Oxidative Stress and Polymorphism in *MTHFR* SNPs (677 and 1298) in Paternal Sperm DNA is Associated with an Increased Risk of Retinoblastoma in Their Children: A Case–Control Study

Shilpa Bisht¹ Bhavna Chawla² Rima Dada¹

¹ Laboratory for Molecular Reproduction and Genetics, Department of Anatomy, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India

² Ocular Oncology and Pediatric Ophthalmology Service, Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India

Address for correspondence Rima Dada, MD, PhD, Laboratory for Molecular Reproduction and Genetics, Department of Anatomy, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India (e-mail: rima_dada@rediffmail.com).

J Pediatr Genet 2018;7:103–113.

Abstract

Keywords

- retinoblastoma
- oxidative stress
- polymorphism
- methylation

Sperm DNA is considered as the most vulnerable to oxidative stress-induced damage that also impairs global sperm DNA methylation leading to sperm-associated pathologies. C677T and A1298C polymorphisms of the methylene tetrahydrofolate reductase (*MTHFR*) gene affect *MTHFR* enzyme activity. This study was planned as a case–control study to determine the *MTHFR* gene polymorphisms in the fathers of children affected with sporadic nonfamilial heritable retinoblastoma in an Indian population. *MTHFR* polymorphisms for single nucleotide polymorphisms 677 and 1298 were also determined in sporadic nonfamilial heritable retinoblastoma patients to estimate the risk for retinoblastoma development and to evaluate the role of *MTHFR* in retinoblastoma pathogenesis.

Introduction

Retinoblastoma (RB) is the most common pediatric intraocular retinal malignancy, which occurs usually before the age of 5 years and represents 2 to 4% of all the pediatric malignancies.¹ Its incidence is approximately 1 in 15,000 to 20,000 live births that translates to approximately 9,000 newly diagnosed RB cases every year worldwide.² The basis for RB development was first elaborated by Alfred G. Knudson's two-hit hypothesis where he explained that the genetic cause behind RB initiation is loss-of-function mutation in both alleles of the *RB1* tumor suppressor gene.^{3,4} RB is distinguished into two clinical forms: unilateral and bilateral. The unilateral RB (60% of all RB cases) affects only one eye and is generally nonheritable, where both the hits in the *RB1* gene occur in a single somatic retinal progenitor cell, but few of (~15%) unilateral RB cases are heritable, where the first hit in the *RB1* gene is constitutional and the second hit

occurs somatically in one or more retinal cells later in due course of development.^{5–7} The bilateral form (40% of all RB cases) is heritable where the patient inherits the first hit in the *RB1* gene from an affected parent in 25% cases and 75% cases may result due to a de novo germline mutation in utero.^{3,8} Unilateral and bilateral sporadic nonfamilial heritable RB patients are without any family history of RB. In these cases, the constitutional mutation (i.e., first hit in the *RB1* gene) mainly occurs as a de novo paternal germline mutation which may occur during spermatogenesis.^{9,10}

Methylene tetrahydrofolate reductase (*MTHFR*) enzyme encoded by *MTHFR* gene is a key regulatory enzyme of one-carbon metabolism pathway involved in folic acid metabolism. *MTHFR* enzyme catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate and functions as a primary methyl donor for the remethylation of homocysteine to methionine. Methionine is further converted into S-adenosyl methionine (SAM) which serves as a

received

March 13, 2018

accepted after revision

June 5, 2018

published online

July 11, 2018

Copyright © 2018 by Georg Thieme
Verlag KG, Stuttgart · New York

DOI <https://doi.org/10.1055/s-0038-1667037>
ISSN 2146-4596.

universal methyl donor for methylation reactions.¹¹ Methylation plays an important role in maintaining the fertilizing potential of the male gamete as well as integrity of sperm DNA.¹² The activity of the *MTHFR* gene is the highest in testis as compared with any other organ that suggests an important role of *MTHFR* during the process of spermatogenesis regulation.¹³ The most common polymorphic form of *MTHFR*, i.e., 677 C > T, encodes a thermolabile form of the enzyme with reduced methylation activity and is the most common underlying cause for hyperhomocysteinemia.¹⁴ The other common polymorphic form of *MTHFR*, i.e., 1298 A > C, is also associated with a decline in enzyme activity but to a lesser extent than the 677 C > T polymorphism.¹⁵ Studies on *MTHFR* knockout animal models have suggested an important role of *MTHFR* in regulating spermatogenesis and fertility.¹⁶

The variable phenotype of RB tumors has suggested an interplay between the genetic and environmental factors in the pathogenesis of RB, which is now considered as an epigenetic disorder rather than genetic.¹⁷ Therefore, to understand the complete pathophysiology of RB, it is important to understand both, genetic and environmental factors. This may help in early detection of this tumor, prompt treatment and better management. The role of paternal factors such as advanced paternal age and lifestyle factors, including environmental factors/exposures, have a direct impact on fertility status, reproductive outcome, embryonic development, and child's lifelong health.¹² Parental age, particularly advanced paternal age, has a negative impact on reproductive outcomes. This is mainly attributed to genome-wide alterations in the methylation patterns of sperm DNA, deterioration in the quality of gametes, and an increased rate of de novo mutations in the sperm DNA and, thus, increase the risk of cancer in the offspring.¹⁸ Environmental factors, such as diet, also have an important role in pregnancy outcome and health status of the resultant offspring. Paternal folic acid deficiency during the preconception period will affect the one-carbon metabolism pathway in particularly the availability of SAM, the universal methyl group donor. This in turn alters the epigenetic reprogramming of the sperm epigenome, affecting the sperm DNA methylation patterns which in turn alters the gene expression profile.¹⁹ Previously, we have documented that paternal usage of tobacco in the form of cigarette smoking and chewing is associated with induction of oxidative stress and oxidative DNA damage to the sperm. This study revealed elevated reactive oxygen species (ROS) levels, increased DNA fragmentation index (DFI), and higher levels of the oxidative base adduct 8-hydroxy-2'-deoxyguanosine (8-OH2dG) in semen samples of fathers of children affected with sporadic nonfamilial heritable RB as compared with the fathers of healthy children. This suggested that oxidative stress and oxidative DNA damage may serve as a possible etiological factor for the causation of sporadic nonfamilial heritable RB.²⁰ Therefore, we may conclude that factors like advanced paternal age and poor lifestyle habits may contribute to the generation of oxidative stress in the male germline. This, together with *MTHFR* polymorphisms, causes aberrant DNA

methylation patterns in the sperm DNA and further increases the oxidative stress by decreasing the glutathione production.²¹ This may cause a de novo germline mutation (first hit in the *RB1* gene) during the disrupted or dysregulated spermatogenesis. This can be one of the causes for the initiation of sporadic nonfamilial heritable RB as a new germline mutation in *RB1* occurs more frequently and primarily during spermatogenesis.⁹

During the preconception period, the paternal folic acid deficiency increases the misincorporation of uracil that causes a nick in the DNA, increases DNA fragility and chromosome breaks, and may increase the risk for a germline mutation in the *RB1* gene and also increases the risk for the development of other secondary tumors later in life.²² The two most common functional polymorphisms in the low-penetrant gene *MTHFR*, that is, 677 C > T (rs1801133; alanine→valine) and 1298 A > C (rs1801131; glutamate→alanine), reduce enzyme activity which in turn causes differential methylation (hypermethylation of tumor suppressor genes and hypomethylation of oncogenes) and affects nucleotide biosynthesis and DNA repair, thereby forming the underlying basis for the development of various cancers.^{23,24} Hypermethylation of CpG islands in the promoter region of tumor suppressor genes is associated with loss-of-function mutation (gene silencing),²⁵ whereas hypomethylation of oncogenes leads to their hyperactivation or overexpression which favors oncogenesis.^{26,27} Hypomethylation of repetitive elements in the genome, such as long interspersed nuclear elements, short interspersed nuclear elements, *Arthrobacter luteus* repeats, and Satellite 2 DNA, is found to be higher in cancer tissues and associated with metastasis and poor prognosis, as well as poor therapy outcomes.²⁸ In unilateral and bilateral sporadic nonfamilial heritable RB, where the exact etiology for germline *RB1* mutation (first hit) is not known, oxidative stress and oxidative DNA damage, as a result of environmental exposure, such as dietary low folic acid intake and adoption of poor lifestyle habits even before the conception, may have a role in increased risk to develop paternal germline mutation.²⁹

This study was undertaken as a case-control study conducted in fathers of children affected with unilateral and bilateral sporadic nonfamilial heritable RB. From our previously conducted studies, we enciphered that in unilateral and bilateral sporadic nonfamilial heritable RB patients, the first hit in the *RB1* gene is inherited predominantly from father's germline. Therefore, we hypothesized that oxidative stress in the paternal germline, together with the *MTHFR* polymorphisms in single nucleotide polymorphisms (SNPs) 677 C > T and 1298 A > C in the sperm and peripheral blood DNA is associated with an increased risk of sporadic nonfamilial heritable RB development in their children. 8-OH2dG is the most predominant form of oxidatively induced lesions in the cell, and therefore its levels in the cell are direct indicative of the endogenously induced damage in the cell, and hence, represents the risk to oxidatively induced diseases including cancer.³⁰ We have correlated these seminal oxidative stress parameters, i.e., ROS, DFI, and 8-OH2dG with *MTHFR* polymorphisms. We have also investigated the

MTHFR polymorphisms in SNPs C677T (rs1801133) and A1298C (rs1801131) in peripheral blood DNA samples of unilateral and bilateral sporadic nonfamilial heritable RB patients to find out the association between these polymorphisms with an increased risk of RB pathogenesis in a cohort of Indian children.

Materials and Methods

Subjects

During this study period (February 2015–February 2017), 103 fathers of children affected with sporadic nonfamilial heritable RB (unilateral and bilateral) were recruited from Ocular Oncology and Pediatric Ophthalmology Service, Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi. Out of the recruited 103 fathers, 8 refused to take part in the study, and 5 were unable to ejaculate. Therefore, a total of 90 fathers and their children affected with sporadic nonfamilial heritable RB were included in this study. Forty-nine (54.44%) patients were affected with unilateral sporadic nonfamilial heritable RB and 41 (45.56%) were affected with bilateral sporadic nonfamilial heritable RB. The control group for fathers of RB patients consisted of 90 age-matched healthy men, who had fathered a child in the previous 1 year and were recruited from the same geographical region. After approval of the study from the Institute's Ethics Committee (Ref. No. IESC/T-319/23.06.2015), semen samples were obtained thrice by masturbation from the fathers of RB patients as well as controls after 3 to 4 days of sexual abstinence. Only those subjects (fathers of RB patients and healthy male controls) who did not have any history of systemic illness, no participation in a clinical trial within 4 weeks before the study, normal constant healthy eating habits during the entire study, and no change in other physical activities during the entire study were enrolled. Written informed consent was obtained from all the participants. Details of each participant regarding lifestyle habits (smoking, alcohol intake), dietary habits (folic acid intake), and occupation were also recorded through a predesigned questionnaire/proforma. *MTHFR* polymorphisms in SNPs C677T and A1298C were analyzed in the sperm and peripheral blood DNA of fathers of RB patients, as well as healthy controls.

Sporadic nonfamilial heritable RB patients ($N = 90$) (unilateral and bilateral) were also analyzed for *MTHFR* polymorphisms in SNPs C677T and A1298C in their peripheral blood DNA samples. Detailed clinical history regarding presenting complaint with duration, age of onset, prenatal and natal history, family history of cancer, and given therapy was also recorded from all the recruited RB patients. Healthy children who did not have any history of cancer were also recruited from the same geographical area. All the subjects and controls under study were North Indians belonging to Indo-Aryans. *RB1* mutation analysis was also performed in peripheral blood DNA samples of fathers and mothers of RB patients to rule out the mutation in *RB1* gene. Only those RB patients whose parents are negative for *RB1* gene mutation were taken for further analysis.

Semen Analysis

Semen samples were collected in a sterile urine culture vial. After liquefaction at 37°C, standard semen analysis was performed using the World Health Organization laboratory manual for the examination and processing of human semen, Fifth Edition (2010).³¹

DNA Isolation from Sperm and Peripheral Blood

Followed by semen analysis, DNA isolation from semen samples was performed using the HiPurA Sperm Genomic DNA Purification Kit (HIMEDIA) using the standard kit protocol. Genomic DNA extraction from peripheral blood was done by salting-out method.³² The extracted DNA samples (sperm and peripheral blood DNA) were stored at -20°C until used for further experiments.

Estimation of Oxidative Stress Biomarkers in the Male Germline

1. Estimation of ROS in neat semen sample

Following liquefaction, 400 μ L aliquots of neat semen sample was taken and 10 μ L of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma, United States) prepared as a 5-mM stock in dimethyl sulfoxide was added.³³ Luminol served as a probe to measure the basal ROS levels in neat semen sample. Positive and negative controls were made by adding 10 μ L of 5 mM luminol to 400 μ L of H₂O₂ and 400 μ L of phosphate-buffered saline, respectively. ROS levels were measured using luminol-dependent chemiluminescence assay by luminometer (Sirius, Berthold Detection Systems GmbH, Pforzheim, Germany) for 10 minutes in the integrated mode. All the samples were run in duplicates and mean of three readings (semen samples were taken thrice from each subject) were taken for analysis. ROS levels were expressed as relative light unit/sec/million sperm.

2. Sperm chromatin structure assay

Sperm chromatin structure assay was done as prescribed by Evenson.³⁴ Briefly, aliquot from each semen ejaculate was taken and diluted to a final concentration of 2×10^6 sperm/mL in tris sodium chloride-ethylenediaminetetraacetic acid (EDTA) buffer to a total of 200 μ L in a falcon tube. Immediately after this, 400 μ L of acid detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% v/v Triton X-100, pH 1.2) was added to the same falcon tube. Followed by this, exactly after 30 seconds, 1.2 mL of acridine orange (AO)-staining solution (6 μ g AO [chromatographically purified] [Polysciences, Inc., United States] per milliliter citrate buffer [0.037 M citric acid, 0.126 M Na₂HPO₄, 1.1 mM EDTA disodium, 0.15 M NaCl, pH 6.0]) was added. The samples were analyzed using a FAC Scan flow cytometer (BD Biosciences, United States), with an air-cooled argon laser operated at 488 nm and a power of 15 mW. The green fluorescence (FL1) was collected through a 515 to 545 nm bandpass filter, and the red fluorescence (FL3) was collected through a 650-nm long pass filter. The sheath/sample was set on "low," adjusted to a flow rate of 200 events/second when analyzing a sample containing 2×10^6 sperm/mL. After complete analysis of the semen samples for DNA damage/fragmentation, the mean of red fluorescence (X-axis) and mean of green fluorescence (Y-axis) was taken for the analyzed sperm cells.

using the FlowJo software (Ashland, Oregon, United States). Extent of DNA damage was expressed as percentage DFI (%DFI), which is the ratio of red to total fluorescence (red and green), that is, the level of denatured DNA over the total DNA. The percentage of sperm cells with high DNA stainability (due to lack of full protamination and an increased amount of retained histones) were also recorded in each sample manually from the graph plot and excluded during the gating procedure as they may interfere with the actual DFI values. All the samples were tested in duplicates and the mean values were used for comparison.

3. 8-hydroxy-2'-deoxyguanosine estimation

8-OH2dG levels were measured in seminal plasma via enzyme-linked immunosorbent assay using the DNA/RNA Oxidative Damage EIA kit from Cayman Chemical, Ann Arbor, Michigan, United States. This assay is based on the competition between oxidatively damaged guanine nucleotide species (present in the sample) and an 8-OH2dG acetylcholinesterase conjugate (Tracer) (provided in the kit) to bind with a limited amount of DNA/RNA oxidative damage monoclonal antibody. Because the amount of tracer remains constant while the amount of oxidatively damaged guanine species may vary, therefore, the amount of tracer that is able to bind to the monoclonal antibody will be inversely proportional to the concentration of oxidatively damaged guanine in the well. The complete protocol as given in the kit manual was followed. Absorbance was measured at 450 nm using a microplate reader (Biotechnique, United States). The results were calculated with the Cayman data analysis system.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism for Genotyping *MTHFR*

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was done to genotype *MTHFR* SNPs 677 C > T (rs1801133) and 1298 A > C (rs1801131) in sperm DNA and peripheral blood DNA of fathers of RB patients and healthy male controls as well as in the peripheral blood DNA of RB patients and healthy children (controls). The sets of forward and reverse primers (previously described) and the PCR conditions used are described in ▶ **Table 1**.

Briefly, 15 µL of *MTHFR* 677 PCR amplicon of 477 base pair (bp) generated using previously described primers was subjected to 1U of *HinfI* (ThermoFisher Scientific) restriction enzyme digestion at 37°C for 15 hours as per the manufacturer's protocol and resolved in 3.5% agarose gel. The C allele is cut by the enzyme and gives 425 and 52 bp products, whereas the T allele introduces a site for *HinfI* digestion leading to digestion products of 260, 165, and 52 bp (▶ **Fig. 1**).

Ladder
(50 bp)

(3.5% TAE Agarose gel)

Fig. 1 *MTHFR* C677T polymorphism was detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The 477 base pair (bp) was digested with *HinfI*. C allele is cut by the enzyme and yields 425 and 52 bp products, whereas the T allele yields 260, 165, and 52 bp products. Lane 1: 50-bp ladder (New England Biolabs); lanes 2 and 3: CC homozygous; lanes 4, 5, 6, and 7: CT heterozygous.

Fifteen µL of *MTHFR* 1298 PCR amplicon of 163 bp generated using previously described primers was subjected to 1 U of *MboII* (ThermoFisher Scientific) restriction enzyme digestion at 37°C for 15 hours as specified by the manufacturer's protocol and resolved in 3.5% agarose gel. The wild-type allele A yields a 56, 31, 30, 28, and 18 bp fragments. The C allele, on the other hand, abolishes a site for *MboII* leading to digestion products of 84, 31, 30, and 18 bp (▶ **Fig. 2**).

Statistical Analyses

Statistical analysis was done using the GraphPad Prism (Version 6.01) software. Comparison between seminal values of ROS, 8-OH2dG, and DFI in fathers of RB patients and controls was done using the Mann-Whitney test (two-tailed). Calculation of genotype and allele frequencies for *MTHFR* 677 C > T and 1298 A > C in sperm and peripheral blood DNA of fathers of RB patients and healthy male controls as well in peripheral blood DNA of RB patients and controls were determined using Fisher's exact test. Odds ratio (OR) were calculated and reported within 95% confidence interval (CI) using a calculator for CIs of OR based upon the null hypothesis (<http://www.hutchon.net/ConfidORnulhypo.htm>). One-way analysis of variance between subjects was conducted to compare the seminal oxidative stress parameters (ROS, DFI, and 8-OHdG) with combined

Table 1 PCR primers and conditions used for the amplification of *MTHFR* SNPs 677 and 1298

Name of SNP	Primer Sequence (5' to 3')	PCR cycle condition	Product size (bp)	Reference
<i>MTHFR</i> 677 C > T	Fp: GGCTGTGCTGTGCTGTTG Rp: CGCTCTGCAAGTTCTGGA	94°C: 60s, 68°C: 60s, 72°C: 60s (30 cycles)	477	54
<i>MTHFR</i> 1298 A > C	Fp: CTTTGGGGAGCTGAAGGACTACTAC Rp: CACTTTGTGACCATTCGGTTTG	94°C: 30s, 65°C: 35s, 72°C: 35s (30 cycles)	163	55

Abbreviations: bp, base pair; *MTHFR*, methylene tetrahydrofolate reductase; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.

Fig. 2 *MTHFR* A1298C polymorphism was detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The 163 base pair (bp) was digested with *MBOII*. The A allele is cut by the enzyme and yields 56, 31, 30, 28, and 18 bp, whereas the C allele yields 84, 31, 30, and 18 bp products. Lane 1: 50-bp ladder (New England Biolabs); lanes 3 and 6: AA homozygous; lanes 2, 4, 5, and 7: AC heterozygous.

genotypes of *MTHFR* 677 and 1298 in fathers of RB patients and healthy controls. A p -value of < 0.05 was considered as statistically significant.

Results

The demographic data pertaining to sporadic nonfamilial heritable RB patients and controls are summarized in ►Table 2. We have found significantly higher levels of ROS, DFI, and 8-OH2dG (p -value < 0.0001 for all the parameters) in fathers of RB patients as compared with healthy controls (►Table 3, ►Figs. 3–5). The cut-off values for seminal oxidative stress biomarkers, that is, ROS, DFI, and 8-OH2dG, were taken from the study previously published by Kumar et al (2016).²⁰ The standard semen parameters such as sperm motility, count, and morphology were found to be comparable in fathers of RB patients and healthy controls.

To investigate the polymorphism in two essential SNPs of *MTHFR* gene, viz., 677 C > T and 1298 A > C, PCR-RFLP analysis was done in sperm DNA and peripheral blood DNA of fathers of RB patients and healthy controls. For

Table 2 Demographic characteristics of sporadic nonfamilial heritable RB patients and controls assessed for *MTHFR* 677 C > T and 1298 A > C polymorphisms

	Number (N)	Mean age (in y)	Sex ratio (Male/Female)
Cases (RB patients)	90	2.2 ± 1.6	53:37
Controls (healthy children)	90	8.7 ± 2.9	49:41

Abbreviations: *MTHFR*, methylene tetrahydrofolate reductase; RB, retinoblastoma.

Table 3 Comparison of seminal oxidative stress parameters: ROS, DFI, and 8-OH2dG levels between fathers of RB patients and controls

Parameters	Fathers of RB patients (N = 90)	Healthy controls (N = 90)	p -Value
ROS (RLU/sec/million sperm)	52.93 ± 39.05	23.20 ± 11.69	< 0.0001
DFI (%)	38.17 ± 6.825	33.68 ± 8.974	< 0.0001
8-OH2dG (pg/mL)	1,000 ± 751.9	516.3 ± 251.2	< 0.0001

Abbreviations: 8-OH2dG, 8-hydroxy-2'-deoxyguanosine; DFI, deoxyribo-nucleic acid fragmentation index; RB, retinoblastoma; RLU, relative light unit; ROS, reactive oxygen species.

MTHFR 677 C > T, the frequency of wild-type genotype CC was found to be lower in fathers of RB patients (21.11%) as compared with the controls (90%). The frequency of the heterozygous mutant genotype CT was found to be higher in fathers of RB patients (78.9%) as compared with the controls (10%) ($p < 0.0001$) (OR = 16.03; 95% CI = 8.9186–28.8141). None of the RB fathers or controls showed the mutant homozygous genotype TT (►Table 4).

For *MTHFR* 1298 A > C, the frequency of wild-type genotype AA was found to be lower in fathers of RB patients (33.33%) as compared with the controls (88.9%). The frequency of heterozygous mutant genotype AC was found to be higher in fathers of RB patients (66.67%) as compared with the controls (11.11%) ($p < 0.0001$) (OR = 10.2234; 95% CI = 5.6237–18.585). None of the fathers of RB patients or controls showed the mutant homozygous genotype CC (►Table 4). Similar genotyping results for *MTHFR* 677 C > T and *MTHFR* 1298 A > C were found in sperm DNA and peripheral blood DNA of fathers of RB patients and healthy controls (►Table 5).

When age and the levels of seminal oxidative stress parameters (ROS, DFI, and 8-OH2dG) were compared between CC (wild-type) and CT (mutant heterozygous) genotypes of *MTHFR* C677T in sperm DNA of fathers of RB patients, a significantly higher level ($p < 0.05$) of all the parameters were found for the CT genotype (mutant heterozygous) as compared with the CC (wild-type) genotype (►Table 6). Similarly, for *MTHFR* A1298C, a significantly higher level of all the parameters (age, ROS, DFI, and 8-OH2dG) ($p < 0.05$) were found for mutant heterozygous AC genotype as compared with wild-type AA genotype (►Table 7).

To investigate the polymorphism in two essential SNPs of *MTHFR* gene, viz., 677 C > T and 1298 A > C, PCR-RFLP analysis was also done in sporadic nonfamilial heritable RB patients and healthy controls. For *MTHFR* 677 C > T, the frequency of wild-type genotype CC was found to be lower in RB patients (26.67%) as compared with the controls (87.78%). The frequency of the heterozygous mutant genotype CT was found to be higher in RB patients (73.33%) as compared with

Fig. 3 Association of *MTHFR* C677T and A1298C combined genotypes with reactive oxygen species (ROS) values in fathers of retinoblastoma (RB) patients. ROS for heterozygous genotype AC/CT and CC/AC genotype was significantly higher compared with wild genotype AA/CC ($p = 0.002$ and $p = 0.004$, respectively).

the controls (12.22%) ($p < 0.0001$) (OR = 11.979, 95% CI = 6.6443–21.5758). None of the RB patients or controls showed the mutant homozygous genotype TT (►Table 8).

For *MTHFR* 1298 A > C, the frequency of wild-type genotype AA was found to be lower in RB patients (46.67%) as compared with the controls (88.9%). The frequency of the heterozygous mutant genotype AC was found to be higher in RB patients (53.33%) as compared with the controls (11.11%) ($p = 0.0002$) (OR = 3.17, 95% CI = 1.7512–5.7699). None of the RB patients or controls showed the mutant homozygous genotype CC (►Table 8).

Post hoc comparisons using the Tukey's honest significant difference test indicated that the mean scores of ROS

and DFI were significantly higher in the heterozygous genotype AC/CT and CC/AC compared with the wild genotype AA/CC ($p < 0.05$), while the mean scores of 8-OHdG were significantly higher in the heterozygous genotype AC/CT, CC/AC and AA/CT compared with the wild genotype AA/CC ($p < 0.05$).

Discussion

RB accounts for nearly 2.5 to 4% of all the childhood cancers in developed countries, where the overall survival rate of children with RB is high because of early detection and prompt treatment, whereas in a developing country like

DFI values in fathers of RB patients per genotype

Fig. 4 Association of *MTHFR* C677T and A1298C combined genotypes with DNA fragmentation index (DFI) values in fathers of retinoblastoma (RB) patients. DFI for heterozygous genotype AC/CT and CC/AC genotype was significantly higher compared with wild genotype AA/CC ($p = 0.0008$ and $p = 0.0009$, respectively).

8-OHdG values in fathers of RB patients per genotype

Fig. 5 Association of *MTHFR* C677T and A1298C combined genotypes with 8-hydroxy-2'-deoxyguanosine (8-OHdG) values in fathers of retinoblastoma (RB) patients. 8-OHdG for heterozygous genotype AC/CT, CC/AC, and AA/CT genotype was significantly higher compared with wild genotype AA/CC ($p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, respectively).

Table 4 Distribution of *MTHFR* A1298C and C677T genotypes in the sperm DNA of fathers of RB patients and controls

<i>MTHFR</i> polymorphism	Fathers of RB patients (N = 90)	Healthy controls (N = 90)	Odds ratio	95% CI	p-Value
<i>MTHFR</i> 677 C > T					
CC genotype	19 (21.11%)	81 (90%)	1	–	
CT genotype	71 (78.9%)	09 (10%)	16.03	8.9186–28.8141	< 0.0001
TT genotype	0	0	–	–	–
<i>MTHFR</i> 1298 A > C					
AA genotype	30 (33.33%)	80 (88.9%)	1	–	–
AC genotype	60 (66.67%)	10 (11.11%)	10.2234	5.6237–18.585	< 0.0001
CC genotype	0	0	–	–	–

Abbreviations: CI, confidence interval; DNA, deoxyribonucleic acid; *MTHFR*, methylene tetrahydrofolate reductase; RB, retinoblastoma.

Table 5 Distribution of *MTHFR* A1298C and C677T genotypes in peripheral blood DNA of fathers of RB patients and controls

<i>MTHFR</i> polymorphism	Fathers of RB patients (N = 90)	Healthy controls (N = 90)	Odds ratio	95% CI	p-Value
<i>MTHFR</i> 677 C > T					
CC genotype	19 (21.11%)	81 (90%)	1	–	
CT genotype	71 (78.9%)	09 (10%)	16.03	8.9186–28.8141	< 0.0001
TT Genotype	0	0	–	–	–
<i>MTHFR</i> 1298 A > C					
AA genotype	30 (33.33%)	80 (88.9%)	1	–	–
AC genotype	60 (66.67%)	10 (11.11%)	10.2234	5.6237–18.585	< 0.0001
CC genotype	0	0	–	–	–

Abbreviations: CI, confidence interval; DNA, deoxyribonucleic acid; *MTHFR*, methylene tetrahydrofolate reductase; RB, retinoblastoma.

Table 6 Association between *MTHFR* C677T genotypes with age and seminal oxidative stress parameters in the fathers of RB patients

<i>MTHFR</i> C677T genotypes	CC genotype (N = 19)	CT genotype (N = 71)	p-Value
Age	30.42 ± 3.23	30.18 ± 6.08	< 0.05
ROS (RLU/sec/million sperm)	49.71 ± 21.42	53.79 ± 42.62	< 0.05
DFI (%)	37.95 ± 8.19	38.23 ± 6.47	< 0.05
8-OH2dG (pg/mL)	584.44 ± 240.20	778.90 ± 637.21	< 0.05

Abbreviations: 8-OH2dG, 8-hydroxy-2'-deoxyguanosine; DFI, deoxyribonucleic acid fragmentation index; *MTHFR*, methylene tetrahydrofolate reductase; RB, retinoblastoma; RLU, relative light unit; ROS, reactive oxygen species.

Table 7 Association between *MTHFR* A1298C genotypes with age and seminal oxidative stress parameters in the fathers of RB patients

<i>MTHFR</i> A1298C genotypes	AA genotype (N = 30)	AC genotype (N = 60)	p-Value
Age	30.16 ± 3.96	30.26 ± 6.27	< 0.05
ROS (RLU/sec/million sperm)	44.64 ± 21.70	58.02 ± 44.05	< 0.05
DFI (%)	36.71 ± 7.38	36.78 ± 6.25	< 0.05
8-OH2dG (pg/mL)	489.81 ± 249.78	845.59 ± 663.24	< 0.05

Abbreviations: 8-OH2dG, 8-hydroxy-2'-deoxyguanosine; DFI, deoxyribonucleic acid fragmentation index; *MTHFR*, methylene tetrahydrofolate reductase; RB, retinoblastoma; RLU, relative light unit; ROS, reactive oxygen species.

India, where there are limited resources and medical facilities, the incidence of RB is two- to threefold higher than that of developed countries. RB ranks 4th in the list of mortalities in Indian children.^{34,35} The exact etiopathology of this tumor in sporadic nonfamilial heritable unilateral and bilateral cases is still not known, and therefore, the dire need is to explore epigenetic factors which may have an underlying role in RB pathophysiology. This can be attributed to the damage caused in the spermatozoa during spermatogenesis in the preconception period due to external and internal insults, as sperm accumulates mutations at a much higher frequency than the oocyte, which may directly or indirectly

affect the outcome of fertilization and increased risk of genetic and epigenetic disorders in the offspring.³⁶ During each step of spermatogenesis, the controlled regulation of sperm epigenome is important so as to maintain the fertility as well as the embryonic development.³⁷ Several environmental factors and unhealthy lifestyle, such as consumption of tobacco (via smoking and chewing), alcohol consumption, exposure to toxic chemicals, sedentary and highly stressed lifestyle, and physical inactivity may cause seminal oxidative stress, which directly affects the sperm epigenome due to oxidative stress-induced aberrant sperm DNA methylation.^{38,39} Most of the de novo germline mutations in sporadic

Table 8 Distribution of *MTHFR* C677T and A1298C genotypes in RB patients and controls

<i>MTHFR</i> polymorphism	RB patients (N = 90)	Healthy controls (N = 90)	Odds ratio	95% CI	p-Value
<i>MTHFR</i> 677 C > T					
CC genotype	24 (26.67%)	79 (87.78%)	1	–	–
CT genotype	66 (73.33%)	11 (12.22%)	11.979	6.6443–21.5758	< 0.0001
TT genotype	0	0	–	–	–
<i>MTHFR</i> 1298 A > C					
AA genotype	42 (46.67%)	67 (88.9%)	1	–	–
AC genotype	48 (53.33%)	23 (11.11%)	3.17	1.7512–5.7699	0.0002
CC genotype	0	0	–	–	–

Abbreviations: CI, confidence interval; *MTHFR*, methylene tetrahydrofolate reductase; RB, retinoblastoma.

nonfamilial heritable RB patients occur mostly on the paternal *RB1* allele.⁹ This suggested an important and inevitable role of paternal diet (mainly folic acid) during the preconception period and a role in RB initiation in the child. Bunin et al⁴⁰ has reported an important implication of paternal diet (dairy-associated nutrients and calcium supplements) with increased risk of de novo germline mutations in sporadic RB cases.

In our study, we have found a very strong association between two most common polymorphisms of *MTHFR* gene in the sperm and peripheral blood DNA of fathers of RB patients as compared with the healthy controls. For *MTHFR* 677 C > T, we have found that the frequency of mutant heterozygous genotype CT was very high in the sperm DNA of fathers of RB children as compared with the healthy controls. It suggests that males with mutant genotype CT may have disrupted spermatogenesis (*MTHFR* 677 C > T encodes for a thermolabile form of the *MTHFR* enzyme with reduced activity and thus dysregulates the methylation during spermatogenesis),⁴¹ showed higher levels of oxidative stress parameters, and are at higher risk of having a child with sporadic RB (OR being 16.03 for CT genotype). Similarly, for *MTHFR* 1298 A > C, we have found that the frequency of mutant heterozygous genotype AC was very high in the sperm DNA of fathers of RB children as compared with the healthy controls. *MTHFR* 1298 A > C also decreases the *MTHFR* enzyme activity and affects spermatogenesis.⁴² Our findings suggested that males with mutant genotype AC for *MTHFR* SNP 1298 are at higher risk of having a child with sporadic RB (OR being 10.2234 for AC genotype).

Advanced paternal age is associated with accumulation of replicative errors, and prolonged environmental exposures to the sperm genome may lead to de novo germline mutations and aberrant methylation in the sperm genome which may activate oncogenes and suppress tumor suppressor genes.⁴³ This suggests an important risk for the development of neuropsychiatric ailments, congenital disorders, and even childhood cancers in the children born to fathers of advanced age.⁴⁴ Single-point mutations such as single base pair substitutions occur more frequently in the paternal germline and are the underlying cause for sporadic cases of achondroplasia and Apert syndrome.⁴⁵ The oxidative stress parameters such as ROS and DFI have important implications in oxidatively induced sperm DNA damage and oxidative stress causes accelerated testicular aging. It impedes the process of methylation and causes aberrant sperm DNA methylation, leading to male infertility and poor reproductive outcomes including childhood cancers in the next progeny. 8-OH2dG is the most common oxidative DNA base lesion and the most potent indicator of the oxidative state of the cell. 8-OH2dG has a potent mutagenic potential and elevated levels of 8-OH2dG in the cell have repercussion in oxidative stress generation and carcinogenesis.³⁰ A deficiency of micronutrients such as folic acid (methyl group donor) is associated with genomic instability and concomitant increase in the activity of the DNA repair glycosylase to correct the DNA base adduct 8-OH2dG. This indicates that folic acid deficiency is a direct indicative of oxidative stress-induced DNA damage in the cell

and, hence, increased activity of DNA repair glycosylases.^{46,47} In this study, the correlation between *MTHFR* 677 C > T and 1298 A > C genotypes with age and seminal oxidative stress parameters in the fathers of RB patients have suggested a higher level of all the parameters (age, ROS, DFI, and 8-OH2dG) in the mutant heterozygous genotype CT (for *MTHFR* 677) and AC (for *MTHFR* 1298) as compared with the wild-type genotypes for both the SNPs, that is, CC (for *MTHFR* 677) and AA (for *MTHFR* 1298). A significant association ($p < 0.05$) between all the oxidative stress parameters (ROS, DFI, and 8-OH2dG) and *MTHFR* combined genotypes were found for the fathers of RB patients but not for healthy controls.

In RB patients, we have found a greater frequency of mutant heterozygous genotype CT for *MTHFR* 677 C > T as compared with healthy controls. This suggests a strong association between *MTHFR* 677 C > T polymorphism with RB pathogenesis (OR for CT genotype is 11.979 in RB patients). Similarly, for *MTHFR* SNP 1298 A > C, we have found a greater frequency of mutant heterozygous genotype AC in the RB patients as compared with healthy controls. This also suggested a strong association between *MTHFR* 1298 C > T polymorphism with RB pathogenesis (OR for AC genotype is 3.17 in RB patients). A large number of previously documented studies have deciphered the important role of methylation as an important epigenetic regulator of the cell functions and cellular physiology and this has important implications in cancer development.^{48–50} This study suggested the role of *MTHFR* 677 C > T and 1298 A > C polymorphisms in RB patients. This may indicate the underlying cause for RB development as *MTHFR* reduced function leads to the global nonavailability of SAM required for various cellular methylation reactions and may lead to global hypomethylation of the genomic DNA. Hypomethylation, when present within gene-coding regions, has been found to be associated with genomic instability leading to deletions of important regulatory regions in the genome and is associated with unmasking of repetitive elements and transposons and thus transcription and replication.⁵¹ Previously, two studies have documented *MTHFR* polymorphic variants in RB patients. The first one was published by de Lima et al in a cohort of RB patients belonging to Northeastern Brazil, where they have found no significant association between RB susceptibility and *MTHFR* polymorphisms 677 C > T and 1298 A > C. On the other hand, they found a strong association between *MTR* 2756 A > G polymorphism and RB susceptibility in RB patients of Northeastern Brazil.⁵² The other study was published by Soleimani et al, who reported a strong association between 677 C > T polymorphism with risk of RB in an Iranian population. In this study, the authors have reported the protective role of allele T which was found to be significantly lower in the RB patient group compared with the control group. The authors found no association between *MTHFR* 1298 A > C polymorphism with RB pathogenesis.⁵³ Our findings of this study indicate a possible and important role of one-carbon metabolism pathway, methylation regulation, and an increased risk of RB initiation in an Indian subpopulation. According to our knowledge, so far this is the first report where an association has been

investigated between *MTHFR* polymorphism in a subset of Indian RB patients. However, the only limitation of this study is the small population size being analyzed for *MTHFR* polymorphic variants 677 C > T and 1298 A > C. Therefore, before translating the findings of this study, it is required to be done in a large subset of RB patients. This study also concluded that oxidative stress and oxidative DNA damage in the male germline together with the *MTHFR* polymorphisms may play an important synergistic role in the impairment of spermatogenesis and causes sperm DNA damage, which may act as “first hit” in the *RB1* gene during the preconception period, and thus, increasing the risk of unilateral and bilateral sporadic nonfamilial heritable RB and predispose these patients to genetic/epigenetic disorders.

Funding

Financial assistance by the Council of Scientific & Industrial Research (Human Resource Development Group), New Delhi, India, in the form of a Senior Research Fellowship to Ms. Shilpa Bisht is thankfully acknowledged.

Acknowledgments

The authors are thankful to all the retinoblastoma patients for providing the blood samples and their fathers and volunteers for providing blood and semen samples.

References

- Dimaras H, Kimani K, Dimba EA, et al. Retinoblastoma. *Lancet* 2012;379(9824):1436–1446
- Kivelä T. The Epidemiological Challenge of the Most Frequent Eye Cancer: Retinoblastoma, an Issue of Birth and Death. London: BMJ Publishing Group Ltd; 2009
- Knudson AG Jr. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 1971;68(04):820–823
- Friend SH, Bernards R, Rogelj S, et al. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* 1986;323(6089):643–646
- Cavenee WK, Hansen MF, Nordenskjöld M, et al. Genetic origin of mutations predisposing to retinoblastoma. *Science* 1985;228(4698):501–503
- Lohmann DR, Gallie BL. Retinoblastoma: revisiting the model prototype of inherited cancer. *Am J Med Genet C Semin Med Genet* 2004; Aug 15;129C(01):23–28
- Thériault BL, Dimaras H, Gallie BL, Corson TW. The genomic landscape of retinoblastoma: a review. *Clin Experiment Ophthalmol* 2014;42(01):33–52
- Shifa JZ, Gezmu AM. Presenting signs of retinoblastoma at a tertiary level teaching hospital in Ethiopia. *Pan Afr Med J* 2017;28:66
- Zhu XP, Dunn JM, Phillips RA, et al. Preferential germline mutation of the paternal allele in retinoblastoma. *Nature* 1989;340(6231):312–313
- Dryja TP, Mukai S, Petersen R, Rapaport JM, Walton D, Yandell DW. Parental origin of mutations of the retinoblastoma gene. *Nature* 1989;339(6225):556–558
- Selhub J. Folate, vitamin B12 and vitamin B6 and one carbon metabolism. *J Nutr Health Aging* 2002;6(01):39–42
- Stuppia L, Franzago M, Ballerini P, Gatta V, Antonucci I. Epigenetics and male reproduction: the consequences of paternal lifestyle on fertility, embryo development, and children lifetime health. *Clin Epigenetics* 2015;7(01):120
- Gupta N, Sarkar S, David A, et al. Significant impact of the *MTHFR* polymorphisms and haplotypes on male infertility risk. *PLoS One* 2013;8(07):e69180
- Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995;10(01):111–113
- Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (*MTHFR*) associated with decreased enzyme activity. *Mol Genet Metab* 1998;64(03):169–172
- Kelly TL, Neaga OR, Schwahn BC, Rozen R, Trasler JM. Infertility in 5,10-methylenetetrahydrofolate reductase (*MTHFR*)-deficient male mice is partially alleviated by lifetime dietary betaine supplementation. *Biol Reprod* 2005;72(03):667–677
- Mastrangelo D, Loré C, Grasso G. Retinoblastoma as an epigenetic disease: a proposal. *J Cancer Ther* 2011;2(03):362
- Urhoj SK, Raaschou-Nielsen O, Hansen AV, Mortensen LH, Andersen PK, Nybo Andersen AM. Advanced paternal age and childhood cancer in offspring: a nationwide register-based cohort study. *Int J Cancer* 2017;140(11):2461–2472
- Lambrot R, Xu C, Saint-Phar S, et al. Low paternal dietary folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes. *Nat Commun* 2013;4:2889
- Kumar SB, Chawla B, Bisht S, Yadav RK, Dada R. Tobacco use increases oxidative DNA damage in sperm-possible etiology of childhood cancer. *Asian Pac J Cancer Prev* 2015;16(16):6967–6972
- Hoffman M. Hypothesis: hyperhomocysteinemia is an indicator of oxidant stress. *Med Hypotheses* 2011;77(06):1088–1093
- Blount BC, Mack MM, Wehr CM, et al. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. *Proc Natl Acad Sci U S A* 1997;94(07):3290–3295
- Alonso-Aperte E, González MP, Póo-Prieto R, Varela-Moreiras G. Folate status and S-adenosylmethionine/S-adenosylhomocysteine ratio in colorectal adenocarcinoma in humans. *Eur J Clin Nutr* 2008;62(02):295–298
- Newman AC, Maddocks ODK. One-carbon metabolism in cancer. *Br J Cancer* 2017;116(12):1499–1504
- To KF, Leung WK, Lee TL, et al. Promoter hypermethylation of tumor-related genes in gastric intestinal metaplasia of patients with and without gastric cancer. *Int J Cancer* 2002;102(06):623–628
- Van Tongelen A, Lorient A, De Smet C. Oncogenic roles of DNA hypomethylation through the activation of cancer-germline genes. *Cancer Lett* 2017;396:130–137
- Ehrlich M. DNA methylation in cancer: too much, but also too little. *Oncogene* 2002;21(35):5400–5413
- Chen D, Zhang XR, Zhang Y, et al. Hypomethylation of repetitive elements in blood leukocyte DNA and risk of gastric lesions in a Chinese population. *Cancer Epidemiol* 2016;41:122–128
- Aitken RJ, Smith TB, Jobling MS, Baker MA, De Iuliis GN. Oxidative stress and male reproductive health. *Asian J Androl* 2014;16(01):31–38
- Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2'-deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 2009;27(02):120–139
- World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Sperm. Geneva, Switzerland: WHO; 2010
- Suguna S, Nandal D, Kamble S, Bharatha A, Kunkulol R. Genomic DNA isolation from human whole blood samples by non enzymatic salting out method. *Int J Pharm Pharm Sci* 2014;6:198–199
- Venkatesh S, Shamsi MB, Dudeja S, Kumar R, Dada R. Reactive oxygen species measurement in neat and washed semen: comparative analysis and its significance in male infertility assessment. *Arch Gynecol Obstet* 2011;283(01):121–126
- Evenson DP. Sperm chromatin structure assay (SCSA®). *Methods Mol Biol* 2013;927:147–164
- Arora RS, Eden TO, Kapoor G. Epidemiology of childhood cancer in India. *Indian J Cancer* 2009;46(04):264–273

- 36 Bisht S, Faiq M, Tolahunase M, Dada R. Oxidative stress and male infertility. *Nat Rev Urol* 2017;14(08):470–485
- 37 Dada R, Kumar M, Jesudasan R, Fernández JL, Gosálvez J, Agarwal A. Epigenetics and its role in male infertility. *J Assist Reprod Genet* 2012;29(03):213–223
- 38 Sharma R, Biedenharn KR, Fedor JM, Agarwal A. Lifestyle factors and reproductive health: taking control of your fertility. *Reprod Biol Endocrinol* 2013;11(01):66
- 39 Baccarelli A, Bollati V. Epigenetics and environmental chemicals. *Curr Opin Pediatr* 2009;21(02):243–251
- 40 Bunin GR, Li Y, Ganguly A, Meadows AT, Tseng M. Parental nutrient intake and risk of retinoblastoma resulting from new germline RB1 mutation. *Cancer Causes Control* 2013;24(02):343–355
- 41 Kumar K, Venkatesh S, Sharma PR, Tiwari PK, Dada R. DAZL 260A > G and MTHFR 677C > T variants in sperm DNA of infertile Indian men. *Indian J Biochem Biophys* 2011;48(06):422–426
- 42 Rezgoune M, Chellat D, Abadi N, Satta D. MTHFR A1298C gene polymorphism and the risk of male infertility in Algerian population. *Int J Pharm Sci Rev Res* 2016;36(01):73–76
- 43 Bennett-Baker PE, Wilkowski J, Burke DT. Age-associated activation of epigenetically repressed genes in the mouse. *Genetics* 2003;165(04):2055–2062
- 44 Sharma R, Agarwal A, Rohra VK, Assidi M, Abu-Elmagd M, Turki RF. Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. *Reprod Biol Endocrinol* 2015;13(01):35
- 45 Conti SL, Eisenberg ML. Paternal aging and increased risk of congenital disease, psychiatric disorders, and cancer. *Asian J Androl* 2016;18(03):420–424
- 46 Duthie SJ. Folic acid deficiency and cancer: mechanisms of DNA instability. *Br Med Bull* 1999;55(03):578–592
- 47 Fenech MF. Dietary reference values of individual micronutrients and nutriones for genome damage prevention: current status and a road map to the future. *Am J Clin Nutr* 2010;91(05):1438S–1454S
- 48 Heijmans BT, Boer JM, Suchiman HE, et al. A common variant of the methylenetetrahydrofolate reductase gene (1p36) is associated with an increased risk of cancer. *Cancer Res* 2003;63(06):1249–1253
- 49 Xu X, Chen J. One-carbon metabolism and breast cancer: an epidemiological perspective. *J Genet Genomics* 2009;36(04):203–214
- 50 Lissowska J, Gaudet MM, Brinton LA, et al. Genetic polymorphisms in the one-carbon metabolism pathway and breast cancer risk: a population-based case-control study and meta-analyses. *Int J Cancer* 2007;120(12):2696–2703
- 51 Stern LL, Mason JB, Selhub J, Choi SW. Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase gene. *Cancer Epidemiol Biomarkers Prev* 2000;9(08):849–853
- 52 de Lima ELS, da Silva VC, da Silva HDA, et al. MTR polymorphic variant A2756G and retinoblastoma risk in Brazilian children. *Pediatr Blood Cancer* 2010;54(07):904–908
- 53 Soleimani E, Saliminejad K, Akbari MT, Kamali K, Ahani A. Association study of the common polymorphisms in the folate-methionine pathway with retinoblastoma. *Ophthalmic Genet* 2016;37(04):384–387
- 54 Mostowska A, Hozyasz KK, Wojcicki P, Dziegielewska M, Jagodzinski PP. Associations of folate and choline metabolism gene polymorphisms with orofacial clefts. *J Med Genet* 2010;47(12):809–815
- 55 Friedman G, Goldschmidt N, Friedlander Y, et al. A common mutation A1298C in human methylenetetrahydrofolate reductase gene: association with plasma total homocysteine and folate concentrations. *J Nutr* 1999;129(09):1656–1661