RESEARCH ARTICLE

Role of MTHFR Gene Polymorphisms in Male Infertility

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Abstract

Background: Folate metabolism plays an important role in appropriate cellular function, DNA methylation, repair, and synthesis. C677T and A1298C variants of methylenetetrahydrofolate reductase (MTHFR) play a role in reduced plasma folate and increase the susceptibility to various multifactorial disorders.

Aim and objective: The present study was aimed to detect the association of C677T polymorphism and A1298C polymorphism in the MTHFR gene with male infertility.

Materials and methods: In the current study, we analyzed a group of 50 infertile men with a clinical history of nonobstructive azoospermia or severe oligozoospermia. For the control group, we also analyze 50 fertile men. Cytogenetic analysis revealed a normal male karyotype in 50 cases of infertile men, which further subjected to molecular analysis. The expected genotype and allele frequencies were calculated for both infertile men and controls. These frequencies were tested when the study group followed Hardy–Weinberg equilibrium. The interaction between the MTHFR genotypes was calculated using the odds ratio for mutant genotypes as compared to the wild types. To evaluate the risk of the different genotypes, 95% confidence intervals (CI) were calculated.

Results: The A1298C polymorphism of the MTHFR gene was present at a statistically increased significance in infertile men.

Interpretation and conclusion: We concluded that MTHFR C677T gene polymorphism is not associated with male infertility whereas A1298C gene polymorphism showed a significant increase in male infertility. To better understanding the causes of male infertility, future studies to be conducted in a large population to obtain a better understanding of the complex gene-to-gene interactions.

Keywords: Case–control study, DNA methylation, Karyotyping, Male infertility, Nonobstructive azoospermia, Severe oligozoospermia, Single-nucleotide polymorphism.

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Introduction

Male infertility is a complex problem that commonly occurs due to a deficiency in semen. Around 15% of the couples are unable to conceive after 1 year of unprotected intercourse.[1,2] Many studies show that male infertility has an important role in genetic factors.[3] In the folic acid metabolism pathway, abnormal spermatogenesis is associated with genetic variants.[4]

Male infertility occurs due to hypogonadism, testicular maldescence, and structural abnormalities of the male genital tract varicoceles, chronic illness, medications, and exposure to chemicals. About 40% of cases were showing no causes associated with male infertility problems. Several studies have reported that genetic factors are associated with idiopathic male infertility cases. Deletion or translocation in the region of azoospermia factor (AZF) in the Y chromosome were identified in men with unexplained oligozoospermia and azoospermia.[5,6] Most of the abnormalities like chromosomal disorder and AZF deletions are *de novo* in the parental germ cells.

Folic acid metabolism is important for the integrity and stability of the genome.[7] Deficiency in folate metabolism may lead to damage in the function of the metabolic pathway resulting in abnormal DNA synthesis and methylation.

Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme that is present in the metabolism of methionine folate and involved in the process of DNA synthesis and regulation of homocysteine levels *in vivo*, which is closely related to spermatogenesis.[8] The MTHFR gene is located in the short arm of chromosome 1 (1p36.3) and is composed of 11 exons.[9,10] Based on the previous reports, C677T (rs1801133) and A1298C (rs1801131) polymorphic variants are two common variants in the gene encoding MTHFR.

The diminishing activity of MTHFR can cause several disorders, including male infertility.[11,12] A1298C polymorphism of MTHFR causes the conversion of glutamine to alanine at codon 429 of the protein which is located at exon 7 resulting in a decrease in enzymatic activity. C677T variants replace alanine with valine which results in increased thermolability and reduced MTHFR specific activity.[9] Our study was aimed to detect the association of C677T

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Conflict of interest: None

polymorphism and A1298C polymorphism in the MTHFR gene with male infertility.

Materials and Methods

The current investigation was studied among cases and control which is executed on 50 infertile patients in those 35 cases with severe oligozoospermia and 15 cases with nonobstructive azoospermia, and a control group of 50 fertile men who had at least one or two children. Peripheral blood samples were collected from Reproductive Medicine and Surgery (SMART) clinic, Sri Ramachandra Institute of Higher Education and Research (Deemed to be University), and Kanmani Fertility Center, T. Nagar, Chennai, respectively, were included in the study.

The study was approved by the Sri Ramachandra Institutional Ethics Committee (Ref. No.: IEC-NI/12/OCT/30/47). The study was conducted at the Department of Human Genetics, Sri Ramachandra Institute of Higher Education and Research (Deemed to be University). Peripheral blood samples were collected from both the patients and controls after obtaining an informed consent duly signed by the subjects who were agreeable to take part in the study.

In our case–control study, genetic analysis was performed on both the cases and controls, and the variation between the cases and controls was utilized for the calculation of relative risk.

The inclusion criteria for cases are infertile men with non-obstructive azoospermia and severe oligozoospermia, whereas men with secondary infertility were exclusion criteria for case subjects. The inclusion criteria for control subjects are men who had fathered at least two children after 1 or 2 years of marriage.

Cytogenetic Analysis

Chromosomal studies are performed by collecting 3 mL of peripheral blood in a sodium heparin vacutainer. Blood cultures were set up in RPMI 1640 media with 20% fetal bovine serum. The cultures were stimulated with phytohemagglutinin (PHA-M) and incubated for 72 hours at 37°C in a CO_2 incubator. The colchicine (1 mg/mL) was added to the culture to arrest the cells at the metaphase stage at the 67th hour, followed by incubation of 1 hour, and were treated with 0.075 M potassium chloride (KCl). The cell pellet was fixed with Carnoy fixative (methanal:acetic acid, 3:1). The chromosomes were prepared on prechilled slides and incubated for the aging of the slides. The chromosome preparations were subjected to GTG-banding using standard procedure.[13] At least 25 metaphase chromosomes per case/slide were analyzed microscopically for structural and/or numerical abnormalities were detected and three metaphases per case were karyotyped using the Cytovision 2.7 Version software.

Cases with numerical and structural abnormalities were excluded from the study subject. Only the normal karyotype subjects were included for the molecular analysis.

Molecular Analysis

Genomic DNA extraction was performed using standard salting out the procedure by collecting blood samples (3 mL) in vacutainer EDTA tubes and 0.8% agarose gel electrophoresis is used to check the qualities of DNA.

The thermocycler was used to perform PCR reactions by using the following primers for C677T forward 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ and reverse 5′-AGGACGGTGCGGTGAGAG TG-3′ and primers for A1298C forward 5′-CTTTGGGGAGCTGAAGGACTACTAC-3′ and reverse 5′-CACTTTGTGACCATTCCGGTTTG-3′. PCR conditions were set up as followed, initial step consisting initial denaturation for 5 minutes at 95°C, denaturation for 45 seconds at 94°C, annealing for 1 minute at 65°C, extension for 1 minute at 72°C, followed by 30 cycles of denaturation for 45 seconds at 94°C, extension for 1 minute at 72°C, and a final extension time of 5 minutes at 72°C and bring it to 4°C. By using a 2% agarose gel, the obtained PCR product was validated and visualized using a gel documentation system (Bio-Rad Laboratories India Pvt. Ltd). Thus, *in vitro* amplification of the gene of interest by PCR was performed.

The PCR products were then added to the wells and subjected to the sequencing PCR reaction. Sequencing PCR was performed using GeneAmp 9600 thermal cycler (Perkin-Elmer Applied Biosystems GeneAmp 9600 PCR System). The reaction conditions are as follows: 95°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. The conditions were repeated for 30 cycles, following which they were subjected to sequencing (ABI 3730).

Statistical Analysis

The expected genotype and allele frequencies were calculated for the cases and controls. These frequencies were used to test when the study group followed Hardy–Weinberg equilibrium. The interaction of the MTHFR genotypes was evaluated by calculating the odds ratio for mutant genotypes as compared to the wild types. To evaluate the risk of the different genotypes, 95% confidence intervals (CI) were calculated. The analysis was done using SPSS (Statistical Package for the Social Sciences v 16.0).

Results

In the present study, C677T and A1298C variants of MTHFR gene polymorphisms were analyzed in a case group of 50 infertile men with nonobstructive azoospermia or severe oligozoospermia and a control group of 50 fertile men who had fathered at least two children. Cytogenetic analysis of case subjects showed a normal karyotype of 46, XY (Fig. 1).

After performing cytogenetic studies, DNA was isolated successfully using the high salting out protocol. After checking the quality of DNA, the PCR amplicons were confirmed by agarose gel electrophoresis.

Sequence Analysis

The PCR products were subjected to DNA sequencing for identifying the genotypes. The genotypes were assigned using the Chromas LITE software v2.1. All three possible genotypes were observed in the present study.

Figure 2 represents the genotype of C677T gene polymorphism and Figure 3 represents the genotype of A1298C gene polymorphism.

Table 1 represents the three different genotypes for the polymorphisms of the MTHFR gene that have been analyzed in the present study.

Genotype distribution of MTHFR C677T gene polymorphism in cases–controls is shown in Table 2.

The expected genotype frequencies were calculated and the distributions of genotypes follow the Hardy–Weinberg equilibrium among both the cases and the controls. Analysis of the samples revealed that the CT (T allele and C allele) genotype was found to be higher (30%) in cases when compared to the controls (12%). However, in both the cases and controls, two mutant TT alleles (6.67%) were also present.

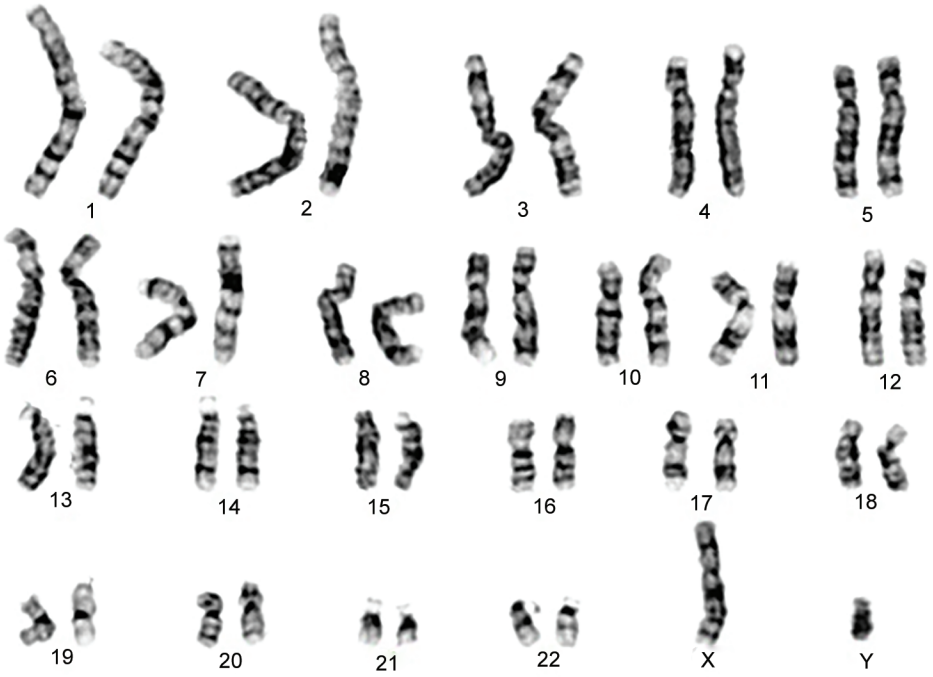

Fig. 1: Normal male karyotype

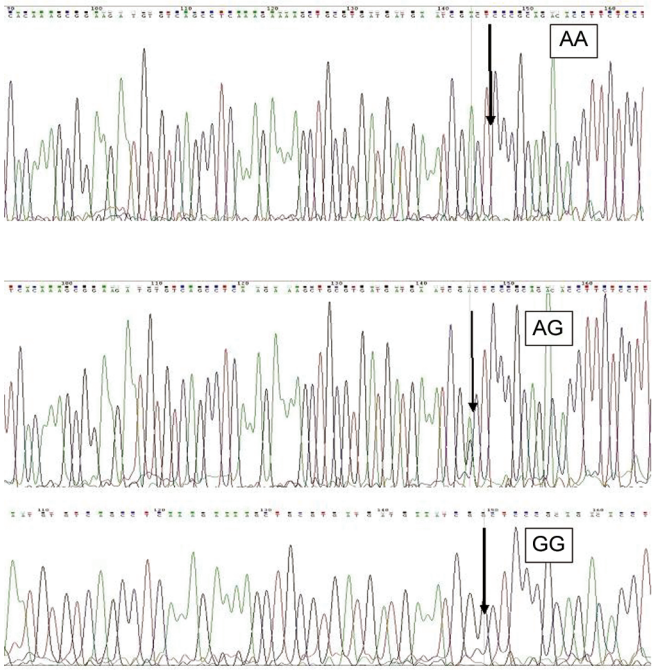

Fig. 2: MTHFR genotypes observed in the present study for C677T polymorphism

Genotype distribution of MTHFR A1298C gene polymorphism in the case–control study is shown in Table 3. For both cases and controls, the distributions of genotypes were followed the Hardy–Weinberg equilibrium. Analysis of the samples revealed that the AC genotype was found to be higher (60%) in cases when compared to the controls (52%). However, in both the cases and controls, mutant CC alleles were also present.

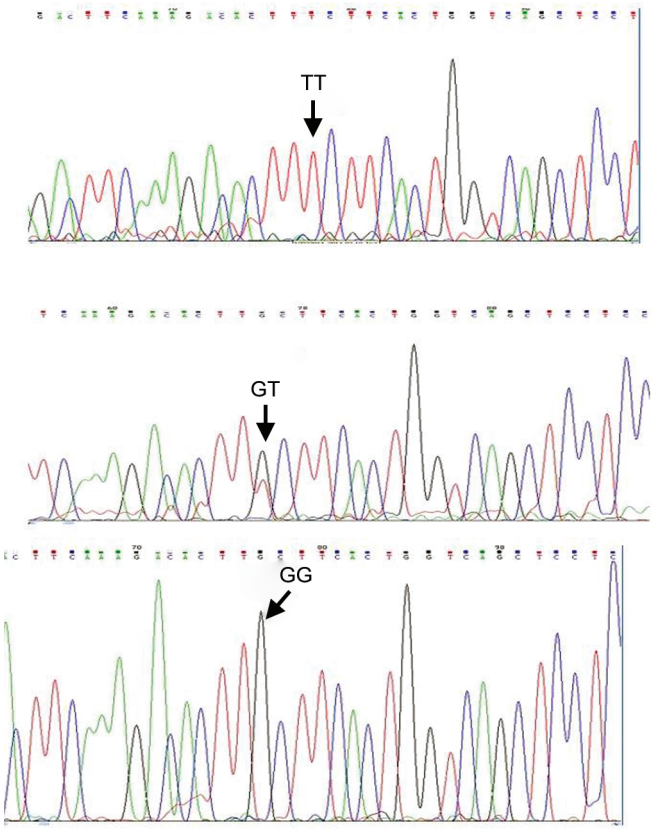

Fig. 3: MTHFR genotypes observed in the present study for A1289C polymorphism

Analysis of MTHFR C677T gene polymorphism in cases–controls is shown in Table 4. With CC as the reference genotype, the OR

(95% CI) for heterozygosity and homozygosity of the T allele was determined. The results revealed no significant increase (*p* value: 0.048) in the frequency of CT allele among the cases and controls (OR: 3.1; 95% CI: 1.11–9.10). Similarly, the TT allele frequency among the cases and controls was not significant (*p* value: 1.00). The results suggest that the two study groups were statistically non-significant.

Analysis of MTHFR A1298C gene polymorphism in cases–controls is shown in Table 5. With AA as the reference genotype, the OR (95% CI) for heterozygosity and homozygosity of the C allele was determined. The results revealed no significant increase (*p* value: 0.63) in the frequency of the CA allele among the cases and controls (OR: 0.66; 95% CI: 0.23–1.92). But, CC allele frequency among the cases and controls revealed significant (OR: 0.19; 95% CI: 0.04–0.793: *p* value: 0.042). The results suggest a statistically significant increase.

Table 1: Different genotypes analyzed in the present study for both C677T and A1298C gene polymorphism

C677T gene polymorphism	
C/C (G/G)	Wild type
C/T (G/A)	Heterozygous
T/T (A/A)	Mutant allele
A1298C gene polymorphism	
A/A(T/T)	Wild type
A/C (T/G)	Heterozygous
C/C (G/G)	Mutant allele

Discussion

In the current study, we aimed to evaluate the possibility of an association between MTHFR gene polymorphisms [two single-nucleotide polymorphisms (SNPs)—the C677T and A1298C polymorphisms] and male infertility. A few previous studies have shown the association of MTHFR C677T polymorphism in infertile patients from Germany, Netherlands, Italy, India, South Korea and China,[14–20] five of them[14,17–19] have reported an association between the polymorphism in the MTHFR gene and male infertility. However, the MTHFR A1298C SNP has been studied less. Varinderpal et al.[21] reported that there is no association between the A1298C SNP and male infertility in an Indian study group; while another study done on Chinese infertile men also[19] concluded that there is no association between this SNP and the idiopathic cases of male infertility. A previous study reported increased sperm

Table 2: Genotype distribution of MTHFR C677T gene polymorphism in cases–controls

		Genotypes			*Allele frequency*		*p value*
C677T (rs1801133)		CC	CT	TT	C	T	
Controls (*N* = 50)	Observed	42	6	2	0.90	0.10	0.018
	Expected	40.5	9.0	0.5			
Cases (*N* = 50)	Observed	33	15	2	0.81	0.19	0.85
	Expected	32.8	15.4	1.8			

Table 3: Genotype distribution of MTHFR A1298C gene polymorphism in cases–controls

		Genotypes			*Allele frequency*		*p value*
A1298C (rs1801131)		AA	AC	CC	A	C	
Controls (*N* = 46)	Observed	8	24	14	0.43	0.57	0.6
	Expected	8.7	22.6	14.7			
Cases (*N* = 40)	Observed	12	24	4	0.60	0.40	0.11
	Expected	14.4	9	6.4			

Table 4: Analysis of MTHFR C677T gene polymorphism in cases–controls

Genotypes	*Controls (N = 50)*	*Cases (N = 50)*	*OR*	*CI*	*p value*
C677T (rs1801133)					
CC	42	33	1.0		
CT	6	15	3.1	1.11–9.10	0.048
TT	2	2	1.27	0.17–9.52	1.00

OR, odds ratio; CI, confidence interval

Table 5: Analysis of MTHFR A1298C gene polymorphism in cases–controls

Genotypes	*Controls (N = 46)*	*Cases (N = 40)*	*OR*	*CI*	*p value*
A1298C (rs1801131)					
AA	8	12	1.0		
AC	24	24	0.66	0.23–1.92	0.63
CC	14	4	0.19	0.04–0.793	0.042

OR, odds ratio; CI, confidence interval

concentration by folic acid and zinc sulfate treatment.[15] This altered folate status occurred due to reduced MTHFR enzyme activity, so important etiological factors are epigenetic alterations in DNA. DNA methylation typically occurs in CpG dinucleotide (i.e., a cytosine directly followed by a guanine) rich regions, CpG islands, highly conserved sequences in promoter regions or first exons of genes.[22] Because of the strong correlation between DNA methylation in promoter regions and transcriptional repression,[22] for epigenetic control of gene expression, DNA methylation plays a vital role in fundamental as well as potentially reversible mechanisms. There is a piece of evidence that hypermethylation is involved in carcinogenesis meanwhile this phenomenon contributes to the suppression of gene transcription.[23] Another study also reported the association of MTHFR A1298C gene polymorphism with male infertility which is correlating with our present study.[24]

In the present study, cytogenetic analysis on 50 infertile males including nonobstructive azoospermia and severe oligozoospermia has shown a normal karyotype of 46, XY. A significant number of infertile men; however, present with a history associated with fertility problems had normal findings on cytogenetics testing.

After confirmation of the cytogenetic analysis as normal for 50 cases, the molecular study was carried out on both the cases and controls to identify the association of C677T polymorphism and A1298C polymorphism in MTHFR with male infertility. PCR-amplified DNA using genomic DNA was carried out in direct sequencing. The PCR-amplified 198 bp DNA fragment of SNP C677T and amplified fragment of 163 bp of SNP A1298C were subjected to DNA sequencing that identified the genotypes. We performed association tests for both SNPs. The observed genotype frequencies among the case groups were in agreement with the Hardy–Weinberg equilibrium. Likewise, the genotype frequencies of controls were in agreement with Hardy–Weinberg equilibrium. Also, for all the association tests the value of odds ratios around 1.0, revealing that 677 C>T SNP in the MTHFR gene is distributed equally in infertile and fertile males. In our study, we have obtained allelic frequency for C677T (TT value is 0.10 for cases and for controls TT value is 0.19) and allelic frequency for A1988C (CC value is 0.40 for cases and for controls CC value is 0.57).

Conclusion

In our present study, the genotype and allelic distribution of the MTHFR gene polymorphisms observed were not significant for C677T gene polymorphism whereas A1298C showed a significant increase in male infertility. We conclude that MTHFR A1298C gene polymorphism supports the hypothesis that it may have a close relationship with male infertility. To better understanding of causes of male infertility, future studies need to be conducted on the large population to focus on identifying new candidate genes to obtain a better understanding of the complex gene-to-gene interactions which have a profound effect on male infertility.

References

1. Hirsh A. Male subfertility. BMJ 2003;327(7416):669–672. DOI: 10.1136/bmj.327.7416.669.
2. Jarvi K, Lo K, Fischer A, et al. CUA guideline: the workup of azoospermic males. Can Urol Assoc J 2010;4(3):163–167. DOI: 10.5489/cuaj.10050.
3. Miyamoto T, Minase G, Okabe K, et al. Male infertility and its genetic causes. J Obstet Gynaecol Res 2015;41(10):1501–1505. DOI: 10.1111/jog.12765.
4. Shen O, Liu R, Wu W, et al. Association of the methylenetetrahydrofolate reductase gene A1298C polymorphism with male infertility: a meta-analysis. Ann Hum Genet 2012;76(1):25–32. DOI: 10.1111/j.1469-1809.2011.00691.x.
5. Waseem AS, Singh V, Makker GC, et al. AZF deletions in Indian populations: original study and meta-analyses. J Assist Reprod Genet 2020;37(2):459–469. DOI: 10.1007/s10815-019-01661-0.
6. Dohle GR, Halley DJ, Van Hemel JO, et al. Genetic risk factors in infertile men with severe oligozoospermia and azoospermia. Hum Reprod 2002;17(1):13–16. DOI: 10.1093/humrep/17.1.13.
7. Ravel C, Chantot-Bastaraud S, Chalmey C, et al. Lack of association between genetic polymorphisms in enzymes associated with folate metabolism and unexplained reduced sperm counts. PloS One 2009;4(8):e6540. DOI: 10.1371/journal.pone.0006540.
8. Ni W, Li H, Wu A, et al. Lack of association between genetic polymorphisms in three folate-related enzyme genes and male infertility in the Chinese population. J Assist Reprod Genet 2015;32(3):369–374. DOI: 10.1007/s10815-014-0423-9.
9. 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(1):111–113. DOI: 10.1038/ng0595-111.
10. Goyette P, Pai A, Milos R, et al. Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR). Mamm Genome 1998;9(8):652–656. DOI: 10.1007/s003359900838.
11. Chen Z, Karaplis AC, Ackerman SL, et al. Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. Hum Mol Genet 2001;10(5):433–443. DOI: 10.1093/hmg/10.5.433.
12. Kelly TLJ, Neaga OR, Schwahn BC, et al. Infertility in 5,10-methylenetetrahydrofolate reductase (MTHFR)-deficient male mice is partially alleviated by lifetime dietary betaine supplementation. Biol Reprod 2005;72(3):667–677. DOI: 10.1095/biolreprod.104.035238.
13. Barch MJ, Knutsen T, Spurbeck JL, ed. The AGT cytogenetics laboratory manual. 3rd ed., 1997.
14. Bezold G, Lange M, Peter RU. Homozygous methylenetetrahydrofolate reductase C677T mutation andmale infertility. New Engl J Med 2001;344(15):1172–1173. DOI: 10.1056/NEJM200104123441517.
15. Ebisch IMW, van Heerde WL, Thomas CMG, et al. C677T methylenetetrahydrofolate reductase polymorphisminterferes with the effects of folic acidand zinc sulftate on sperm concentration. Fertil Steril 2003;80(5):1190–1194. DOI: 10.1016/S0015-0282(03)02157-5.
16. Stuppia L, Gatta V, Scarciolla O, et al. The methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism and male infertility in Italy. Jendocrinol Invest 2003;26(7):620–622. DOI: 10.1007/BF03347018.
17. Singh K, Singh SK, Sah R, et al. MutationC677T in the methylenetetrahydrofolate reductase gene is associated with male infertility in an Indian population. Int J Androl 2005;28(2):115–119. DOI: 10.1111/j.1365-2605.2004.00513.x.
18. Park JH, Lee HC, Jeong Y-M, et al. MTHFR C677T polymorphism associates with unexplained infertile male factors. J Assis Reprod Genet 2005;22(9-10):361–368. DOI: 10.1007/s10815-005-6795-0.
19. Lee HC, Jeong YM, Lee SH, et al. Association study of four polymorphisms in three folate-related enzyme genes with non-obstructive male infertility. Hum Reprod 2006;21(12):3162–3170. DOI: 10.1093/humrep/del280.
20. Zhou-Cun A, Yang Y, Si-Zhong Z, et al. Single nucleotide polymorphism C677T in the methylenetetrahydrofolate reductase gene might be a genetic risk factor for male infertility for Chinese men with azoospermia or severe oligozoospermia. Asian J Androl 2007;9(1):57–62. DOI: 10.1111/j.1745-7262.2007.00225.x.

21. Varinderpal SD, Mohd S, Syed AH. Associations of MTFR DNMT3b 4977 bp deletion in mtDNA and GSTM1 deletion, and aberrant CpG island hypermethylayion of GSTM1in non-obstructive infertility in Indian men. Mol Hum Reprod 2007;13(4):213–222. DOI: 10.1093/molehr/gal118.
22. Robertson KD, Wolfe AP. DNA methylation in health and disease. NatRev Genet 2000;1(1):11–19. DOI: 10.1038/76862.
23. Siegfried Z, Eden S, Mendelsohn M, et al. Gene silencing in mammalian cells and the spread of DNA methylation. Nat Genet 1999;22(2):203–206. DOI: 10.1038/9727.
24. Kumar J, Das SK, Sharma P, et al. Homocysteine levels are associated with MTHFR A1298C polymorphism in Indian population. J Hum Genet 2005;50(12):655–663. DOI: 10.1007/s10038-005-0313-1.