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Association between methionine synthase reductase A66G polymorphism and primary infertility in Chinese males

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ABSTRACT. We examined the association between the methionine synthase reductase (*MTRR* A66G), methylenetetrahydrofolate reductase (*MTHFR* C677T and A1298C), and methionine synthase (*MS* A2756G) genotypes and non-obstructive male infertility in a Chinese population. This case-control study included 162 infertile Chinese patients with azoospermia (N = 100) or oligoasthenozoospermia (N = 62) and 120 fertile men as controls. The polymorphisms *MTRR* A66G, *MTHFR* C677T, A1298C, and *MS* A2756G were identified by direct DNA sequencing and the results were statistically analyzed. We found no association between the incidence of any of these variants in azoospermia patients and control populations. The frequency of the *MTRR*66 polymorphic genotypes (AG, AG+GG) was significantly higher in the oligoasthenozoospermia group compared to the controls

(P = 0.013, 0.012). Our findings revealed an association between the single-nucleotide polymorphism A66G in the *MTRR* gene and male infertility, particularly in oligoasthenozoospermia males, suggesting that this polymorphism is a genetic risk factor for male infertility in Chinese men.

Key words: Male infertility; Methionine synthase reductase; Methylenetetrahydrofolate reductase; Methionine synthase; Single-nucleotide polymorphism

INTRODUCTION

Infertility is defined as the failure of a couple to conceive after 12 months of unprotected regular sexual intercourse, which affects 15-30% of couples attempting to conceive (Kara and Simoni, 2010). Male factor infertility is partially or fully responsible for approximately 50% of infertility cases (Ammar et al., 2012). Infertility can be classified as primary or secondary, depending on whether a pregnancy was once induced or was not induced (Simoni and Wieacker, 2010). There are endocrinological (e.g., trauma to the pituitary), infectious (e.g., mumps orchitis), toxicological (e.g., lead exposure, exogenous androgen, chemotherapeutic drugs), and radiological (e.g., therapeutic radiation or occupational exposure) etiologies for male infertility, which is related to several risk factors such as chromosomal abnormalities (Kara and Simoni, 2010), Y chromosome micro/macrodeletions (Lee et al., 2011), cystic fibrosis transmembrane conductance regulator mutations (Augarten et al., 1994), and other genetic factors (Lee et al., 2003).

Folate is essential for DNA synthesis and methylation reactions (Fang and Xiao, 2003). DNA methylation plays an important role in spermatogenesis, and folate deficiency reduces hyperhomocysteinemia, which is considered to be a risk factor for various diseases, including infertility. Methionine synthase reductase (MTRR), methylenetetrahydrofolate reductase (MTHFR), and methionine synthase (MS) play essential and interrelated roles in folate metabolism (Figure 1). MTHFR catalyzes the conversion of methylenetetrahydrofolate to methyltetrahydrofolate, which is the main circulating form of folate and also forms precursors for purines and pyrimidines for DNA synthesis (Singh and Jaiswal, 2013). Methionine is a methyl donor and the methyl group is produced from methyltetrahydrofolate. This reaction is regulated by 2 enzymes: MS, which catalyzes the transfer of a methyl group from methyltetrahydrofolate to homocysteine and concurrently generates methionine and THF, and MTRR, which restores MS activity and is thus a critical determinant of homocysteine levels (Gaughan et al., 2001).

Several single-nucleotide polymorphisms (SNPs) of folate metabolism-related genes have been identified, including *MTHFR* C677T (rs1801133) (Frosst et al., 1995), *MTHFR* A1298C (rs1801131) (van der Put et al., 1998), *MS* A2756G (rs1805087) (Chen et al., 1998; Ma et al., 1999), and *MTRR* A66G (rs1801394) (Olteanu et al., 2002). These SNPs can modulate homocysteine levels, which may affect DNA synthesis and methylation by restricting the activity of folate metabolism-related enzymes.

Lee et al. (2006) analyzed a large number of subjects and found the first genetic evidence that the *MTHFR* C677T, *MS* A2756G, and *MTRR* A66G genotypes were independently associated with male infertility. In addition, SNP of the 3 enzymes may have a different impact on the folate cycle during spermatogenesis in the Korean population.

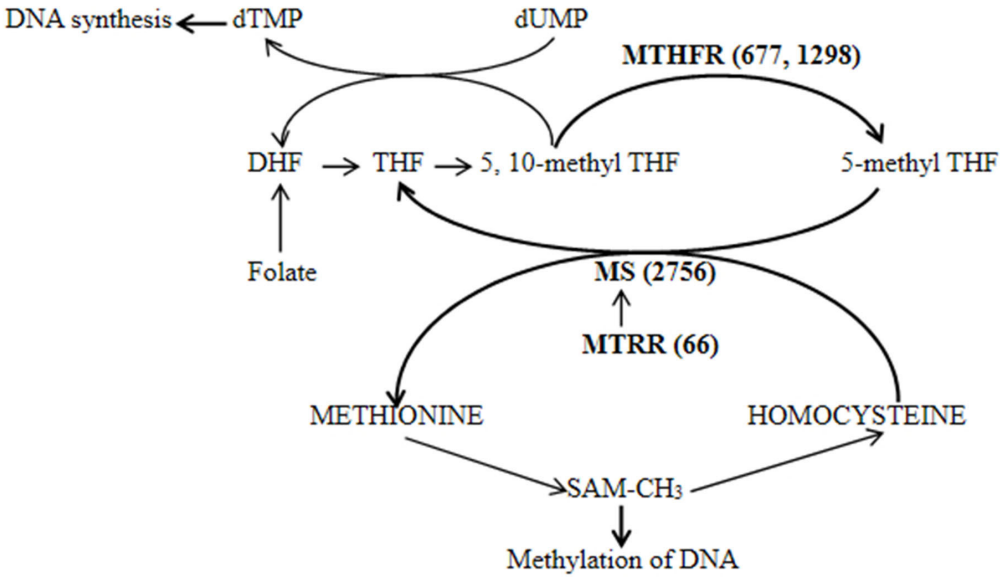

Figure 1. Overview of the human folic acid metabolic pathway and the role of methionine synthase reductase (MTRR), methylenetetrahydrofolate reductase (MTHFR), and methionine synthase (MS).

In this study, we examined the association between 4 SNPs (*MTHFR* C677T, *MTHFR* A1298C, *MS* A2756G, and *MTRR* A66G) and non-obstructive male infertility in China.

MATERIAL AND METHODS

Patients and controls

The study was approved by the Ethical Committee of Sichuan University. A total of 162 infertile men with non-obstructive azoospermia (N = 100) or oligoasthenozoospermia (OA group; N = 62, sperm count <20 x 10^6/mL; progressive sperm motility <50%), and 120 men with proven fertility (at least 1 child) without assisted reproductive technologies were recruited for the study from July 2010 to June 2012. Semen analysis for sperm concentration, motility, and morphology was performed following the World Health Organization criteria (WHO, 1999). In addition, all patients underwent at least 2 semen analyses, and patients were verified to be free from chromosomal abnormalities, Y chromosome micro/macro-deletion, hypo-gonadotropic hypogonadism, infections, and obstructive azoospermia. Chromosomal abnormalities and Y chromosome micro/macro-deletion in the controls were also ruled out. The comprehensive clinical history of all patients was recorded, and other possible causes of male infertility such as clinical history of congenital bilateral absence of the vas deferens and varicocele endocrine were excluded. Written informed consent was obtained from all subjects.

Mutation analysis

Genomic DNA was extracted from whole blood using H.Q.&.Q.Blood DNA Kit

(AnHui U-gene Biotechnology Co., Ltd., Hefei, China) according to manufacturer instructions. Spectroscopic methods were performed to quantify the extracted genomic DNA and the DNA samples were stored at -20°C. A polymerase chain reaction protocol was used to genotype all SNPs. Primers were designed using PRIMER3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Amplifications were performed in a total volume of 25 mL buffered solution containing approximately200 ng genomic DNA, 0.25 mM dNTPs (TransGen, Beijing, China), 1.5 mM Mg^{2+} (Fermentas, Vilnius, Lithuania), 0.2 mM of each primer (Invitrogen, Carlsbad, CA, USA), and 2.5 U *Taq* polymerase (Fermentas). Thermocycling consisted of 95°C for 2 min; 35 cycles of 95°C for 1 min, annealing temperature (Table 1) for 30 s, and 72°C for 30 s; and a final 7-min extension at 72°C. After the reaction, samples were stored at 4°C. Products were sequenced using an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

The allele and genotype frequencies of the patients and controls were calculated by counting. The chi-squared or Fisher exact test (2-sided) was performed to compare differences in mutation rates. The statistical package was used to estimate the odds ratio and 95% confidence intervals were the SPSS15.0 statistical software (SPSS Inc., Chicago, IL, USA). P values < 0.05 were considered to be statistically significant.

RESULTS

We analyzed the 4 polymorphisms in the *MTRR*, *MS*, and *MTHFR* genes in infertile and fertile men by direct DNA sequencing. Table 1 and Figure 2 show the results of the *MTHFR* C677T, *MTHFR* A1298C, *MS* A2765G, and *MTRR* A66G analyses performed using direct DNA sequencing.

Table 1. List of SNPs analyzed along with PCR primers used for genotyping.

SNP	Chromosome	Forward primer/Reverse primer (5'-3')	Annealing temperature (°C)	Fragment size (bp)
A66G	5	GATTCAAGCCCAAGTAGT TGCAGAAAATCCATGTAC	53	383
C677T	1	CAGGACAGTGTGGGAGTTT GCTGCGTGATGATGAAATC	54	371
A1298C	1	CAGACCTTCCTTGCAAATA ACTCCAGCATCACTCACTT	53	377
A2756G	1	TGTTATCAGCATTGACCATTACTAC CAGAAATTCTCTAAAATGATCCAAA	54	301

The results of statistical analysis of 4 polymorphisms in the fertile and non-obstructive infertile men are summarized in Table 2. All polymorphisms were in Hardy-Weinberg equilibrium (P = 0.922, 0.797, 0.512, and 0.333, respectively). Allelic frequencies for fertile men with the 66GG and 677TT variations in this study were similar to those in healthy Chinese men (Yang et al., 2013). The *MTRR* A66G, *MTHFR* C677T, *MTHFR* A1298C, and *MS* A2756G polymorphism data, according to our case-control analysis, showed no significant differences for individuals with infertility (P > 0.05).

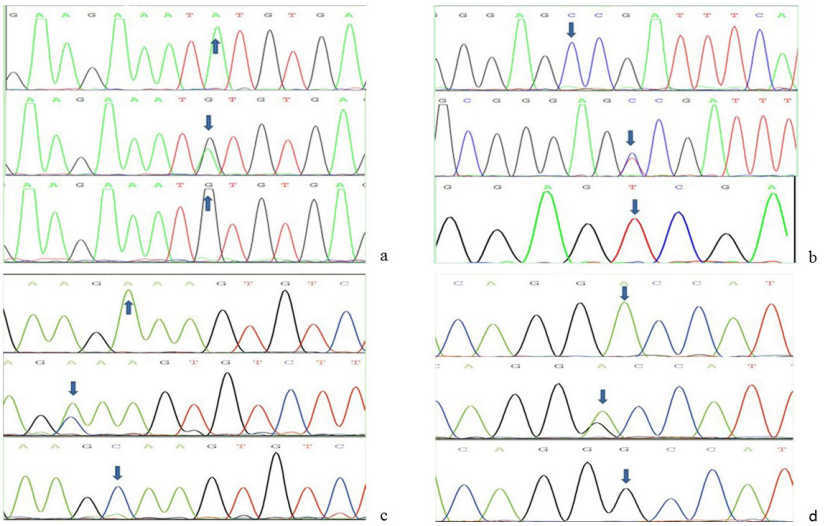

Figure 2. Sequence results of the *MTRR*, *MTHFR*, and *MS* genes. **a.** *MTRR* 66 (AA, AG, GG); **b.** *MTHFR* 677 (CC, CT, TT); **c.** *MTHFR* 1298 (AA, AC, CC); **d.** *MS* 2756 (AA, AG, GG).

Table 2. Distribution of the *MTRR* A66G, *MTHFR* C677T, *MTHFR* A1298C, and *MS* A2756G genotypes in infertile and fertile men.

	Fertile men (N = 120)	Infertile men (N = 162)	OR (95%CI)	P
*MTRR*66				
66AA	(58.33%) 70	(51.23%) 83		
66AG	(36.67%) 44	(40.12%) 65	1.246 (0.758-2.049)	0.386
66GG	(5.00%) 6	(8.64%) 14	1.968 (0.718-5.391)	0.182
AG+GG	(41.67%) 50	(48.76%) 79	1.333 (0.828-2.145)	0.237
A	(76.67%) 184	(71.30%) 231		
G	(23.33%) 56	(28.70%) 93	1.323 (0.901-1.942)	0.153
*MTHFR*677				
677CC	(40.00%) 48	(37.65%) 61		
677CT	(45.00%) 54	(47.53%) 77	1.122 (0.671-1.876)	0.660
677TT	(15.00%) 18	(14.81%) 24	1.049 (0.511-2.153)	0.896
CT+TT	(60.00%) 72	(62.34%) 101	1.104 (0.680-1.791)	0.689
C	(62.50%) 150	(61.42%) 199		
T	(37.50%) 90	(38.58%) 125	1.047 (0.742-1.477)	0.794
*MTHFR*1298				
1298AA	(66.67%) 80	(62.35%) 101		
1298AC	(31.67%) 38	(33.33%) 54	1.126 (0.677-1.871)	0.648
1298CC	(1.67%) 2	(4.32%) 7	2.772 (0.560-13.712)	0.304
AC+CC	(33.33%) 40	(37.65%) 61	1.208 (0.736-1.982)	0.454
A	(82.50%) 198	(79.01%) 256		
C	(17.50%) 42	(20.99%) 68	1.252 (0.817-1.919)	0.301
*MS*2756				
2756AA	(84.17%) 101	(76.54%) 124		
2756AG	(14.17%) 17	(21.60%) 35	1.677 (0.888-3.168)	0.109
2756GG	(1.67%) 2	(1.85%) 3	1.222 (0.200-7.453)	1.000
AG+GG	(15.84%) 19	(23.45%) 38	1.629 (0.885-2.999)	0.115
A	(91.25%) 219	(87.35%) 283		
G	(8.75%) 21	(12.65%) 41	1.511 (0.868-2.631)	0.143

OR = odds ratio; CI = confidence interval.

Variations in the polymorphisms analyzed in the azoospermia group are summarized in Table 3. Differences in *MTRR* A66G, *MTHFR* C677T, *MTHFR* A1298C, and *MS* A2756G

were not statistically significant.

Table 3. Genotype and allelic frequencies for *MTRR* A66G, *MTHFR* C677T, *MTHFR* A1298C, and *MS* A2756G in the azoospermia group (N = 100).

Gene	Azoospermia	Allele type			
*MTRR*66		66AA	66AG	66GG	AG+GG
	Frequency (%) (N = 100)	(59.00) 59	(31.00) 31	(10.00) 10	(41.00) 41
	OR (95%CI)		0.836 (0.470-1.486)	1.977 (0.678-5.763)	0.973 (0.568-1.668)
	P		0.541	0.205	0.920
	Fertile men (%) (N = 120)	(58.33) 70	(36.67) 44	(5.00) 6	(41.67) 50
*MTHFR*677		677CC	677CT	677TT	CT+TT
	Frequency (%) (N = 100)	(36.00) 36	(49.00) 49	(15.00) 15	(64.00) 64
	OR (95%CI)		1.210 (0.677-2.161)	1.111 (0.494-2.498)	1.185 (0.685-2.050)
	P		0.519	0.799	0.543
	Fertile men (%) (N = 120)	(40.00%) 48	(45.00%) 54	(15.00%) 18	(60.00%) 72
*MTHFR*1298		1298AA	1298AC	1298CC	AC+CC
	Frequency (%) (N = 100)	(66.00) 66	(31.00) 31	(3.00) 3	(34.00) 34
	OR (95%CI)		0.989 (0.556-1.758)	1.818 (0.295-11.206)	1.030 (0.588-1.806)
	P		0.970	0.660	0.917
	Fertile men (%) (N = 120)	(66.67%) 80	(31.67%) 38	(1.67%) 2	(33.33%) 40
*MS*2756		2756AA	2756AG	2756GG	AG+GG
	Frequency(%) (N = 100)	(74.00) 74	(24.00) 24	(2.00) 2	(26.00) 26
	OR (95%CI)		1.927 (0.967-3.841)	1.365 (0.188-9.913)	1.868 (0.962-3.625)
	P		0.060	1.000	0.067
	Fertile men (%) (N = 120)	(84.17%) 101	(14.17%) 17	(1.67%) 2	(15.84%) 19

The allelic frequencies of the 4 SNPs in the OA group are listed in Table 4. Three SNPs (*MTHFR* C677T, *MTHFRA*1298C, and *MS* A2756G) were not associated with the OA group, but *MTRR* A66G was significantly associated with the OA group. The frequencies of *MTRR* A66G genotypes AG and AG+GG were associated with the OA group (P = 0.013, 0.012).

Table 4. Genotype and allele frequencies for *MTRR* A66G, *MTHFR* C677T, *MTHFR* A1298C, and *MS* A2756G in the oligoasthenozoospermia (OA) group (N = 62).

Gene	OA	Allele type			
*MTRR*66		66AA	66AG	66GG	AG+GG
	Frequency (%) (N = 62)	(38.71) 24	(54.84) 34	(6.45) 4	(61.29) 38
	OR (95%CI)		2.254 (1.183-4.293)	1.944 (0.505-7.481)	2.217 (1.184-4.149)
	P		0.013[a]	0.453	0.012[a]
	Fertile men (%) (N = 120)	(58.33) 70	(36.67) 44	(5.00) 6	(41.67) 50
*MTHFR*677		677CC	677CT	677TT	CT+TT
	Frequency (%) (N = 62)	(40.32) 25	(45.16) 28	(14.52) 9	(59.68) 37
	OR (95%CI)		0.996 (0.512-1.936)	0.960 (0.377-2.445)	0.987 (0.528-1.844)
	P		0.990	0.932	0.966
	Fertile men (%) (N = 120)	(40.00%) 48	(45.00%) 54	(15.00%) 18	(60.00%) 72
*MTHFR*1298		1298AA	1298AC	1298CC	AC+CC
	Frequency (%) (N = 62)	(56.45) 35	(37.10) 23	(6.45) 4	(43.55) 27
	OR (95%CI)		1.383 (0.720-2.657)	4.571 (0.800-26.129)	1.543 (0.822-2.895)
	P		0.329	0.084	0.176
	Fertile men (%) (N = 120)	(66.67%) 80	(31.67%) 38	(1.67%) 2	(33.33%) 40
*MS*2756		2756AA	2756AG	2756GG	AG+GG
	Frequency (%) (N = 62)	(80.65) 50	(17.74) 11	(1.61) 1	(19.35) 12
	OR (95%CI)		1.307 (0.570-3.000)	1.010 (0.089-11.407)	1.276 (0.574-2.834)
	P		0.527	1.000	0.549
	Fertile men (%) (N = 120)	(84.17%) 101	(14.17%) 17	(1.67%) 2	(15.84%) 19

[a]Statistically significant P values (P < 0.05) *vs* the fertile male group.

We analyzed the gene-to-gene correlation between 3 genes (*MTRR* A66G, *MTHFR* C677T, and *MS* A2756G) in the OA groups (Table 5). The genotypes *MTHFR* C677T and *MS* A2756G were not associated with the *MTRR* A66G genotype. However, the *MTHFR* 677TT genotype was generally increased for *MTRR* 66AA genotypes (P = 0.078).

Table 5. Gene-to-gene interaction analyses for *MTRR* A66G, *MTHFR* C677T, and *MS* A2756G in the oligoasthenozoospermia (OA) group.

Patient group	SNP		Allele type											
OA group	66		AA				AG				GG			
			C%[b] (N)	P%[c] (N)	P	OR	C% (N)	P% (N)	P	OR	C% (N)	P% (N)	P	OR
	677	CC	53.3 (16)	41.7 (10)			22.2 (4)	44.1 (15)			(50.00) 1	0.0 (0)		
		CT	46.6 (14)	45.8 (11)	0.779	1.26 (0.4-3.8)	55.6 (10)	44.1 (15)	0.211	0.40 (0.1-1.5)	(50.00) 1	50.0 (2)	1	3.0 (0.7-14.9)
		TT	0.0 (0)	12.5 (3)	0.078[a]		22.2 (4)	11.8 (4)	0.183	0.27 (0.0-1.6)	(0.00) 0	50.0 (2)	0.333	
		CT+TT	46.7 (14)	58.3 (14)	0.425	1.60 (0.5-4.7)	77.8 (14)	55.9 (19)	0.142	0.36 (0.1-1.3)	(50.00) 1	100.0 (4)	0.333	
	2756	AA	80.0 (24)	87.5 (21)			83.3 (15)	73.5 (25)			(50.00) 1	100.0 (4)		
		AG	20.0 (6)	12.5 (3)	0.715	0.57 (0.1-2.6)	11.1 (2)	23.5 (8)	0.461	2.40 (0.4-12.8)	(50.00) 1	0.0 (0)	0.333	
		GG	0.0 (0)	0.0 (0)			5.6 (1)	2.9 (1)	1	0.60 (0.0-10.3)	(0.00) 0	0.0 (0)		
		AG+GG	20.0 (6)	12.5 (3)	0.715	0.57 (0.1-2.6)	16.7 (3)	26.5 (9)	0.507	1.80 (0.4-7.7)	(50.00) 1	0.0 (0)	0.333	

[a]Not significant (P > 0.05) but showed a tendency toward significance. [b]Percentage of fertile men. [c]Percentage of infertile men.

DISCUSSION

Folate level may affect spermatogenesis by inducing DNA hypomethylation. This can disrupt gene expression and lead to uracil misincorporation during DNA synthesis, causing errors in DNA repair, strand breakage, and chromosomal anomalies. Mice lacking the *MTHFR* gene showed delayed maturation of the external genitalia and spermatogenic failure, which may explain some degree of human male infertility (Kelly et al., 2005). In humans, an improvement in spermatozoa number and motility as well as a decrease in round cell number were observed after 1 cycle of spermatogenesis with folinic acid treatment (Bentivoglio et al., 1993). These data indicate that folate metabolism plays an important role in maintaining spermatogenesis.

In our study, we analyzed the association between the 4 SNPs of 3 genes and the folate cycle in infertile men. We selected patients by performing karyotyping and azoospermia factor microdeletion detection, and classified patients without any chromosomal abnormalities and azoospermia factor microdeletion into 2 groups (azoospermia and OA).

Previous studies (Bezold et al., 2001; Ebisch et al., 2003; Stuppia et al., 2003; Park et al., 2005; Singh et al., 2005; A et al., 2007; Tetik et al., 2008; Ravel et al., 2009; Gava et al., 2011a,b; Safarinejad et al., 2011) evaluated the association between the *MTHFR* C677T polymorphism and infertility in patients from Europe, Asia, and Brazil. Bezold et al. (2001), Singh et al. (2005), and Park et al. (2005) showed that the frequency of the TT homozygote and CT heterozygote in *MTHFR* C677T was significant in infertile patients. Singh et al. (2005) also found that there were no T homozygotes in the control population, which showed that the T allele occurs at a very low frequency in the control population. However, Ebish et al. (2003), Stuppia et al. (2003), and Ravel et al. (2009) found no statistical significance of the C677T variation in infertile males and indicated the importance of folate in spermatogenesis. Our

results agree with those of previous studies. Some previous studies (Park et al., 2005; Lee et al., 2006; Ravel et al., 2009; Singh et al., 2010; Gava et al., 2011a,b; Safarinejad et al., 2011; Gupta et al., 2013) examined the influence of the *MTHFR* A1298C polymorphisms in infertile patients. Singh et al. (2010) reported that *MTHFR* A1298C may be an additional genetic risk factor for primary male infertility in the Indian population. However, Park et al. (2005), Lee et al. (2006), Ravel et al. (2009), Safarinejad et al. (2011), and Gupta et al. (2013) found no statistical significance for the A1298C variation in unexplained infertile males. Most studies suggested that some gene(s) other than *MTHFR* in the DNA methylation pathway affected male infertility.

Little is known regarding the role of *MTRR* A66G and *MS* A2756G in male infertility. Lee et al. (2006) reported that the frequencies of *MTRR* 66GG genotypes in non-obstructive infertile men, *MS* 2756GG genotypes in the azoospermia group, and *MTRR* 66GG in the OA group were higher compared with those in fertile Korean men. Gava et al. (2011b) showed that *MS* A2756G polymorphisms may be important in the predisposition to primary infertility in Brazilian men. However, Farcas et al. (2009) and Ravel et al. (2009) suggested that these polymorphisms in infertile men were not significantly more common than in controls.

Interesting, Singh et al. (2010) and Gupta et al. (2013) found very different results in the same region of *MTHFR* A1298C. Contrasting outcomes also have been shown in Brazilian men in 2 studies in the same ethnic background (Gava et al., 2011a,b). Our study and that of A et al. (2007) also found contrasting outcomes for *MTHFR* C677T. Ethnic and geographic variation may explain these results. *MTHFR* C677T was less frequent among Africans than among Caucasians (Rozen, 1997; Stevenson et al., 1997). The frequency of *MTHFR* A1298C also differed across populations (Botto and Yang, 2000). Moreover, gene-nutrient and gene-environmental factors have been shown to affect the impact of these *MTHFR* genetic variants (Toffoli and De Mattia, 2008). Ravel et al. (2009) have suggested that the different outcomes are due to population stratification rather than a causal link with the phenotype. Different methods with different sensitivities and accuracies may contribute to the contrasting outcomes.

Our results revealed an association between the *MTHFR* and *MTRR* genotypes and OA group. Although A66G and other SNPs were not associated with the OA group, the *MTHFR* 677TT genotype showed a trend toward increased *MTRR* 66AA types ($P = 0.078$). Additional studies including a larger number of subjects are necessary. In conclusion, the SNP *MTRR* A66G may be a genetic risk factor for infertility for Chinese men, particularly in the OA patient group. The exact mechanism underlying the folate pathway remains unclear, but mechanisms have been suggested to explain the cause of infertility. Lee et al. (2006) concluded that these SNPs were independently associated with male infertility. The SNP of each enzyme may affect enzyme activity to varying degrees by influencing the three-dimensional structure and amino acid substitution. Previous studies showed that the *MTRR* 66GG genotype contributed more than the *MTHFR* 677TT and *MS* 2756GG genotypes to the regulation of homocysteine levels (Gaughan et al., 2001; Jacques et al., 2003; Zijno et al., 2003). Our findings are partly consistent with these results. Future studies including subjects of different ethnic and geographic origins should be conducted for other genes encoding crucial enzymes in the folate metabolic pathway.

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