Lack of association between genetic polymorphisms in three folate-related enzyme genes and male infertility in the Chinese population

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Abstract

Purpose This study explored the possible association of four single nucleotide polymorphisms (SNPs) of the three folate-related enzyme genes: *MTHFR* C677T and A1298C, *MTR* A2756G and *MTRR* A66G, with male infertility in the Chinese population.

Methods The polymorphic distributions of the four SNPs (*MTHFR* C677T and A1298C, *MTR* A2756G and *MTRR* A66G) were investigated by the method of SNaPshot in a Chinese cohort including 296 idiopathic infertile males with azoospermia or oligozoospermia and 204 fertile males.

Results We found no evidence for an association between any of these variants (*MTHFR* C677T and A1298C, *MTR* A2756G and *MTRR* A66G) and male infertility.

Conclusions There is no evidence for an association between male infertility and polymorphism of the three folate-related enzyme genes in the Chinese population.

Keywords Male infertility · Single nucleotide polymorphisms · *MTHFR* · *MTR* · *MTRR*

Capsule The four SNPs of the three folate-related enzyme genes: *MTHFR* C677T and A1298C, *MTR* A2756G and *MTRR* A66G, are not associated with male infertility in the Chinese population

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Introduction

Infertility is estimated to affect 10–15 % of couples, and approximately half of these infertile couples are due to male infertility [1]. Despite significant advances in technologies and diagnostic methods in the field of male infertility, the etiology of about 50 % of cases remains unknown [2]. It has been suggested that genetic factors contribute up to 15–30 % of male factor infertility [3]. The genetic factors involved in male infertility include Y chromosome microdeletions, chromosomal or monogenic disorders, mitochondrial DNA (*MTDNA*) mutations, imprinting disorders, or endocrine disorders of genetic origin [4]. Spermatogenesis impairment is the most common form of male infertility, and many genetic factors are involved in impaired spermatogenesis [4, 5].

Folates are essential for DNA synthesis, RNA synthesis, methylation reactions, and protein synthesis [6]. The metabolism of folates play a key role in the maintenance of genome integrity due to its role in DNA synthesis, repair, and methylation [7]. The methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*) and *MTR* reductase (*MTRR*) enzymes are key regulatory enzymes in folate metabolism and are critical for DNA methylation and spermatogenesis. *MTHFR* knockout mice show phenotypes with abnormalities in general development, severe oligospermia and infertility [8]. Long-term treatment of betaine, an alternative methyl donor for remethylation of homocysteine, partially restored both spermatogenesis and fertility in *MTHFR*^{-/-} male mice [8]. Boxmeer et al. have demonstrated that low concentrations of folate in seminal plasma is associated with increased levels of sperm DNA damage in fertile men, and suggested that low folate in the microenvironment of spermatozoa may have adverse effects on sperm DNA stability [9]. The human *MTHFR* gene is composed of 11 exons and located on the short (p) arm of chromosome one (1p36.3) [10], catalyzing the reduction of

methylene tetrahydrofolate (5,10-methyl THF) to methyl tetrahydrofolate (5-methyl THF) [11]. *MTHFR* C677T (rs1801133) and *MTHFR* A1298C (rs1801131) are common polymorphisms in the *MTHFR* gene. The 677C>T (Ala222Val) SNP results in alanine-to-valine substitution within the catalytic domain of the *MTHFR* enzyme at codon 222 and the 1298A>C (Glu429Ala) SNP results in glutamate-to-alanine substitution at codon 429 [12]. Both polymorphisms have been shown to result in some impairment of enzymatic activity. Methionine synthase (MS) catalyzes the transfer of a methyl group from 5-methyl THF to homocysteine, which produces methionine and THF [13]. A common polymorphism at position 2756 within the *MTR* gene (A2756G, rs1805087) results in a transition from adenine to guanine that was thought to be associated with enzyme activity [14]. *MTRR* catalyzes the reductive methylation of MS using *S*-adenosylmethionine providing the methyl group, and may also participate in DNA methylation [15]. A 66A→G substitution specifying an isoleucine to methionine substitution (I22M) is a common *MTRR* polymorphism [15].

A large number of molecular epidemiological studies have been conducted to evaluate the role of the three folate-related enzyme gene (*MTHFR*, *MTR* and *MTRR*) polymorphisms in male infertility [11, 13, 15–31]. However, the association between polymorphism and the risk of male infertility is still controversial. Here, our study assesses the association of four polymorphisms in three folate-related enzyme genes with male infertility in the Chinese population.

Materials and methods

Subjects

A total of 296 idiopathic infertile male with azoospermia or oligozoospermia (sperm concentration < 20×10⁶/ml) in the East China population were recruited from The First Affiliated Hospital of Wenzhou Medical University, China. These patients were seeking infertility care at the hospital. At least two semen samples were tested for initial evaluation of the patient according to the WHO guidelines. Astheno or teratozoospermia was seen in 79.8 % of the samples. The average sperm motility is 30.9 %. Patients with recognizable genetic causes of male infertility such as chromosome anomalies and microdeletions in the AZF region of the Y chromosome were excluded. The control group consisted of 204 fertile men who had at least one child from the general population in area of East China (Zhejiang province). Blood samples were collected from all 500 subjects, and total genomic DNAs were isolated by QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. Written informed consent was obtained from all study subjects. The study was approved by

the Medical Ethics Committee of The First Affiliated Hospital of Wenzhou Medical University.

Genotyping

Four SNPs [rs1801133 (C677T, A222V), rs1801131 (A1298C, E429A), rs1805087 (A2756G, D919G), rs1801394 (A66G, I22M)] were genotyped using SNaPshot, with technical support from Center for Genetic & Genomic Analysis, Genesky Biotechnologies Inc. Specific oligonucleotide primers were designed by the ABI prism SNaPshot Multiplex Kit (ABI). Single base extension using gene-specific primers was performed according to the manufacturer's protocol (ABI Prism SNaPshot multiplex system; Applied Biosystems).

For the detection of rs1801133 polymorphism, the primer pairs were: 5' - CCC CTC ACC TGG ATG GGA AA -3' (sense) and 5' - GAG GCC AGC CTC TCC TGA CTG -3' (antisense). The primer pairs for rs1801131 polymorphism were: 5' - CTT CTC CCT TTG CCA TGT CCA C -3' (sense) and 5' - GAA GAG CAA GTC CCC CAA GGA G -3' (antisense). The primer pairs for rs1805087 polymorphism were: 5' - TTC CTG AAG GAG GTG TTA TCA GCA -3' (sense) and 5' - TCC AAA GCC TTT TAC ACT CCT CAA A -3' (antisense). For the rs1801394 polymorphism: 5' - ACA GCA GGG ACA GGC AAA GG -3' (sense) and 5' - CCC AAC CAA AAT TCT TCA AAG CAC -3' (antisense) primers were used.

PCR consisted of 1×HotStarTaq buffer, 3 mM Mg²⁺, 0.3 mM of each dNTP, 1 μM of each primer and 1 U of HotStarTaq polymerase (Qiagen) in a 20 μL reaction volume. Thermal cycling conditions were 95 °C 2 min; 11 cycles×(94 °C 20 s, 65 °C-0.5 °C /cycle 40 s, 72 °C 1.5 min); 24 cycles×(94 °C 20 s, 59 °C 30 s, 72 °C 1.5 min); 72 °C 2 min; 4 °C forever. Following amplification, 2 U Exonuclease I and 5 U SAP (Shrimp alkaline phosphatase) were added to 15 μL PCR products and incubated at 37 °C for 1 h, followed by heat inactivation at 75 °C for 15 min. The single nucleotide primer extension assay consisted of 5 μL SNaPshot Multiplex Mix (Applied Biosystems), 2 μL purified PCR product and 0.8 μM of each primer in a final reaction volume of 10 μL. The following specific primers were used: rs1801131SR, 5' - T₂₉GG GGA GGA GCT GAC CAG TGA AG -3'; rs1801133SF, 5' - T₄₄AA AAG CTG CGT GAT GAT GAA ATC G -3'; rs1805087SR, 5' - T₁₈CC ACT TAC CTT GAG AGA CTC ATA ATG G -3' and rs1801394SR, 5' - T₁₇CC ATG TAC CAC AGC TTG CTC ACA -3'. The amplification consisted of 28 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. Then, 1 μL of SAP was added to each reaction and incubated at 37 °C for 1 h followed by heat inactivation at 75 °C for 15 min. The SNaPshot products were mixed with HiDiFormamide and LIZ 120 (Applied Biosystems) size

Table 1 Genotype and allele distribution of *MTHFR* SNPs in infertile patients and fertile controls

SNP	Genotype	All subjects (N=500)	Control (N=204)	Patients (N=296)	P value	OR (95 % CI)
C677T (rs1801133)	CC	201 (40.2 %)	84 (41.2 %)	117 (39.5 %)	0.917	1.03 (0.70–1.51)
	CT	229 (45.8 %)	94 (46.1 %)	135 (45.6 %)		
	TT	70 (14.0 %)	26 (12.7 %)	44 (14.9 %)		
	C allele	629 (62.9 %)	260 (63.7 %)	369 (62.3 %)	0.444	1.08 (0.84–1.40)
	T allele	367 (36.7 %)	144 (35.3 %)	223 (37.7 %)		
A1298C (rs1801131)	AA	318 (63.6 %)	137 (67.2 %)	181 (61.1 %)	0.207	1.29 (0.88–1.9)
	AC	168 (33.6 %)	62 (30.4 %)	106 (35.8 %)		
	CC	14 (2.8 %)	5 (2.5 %)	9 (3.0 %)		
	A allele	804 (80.4 %)	336 (82.4 %)	468 (79.1 %)	0.695	1.36 (0.45–4.16)
	C allele	196 (19.6 %)	72 (17.6 %)	124 (20.9 %)		

standard, and denatured at 95 °C for 2 min, then placed on ice for at least 2 min before injection to the ABI 3730xl (96 Capillary) DNA analyzer (Applied Biosystems). The resulting data were analyzed by GeneMapper 4.1 (Applied Biosystems).

Statistical analyses

The deviation from the Hardy-Weinberg equilibrium was examined using Chi-square test. SNP Stats software (<http://bioinfo.iconcologia.net/SNPStats>) was used to analyze genetic data. The differences in genotype distribution between control and patients were calculated using a two-tailed χ^2 test. An unconditional logistic regression method was used to calculate the odds ratio (OR) and 95 % confidence intervals (CIs). $P < 0.05$ was taken as statistically significant.

Results

We analyzed *MTHFR* rs1801133 (C677T, A222V) and rs1801131 (A1298C, E429A), *MTR* rs1805087 (A2756G, D919G) and *MTRR* rs1801394 (A66G, I22M) polymorphisms in 296 infertile and 204 fertile (control) men. The genotype distribution of C677T, A1298C, A2756G and A66G is summarized in Tables 1, 2 and 3. The results are in Hardy-Weinberg equilibrium in controls and infertile men.

Table 1 shows the prevalence of *MTHFR* C677T and A1298C polymorphism in control and infertile men. The frequencies of the CC, TC and TT genotypes of *MTHFR* C677T polymorphism in the control group are 41.2, 46.1 and 12.8 % versus 39.5, 45.6 and 14.9 % in the infertile group. The frequency of the *MTHFR* C677T genotypes in fertile and infertile men was not statistically different. Likewise, there was no significant difference between the control and infertile groups with regards to genotypic and allelic frequencies of A1298C polymorphism (Table 1).

The association between *MTR* A2756G polymorphism and male infertility is presented in Table 2. As shown in this table, no significant association is found between *MTR* A2756G polymorphism and risk of male infertility.

The polymorphism distribution of A66G in the *MTRR* gene is summarized in Table 3. The frequencies of heterozygous (AG) and homozygous (GG) in the control and infertile groups are 44.6 and 6.9 %, and 40.2 and 6.4 %, respectively. These data showed no statistically significant difference between control and infertile men.

Discussion

Folates are group B vitamins that play essential roles in cell metabolism, including the synthesis of nucleic acids and epigenetic regulation of gene expression through remethylation of homocysteine into methionine [32]. Impaired folate status

Table 2 Genotype and allele distribution of *MTR* SNP (Rs1805087, A2756G) in infertile patients and fertile controls

SNP	Genotype	All subjects (N=500)	Control (N=204)	Patients (N=296)	P value	OR (95 % CI)
A2756G (Rs1805087)	AA	408 (81.6 %)	163 (79.9 %)	245 (82.8 %)	0.507	0.85 (0.53–1.36)
	AG	84 (16.8 %)	37 (18.1 %)	47 (15.9 %)		
	GG	8 (1.6 %)	4 (2.0 %)	4 (1.4 %)		
	A allele	900 (90.0 %)	363 (89.0 %)	537 (90.7)	0.689	0.84 (0.56–1.25)
	G allele	100 (10.0 %)	45 (11.0 %)	55 (9.3 %)		

Table 3 Genotype and allele distribution of *MTRR* SNP (Rs1801394, A66G) in infertile patients and fertile controls

SNP	Genotype	All subjects (N=500)	Control (N=204)	Patients (N=296)	P value	OR (95 % CI)
A66G (Rs1801394)	AA	257 (51.4 %)	99 (48.5)	158 (53.4 %)		
	AG	210 (42.0 %)	91 (44.6 %)	119 (40.2 %)	0.286	0.82 (0.57–1.19)
	GG	33 (6.6 %)	14 (6.9 %)	19 (6.4 %)	0.844	0.85 (0.41–1.76)
	A allele	724 (72.4 %)	289 (70.8 %)	435 (73.5 %)		
	G allele	276 (27.6 %)	119 (29.2 %)	157 (26.5 %)	0.358	0.87 (0.65–1.16)

has been associated with several pathologies such as cardiovascular, neurodegenerative diseases and pregnancy complications [32]. Recent studies suggest that the key enzymes in folate metabolism are critical for male spermatogenesis [24, 33]. Impaired folate status causes DNA hypomethylation that disrupts gene expression by inducing uracil misincorporation during DNA synthesis thereby leading to errors in DNA repair, strand breakage and chromosomal anomalies. Mice deficient in *MTHFR* saw hyper-homocystenemia, decreased S-adenosylmethionine levels, global DNA hypomethylation and developmental retardation with severe neuropathology [33]. These mice also showed delayed maturation of the external genitalia [33]. Kelly et al. reported that severe *MTHFR* deficiency in male mice results in abnormal spermatogenesis and infertility [8], suggesting that the *MTHFR* gene is involved in male fertility. Folate has antioxidant properties that counteract reactive oxygen species (ROS) [34]. It has been reported that low concentrations of folate in seminal plasma is associated with increased sperm DNA damage [9]. Low seminal plasma folate concentrations correlated significantly with low sperm density and total sperm count in male smokers and non-smokers [35]. These data suggest that folate metabolism plays an important role in the maintenance of spermatogenesis.

Single nucleotide polymorphisms (SNPs) have been found to be associated with the activities of the folate metabolism-related enzymes. In this study, we conducted a case-control study to investigate the association of the four SNPs (*MTHFR* C677T and A1298C, *MTR* A 2756G and *MTRR* A66G) of three folate-related enzyme genes with male infertility in the Chinese population. *MTHFR* C677T and A1298C are associated with decreased enzyme activity [36, 37]. The distribution of alleles varies among different ethnic population [31]. A number of studies have evaluated the association of *MTHFR* gene polymorphism with male infertility [13, 38–40, 22, 27, 17, 16, 18, 29, 25, 41, 28, 42, 26, 21, 23]. The *MTHFR* C677T polymorphism was reported to be associated with male infertility in the German [42], Indian [26, 18, 17, 38], Korean [23], Chinese [19] and Italian populations [41]. In contrast, Stuppia et al. [28] and Ebisch et al. [21] did not find any statistically significance of C677T variation in infertile men from Italy and the Netherlands. These apparently contradictory data from studies on different populations suggest that the role of C677T insusceptibility to male infertility might depend on

ethno geographic factors [19]. It should be noted that contrasting results from the Italian population were also observed by two studies [41, 28]. In our study, we did not find any association of C677T polymorphism with male infertility in East China population. The frequency of the *MTHFR* A1298C variant allele also differs across populations, the frequency of the C allele is less frequent among Chinese than among Caucasians [20]. Lack of association between *MTHFR* A1298C and male infertility was observed in French, Indian, Korean and Moroccan populations [17, 13, 24, 16]. Our results are in concordance with previous studies. The A2756G transition in the *MTR* gene results in the substitution of the Asp919Gly in the *MTR* enzyme and leads to more effective homocysteine remethylation and methionine production [30]. *MTRR* A66G has a lower affinity for *MTR* and is inconsistently associated with homocysteine level [37]. However, the lack of association was also observed between male infertility and *MTR* A2756G or *MTRR* A66G. Montjean et al. reported that no association was found between *MTHFR* (A1286C, C677T, and G203A), *MTRR* (A66G and C524T) genetic variants and sperm counts in French population [43]. Ravel et al. failed to detect an association between among three variants in *MTHFR* (G203A, C665T, and A1286C), two variants in *MTRR* (A66G and C524T), a mutation in the CBS gene (G919A) and reduced sperm counts in infertile individuals of French ethnic origin [24]. On the other hand, Lee et al. [13] investigated the relationship between polymorphisms of three folate metabolism enzymes (*MTHFR* C677T and A1298C, *MTR* A2756G, and *MTRR* A66G) and non-obstructive Korean male infertility. They found that *MTHFR* C677T, *MTR* A2756G and *MTRR* A66G genotypes were independently associated with male infertility [13]. Geographic variation, racial, and ethnic differences in the distribution of the polymorphisms in folate-related enzyme genes could be one of the explanations for conflicting findings [44]. Moreover, gene-nutrient/environmental and gene racial/ethnic interactions have been shown to influence the impact of *MTHFR* polymorphisms [45].

Some limitations of our study must be addressed. One is that we did not do routine semen analysis for the control group and thus, could not compare the semen parameters between control and infertile men. A certain percentage of the control group might have abnormal sperm counts yet produce normal

children. Furthermore, infertile men were not classified into subtypes (using semen analysis) in this study. The association between the four SNPs and different subtypes of infertile men (including azoospermia, oligozoospermia, asthenozoospermia, oligo-teratozoospermia, astheno-teratozoospermia and oligo-astheno-teratozoospermia) requires further evaluation.

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Conflict of interest None.

References

- Lu C, Zhang J, Li Y, Xia Y, Zhang F, Wu B, et al. The b2/b3 subdeletion shows higher risk of spermatogenic failure and higher frequency of complete AZFc deletion than the gr/gr subdeletion in a Chinese population. *Hum Mol Genet*. 2009;18(6):1122–30. doi:10.1093/hmg/ddn427.
- Patrizio P, Leonard DG, Chen KL, Hernandez-Ayup S, Trounson AO. Larger trinucleotide repeat size in the androgen receptor gene of infertile men with extremely severe oligozoospermia. *J Androl*. 2001;22(3):444–8. doi:10.1002/j.1939-4640.2001.tb02200.x.
- Ferlin A, Raicu F, Gatta V, Zuccarello D, Palka G, Foresta C. Male infertility: role of genetic background. *Reprod BioMed Online*. 2007;14(6):734–45. doi:10.1016/S1472-6483(10)60677-3.
- Shamsi MB, Kumar K, Dada R. Genetic and epigenetic factors: Role in male infertility. *Indian J Urol IJU J Urol Soc India*. 2011;27(1):110–20. doi:10.4103/0970-1591.78436.
- Chen H, Pu XY, Zhang RP, A ZC. Association of common SNPs rs1136410 in PARP1 gene with the susceptibility to male infertility with oligospermia. *J Assist Reprod Genet*. 2014. doi:10.1007/s10815-014-0311-3.
- Crider KS, Yang TP, Berry RJ, Bailey LB. Folate and DNA methylation: a review of molecular mechanisms and the evidence for folate's role. *Adv Nutr*. 2012;3(1):21–38. doi:10.3945/an.111.000992.
- Fowler B. Homocysteine: overview of biochemistry, molecular biology, and role in disease processes. *Semin Vasc Med*. 2005;5(2):77–86. doi:10.1055/s-2005-872394.
- 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(3):667–77. doi:10.1095/biolreprod.104.035238.
- Boxmeer JC, Smit M, Utomo E, Romijn JC, Eijkemans MJ, Lindemans J, et al. Low folate in seminal plasma is associated with increased sperm DNA damage. *Fertil Steril*. 2009;92(2):548–56. doi:10.1016/j.fertnstert.2008.06.010.
- Goyette P, Pai A, Milos R, Frosst P, Tran P, Chen Z, et al. Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR). *Mamm Genome Off J Int Mamm Genome Soc*. 1998;9(8):652–6. doi:10.1007/s003359900838.
- Shen O, Liu R, Wu W, Yu L, Wang X. 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.
- Neto AI, de Moura Jr JR, Persuhn DC. Frequency of MTHFR G1793A polymorphism in individuals with early coronary artery disease: cross-sectional study. *Sao Paulo Med J Rev Paul Med*. 2013;131(5):296–300. doi:10.1590/1516-3180.2013.1315500.
- Lee HC, Jeong YM, Lee SH, Cha KY, Song SH, Kim NK, et al. Association study of four polymorphisms in three folate-related enzyme genes with non-obstructive male infertility. *Hum Reprod*. 2006;21(12):3162–70. doi:10.1093/humrep/del280.
- Matsuo K, Suzuki R, Hamajima N, Ogura M, Kagami Y, Taji H, et al. Association between polymorphisms of folate- and methionine-metabolizing enzymes and susceptibility to malignant lymphoma. *Blood*. 2001;97(10):3205–9. doi:10.1182/blood.V97.10.3205.
- Gaughan DJ, Kluijtmans LA, Barbaux S, McMaster D, Young IS, Yarnell JW, et al. The methionine synthase reductase (MTRR) A66G polymorphism is a novel genetic determinant of plasma homocysteine concentrations. *Atherosclerosis*. 2001;157(2):451–6. doi:10.1016/S0021-9150(00)00739-5.
- Eloualid A, Abidi O, Charif M, El Houate B, Benrahma H, Louanjli N, et al. Association of the MTHFR A1298C variant with unexplained severe male infertility. *PLoS One*. 2012;7(3):e34111. doi:10.1371/journal.pone.0034111.
- Gupta N, Sarkar S, David A, Gangwar PK, Gupta R, Khanna G, et al. Significant impact of the MTHFR polymorphisms and haplotypes on male infertility risk. *PLoS One*. 2013;8(7):e69180. doi:10.1371/journal.pone.0069180.
- Gupta N, Gupta S, Dama M, David A, Khanna G, Khanna A, et al. Strong association of 677 C>T substitution in the MTHFR gene with male infertility—a study on an indian population and a meta-analysis. *PLoS One*. 2011;6(7):e22277. doi:10.1371/journal.pone.0022277.
- A ZC, Yang Y, Zhang SZ, Li N, Zhang W. Single nucleotide polymorphism C677T in the methylenetetrahydrofolate reductase gene might be a genetic risk factor for 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.
- Botto LD, Yang Q. 5,10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. *Am J Epidemiol*. 2000;151(9):862–77. doi:10.1093/oxfordjournals.aje.a010290.
- Ebisch IM, van Heerde WL, Thomas CM, van der Put N, Wong WY, Steegers-Theunissen RP. C677T methylenetetrahydrofolate reductase polymorphism interferes with the effects of folic acid and zinc sulfate on sperm concentration. *Fertil Steril*. 2003;80(5):1190–4. doi:10.1016/S0015-0282(03)02157-5.
- Mfady DS, Sadiq MF, Khabour OF, Fararjeh AS, Abu-Awad A, Khader Y. Associations of variants in MTHFR and MTRR genes with male infertility in the Jordanian population. *Gene*. 2014;536(1):40–4. doi:10.1016/j.gene.2013.12.001.
- Park JH, Lee HC, Jeong YM, Chung TG, Kim HJ, Kim NK, et al. MTHFR C677T polymorphism associates with unexplained infertile male factors. *J Assist Reprod Genet*. 2005;22(9–10):361–8. doi:10.1007/s10815-005-6795-0.
- Ravel C, Chantot-Bastaraud S, Chalmey C, Barreiro L, Aknin-Seifer I, Pfeffer J, 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.
- Singh K, Singh SK, Raman R. MTHFR A1298C polymorphism and idiopathic male infertility. *J Postgrad Med*. 2010;56(4):267–9. doi:10.4103/0022-3859.70935.
- Singh K, Singh SK, Sah R, Singh I, Raman R. Mutation C677T in the methylenetetrahydrofolate reductase gene is associated with male infertility in an Indian population. *Int J Androl*. 2005;28(2):115–9. doi:10.1111/j.1365-2605.2004.00513.x.
- Stangler Herodez S, Zagradisnik B, Erjavec Skerget A, Zagorac A, Takac I, Vlaisavljevic V, et al. MTHFR C677T and A1298C genotypes and haplotypes in Slovenian couples with unexplained infertility problems and in embryonic tissues from spontaneous abortions. *Balk J Med Genet BJMG*. 2013;16(1):31–40. doi:10.2478/bjmg-2013-0015.
- Stuppia L, Gatta V, Scarciolla O, Colosimo A, Guanciali-Franchi P, Calabrese G, et al. The methylenetetrahydrofolate reductase

- (MTHFR) C677T polymorphism and male infertility in Italy. *J Endocrinol Investig.* 2003;26(7):620–2. doi:[10.1111/j.1365-2605.2011.01147.x](https://doi.org/10.1111/j.1365-2605.2011.01147.x).
29. Wei B, Xu Z, Ruan J, Zhu M, Jin K, Zhou D, et al. MTHFR 677C>T and 1298A> polymorphisms and male infertility risk: a meta-analysis. *Mol Biol Rep.* 2012;39(2):1997–2002. doi:[10.1007/s11033-011-0946-4](https://doi.org/10.1007/s11033-011-0946-4).
 30. Weiner AS, Boyarskikh UA, Voronina EN, Tupikin AE, Korolkova OV, Morozov IV, et al. Polymorphisms in folate-metabolizing genes and risk of idiopathic male infertility: a study on a Russian population and a meta-analysis. *Fertil Steril.* 2014;101(1):87–94 e3. doi:[10.1016/j.fertnstert.2013.09.014](https://doi.org/10.1016/j.fertnstert.2013.09.014).
 31. Wilcken B, Bamforth F, Li Z, Zhu H, Ritvanen A, Renlund M, et al. Geographical and ethnic variation of the 677C>T allele of 5,10-methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas world wide. *J Med Genet.* 2003;40(8):619–25. doi:[10.1136/jmg.40.8.619](https://doi.org/10.1136/jmg.40.8.619).
 32. Forges T, Pellanda H, Diligent C, Monnier P, Gueant JL. Do folates have an impact on fertility? *Gynecol Obstet Fertil.* 2008;36(9):930–9. doi:[10.1016/j.gyobfe.2008.07.004](https://doi.org/10.1016/j.gyobfe.2008.07.004).
 33. Chen Z, Karaplis AC, Ackerman SL, Pogribny IP, Melnyk S, Lussier-Cacan S, 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):433–43. doi:[10.1093/hmg/10.5.433](https://doi.org/10.1093/hmg/10.5.433).
 34. Ebisch IM, Thomas CM, Peters WH, Braat DD, Steegers-Theunissen RP. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. *Hum Reprod Update.* 2007;13(2):163–74. doi:[10.1093/humupd/dml054](https://doi.org/10.1093/humupd/dml054).
 35. Wallock LM, Tamura T, Mayr CA, Johnston KE, Ames BN, Jacob RA. Low seminal plasma folate concentrations are associated with low sperm density and count in male smokers and nonsmokers. *Fertil Steril.* 2001;75(2):252–9. doi:[10.1016/S0015-0282\(00\)01697-6](https://doi.org/10.1016/S0015-0282(00)01697-6).
 36. Finnell RH, Shaw GM, Lammer EJ, Volcik KA. Does prenatal screening for 5,10-methylenetetrahydrofolate reductase (MTHFR) mutations in high-risk neural tube defect pregnancies make sense? *Genet Test.* 2002;6(1):47–52. doi:[10.1089/109065702760093915](https://doi.org/10.1089/109065702760093915).
 37. van der Put NM, Gabreels F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *Am J Hum Genet.* 1998;62(5):1044–51. doi:[10.1086/301825](https://doi.org/10.1086/301825).
 38. Naqvi H, Hussain SR, Ahmad MK, Mahdi F, Jaiswar SP, Shankhwar SN, et al. Role of 677C>T polymorphism a single substitution in methylenetetrahydrofolate reductase (MTHFR) gene in North Indian infertile men. *Mol Biol Rep.* 2014;41(2):573–9. doi:[10.1007/s11033-013-2894-7](https://doi.org/10.1007/s11033-013-2894-7).
 39. Dhillon VS, Shahid M, Husain SA. Associations of MTHFR DNMT3b 4977 bp deletion in mtDNA and GSTM1 deletion, and aberrant CpG island hypermethylation of GSTM1 in non-obstructive infertility in Indian men. *Mol Hum Reprod.* 2007;13(4):213–22. doi:[10.1093/molehr/gal118](https://doi.org/10.1093/molehr/gal118).
 40. Li SS, Li J, Xiao Z, Ren AG, Jin L. Prospective study of MTHFR genetic polymorphisms as a possible etiology of male infertility. *Genet Mol Res GMR.* 2014;13(3):6367–74. doi:[10.4238/2014.March.24.26](https://doi.org/10.4238/2014.March.24.26).
 41. Paracchini V, Garte S, Taioli E. MTHFR C677T polymorphism, GSTM1 deletion and male infertility: a possible suggestion of a gene-gene interaction? *Biomark Biochem Indic Expo Response Susceptibility Chem.* 2006;11(1):53–60. doi:[10.1080/13547500500442050](https://doi.org/10.1080/13547500500442050).
 42. Bezold G, Lange M, Peter RU. Homozygous methylenetetrahydrofolate reductase C677T mutation and male infertility. *N Engl J Med.* 2001;344(15):1172–3. doi:[10.1056/NEJM200104123441517](https://doi.org/10.1056/NEJM200104123441517).
 43. Montjean D, Benkhalifa M, Dessolle L, Cohen-Bacrie P, Belloc S, Siffroi JP, et al. Polymorphisms in MTHFR and MTRR genes associated with blood plasma homocysteine concentration and sperm counts. *Fertil Steril.* 2011;95(2):635–40. doi:[10.1016/j.fertnstert.2010.08.054](https://doi.org/10.1016/j.fertnstert.2010.08.054).
 44. Gava MM, Kayaki EA, Bianco B, Teles JS, Christofolini DM, Pompeo AC, et al. Polymorphisms in folate-related enzyme genes in idiopathic infertile Brazilian men. *Reprod Sci.* 2011;18(12):1267–72. doi:[10.1177/1933719111411729](https://doi.org/10.1177/1933719111411729).
 45. Toffoli G, De Mattia E. Pharmacogenetic relevance of MTHFR polymorphisms. *Pharmacogenomics.* 2008;9(9):1195–206. doi:[10.2217/14622416.9.9.1195](https://doi.org/10.2217/14622416.9.9.1195).