ABSTRACT. The aim of this study was to explore the relationship between 2 genetic polymorphisms of the methylenetetrahydrofolate reductase gene (MTHFR), C677T and A1298C, and determine the long-term reproductive outcome in infertile men. This was a prospective study conducted in an andrology clinic. Men with a 1-year history of infertility were assessed for the MTHFR polymorphisms at 5-year follow-up. We compared the MTHFR C677T and A1298C polymorphisms by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) between men who did and did not bear children during follow-up. Of the 215 men who were infertile at 1 year, 82 (38.1%) remained infertile and 133 (61.9%) achieved natural conception during the 5-year follow-up, with the highest rate in the first year (32.6%). The MTHFR 677TT genotype (homozygote) was associated with a substantially increased risk of infertility during
follow-up [odds ratio (OR) = 10.242; 95% confidence interval (CI) = 1.257-83.464] relative to the \textit{MTHFR} 677CC genotype (wild-type). Risk of infertility was not increased by the \textit{MTHFR} A1298C polymorphism alone, but was increased by the combination of polymorphisms \textit{MTHFR} C677T and \textit{MTHFR} A1298C (OR = 11.818; 95%CI = 1.415-98.674). The homozygous \textit{MTHFR} C677T genotype was a risk factor for male infertility during 5-year follow-up, whereas a correlation between \textit{MTHFR} A1298C and infertility was not observed. The \textit{MTHFR} C677T and \textit{MTHFR} A1298C polymorphisms had additive effects on male infertility.

\textbf{Key words:} Gene polymorphism; Male infertility; Prospective study; Methylenetetrahydrofolate reductase

\section*{INTRODUCTION}

With the continued development of medical genetics, an increasing number of studies have reported that genetic factors play an important role in male infertility. Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme involved in the metabolism of methioninefolate and participates in the process of DNA synthesis and regulation of homocysteine levels \textit{in vivo}. Researchers have shown that 2 genetic polymorphisms, \textit{MTHFR} C677T and \textit{MTHFR} A1298C, may reduce the activity of MTHFR and are related to several disorders, including male infertility (Chen et al., 2001; Kelly et al., 2005). While several case-control studies have described an association between \textit{MTHFR} genetic polymorphisms and male infertility, data regarding the relationship between the \textit{MTHFR} polymorphisms and long-term reproductive outcome in infertile patients are rare. Thus, we conducted a prospective study to explore the effects of the \textit{MTHFR} C677T and \textit{MTHFR} A1298C polymorphisms on the long-term reproductive outcome of infertile men.

\section*{MATERIAL AND METHODS}

\subsection*{Study subjects}

Subjects were recruited at the Andrology Clinic of Beijing Obstetrics and Gynecology Hospital from December 2003 to December 2004. Subjects were diagnosed as infertile if they had been married for more than 1 year but their spouses had not become pregnant over the course of a normal sex life without the use of any contraceptive methods. Systemic organic disease, endocrine factors, specific genetic abnormalities, reproductive tract disease, and abnormal ejaculation were excluded as causes of infertility. The results of infertility tests in spouses were normal.

Semen was collected through masturbation after abstinence for 3-7 days. Semen samples were inspected within 30 min after collection, and the results were analyzed according to World Health Organization guidelines. Each study subject also donated 5 mL blood, which was centrifuged, and the blood cell sediment was cryopreserved at -20°C.

At the end of 2008, all subjects were contacted by telephone to determine if they
had borne children in the past 5 years. A total of 226 subjects agreed to participate in the study, with the exclusion of 11 men who had divorced during the intervening period. The final number of study subjects was therefore 215, which included 133 men whose spouses had conceived naturally (classified as the “control group”) and another 82 men whose spouses had not conceived naturally, including 5 subjects who achieved conception through assisted procedures (the “patient group”). The average ages of the control group and the patient group were 30.89 ± 4.42 and 32.49 ± 4.35 years, respectively.

Genomic DNA extraction

DNA was extracted from blood cell specimens of the 215 study subjects using the Wizard Genomic DNA Purification Kit (Promega Corporation; Madison, WI, USA) following the manufacturer protocol.

MTHFR C677T and MTHFR A1298C genotype determination

The MTHFR genotype was detected by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP). Specific primer pairs were selected according to the related reference sequence (5'-TGA AGG AGA AGG TGT CTG CGG GA-3' as the upstream primer and 5'-AGG ACG GTG CGG TGA GAG TG-3' as the downstream primer for MTHFR C677T (Frosst et al., 1995); 5'-CTT TGG GGA GCT GAA GGA CTA CTA C-3' as the upstream primer and 5'-CAC TTT GTG ACC ATT CCG GTT TG-3' as the downstream primer for MTHFR A1298C (van der Put, 1998).

The PCR volume was 25 μL and contained 2.5 μL 10X PCR buffer, 2 μL 200 μM deoxynucleoside triphosphates, 0.5 μL 10 μM each primer for MTHFR C677T or 0.25 μL 10 μM each primer for MTHFR A1298C, 1.5 U Taq DNA polymerase, and 50 ng DNA as a template. PCR was carried out over 35 cycles for MTHFR C677T and over 36 cycles for MTHFR A1298C at a denaturing temperature of 95°C for 5 min, an annealing temperature of 60°C for 35 s, and a primer extension temperature of 72°C for 7 min. PCR products for the MTHFR C677T polymorphism were digested by HinfI restriction enzyme in a 20 μL reaction volume containing 10 μL PCR fragment, 2 μL 10X buffer 2, and 5 U HinfI at 37°C overnight. The product of PCR analysis of the MTHFR A1298C polymorphism was digested by MboII restriction enzyme in a 20 μL reaction volume containing 5 μL PCR fragment, 2 μL 10X buffer 2, and 1.5 U MboII at 37°C for 1 h. The digestion products were evaluated on an ethidium bromide-stained agarose gel (3% for MTHFR C677T, 4% for MTHFR A1298C), and then the genotype was determined.

Each genotype determined by electrophoresis was evaluated by DNA sequencing for verification.

Statistical analysis

Data were analyzed using the SPSS statistical package, version 13.0 (SPSS, Inc.; Chicago, IL, USA). A chi-square test was used to compare genotype and allele frequencies, with a P-value <0.05 considered to be statistically significant. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated.
RESULTS

Characteristics of mutations in the MTHFR gene

The MTHFR C677T polymorphism results in a substitution of thymidine for cytosine in exon 4 of the MTHFR gene. The length of the amplified products of MTHFR C677T was 198 base pairs (bp), and the digested products contained 3 genotypes. The first genotype was CC (wild)-type, which had 1 gene fragment with a length of 198 bp. The second genotype was TT, which was the homozygote and included 2 gene fragments with lengths of 175 and 23 bp. The third genotype was CT, which was the heterozygote and included 3 gene fragments whose lengths were 198, 175, and 23 bp (Figure 1A).

The MTHFR A1298C polymorphism results in a substitution of cytosine for adenine in exon 7 of the MTHFR gene. The length of the amplified products of MTHFR A1298C was 163 bp, and the digested products contained 3 genotypes. The first genotype was AA, which had 5 gene fragments with lengths of 56, 31, 30, 28, and 18 bp. The second genotype was CC, which was the homozygote and included 4 gene fragments whose lengths were 84, 31, 30, and 18 bp. The third genotype was AC, which was the heterozygote and included 6 gene fragments whose lengths were 84, 56, 31, 30, 28, and 18 bp (Figure 1B).

Figure 1. A. Restriction fragment length polymorphism (RFLP) analysis of the C677T mutation on a 198-bp PCR fragment with Hinfl. The C677T mutation adds a Hinfl restriction site. Digestion of the 677TT genotype yields 2 fragments of 175 and 23 base pairs (bp), whereas the 677CT genotype results in 3 fragments of 198, 175, and 23 bp, and the 677CC genotype shows only 1 fragment of 198 bp. The figure depicts the 3 possible genotypes. The 23-bp fragment ran off the gel. B. RFLP analysis of the A1298C mutation on a 163-bp PCR fragment with MboII. The A1298C mutation abolishes the MboII restriction site. Digestion of the 1298AA genotype yields 5 fragments of 56, 31, 30, 28, and 18 bp, whereas the 1298CC genotype results in 4 fragments of 84, 31, 30, and 18 bp, and the 1298AC genotype shows 6 fragments of 84, 56, 31, 30, 28, and 18 bp. The figure depicts the 3 possible genotypes. The 18-bp fragment ran off the gel. As the molecular weights of 31, 30, and 28 bp are very similar, they could not be distinguished on the gel.
Possible etiology of male infertility

Five-year follow-up

At the end of the 5-year follow-up, the natural conception rate was 61.9% (133 of 215 subjects). The natural conception rate was highest during the first year of follow-up, at 32.6%, and then declined progressively, which was calculated by using direct survival analysis.

Genotype analysis

The genotype distributions of MTHFR C677T and MTHFR A1298C in all study subjects were tested according to the Hardy-Weinberg law of genetic equilibrium (P > 0.05).

The association between the MTHFR C677T genetic polymorphism and infertility status is shown in Table 1. The frequency of the MTHFR 677T allele was higher in the patient group than in the control group. Compared with the MTHFR 677CC genotype, the MTHFR 677TT genotype was associated with a greater likelihood of infertility. With regard to the frequencies of the 3 genotypes and alleles of MTHFR A1298C, there were no statistically significant differences between the patient group and the control group (Table 2).

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<th>Table 1. Association between MTHFR C677T genotypes and infertility status.</th>
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*Chi-square test was used; P < 0.05 was considered to be statistically significant (values in bold).

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<th>Table 2. Association between MTHFR A1298C genotypes and infertility status.</th>
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*Chi-square test was used; P < 0.05 was considered to be statistically significant.

In this study, 42 men had the combined mutation of MTHFR C677T and MTHFR A1298C, but there were no cases of combined mutation of the MTHFR C677T homozygote (TT) and the MTHFR A1298C homozygote (CC). Excluding the interference of the mutation of MTHFR A1298C, the risk of male infertility associated with the MTHFR 677TT genotype was increased. Analysis of the combined polymorphism showed that the risk of male infertility was clearly increased (Table 3).
DISCUSSION

Chen et al. (2001) showed that expression of MTHFR is higher in the testicles than in other organs in the adult male mouse. This implies that MTHFR may play an important role in spermatogenesis. Another study (Kelly et al., 2005) showed that the male mouse has irregular spermatogenesis and becomes infertile if the \textit{MTHFR} gene is knocked out, which supports a role for MTHFR in spermatogenesis.

To date, research regarding the association between \textit{MTHFR} genetic polymorphisms and male infertility has not been extensive and has mostly included case-control studies. The current study explored the interaction between \textit{MTHFR} genetic polymorphisms and male infertility through a prospective design and 5-year follow-up. The results showed that the \textit{MTHFR} $677TT$ (homozygous) genotype significantly increases the risk of infertility compared with the wild-type or heterozygous genotype. This finding is consistent with a 1-year follow-up study of idiopathic male infertility conducted by Paracchini et al. (2006). Some case-control studies, such as those conducted by Bezold et al. (2001) and others (Park et al., 2005; Singh et al., 2005; Lee et al., 2006; A et al., 2007), also described a higher rate of \textit{MTHFR} C677T in infertile men, whereas the studies of Ebisch et al. (2003), Stuppia et al. (2003), and Dhillon et al. (2007) reported no obvious association between the \textit{MTHFR} C677T mutation and male infertility.

Our study found no clear relationship between the \textit{MTHFR} A1298C polymorphism and male infertility, as shown previously by Park et al. (2005), Lee et al. (2006), and Dhillon et al. (2007). In addition, our study revealed an additive effect between the \textit{MTHFR} C677T and \textit{MTHFR} A1298C polymorphisms on male infertility. We did not observe cases of a combined homozygous mutation of \textit{MTHFR} C677T and A1298C, which is consistent with a study conducted by van der Put et al. (1998), who concluded that the combined homozygous mutation of \textit{MTHFR} C677T and \textit{MTHFR} A1298C is catastrophic to the function of MTHFR.

It has been demonstrated that the \textit{MTHFR} C677T mutation may decrease the activity of MTHFR, but that the single mutation of \textit{MTHFR} A1298C only minimally affects MTHFR activity. Other studies (van der Put et al., 1998; Robien and Ulrich, 2003) have shown that activity of MTHFR is weakened by the \textit{MTHFR} C677T mutation when combined with the \textit{MTHFR} A1298C mutation. MTHFR is considered to participate in spermatogenesis by regulating DNA methylation (Friso et al., 2002). Low DNA methylation is thought to be associated with low fertility rates (Cisneros, 2004). Therefore, men with the \textit{MTHFR} C677T homozygote genotype or the mutation of \textit{MTHFR} C677T combined with \textit{MTHFR} A1298C may have lower genomic DNA methylation rates, thereby affecting their fertility rates. However, the details of this mechanism require further study.

\begin{table}[h]
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\begin{tabular}{|c|c|c|c|c|}
\hline
677 Genotype & 1298 Genotype & Infertility (N, %) (patient group) & Fertility (N, %) (control group) & OR (95%CI) & P value$^a$ \\
\hline
CC & AA & 1 (1.2) & 13 (9.8) & 1 (Reference) & \\
CT & AA & 22 (26.8) & 42 (31.6) & 6.810 (0.835-55.515) & 0.089 \\
TT & AA & 26 (31.7) & 33 (24.8) & 10.242 (1.257-83.464) & 0.010 \\
CC & CC+AC & 13 (15.9) & 23 (17.3) & 7.348 (0.861-62.743) & 0.090 \\
CT/TT & AC/CC & 20 (24.4) & 22 (16.5) & 11.818 (1.415-98.674) & 0.007 \\
\hline
\end{tabular}
\caption{Association between the \textit{MTHFR} C677T polymorphism combined with the \textit{MTHFR} A1298C polymorphism and infertility status.}
\end{table}

$^a$Chi-square test was used; $P < 0.05$ was considered to be statistically significant (values in bold).
The natural conception rate in the first year of follow-up in our study was 32.6%, which was slightly lower than that determined in a study by Paracchini et al. (2006). The conception rate over the entire 5-year period was 61.9%, which is higher than expected. A possible explanation for this may be the limitations of the method of telephone follow-up. Improved methods for direct communication with patients are required in future studies in order to precisely determine the natural conception rate.

In conclusion, we used a long-term follow-up design to study the role of MTHFR genetic polymorphisms in infertile men. The results suggest that the MTHFR 677TT (homozygous) genotype increases the risk of male infertility in the long term, and that there is an additive effect between the MTHFR C677T and MTHFR A1298C polymorphisms. The results did not show a clear relationship between the MTHFR A1298C polymorphism and male infertility. This indicates that infertile patients with different MTHFR genetic polymorphisms have varying reproductive outcomes.

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