

·Original Article·

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

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Abstract

Aim: To analyze the distribution of the single nucleotide polymorphism (SNP) C677T in the methylenetetrahydrofolate reductase (*MTHFR*) gene in 355 infertile Chinese patients with idiopathic azoospermia or severe oligozoospermia and 252 fertile Chinese men as controls to explore the possible association of the SNP and male infertility. **Methods:** Using the polymerase chain reaction (PCR)-restriction fragment length polymorphism technique, the allele and genotype distribution of SNP C677T in the *MTHFR* gene were investigated in both patients and controls. **Results:** The frequencies of allele T (40.9% vs. 30.4%, $P = 0.002$, odds ratio [OR] = 1.58, 95% confidence interval [CI]: 1.24–2.02) and mutant homozygote (TT) (18.3% vs. 11.5%, $P = 0.023$, OR = 1.72, 95% CI: 1.07–2.76) as well as carrier with allele (TT + CT) (63.4% vs. 49.2%, $P = 0.0005$, OR = 1.79, 95% CI: 1.29–2.48) in infertile patients were significantly higher than those in controls. After patient stratification, the significant differences in distribution of the SNP between each patient subgroup and control group still remained. **Conclusion:** Our findings indicate that there is an association of SNP C677T in the *MTHFR* gene with male infertility, suggesting that this polymorphism might be a genetic risk factor for male infertility in Chinese men. (*Asian J Androl* 2007 Jan; 9: 57–62)

Keywords: male infertility; methylenetetrahydrofolate reductase gene; single nucleotide polymorphism; C677T

1 Introduction

Methylenetetrahydrofolate reductase (MTHFR) is one of the key enzymes in folate metabolism that is essential

for numerous cellular functions. The enzyme reduces the 5-10-methylenetetrahydrofolate to its biologically active form 5-methyltetrahydrofolate, and the latter then donates its methyl group for changing the homocysteine into methionine. Subsequently, methionine provides the methyl group for the formation of S-adenosylmethionine, the methyl donor for DNA and protein methylation, and the DNA methylation is crucial for spermatogenesis [1].

The C677T polymorphism in the coding region of human *MTHFR* gene that changes an alanine to a valine

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residue is a common single nucleotide polymorphism (SNP). Its polymorphic distribution varies greatly in different populations [2]. This gene variant encodes a thermostable form of MTHFR, which decreases the enzyme activity by approximately 35% in heterozygote (CT) and 70% in mutant homozygote (TT) [3]. The homozygous C667T in the *MTHFR* gene is reported to be associated with the risk of certain human diseases, including some cardiovascular disorders, cancers and neural tube defects [3–6]. The activity of MTHFR is much higher in testis than in other major organs in the adult mouse, suggesting that it might play an important role in spermatogenesis [7]. Moreover, in the *Mthfr* gene knockout mice, the MTHFR-deficiency resulted in abnormal spermatogenesis and infertility in males, providing further evidence that *Mthfr* gene is involved in male infertility [8]. In humans, the combined treatment with zinc sulfate and folic acid could increase total sperm count [9]. Therefore, it is reasonable to hypothesize that C677T mutation in the *MTHFR* gene might also be associated with spermatogenesis and male infertility in humans.

In the present study, we investigated the frequency distribution of the common SNP C677T in the *MTHFR* gene in 355 infertile patients with idiopathic azoospermia or severe oligozoospermia and compared it with those in 252 fertile controls to explore the possible association between this gene variation and male infertility.

2 Materials and methods

2.1 Subjects

The total 355 infertile patients, including 228 with idiopathic azoospermia and 127 with severe oligozoospermia (semen count less than $5 \times 10^6/\text{mL}$) aged from 25 to 38 years, were recruited from the Department of Urology and Department of Andrology, West China Hospital, Sichuan University between November 2001 and January 2005. After examination by specialists, a history of orchitis, maldescensus of testis, varicocele and obstruction of vas deferens were excluded. Then patients with chromosomal abnormalities and microdeletions in the azoospermia factor region on Y chromosomes were excluded by chromosomal G-banding [10] and corresponding molecular analysis, respectively [11]. All patients underwent at least two semen analyses according to World Health Organization guidelines after 3–5 days of sexual abstinence. The control group consisted of 252 men who were proven fertile with normozoospermia aged from 26 to 51 years. All of

the study subjects are of Han nationality, which makes up more than 90% of the Chinese population and informed consent was obtained from all subjects.

2.2 Polymerase chain reaction (PCR) amplification

Genomic DNA was extracted from the peripheral blood leucocytes of patients and controls using a salting out procedure [12]. Primers 5'-CATCCCTATTGGCAG GTTAC-3' (forward) and 5'-GACGGTGC GG TGAG AGTG-3' (reverse) were used to amplify the 265 bp fragment around the polymorphic site studied. Polymerase chain reaction (PCR) amplification was carried out in a total volume of 25 μL containing approximately 100 ng of genomic DNA, 200 $\mu\text{mol/L}$ dNTPs, 8 pmol of each primer, 1.5 mmol/L MgCl_2 and 1 U Taq polymerase (Takara, Shiga, Japan), and 2.5 μL of $10 \times$ PCR buffer. The reaction profile was: predenaturation at 94°C for 5 min followed by denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s for 35 cycles, with a final extra extension at 72°C for 5 min.

2.3 Genotyping of SNP C677T in the MTHFR gene

PCR amplicons were digested with restriction enzyme *HinfI* (MBI, Vilnius, Lithuania). Then the products of digestion were electrophoresed on a 2.5% agarose gel and observed with Gel Doc 1000 system (Bio-Rad, Hercules, CA, USA). The wild type homozygote (CC), heterozygote (CT) and mutant homozygote (TT) showed one band (265 bp), three bands (265, 171 and 94 bp) and two bands (171 and 94 bp), respectively, because allele T produced a cut site for *HinfI*. The genotypes were further confirmed by DNA sequencing of PCR products of some samples.

2.4 Statistical analysis

The allele and genotype frequencies of the patients and controls were calculated by counting. The Hardy-Weinberg equilibrium was tested using HWE software. The differences in allelic and genotypic frequencies of C667T locus between groups were evaluated using the χ^2 test with odds ratio (OR). The differences in average sperm count among the different genotype groups in controls were evaluated with one-way analysis of variance (ANOVA).

3 Results

The polymorphism distribution of C677T in the

Table 1. Distribution of C677T polymorphism in methylenetetrahydrofolate reductase (*MTHFR*) gene in fertile controls and infertile patients with azoospermia or severe oligozoospermia. *Controls vs. [1] total patients, [2] azoospermic patients and [3] severe oligozoospermic patients. CC, wild type homozygote; CT, heterozygote; TT, mutant homozygote; OR, odds ratio; CI, confidence interval.

	Controls		Patients		P-value* OR (95% CI)		
	Total (n = 252)	Total (n = 355)	Azoospermia (n = 228)	Severe Oligozoospermia (n = 127)	[1]	[2]	[3]
CC	0.508 (128)	0.366 (130)	0.364 (83)	0.370 (47)			
CT	0.377 (95)	0.451 (160)	0.426 (97)	0.496 (63)	0.070	0.279	1.63 (1.06–2.50)
TT	0.115 (29)	0.183 (65)	0.210 (48)	0.134 (17)	0.023	0.004	0.597
CT + TT	0.492 (124)	0.634 (225)	0.636 (145)	0.630 (80)	1.72 (1.07–2.76)	2.05 (1.24–3.39)	
C	0.696 (351)	0.591 (420)	0.577 (263)	0.618 (157)	0.0005	0.002	0.011
T	0.304 (153)	0.409 (290)	0.423 (193)	0.382 (97)	1.79 (1.29–2.48)	1.80 (1.25–2.60)	1.76 (1.14–2.72)
					0.0002	0.0001	0.030
					1.58 (1.24–2.02)	1.68 (1.29–2.20)	1.42 (1.03–1.9)

MTHFR gene is summarized in Table 1. As shown in the table, the allele T, mutant homozygotes and the allele T carrier in infertile patients were (TT + CT) significantly more than those in controls. The frequency of heterozygotes was also higher in infertile patients than that in controls, but the difference was not statistically significant. After classifying the patients into azoospermic and severe oligozoospermia groups, the frequencies of allele T, mutant homozygotes and allele T carrier still remained significantly higher in patients with azoospermia than those in controls. Also, similar significant differences in frequencies of allele T, heterozygotes and the allele T carrier between patients with severe oligozoospermia and controls were observed. The distributions of genotypes followed the Hardy–Weinberg equilibrium in the total patients, the patients with azoospermia or severe oligozoospermia as well as in the controls (data not shown).

We also analyzed a possible association between the gene polymorphism and the sperm count in controls. The average sperm count of genotype CC, CT and TT was $(70 \pm 35) \times 10^6$, $(68 \pm 32) \times 10^6$ and $(63 \pm 29) \times 10^6$, respectively. No significant differences in average sperm counts were detected among the three genotype groups ($P > 0.05$), but the average sperm count of genotype TT was slight lower than that of genotype CC.

The representative results of genotyping for C677T locus in the *MTHFR* gene by electrophoresis and DNA sequencing of genotypes are shown in Figures 1 and 2, respectively.

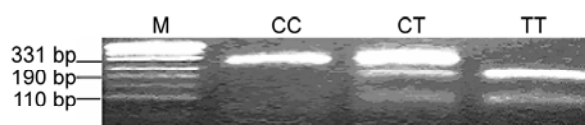


Figure 1. The results of genotyping of C677T in methylenetetrahydrofolate reductase (*MTHFR*) gene. M, DNA size marker (pUC19 DNA/*MspI*); CC, wild type homozygote; CT, heterozygote; TT, mutant homozygote.

4 Discussion

Several studies, summarized in Table 2, reported the association of SNP C677T polymorphism in the *MTHFR* gene with male infertility. As shown in Table 2, the prevalence of the homozygous mutation was significantly higher in the infertile subjects (18.8%) than in the control group (9.5%) in German men, which suggests that mutant homozygote of the SNP could increase the risk of male infertility and decreased activity of *MTHFR* might lead to male infertility [13]. In other two studies on Indian and Korean men, respectively, not only homozygote but also heterozygote of C667T is associated with male infertility in men with azoospermia or severe oligozoospermia, providing further evidence that the mutation of *MTHFR* gene might predispose to male infertility and that the gene might be involved in spermatogenesis im-

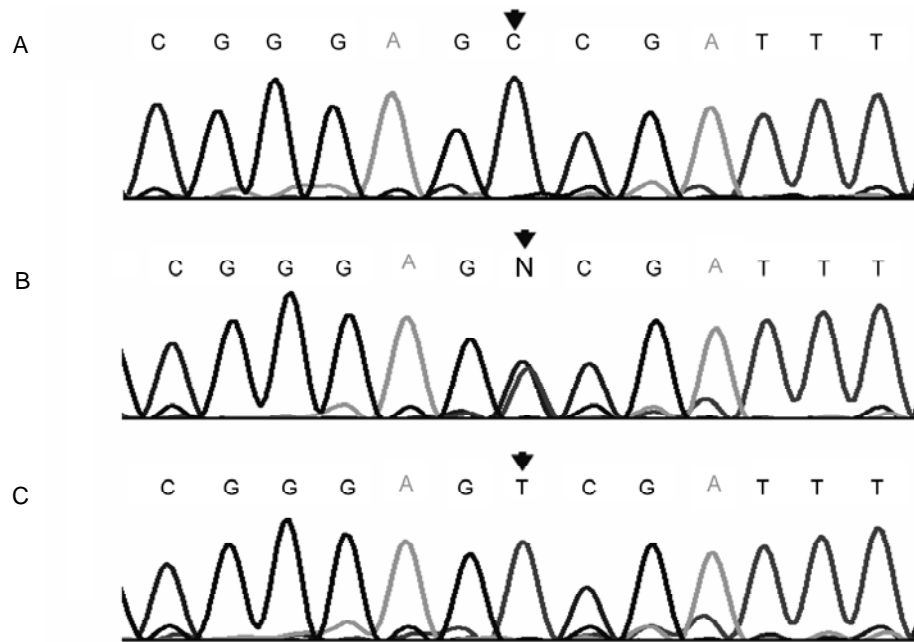


Figure 2. The results of DNA sequencing of the three genotypes of single nucleotide polymorphism (SNP) C677T in methylenetetrahydrofolate reductase (*MTHFR*) gene. Arrows indicate the position of the SNP. (A): Wild homozygote (CC); (B): Heterozygote (CT); (C): Mutant homozygote (TT).

Table 2. Genotype distribution of MTHFR C677T in infertile patients and controls from different studies. *Significant difference between patient and control group was observed. CC, wild type homozygote; CT, heterozygote; TT, mutant homozygote.

Population	Group	Number of cases	Frequency of genotype (%)			References
			CC	CT	TT	
German	Controls	200	46.0	44.5	9.5	Bezold <i>et al.</i> [13]
	Patients	225	44.7	36.5	18.8*	
Netherlandic	Controls	112	44.2	42.5	13.3	Ebisch <i>et al.</i> [9]
	Patients	77	54.5	36.4	9.1	
Italian	Controls	105	31.4	41.0	27.6	Stuppia <i>et al.</i> [16]
	Patients	93	39.8	38.9	20.4	
Indian	Controls	200	81.5	18.5	0	Singh <i>et al.</i> [14]
	Patients	151	69.5	26.5*	4.0*	
Korean	Controls	105	36.6	50.5*	12.9*	Park <i>et al.</i> [15]
	Patient	205	28.1	55.0	16.9	

pairment [14, 15]. In contrast, similar studies from the Netherlands and Italy did not find any significant difference in polymorphic distribution of SNP C677T between male infertile patients and fertile controls, which does not support the role played by the SNP in male infertility [9, 16]. These contradictory results from studies on different populations suggest that the role of C677T in

susceptibility to male infertility might depend on ethnic or geographic factors. It is reported that the detrimental effects of the C677T mutation on enzymatic activity of MTHFR depend on status of folate [17]. The mutation might be tolerated in subjects with rich folate supply, whereas in individuals with insufficient folate intake it might result in some biochemical or clinical phenotypic

consequences [17]. Therefore, diverse levels of dietary folate uptake might explain the discrepancy in the effect of SNP C677T on male infertility in different populations.

In the present study, we investigated the relationship between the SNP C677T in the *MTHFR* gene and male infertility in patients with idiopathic azoospermia or severe oligozoospermia and fertile controls and found that the frequencies of allele T, mutant homozygote and the allele T carrier were significantly higher in the infertile patients studied than those in the controls. After stratifying the patients by semen count, there was still a statistical difference in polymorphic distribution of the SNP between each patient subgroup and control group. These results indicate that there is an association of SNP C677T in the *MTHFR* gene with male infertility, suggesting that this mutation might be a genetic risk factor for infertility in Chinese men. In the controls, although there was no statistical difference in average sperm count between genotype CC and TT, the average sperm count of genotype TT was slightly lower than that of CC, which suggests that the C677T variation might exert some effect on spermatogenesis.

Although the susceptibility to male infertility related to SNP C677T is not yet clear, there are several possible mechanisms that might explain the increase of the risk for male infertility. First, spermatogenesis is a complex process involving highly regulated expression of numerous genes and DNA methylation is essential for such an expression [1]. Indeed, the hypomethylation of DNA is reported to lead to the failure of differentiation of germ cells into spermatocyte and sperm count decrease [18]. In humans, allele T of SNP C677T can reduce *MTHFR* activity and subjects with genotype TT are associated with decreased global genomic methylation compared those with genotype CC [5]. Second, the C677T mutation in the *MTHFR* gene might result in hyperhomocysteinemia in low folate status [17] and a high level of homocysteine can induce the auto-oxidation that might cause DNA damage. Besides possible damage to germ line DNA, oxidative stress could also damage the cell membranes [19]. Finally, the C677T mutation in the *MTHFR* gene could lead to hyperhomocysteinemia and increase the risk of vascular disease [3, 17]. In subjects with hyperhomocysteinemia, the precocious atherosclerotic vascular alterations could lead to testicular arterial blood flow reduction and impairment of spermatogenesis [20].

In summary, the present study found an association

between the SNP C677T in the *MTHFR* gene and infertility in men with azoospermia and severe oligozoospermia, suggesting that this mutation might be a genetic risk factor for infertility in Chinese men. Because the variation is also involved in cardiovascular diseases as well as some cancers, but no relevant data of the subjects studied were available at present, whether these diseases affect the results of the present study is in need of further evaluation. In addition, a further functional study is needed to elucidate the role of SNP in the pathogenesis of male infertility.

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