequency of indels was 70% (7/10).
The level of global genome methylation ranged between
131.859 and 270.647 DPM/µg in patients carrying RB1
mutations. The mean and median DPM/µg in patients with
RB1 mutations were 123.732 and 138.356, respectively. These
values in patients with RB1 mutations were lower compared
with the overall median and mean DPM/µg levels of global
genome methylation in the peripheral blood of 69 patients with
retinoblastoma.

Discussion

Retinoblastoma, which is a prototype of hereditary cancer,
is the most common intraocular tumor in children (27). The
incidence of retinoblastoma is ~11 new cases per million
individuals, particularly for early age (under 5-years of age)
In America and Europe (28). The disease may be fatal if left untreated. Therefore, understanding the genetic risks involves can assist with early diagnosis of the disease and also treatment, which can save both the vision and life of the patients. Most clinical phenotypes can be explained by double mutational inactivation of RB1 (29). However, additional mutations or alterations such as epigenetic changes may be involved in the etiology of retinoblastoma (30,31). In addition, a detailed analysis of the association between genetic alterations such as loss of heterozygosity and mutations in RB1 are frequently observed in tumors of multiple tissue types and are typically considered to serve an oncogenic role (32), the precise mechanism underlying tumorigenesis has not been fully elucidated. Inactivation of RB1 has been associated with increased tumor susceptibility in several model systems (33,34). Abnormal pRb leads to deregulation of E2F target genes, such as cyclin E, thymidine kinase and DNA polymerase α, which control the cell cycle (35-38). In addition, McCabe et al and Kooi et al (12,13) demonstrated that the aberrant regulation of DNMT1 in RB1−/− prostate epithelium was associated with increased DNA hypermethylation. Additionally, the DNMT1 promoter has been demonstrated to be regulated by the pRb/E2F pathway in murine and human cell lines of an epithelial or fibroblast origin (12,13). In the absence of pRb, DNMT1 transcripts exhibited aberrant cell cycle regulation (12). Upregulation of DNA methyltransferases has been reported in multiple types of tumor, including breast, colon, lung and prostate cancer, as well as retinoblastoma (15). Increased expression of DNMTs in tumors was significantly correlated with increased methylation of CpG islands within the promoters of tumor suppressor genes, which suggested a functional role for DNMT upregulation in cancer (39-43). In addition, several studies have demonstrated that the expression levels of DNMTs are progressively elevated in samples representing colon cancer progression with increasing expression from normal tissue in healthy subjects to polyps and finally to carcinoma (41-44). It was reported cases of retinoblastoma in which one allele of the RB1 was mutated, whereas the other was apparently normal in sequence, but not expressed in tumor cells; treatment of the cells with the demethylating drug 5-azacytidine induced the reactivation of the silent allele. This was developed into a hypothesis that tumor suppressor genes were inactivated by epigenetic events, rather than by mutation, in certain cases (14,45). Greger et al (46) demonstrated that hypermethylation of RB1 occurred in 13% of sporadic unilateral tumors and may reduce gene activity. Quinonez-Silva et al (47) reported that the inactivation of the second allele in RB1 occurred via methylation. Additionally, Livide et al (17) identified epigenetic alterations in tumor protein p53, cadherin 13, GATA-binding protein 5, checkpoint with forkhead and ring finger domains and immunoglobulin superfamily member 4. Another study reported that Ras association domain family member 1 was methylated in 82% of patients with retinoblastoma (48). Mol et al (49) suggested that retinoblastoma tumors may exhibit highly variable levels of genome stability, even if genetic alterations such as loss of heterozygosity (LOH), local amplification or copy number variation were rare in patients with retinoblastoma. Zhang et al (4) performed whole genome sequencing of four primary retinoblastoma tumors and matched normal tissues and revealed that the average number of structural alterations was 10 per case; their data on genomic instability was insufficient to explain retinoblastoma development, which further indicated that epigenetic mechanisms may contribute to retinoblastoma tumorigenesis in addition to structural events. In addition, their results revealed that without epigenetic alterations and genomic instability, the inactivation of both RB1 alleles is insufficient to result in retinoblastoma (33). A study conducted by Dimaras et al (50) explored the molecular

### Table I. Distribution of global genome methylation levels in the tumor tissues (n=39) and peripheral blood (n=69) of patients with retinoblastoma and the peripheral blood of all healthy controls (n=44), including unaffected healthy siblings and unrelated healthy controls.

| Variable       | Tumor tissues of patients, n | Peripheral blood of patients, n | Peripheral blood of healthy controls, n |
|----------------|------------------------------|---------------------------------|----------------------------------------|
| Mean DPM/µg    | 99,197                       | 14,9170                         | 38,9569                                |
| Median DPM/µg  | 19,164                       | 13,0726                         | 34,699                                 |
| Q3-Q1          | 13,8273                      | 23,4096                         | 53,5779                                |
| P-value        | 0.044*                       | <0.0001*                        | -                                      |

*Compared with the peripheral blood of healthy controls. The DPM/µg value is inverse of the genome methylation level. DPM, the ratio of radioactivity counts per min inside the sample compositions in scintillation counters; Q, quartile.

### Table II. Global genome methylation levels in peripheral blood DNA of patients with retinoblastoma (n=26) and their unaffected healthy siblings (n=26).

| Variable       | Patients, n | Siblings, n |
|----------------|-------------|-------------|
| Mean DPM/µg    | 186,536     | 329,189     |
| Median DPM/µg  | 185,859     | 323,112     |
| Q3-Q1          | 240,984     | 472,568     |
| P-value        | 0.02        | -           |

The DPM/µg value is inverse of the genome methylation level. DPM, the ratio of radioactivity counts per min inside the sample compositions in scintillation counters; Q, quartile.
definition of retinoma; the results demonstrated that the expression levels of oncogenes and tumor suppressor genes in retinoma were different compared with those in retinoblastoma. These results may suggest that methylation is one of the molecular switches that serve a role in genomic instability in the progression of retinoma to retinoblastoma. Since tumor tissues of patients with retinoblastoma have a biallelic alteration in the \( RB1 \) gene, extra alterations in the second allele of \( RB1 \) gene are required for retinoblastoma progression (16). \( DNMT1 \) activation is controlled by pRb and E2F transcription factors. McCabe et al (12) and Kooi et al (13) revealed that \( DNMT1 \) was highly expressed in the cells lacking the \( RB1 \) gene. However, the altered \( DNMT1 \) expression can change the methylation status of proto-oncogenes, tumor suppressor genes and other intergenic regions in the genome for the progression of retinoblastoma (51-53). In agreement with studies that emphasized the importance of methylation, the results of the present study suggested that the level of global genome methylation may be important for the oncogenesis and inheritance of retinoblastoma. Berdasco et al (16) have suggested that retinoblastoma may have a specific methylation signature, which needs to be further investigated.

In the present study, the level of global genome methylation was measured in the tumor tissues of patients with retinoblastoma and peripheral blood samples of patients with retinoblastoma, their unaffected siblings and unrelated healthy controls to evaluate whether global genome methylation may be a marker for the inheritance of familial retinoblastoma. The levels of global genome methylation were 1.5-fold higher in DNA derived from the tumor tissue compared with that derived from the peripheral blood in patients with retinoblastoma.

The germline inactivation of \( RB1 \) gene has been found in 25-35% of retinoblastoma (54). Expression of \( DNMT1 \), which is responsible for global genome methylation in cells, is elevated by the loss of \( RB1 \) (12,15). The present study demonstrated that the methylation levels in peripheral blood samples of patients with retinoblastoma were 2.6-fold elevated compared with those in the peripheral blood of their siblings and unrelated healthy controls. The higher global genome methylation levels in peripheral blood samples were associated with a 3.33-fold increased risk for retinoblastoma (P<0.0001). These results suggested that global genome methylation level was higher in patients with retinoblastoma. There was a correlation between the global genome methylation levels in the paired tumor tissues and peripheral blood samples of the patients. The level of global genome methylation in peripheral blood may be used as an alternative biomarker for the inheritance of retinoblastoma following confirmation of these results in a larger study.

The correlations between the expression levels of \( DNMT1, DNMT2, DNMT3a \) and \( DNMT3b \) and the levels of global genome methylation could not be in patients in the present study as the RNAs from the tissues or peripheral blood samples of patients with retinoblastoma were unavailable. Instead, the expression levels of \( DNMT \) genes and the levels of global genome methylation were investigated in a commercial human retinoblastoma tumor cell line which has defective \( RB1 \) and a cell line which has an intact \( RB1 \). High levels of expression of all four \( DNMT \) genes were identified in the cell line with mutant \( RB1 \) compared with the cell line with intact \( RB1 \). In addition, the mean and median values of global genome methylation levels in 27 patients with \( RB1 \) mutations were lower compared with those in all patients with retinoblastoma. These results suggested that

### Table III. Distribution of global genome methylation levels in patients with unilateral (n=46) and bilateral (n=23) retinoblastoma and all healthy controls (n=44), including healthy siblings and unrelated healthy controls.

| Variable            | Patients with bilateral RB, n | Patients with unilateral RB, n | Healthy controls, n |
|---------------------|-------------------------------|-------------------------------|---------------------|
| Mean DPM/µg         | 112,938                       | 159,142                       | 389,569             |
| Median DPM/µg       | 19,988                        | 155,517                       | 346,699             |
| Q3-Q1               | 125,534                       | 233,443                       | 535,779             |
| P-value             | 0.0084                        | 0.0007                        | -                   |

*Compared to healthy controls. The DPM/µg value is inverse of the genome methylation level. RB, Retinoblastoma; DPM, the ratio of radioactivity counts per min inside the sample compositions in scintillation counters; Q, quartile.

### Table IV. Distribution of global genome methylation levels according to the median value in the peripheral blood of patients (n=69) with retinoblastoma and all healthy controls (n=44).

| Variable                        | Patients, n | Healthy controls, n | OR (95% CI)   |
|---------------------------------|-------------|---------------------|---------------|
| Low methylation level, DPM/µg ≥346,699 | 31          | 31                  | 1             |
| High methylation level, DPM/µg <346,699    | 38          | 13                  | 3.33 (0.98-11.35) |

DPM, the ratio of radioactivity counts per min inside the sample compositions in scintillation counters. High DPM/µg value is shown the inverse of methylation levels in the genome.
the levels of DNMT expression were affected by alterations in RB1, which in turn affected the global genome methylata-

Based on these results, high levels of genome-wide methylation may result in inactivation of other genes and regions in the genome and lead to LOH in the second allele of RB1 or other genes in familial and sporadic cases of retinoblastoma. This may explain why retinoblastoma develops rapidly at an early age. In addition, epigenetic alterations in addition to mutations in RB1 are important in the development and inheritance of retinoblastoma (7). The repression of genes that interact with RB1, such as the DNMT gene family, which is involved in de novo methylation, may be important for inactivation of the RB1 gene and development of retinoblastoma disease in patients with familial and sporadic retinoblastoma and may serve a role in the treatment of retinoblastoma and tumor regression. In agreement with previous retinoblastoma methylation studies, the results of the present study emphasized that global genome hypermethylation may serve a functional role in molecular switching and may be responsible for genomic instability during retinoblastoma oncogenesis, which occurs at an early age and progresses rapidly.

In conclusion, the results of the present study demonstrated that retinoblastoma exhibited a unique methylation profile in the entire genome of the peripheral blood in patients with retinoblastoma, highlighted the importance of epigenetics and demonstrated an association between global genome methylation and the expression of DNMT genes, which have not previously been associated with the risk of retinoblastoma and familial retinoblastoma. Therefore, the correlations between RB1 mutation and DNMT gene expression and between DNMT expression and global methylation levels have been shown for the cell lines investigated in the present study. The results are suggested that there are interactions between RB1, DNMTs genes and global methylation in the genome for developing retinoblastoma. The present study had several limitations; the expression levels of DNMT genes could not be measured in due to of the absence of RNA materials in Istanbul University-Oncology Institute, and the RB1 mutation screening could only be performed in 27 of the 69 patients. However, the results demonstrated that global genome methylation may be involved in retinoblastoma oncogenesis, and that this may be determined in the peripheral blood samples of patients with familial retinoblastoma. Understanding the molecular events that initiate and maintain epigenetic gene silencing may lead to the development of clinical strategies for cancer prevention and therapies that reverse the silencing process. The results of the present study present a different perspective of the oncogenesis and inheritance of reti-

Authors’ contributions

RK recruited and referred patients to the study and helped with their follow-up. HY performed the experiments and wrote the manuscript. HCW performed statistical analysis. All the radioactive material required for the experimental processes of the study was provided by RMS. RMS also contributed towards the interpretation of data, writing of the manuscript and language editing of the manuscript. RK edited the manuscript and contributed to criticism and increased the scientific value of writing. HT and EZY recruited healthy cases to the study, collected the data, drew pedigrees, filled in demographic information forms and prepared all documents to transfer the samples to the laboratory in the USA from Turkey and also performed all DNA extractions of specimens in the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in studies involving human particip-

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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