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Relationship Between Genetic Polymorphisms of Methylenetetrahydrofolate Reductase (C677T, A1298C, and G1793A) as Risk Factors for Idiopathic Male Infertility

Mohammad Reza Safarinejad, MD[1], Nayyer Shafiei, BS[1], and Shiva Safarinejad, BS[1]

Abstract

Objective: The human methylenetetrahydrofolate reductase (*MTHFR*) gene plays a crucial role in folate metabolism. Data regarding the influence of *MTHFR* gene polymorphisms on male fertility status are scarce and conflicting. We determined associations between 3 *MTHFR* gene polymorphisms (C677T, A1298C, and G1793A), serum folate, and total homocysteine (tHcy) levels, with male fertility status and semen parameters. **Methods:** *MTHFR* genotypes were determined using polymerase chain reaction restriction fragment length polymorphism (PCR-RLFP) technique and serum tHcy, folate, and vitamin B12 concentrations were measured in 164 men with idiopathic infertility and 328 healthy participants. **Results:** There was a significant difference in genotype frequency distribution of *MTHFR* C677T polymorphism between infertile patients and controls ($P = .004$). The 677T allele carriers (TC or TT) had a significantly increased risk of infertility compared with the CC homozygotes (odds ratio [OR] 1.60, 95% confidence interval [CI] 1.21-2.75, and OR = 2.68, 95% CI = 1.84-3.44, respectively), in a logistic regression model after adjustment for confounding factors. Men with the 677T, 1298C, and 1793G alleles showed significantly higher serum tHcy and lower folate levels (all *P*s < .01). We found a positive correlation between serum folate concentrations and sperm density ($r = .74$, $P = .001$), percentage of sperm with progressive motility ($r = .68$, $P = .001$), as well as percentage of sperm with normal morphology ($r = .72$, $P = .001$). **Conclusion:** *MTHFR* C677T polymorphism is associated with an increased risk of idiopathic male infertility. Further study on the biologic role that this polymorphism plays in the development of infertility may lead to better understanding of the etiology of impaired spermatogenesis.

Keywords

infertility, male factor, MTHFR, gene, polymorphism

Introduction

The human methylenetetrahydrofolate reductase (*MTHFR*) gene is composed of 11 exons[1] and is located on the short arm of chromosome 1 (1p36.3).[2] The MTHFR enzyme catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate.[3] The MTHFR enzyme plays an essential role in folate metabolism, a critical factor in DNA methylation and spermatogenesis.[4] There are 2 common polymorphisms in the *MTHFR* gene[5,6]: *MTHFR* C677T (rs1801133) and *MTHFR* A1298C (rs1801131). The 677C>T (Ala222Val) polymorphism results in alanine-to-valine substitution at codon 222.[1] The 1298A>C (Glu429Ala) results in glutamate-to-alanine substitution at codon 429.[1] Recently a third and novel polymorphic site of the *MTHFR* gene in exon 11 has been reported by Rady et al.[7] The 1793G>A polymorphism results in an arginine-to-glutamine change at codon 594. The *MTHFR* 677CT and 1298AC polymorphisms are associated with up to a 70% reduction in folate metabolism and hyperhomocysteinemia.[8,9] Folate status is correlated with a variety of diseases, including neural tube defects,[10] neurological and cognitive disorders,[11] cancer,[12] and cardiovascular disease.[13] Folate plays a crucial role in the regulation of homocysteine homeostasis, in providing methyl groups to maintain transmethylation reactions, and in supplying 1 carbon units to DNA/RNA synthesis.[14] Several mechanisms may explain an association

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between folate and infertility. It has been reported that folate has antioxidant properties.[15] Low folate in seminal plasma is associated with increased sperm DNA damage.[16] In addition, Wallock et al reported that low seminal plasma folate concentrations are associated with low sperm density and count in male smokers and nonsmokers.[17] In addition, it has been reported that folate deficiency increases DNA fragility due to the misincorporation of uracil instead of thymine.[18] Serum folate level inversely correlated with serum total homocysteine (tHcy) concentration.[19] Decreased serum B-vitamin levels cause elevated homocysteine concentrations and impairment of the remethylation cycle. This metabolism is involved in the synthesis repair and methylation of DNA and RNA.[20] These processes are crucial in spermatogenesis.[16] Ebisch et al demonstrated a deleterious effect of an elevated tHcy concentration in ejaculated sperm.[21] Studies addressing the effect of *MTHFR* polymorphisms of male factor infertility are scarce and conflicting. The *MTHFR* polymorphism is correlated with male infertility in African, Indian, and South East Asian populations,[22,23] however these results were not reproduced in the European populations.[24] The relationship between all 3 MTHFR polymorphisms (C677T, A1298C, and G1793A) and the risk of idiopathic male infertility has not been previously investigated, as to our knowledge; our study is the first to report an association.

Materials and Methods

Study Participants

This case–control study included 463 unrelated men of reproductive age. Case patients consisted of 164 men with idiopathic oligoasthenoteratozoospermia (OAT). For each case, 2 controls ($n = 328$) were randomly selected matching the index case for age (± 2 years). They were volunteers who selected prospectively after consent from the Andrology Outpatient Clinic. The whole study cases were compared to whole matched controls. Infertility was defined as unsuccessful attempts to become pregnant after at least 24 consecutive months of unprotected intercourse with the same partner. All of the participants in control groups were healthy fertile men, had fathered at least 1 child, and had normal sperm parameters at the time of recruitment to the study. All patients gave informed consents and the investigation was conducted in accordance with the International Conference on Harmonisation−Good Clinical Practice (ICH-GCP) guidelines and the principles of the Declaration of Helsinki. Diagnosis of OAT in infertile men, and normal semen parameters in control participants was based on at least 2 semen analyses performed 4 weeks apart. Normal semen values according to World Health Organization (WHO, 1999)[25] criteria were sperm density greater than 20×10^6/mL, sperm motility grade A + B greater than 50%, normal morphology greater than 30%, and/or semen volume greater than 2 mL.

Inclusion and Exclusion Criteria

Inclusion criteria for cases were as follows: stable relationship, infertility for at least 2 years of unprotected intercourse with the same partner, confirmed abnormal semen parameters, no indication of hormonal, medical, or surgical causes for infertility, origin and dwelling in the studied geographic area (Tehran), lack of family history of infertility, a normal fertile female partner according to investigations, and a total testicular volume ≥ 12 mL on ultrasound. Control participants had no personal or family history of infertility. Exclusion criteria for infertile cases included age above 45 years; azoospermia; urinary tract infections; associated varicocele, hydrocoele, undescended testis or their corrective surgery; use of cytotoxic drugs, immunosuppressants, anticonvulsives, androgens, or antiandrogens; leukocytospermia (more than 10^6 white blood cells per mL), or positive mixed agglutination reaction test, and abnormal hormonal profile or presence of any endocrinopathy. Participants with a history of hepatobiliary disease, significant renal insufficiency, drug or alcohol abuse or dependence, tobacco use, occupational and environmental exposures to potential reproductive toxins, and a body mass index (BMI) of ≥ 30 kg/m^2 were also excluded.

Evaluations

All these participants underwent a comprehensive evaluation, including detailed medical, andrological, and surgical histories, physical examination, and laboratory analysis. Information on demographic variables, educational level, socioeconomic conditions, and smoking status were also gathered simultaneously. Body mass index was calculated as weight (in kilograms) divided by standing height (in meters squared). Obesity was defined as BMI ≥ 30 kg/m^2. Laboratory analysis included measurement of serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), total testosterone (T), prolactin (PRL), thyroid-stimulating hormone (TSH), free thyroxin (FT4), free triiodothyronin (FT3), Inhibin B levels, karyotype, and Y chromosome microdeletion screening. Hematological and routine biochemistry analyses were also performed. Total testicular volume was measured using scrotal ultrasound. Laboratory examination also included at least 2 semen analyses after 3 days of abstinence with an interval of 4 weeks between them. Values for semen parameters were calculated as means of 2 analyses.

Hormonal Assays

Total serum T was measured using radioimmunoassay (RIA) kit (3H Testosterone; Biomerieux, Lyon, France), with a sensitivity of detection of 4 pg/mL. Normal reference range for T was 3.5 to 9.5 ng/mL. Serum LH and FSH levels were determined using time-resolved immunofluorometric assay kits (DELFIA hLH for LH and DELFIA hFSH for FSH; Wallac Co, Turku, Finland). The intra- and inter-assay coefficients of variation of individual immunofluorometric assay method was below 9% within the reference ranges. Reference ranges were as follows: LH, 1.0 to 8.4 IU/L; and FSH, l.0 to 10.5 IU/L. Serum levels of PRL were measured by commercial RIA kit. This commercial kit has been used previously with

inter- and intra-assay variations of less than 10%. Reference ranges for PRL was 92 to 697 pmol/L. Serum inhibin B was determined by enzyme-linked immunosorbent assay [ELISA] method using kit reagents and inhibin B standard (Oxford Bio-innovation Ltd, Oxon, UK). The assay sensitivity was 4 pg/mL and the between-assay variation was 15%. Ciba Corning kits (Ciba Corning Diagnostics SA, Spain) were used to determine by chemoluminescence TSH (intra-assay <4.7%, interassay <6.25%), FT3 (intra-assay <3.8%, interassay <6.2%), and FT4 (intra-assay <3.26%, interassay <4.95%). Reference ranges were as follows: 0.35 to 5.5 mU/L for TSH, 3.5 to 6.5 pmol/L for FT3, and 10.3 to 23.2 pmol/L for FT4.

Measurement of plasma, folate, vitamin B12, and homocysteine. Total Hcy (free plus protein-bound Hcy) in plasma was determined by automated high-performance liquid chromatography ([HPLC] intra-assay variability = 1.4%-1.9%, interassay variability = 1.5%-1.9%). Plasma folate (interassay variability = 4%-5%) and vitamin B12 (interassay variability = 4%-6%) were measured using a chemiluminescent immunoassay with a sensitivity of 0.3 ng/mL for folate and 34 pg/mL for vitamin B12. Reference ranges were as follows: tHcy, 4.4 to 12.4 μmol/L; vitamin B12, 182 to 912 pg/mL; and folate, 5.3 to 14.2 ng/mL.

MTHFR Genotyping

Blood specimens were collected in tubes containing EDTA, and DNA samples were extracted from whole blood using salting-out procedure. *MTHFR* genotypes at C677T, A1298C, and G1793A sites were analyzed by polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) method. *MTHFR* genotyping was performed exactly as described in our previous study.[26]

MTHFR C677T genotype determination. *MTHFR* genotypes at C677T were analyzed by PCR-based RFLP methods, as described by Frosst et al,[1] with some modifications. Polymerase chain reaction was performed in a reaction mixture of 10 μL containing 0.5 units of Taq and 1 μL of template DNA with a concentration of around 50 to 150 ng/μL. Polymerase chain reaction primers for the C677T site were 5′-TGAAG GAGAA GGTGT CTGC GGGA-3′ and 5′-AGGAC GGTGC GGTGA GAGTG-3′, which yield a 198-bp fragment. Polymerase chain reaction conditions consisted of initial denaturation at 94°C for 5 minutes, followed by 30 cycles at 94°C for 30 seconds, 62°C for 30 seconds and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. Then 198-bp PCR product was digested with the enzyme *Hin*fI (12 units) for 3 hours at 37°C. Digestion products were separated by electrophoresis on a 3% agarose gel and visualized with ethidium bromide. The *MTHFR* 677CC genotype, which was regarded as wild type, produced a single band at 198 bp. The heterozygotes (677CT) yielded 198-, 175-, and 23-bp fragments. The mutant *MTHFR* 677TT genotype produced 175- and 23-bp fragments.

MTHFR A1298C genotype determination. The *MTHFR* A1298C genotype was determined using the method described by van der Put et al,[27] with some modifications. The sequences used were 5′-CTTTG GGGAG CTGAA GGACT ACTAC-3′ (sense) and 5′-CACTT TGTGA CCATT CCGGT TTG-3′ (antisense). Polymerase chain reaction amplification procedure was an initial denaturation at 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. Then PCR product of 163 bp was digested at 37°C for 3 hours with 10 units of MboII in a reaction mixture of 20 μL. Finally, digested products were separated by the electrophoresis on an ethidium bromide-contained 4% agarose gel, and the results were visualized under UV light. The 1298AA wild-type homozygotes produced 5 fragments of 56, 31, 30, 28, and 18 bp; 1298AC heterozygotes yielded 6 fragments of 84, 56, 31, 30, 28, and 18 bp; and 1298CC homozygotes produced 4 fragments of 84, 31, 30, and 18 bp.

MTHFR G1793A genotype determination. The 1793G>A mutation was analyzed in PCR products using the primers published by Rady et al.[7] These primers included 5′-CTCTGTG TGTGTGTGCATGTGTGCG-3′ and 5′-GGGACAGGAGTG GCTCCAACGCAGG-3′. Polymerase chain reaction parameters were as follows: an initial denaturation at 94°C for 2 minutes, followed by 40 cycles of 94°C for 1 minutes, 62°C for 1 minutes (annealing), 72°C for 1 minutes (extension), and an extra extension step at 72°C for 10 minutes. After PCR amplification, the product was digested for 5 hour by *Bsr*bI. The digested products were checked on 2% agarose gels and analyzed by ethidium bromide stained 3%. Digestion of the 310-bp fragment with BsrbI enzyme produced 2 bands of 233 and 77 bp for normal 1793GG genotype and 3 bands of 370, 233, and 77 bp for G1793A heterozygote. The mutant 1793AA homozygous genotype only had 1 (310 bp) band.

Quality Control

For the *MTHFR* polymorphisms, genotyping was carried out with blinding to case/control status. In addition, genotyping was repeated in 10% randomly selected samples. Two investigators, who were blinded to clinical data, performed the assay independently. In case of any discordance between them (<1%), a third investigator would carry out the genotyping and determine whether it was necessary to repeat the genotyping. In addition, 10% of all samples were randomly selected for repeated genotyping. Moreover, 5% of the samples were randomly selected for direct sequencing. The direct sequencing confirmed the genotyping in all.

Statistical Analysis

To detect a 10% difference in the prevalence of C677T genotypes, a total of 160 patients in each group would be required to achieve a power of 80% at a significance level of $\alpha = .05$. Differences between groups were examined by χ^2 test and

Students t test when appropriate. The allelic frequencies were calculated by direct counting the alleles. The matching of genotypic frequencies with Hardy-Weinberg equilibrium was examined using χ^2 test ($P < .05$ = equilibrium and $P > .05$ = disequilibrium). Two-tailed χ^2 test was used to determine the differences in genotype distribution between patients and control participants. The association between 3 *MTHFR* polymorphisms and semen parameters was evaluated by binary logistic regression analysis. Factors with a continuous value were adjusted as a continuous variable by logistic model. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using an unconditional logistic regression model. The Haploview program (Daly Lab) was used to estimate pairwise linkage disequilibrium (LD) and construct haplotypes. Haplotype frequencies were calculated using PHASE 2.0 software.[28] Two-sided P values less than .05 were considered statistically significant. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS Inc, Chicago, Illinois).

Results

Characteristic of Study Participants

Clinical and demographic characteristics of the study population are shown in Table 1. No significant differences were seen between 2 groups with regard to age distribution, occupational status, education level, serum hormones, serum biochemistry, and BMI.

Genotypes

Genotypic and allelic frequencies of C677T, A1298C, and G1793A in *MTHFR* gene are shown in Table 2. The genotype distribution of the C677T (controls: $\chi^2 = .05$, $P = .817$; infertile participants: $\chi^2 = 1.75$, $P = .186$), A1298C (controls: $\chi^2 = .07$, $P = .787$; infertile participants: $\chi^2 = 2.24$, $P = .096$), and G1793C (controls: $\chi^2 = 1.51$, $P = .218$; infertile participants: $\chi^2 = .65$, $P = 0.414$) were in agreement with Hardy-Weinberg equilibrium.

Association between MTHFR polymorphism C677T and infertility risk. The frequencies of CC, CT, and TT genotypes of the *MTHFR* C677T polymorphism were 35.4%, 48.8%, and 15.8%, respectively, in the cases and 43.9%, 45.1%, and 11%, respectively, in the controls; the difference was statistically significant ($P = .004$). The frequency of the 677C allele of MTHFR C677T was 59.8% in the patient group and 66.5% in the control group. The frequency of the 677T allele was 40.2% in the patient group and 33.5% in the control group. Comparison of frequencies between patients and control groups showed that the frequency of 677T allele was significantly higher in the patient group than in the control group (OR = 2.37; 95% CI = 1.78-3.73; $P = .017$; Table 3). Therefore, persons with 677T allele have higher risk of developing infertility. The OR for infertility in patients who were heterozygotic for 677T allele (677CT) was 1.60 (95% CI = 1.21-2.75; $P = .039$) when compared with referent participants (677CC). The OR was even higher (2.68, 95% CI = 1.84-3.44; $P = .014$) for patients who were homozygotic for 677T alleles (Table 3).

Table 1. Baseline Demographics, Serum Hormones, and Semen Parameters of Study Groups

Characteristics	Patients (n = 164)	Controls (n = 328)	P Value
Age (year)	31.7 ± 4.4	32.4 ± 4.6	.08
Duration of marriage	5.8 ± 3.3	6.2 ± 3.4	.08
BMI (kg/m^2)	27.4 ± 2.6	26.9 ± 2.8	.08
Occupational status, no (%)			
Employed	144 (87.8)	282 (86.6)	.08
Unemployed	20 (12.2)	44 (13.4)	.08
Educational level			
None	0	0	—
Primary school	9 (5.5)	20 (6.1)	.07
High school	111 (67.7)	226 (68.9)	.07
Graduate	44 (26.8)	82 (25.0)	.07
Serum hormones			
Testosterone (ng/mL)	5.2 ± 1.27	6.2 ± 2.15	.06
LH (IU/L)	6.7 ± 2.4	7.1 ± 2.2	.08
FSH (IU/L)	7.5 ± 2.1	6.7 ± 2.2	.08
PRL (pmol/L)	365 ± 118	372 ± 117	.09
TSH (mIU/mL)	2.2 ± 1.1	2.2 ± 1.2	.1
Free thyroxine (pmol/L)	14.3 ± 2.5	14.4 ± 2.7	.09
Free triiodothyronine (pmol/L)	3.6 ± 1.2	3.6 ± 1.2	.08
Inhibin B (pg/mL)	171.7 ± 20.2	187.6 ± 21.2	.06
Serum biochemistry			
Folic acid (ng/mL)	8.22 ± 2.36	8.08 ± 2.34	.08
Homocysteine (μmol/L)	10.89 ± 2.34	10.84 ± 2.37	.08
Vitamin B12 (pg/mL)	705 ± 51	716 ± 49	.08
Blood glucose (mg/dL)	101 ± 10	99 ± 11	.1
Alanine aminotransferase (IU/L)	28 ± 15	28 ± 12	.09
Aspartate aminotransferase (IU/L)	28 ± 14	26 ± 14	.08
Alkaline phosphatase (IU/L)	237 ± 52	239 ± 53	.08
Total bilirubin (mg/dL)	0.8 ± 0.2	0.8 ± 0.2	.09
Blood urea nitrogen (mg/dL)	14.8 ± 4.2	14.6 ± 4.2	.08
Creatinine (mg/dL)	1.1 ± 0.2	1.0 ± 0.4	.1
Serum hematological parameter			
Hemoglobin (mg/dl)	14.7 ± 0.34	14.5 ± 0.37	0.08
White blood cells (10^3)	7.72 ± 2.14	7.68 ± 2.16	0.08
Red blood cells (10^9)	8.17 ± 0.52	8.16 ± 0.53	0.1
Platelets (10^3)	862 ± 115	866 ± 117	0.09
Semen parameters			
Ejaculate volume (mL)	2.7 ± 1.4	2.7 ± 1.2	.1
Total sperm/ejaculate ($\times 10^6$)	46.6 ± 12.4	179.7 ± 25.8	.001
Sperm density ($\times 10^6$/mL)	14.3 ± 4.6	61.7 ± 12.8	.001
Motility (% motile)	22.3 ± 4.4	61.2 ± 7.6	.001
Morphology (% normal)	20.3 ± 4.2	57.1 ± 8.5	.001

Abbreviations: BMI, body mass index; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TSH, thyroid-stimulating hormone; PRL, prolactin.

Table 2. Frequency Distribution of the MTHFR C677T, A1298C, G1793A Alleles, Genotypes, and Haplotypes in all Cases and Controls, No (%)

Variables	All participants (n = 492)	Cases (n = 164)	Controls (n = 328)
MTHFR C677T			
CC	202 (41.1)	58 (35.4)	144 (43.9)
CT	228 (46.3)	80 (48.8)	148 (45.1)
TT	62 (12.6)	26 (15.8)	36 (11.0)
C allele	632 (64.2)	196 (59.8)	436 (66.5)
T allele	352 (35.8)	132 (40.2)	220 (33.5)
MTHFR A1298C			
AA	224 (45.5)	75 (45.7)	149 (45.4)
AC	211 (42.9)	70 (42.7)	141 (43.0)
CC	57 (11.6)	19 (11.6)	38 (11.6)
A allele	659 (67.0)	220 (67.1)	439 (66.9)
C allele	325 (33.0)	108 (32.9)	217 (33.1)
MTHFR G1793A			
GG	455 (92.5)	151 (92.1)	304 (92.7)
GA	37 (7.5)	13 (7.9)	24 (7.3)
AA	0 (0.0)	0 (0.0)	0 (0.0)
A allele	37 (3.8)	13 (4.0)	24 (3.7)
G allele	947 (96.2)	315 (96.0)	632 (96.3)
MTHFR haplotype			
677C-1298C-1793G	102 (10.4)	19 (5.8)	83 (12.7)
677T-1298C-1793G	194 (19.7)	77 (23.5)	117 (17.8)
677C-1298A-1793G	493 (50.1)	164 (50.0)	329 (50.1)
677C-1298C-1793A	29 (2.9)	12 (3.6)	17 (2.6)
677T-1298A-1793G	158 (16.1)	55 (16.8)	103 (15.7)
677C-1298A-1793A	8 (0.8)	1 (0.3)	7 (1.1)
677T-1298C-1793A	0 (0.00)	0 (0.00)	0 (0.00)
677T-1298A-1793A	0 (0.00)	0 (0.00)	0 (0.00)

Abbreviation: MTHFR, methylenetetrahydrofolate reductase.

Association between MTHFR polymorphism A1298C and infertility risk. As can be seen from Table 3, there were no significant associations between A1298C genotypes and risk of infertility ($P = .32$).

Association between MTHFR polymorphism G1793A and infertility risk. There was no significant difference in genotypic and allelic frequencies of G1793A polymorphism between infertile patients and controls ($P = .46$; Table 3).

Association of combined C677T/A1298C and C677T/G1793A polymorphisms with infertility. With combined genotype distribution for MTHFR C677T/A1298C, the frequency of combined 677CC+1298 AC variant was significantly higher in the controls (15.5%) than the cases (5.5%), which decreased more than 40% the risk of developing unexplained infertility (OR = 0.36, 95% CI = 0.19-0.56; $P = .001$). When we used 677CC+1298AA variant as the reference, we found that 677CT+1298AC and 677TT+1298AC variants were associated with a higher risk of infertility (OR = 2.64, 95% CI = 1.87-3.78; $P = .016$ and OR = 3.62, 95% CI = 2.79-5.18; $P = .001$, respectively; Table 4). We also found that the combined 677CT+1793GG and 677TT+1793GG variants were associated with a higher risk of infertility (OR = 1.76, 95% CI = 1.22-2.64; $P = .026$ and OR = 2.47, 95% CI = 1.69-3.76; $P = .018$, respectively).

Distribution of MTHFR haplotype frequencies in the infertile men and controls. The polymorphisms in the *MTHFR* gene were in LD. Linkage disequilibrium between C677T and A1298C ($D' = .967$, $r^2 = .226$) were stronger than that between C677T and G1793A ($D' = .569$, $r^2 = .127$). The haplotype frequencies of the 3 polymorphisms in the *MTHFR* gene were each estimated to be >1% (except 1 haplotype). Eight major haplotypes of *MTHFR* were present in the study population (Table 2). 677C-1298A-1793G and 677T-1298C-1793G were the most common haplotypes both in infertile patients (50.0 and 23.5%, respectively) and in healthy controls (50.1 and 17.8%, respectively). When we compared the overall distribution of haplotype frequencies between infertile patients and controls, the P value is .0298 and the confidence interval 1.67 to 2.89. Therefore, the relationship was statistically significant. The 677C-1298C-1793G haplotypes occurred at greater frequencies in controls versus cases, suggesting that it is protective for infertility (OR = 0.36, 95% CI = 0.21-0.64, $P = .001$) whereas 677T-1298C-1793G haplotype was at higher frequency in infertile cases (OR = 2.71, 95% CI = 1.62-3.89, $P = .004$; Table 3).

Association between MTHFR genotypes and semen parameters. In contrast to fertility status, all 3 *MTHFR* gene polymorphisms were associated with semen parameters, and these associations were similar in both groups. We observed that the presence of risk alleles (677T, 1298C, and 1793G) were associated with impaired semen parameters in proven fertile and infertile men (Tables 5, and 6).

For 677TT genotype, all 3 semen parameters (sperm count, motility, and morphology) were significantly lower than those in the 677CC genotype (each $P = .01$). A negative correlation was found between 677TT genotype and sperm count ($r = -.68$, $P = .01$) as well as mean sperm motility ($r = -.67$, $P = .01$). Mean sperm density was $11.8 \pm 4.1 \times 10^6$/mL, and $17.2 \pm 3.5 \times 10^6$/mL in OAT participants with 677TT and 677CC genotypes, respectively ($P = .012$). The mean percentage motility in infertile patients with 677TT genotype (21.4 ± 4.2) was significantly lower than the mean for 677CC genotype participants (26.8 ± 4.7; $P = .024$; Table 5). The percentage of sperm with normal morphology was 19.2% ± 3.9% in patients with 677TT genotype, and 24.5% ± 4.7% in patients with 677CC genotype ($P = .024$; Table 4). Sperm concentration, motility, and morphology had a significant negative correlation with the presence of 1298C allele ($r = -.62$, $P = .01$; $r = -.67$, $P = .01$, and $r = -.77$, $P = .001$, respectively). The mean sperm density ($\times 10^6$/mL) in infertile patients with 1298AA and 1298CC genotypes was 16.1 ± 4.7 and 13.4 ± 3.4 ($P = .034$; Table 5). Patients with 1298AA genotype had a mean motility of 24.8% ± 5.4%, which was significantly higher than the mean in patients with 1298TT genotype (21.6% ± 3.8%,

Table 3. Frequency Distribution of the MTHFR C677T, A1298C, G1793A Alleles, Genotypes and Haplotypes, and Their Associations With the Risk of Infertility[a]

Polymorphism	Cases (N = 164)	Controls (N = 328)	Crude OR (95% CI)	*P* Value	Adjusted OR[a] (95% CI)	*P* Value
MTHFR C677T						
CC	58 (35.4%)	144 (43.9%)	1.0 (Referent)			
CT	80 (48.8%)	148 (45.1%)	1.63 (1.17-2.87)	.032	1.60 (1.21-2.75)	.039
TT	26 (15.8%)	36 (11.0%)	2.76 (1.86-3.57)	.012	2.68 (1.84-3.44)	.014
C allele	196 (59.8%)	436 (66.5%)	1.0 (Referent)			
T allele	132 (40.2%)	220 (33.5%)	2.41 (1.68-3.83)	.012	2.37 (1.78-3.73)	.017
MTHFR A1298C						
AA	75 (45.7%)	149 (45.4%)	1.0 (Referent)			
AC	70 (42.7%)	141 (43.0%)	0.97 (0.83-1.61)	.94	1.04 (0.84-1.57)	.78
CC	19 (11.6%)	38 (11.6%)	1.05 (0.82-1.64)	.75	1.06 (0.87-1.62)	.99
A allele	220 (67.1%)	439 (66.9%)	1.0 (Referent)			
C allele	108 (32.9%)	217 (33.1%)	0.94 (0.84-1.74)	.84	0.97 (0.87-1.77)	.89
MTHFR G1793A						
GG	151 (92.1%)	304 (92.7%)	1.0 (Referent)			
GA	13 (7.9%)	24 (7.3%)	1.17 (0.72-1.48)	.79	1.11 (0.74-1.54)	.82
AA	0 (0.00)	0 (0.00)	NA			
A allele	13 (4.0%)	24 (3.7%)	1.0 (Referent)			
G allele	315 (96.0%)	632 (96.3%)	0.98 (0.88-1.69)	1.04	0.94 (0.87-1.62)	1.02
MTHFR haplotypes						
677C-1298C-1793G	19 (5.8)	83 (12.7)	1.0 (Referent)			
677T-1298C-1793G	77 (23.5)	117 (17.8)	2.76 (1.64-4.11)	.004	2.71 (1.62-3.89)	.004
677C-1298A-1793G	164 (50.0)	329 (50.1)	0.98 (0.81-1.72)	.82	0.95 (0.87-1.78)	.83
677C-1298C-1793A	12 (3.6)	17 (2.6)	1.06 (0.92-1.21)	1.05	1.00 (0.93-1.17)	1.0
677T-1298A-1793G	55 (16.8)	103 (15.7)	1.12 (0.82-1.86)	.64	1.06 (0.85-1.80)	.68
677C-1298A-1793A	1 (0.3)	7 (1.1)	0.84 (0.65-1.43)	.77	0.87 (0.72-1.41)	.72
677T-1298C-1793A	0 (0.00)	0 (0.00)	NA			
677T-1298A-1793A	0 (0.00)	0 (0.00)	NA			

Abbreviation: MTHFR, methylenetetrahydrofolate reductase, OR, odds ratio, CI, confidence interval, NA, not applicable.
[a] Adjusted OR: adjusted in multivariate logistic regression models including age, BMI, occupational status, educational level, plasma folate, vitamin B12, homocysteine, smoking status, and MTHFR genotypes.

Table 4. Combined Genotype Distribution for MTHFR C677T/A1298C, and MTHFR C677T/G1793A Polymorphisms in all Cases and Controls

MTHFR genotypes	Cases (n = 164) (%)	Controls (n = 328) (%)	Odds ratio (OR)[a]	95% CI	*P* Value
C677T/A1298C					
CC + AA	40 (24.4)	95 (29.0)	1.0 (Referent)		
CC + AC	9 (5.5)	51 (15.5)	0.36	0.19-0.56	.001
CC + CC	9 (5.5)	14 (4.3)	1.24	0.77-1.69	.67
CT + AA	20 (12.2)	48 (14.6)	0.81	0.65-1.84	.25
CT + AC	50 (30.5)	77 (23.5)	2.64	1.87-3.78	.016
CT + CC	10 (6.1)	19 (5.8)	1.07	0.92-1.38	1.0
TT + AA	15 (9.1)	16 (4.9)	2.91	1.89-4.25	.001
TT + AC	11 (6.7)	8 (2.4)	3.62	2.79-5.18	.001
TT + CC	0 (0.00)	0 (0.00)	NA		
(CT + AC) + (TT + AC)	61 (37.2)	85 (25.9)	2.67	1.74-3.83	.001
C677T/G1793A					
CC + GG	51 (31.1)	140 (42.7)	1.0 (Referent)		
CC + GA	7 (4.3)	20 (6.1)	1.32	0.78-1.64	.63
CC + AA	0 (0.00)	0 (0.00)	NA		
CT + GG	74 (45.1)	132 (40.3)	1.76	1.22-2.64	.026
CT + GA	6 (3.6)	12 (3.6)	1.0		
CT + AA	0 (0.00)	0 (0.00)	NA		
TT + GG	26 (15.9)	24 (7.3)	2.47	1.69-3.76	.018
TT + GA	0 (0.00)	0 (0.00)	NA		
TT + AA	0 (0.00)	0 (0.00)	NA		
(CT + GA) + (TT + GA)	6 (3.6)	12 (3.6)	1.0		

Abbreviations: MTHFR, methylenetetrahydrofolate reductase; OR, odds ratio; CI, confidence interval; NA, not applicable.
[a] Adjusted OR: adjusted in multivariate logistic regression models including age, BMI, occupational status, educational level, plasma folate, vitamin B12, homocysteine, smoking status and MTHFR genotypes.

Table 5. Relationship Between MTHFR C677T, A1298C, G1793A Alleles, Genotypes and Haplotypes, and Semen Parameters in Infertile Men

		Sperm Density (mean)			Sperm Motility (mean)			Sperm Morphology		
Polymorphism	n	$\times 10^6$/mL	95% CI[a]	*P* Value	%	95% CI[a]	*P* Value	%	95% CI[a]	*P* Value
MTHFR C677T										
CC	58	17.2	1.0 (Referent)		26.8	1.0 (Referent)		24.5	1.0 (Referent)	
CT	80	15.0	10.7-19.7	.084	24.2	22.3-26.7	.068	21.6	17.6-25.5	.041
TT	26	11.8	9.7-13.7	.012	21.4	17.9-25.6	.024	19.2	16.1-22.3	.024
C allele	196	15.8	1.0 (Referent)		23.4	1.0 (Referent)		21.4	1.0 (Referent)	
T allele	132	11.2	9.8-13.7	.034	20.3	17.9-23.4	.036	18.5	16.2-23.3	.042
MTHFR A1298C										
AA	75	16.1	1.0 (Referent)		24.8	1.0 (Referent)		22.6	1.0 (Referent)	
AC	70	14.1	10.2-18.9	.083	22.1	18.2-26.7	.084	20.4	16.2-24.9	.078
CC	19	13.4	9.7-16.8	.034	21.6	17.9-25.2	.036	19.8	15.3-23.2	.033
A allele	220	15.8	1.0 (Referent)		24.4	1.0 (Referent)		22.9	1.0 (Referent)	
C allele	108	11.6	9.5-13.8	.028	19.6	15.7-24.7	.018	17.4	13.1-22.2	.024
MTHFR G1793A										
GG	151	14.3	1.0 (Referent)		22.1	1.0 (Referent)		20.2	1.0 (Referent)	
GA	13	17.6	143.6-18.9	.029	26.8	22.9-29.7	.022	24.7	20.4-27.6	.031
AA	0		NA		NA			NA		
A allele	13	17.8	1.0 (Referent)		26.7	1.0 (Referent)		24.7		1.0 (Referent)
G allele	315	14.1	9.7-18.2	.027	22.1	18.8-26.6	.024	20.1	16.1-24.5	.034
MTHFR haplotypes										
677C-1298C-1793G	19	15.2	1.0 (Referent)		26.8	1.0 (Referent)		24.6	1.0 (Referent)	
677T-1298C-1793G	77	13.2	9.5-15.8	.016	22.1	17.8-26.5	.013	18.4	16.1-22.2	.012
677C-1298A-1793G	164	14.1	10.2-18.9	.68	25.6	18.6-29.2	.36	23.8	17.3-27.3	.76
677C-1298C-1793A	12	14.2	10.3-18.8	.64	25.8	18.7-29.7	.37	23.6	17.7-27.6	.62
677T-1298A-1793G	55	14.4	10.5-18.9	.71	25.7	18.6-29.2	.35	23.7	17.9-27.5	.64
677C-1298A-1793A	1	14.6	10.5-18.8	.88	25.8	19.4-29.8	.37	24.1	20.3-27.2	.83
677T-1298C-1793A	0		NA							
677T-1298A-1793A	0		NA							

Abbreviations: MTHFR, methylenetetrahydrofolate reductase; OR, odds ratio; CI, confidence interval; NA, not applicable.
[a] Adjusted OR: adjusted in multivariate logistic regression models including age, BMI, occupational status, educational level, plasma folate, vitamin B12, homocysteine, smoking status and MTHFR genotypes.

$P = .036$). There was a significant difference in sperm morphology in patients with 1298AA and 1298CC genotypes. The mean percentage of normal forms in patients with 1298AA and 1298CC genotypes was $22.6\% \pm 4.3\%$ and $19.8\% \pm 4.6\%$, respectively ($P = .033$). Based on Pearson correlations, each semen analysis parameter significantly correlated negatively with 1793G allele presence, including sperm concentration ($r = -.46$, $P = .01$), percentage of motile sperm ($r = -.54$, $P = .01$), and percentage of sperm with normal morphology ($r = -.061$, $P = .001$; Table 5).

In normal fertile men, the mean sperm density, sperm motility, and sperm morphology was also significantly lower in the 677TT genotype, compared with 677CC genotype (Table 6). There was a statistically significant trend toward higher percentage of sperm density, sperm motility, and percentage of the sperm with normal morphology in the 1298AA and 1793GA genotype carriers, compared with 1298CC and 1793GG carriers.

Serum biomarkers. We analyzed the group distribution of *MTHFR* genotypes according to plasma biochemical parameters, and we found that the presence of the 677T, 1298C, and 1793G alleles both in infertile patients and controls were associated with higher serum tHcy levels and lower serum folate levels (all *P*s < .01), as compared with the referent alleles (Table 7). However, serum tHcy, folate, and vitamin B12 levels were not significantly different in infertile patients compared to the controls ($P = .45$, $P = .68$, and $P = .72$, respectively). Next, we wanted to know whether the levels of serum tHcy and folate were related to the different *MTHFR* genotypes. As shown in Table 7, participants carrying the 677T allele (TC and TT) and 1298C allele (AC and CC) had significantly higher mean serum tHcy levels and lower mean serum folate levels than participants with 677CC and 1298AA homozygotes in both control and infertile groups (all *P*s < .01; for details see Table 7). Participants with combined 677CT+1298CC and 677CT+1793GA variants had highest and participants with 677CC+1298AA and 677CC+1793GA variants had lowest serum tHcy and folate levels, respectively (all *P*s < .01; for details see Table 8). We observed that serum folate levels were negatively correlated with tHcy levels in serum from infertile patients ($r = -.68$, $P = .01$) and healthy controls ($r = -.73$, $P = .01$). Highest and lowest serum tHcy levels in both groups were observed with

Table 6. Relationship Between MTHFR C677T, A1298C, G1793A Alleles, Genotypes and Haplotypes, and Semen Parameters in Proven Fertile Men

		Sperm Density (Mean)			Sperm Motility (Mean)			Sperm Morphology		
Polymorphism	n	× 106/mL	95% CI[a]	*P* Value	%	95% CI[a]	*P* Value	%	95% CI[a]	*P* Value
MTHFR C677T										
CC	160	74.2	1.0 (Referent)		74.6	1.0 (Referent)		70.4	1.0 (Referent)	
CT	144	68.3	57.6-80.1	.026	67.8	60.3-74.6	.018	51.6	43.4-59.5	.021
TT	24	61.8	50.4-72.5	.004	61.5	54.7-68.8	.004	57.4	49.1-65.4	.004
C allele	464	72.4	1.0 (Referent)		73.5	1.0 (Referent)		65.6	1.0 (Referent)	
T allele	192	61.2	50.8-71.7	.006	60.8	53.4-67.4	.004	52.7	44.2-60.3	.004
MTHFR A1298C										
AA	159	74.2	1.0 (Referent)		74.4	1.0 (Referent)		71.0	1.0 (Referent)	
AC	136	67.8	55.2-68.9	.027	68.1	61.2-75.7	.022	57.8	49.2-65.9	.017
CC	33	61.7	50.7-72.8	.004	61.6	54.9-68.2	.004	57.8	49.3-65.2	.004
A allele	454	72.4	1.0 (Referent)		73.4	1.0 (Referent)		65.9	1.0 (Referent)	
C allele	202	62.1	51.5-73.1	.004	61.2	54.7-68.7	.004	52.4	44.2-60.3	.004
MTHFR G1793A										
GG	296	61.2	1.0 (Referent)		62.2	1.0 (Referent)		58.2	1.0 (Referent)	
GA	32	64.8	54.6-73.9	.78	64.6	56.4-72.7	.34	60.7	52.4-68.6	.38
AA	0		NA		NA			NA		
A allele	32	64.6	1.0 (Referent)		64.8	1.0 (Referent)		60.2	1.0 (Referent)	
G allele	624	61.4	50.7-71.2	.37	62.7	17.8-25.6	.36	58.8	50.1-66.5	.37
MTHFR haplotypes										
677C-1298C-1793G	88	70.2	1.0 (Referent)		70.8	1.0 (Referent)		66.6	1.0 (Referent)	
677T-1298C-1793G	89	56.2	50.5-72.8	.004	56.1	49.8-63.5	.004	52.4	44.1-60.2	.004
677C-1298A-1793G	344	69.1	57.2-78.9	.78	68.6	61.6-75.2	.36	64.6	56.3-72.4	.36
677C-1298C-1793A	25	68.2	60.3-78.8	.64	69.7	61.7-76.7	.57	65.7	57.7-73.7	.42
677T-1298A-1793G	103	67.4	55.5-77.9	.61	67.4	60.6-74.2	.45	63.6	55.9-71.5	.34
677C-1298A-1793A	7	67.6	56.5-78.8	.68	68.4	61.4-76.8	.37	63.2	55.3-71.3	.33
677T-1298C-1793A	0		NA							
677T-1298A-1793A	0		NA							

Abbreviations: MTHFR, methylenetetrahydrofolate reductase; OR, odds ratio; CI, confidence interval; NA, not applicable.
[a] Adjusted OR: adjusted in multivariate logistic regression models including age, BMI, occupational status, educational level, plasma folate, vitamin B12, homocysteine, smoking status, and *MTHFR* genotypes.

677C-1298C-1793G and 677C-1298A-1793A haplotypes, respectively.

Correlation between serum biomarkers and semen parameters. The relationship between serum tHcy and semen parameters was analyzed using Pearson correlation coefficient (Table 9). In the all participants, significant negative correlations were observed between the serum tHcy levels and sperm density ($r = -.71$, $P = .001$), sperm motility ($r = -.64$, $P = .001$), as well as sperm morphology ($r = -.72$, $P = .001$). For the serum folate levels, significant positive correlations were found between serum folate levels and sperm density ($r = .78$, $P = .001$), sperm motility ($r = .74$, $P = .001$), as well as sperm morphology ($r = .72$, $P = .001$).

Discussion

In current study, we found that the functional polymorphism of the *MTHFR* C677T is strongly associated with risk of idiopathic male infertility. The 677TT genotype and 677TT +1298AC combined genotype had a 2.68- and 3.62-fold increased risk of unexplained male infertility, respectively. Likewise, the 677TT+1793GG combined genotype had a 2.47-fold increased risk of infertility. On the contrary, we did not find any association between *MTHFR* A1298C polymorphism nor between *MTHFR* G1793A polymorphism and fertility status. However, when we evaluated these 3 polymorphisms together based on their reported LD,[29] the risk of idiopathic male infertility increased as the number of risk alleles increased in a dose-dependent manner.

The frequency of the 677T allele varies significantly among populations, ranging from 30% to 40% in Europe and America to 5% to 10% in Africa and Sri Lanka.[30,31] The result of the most common phenotype of C677T mutation is the accumulation of homocysteine and depletion of folate, leading to hyperhomocysteinemia and decreased serum folate levels. It has been reported that folate insufficiency increases DNA fragility due to the misincorporation of uracil instead of thymine.[18,32] During DNA repair processes, when removal of the misincorporated uracil fails, double-strand breaks occur.[18,31] Therefore, possible deleterious effects of the *MTHFR* C677T polymorphism on male fertility status might be due to undermethylation. In previous studies, participants homozygous for the 677T allele exhibited more pronounced deficits in intracellular

Table 7. Comparison of Serum Homocysteine, Folate and Vitamin B12 Levels With C677T, A1298C, and G1793A Genotypes of *MTHFR* Gene in all Participants[a]

	Biochemical Variables								
	Homocysteine (μmol/L)			Folate (ng/mL)			Vitamin B12 (pg/mL)		
MTHFR Genotypes	Patients	Controls	*P* Value[b]	Patients	Controls	*P* Value[b]	Patients	Controls	*P* Value[b]
MTHFR C677T									
CC	8.56 ± 2.63[c]	8.96 ± 2.24[c]	.45	8.42 ± 2.34[c]	8.23 ± 2.19[c]	.42	730 ± 42[c]	744 ± 48[c]	.44
CT	11.31 ± 2.24[d]	11.23 ± 1.94[d]	.64	7.33 ± 2.15[d]	7.33 ± 2.12[d]	.77	693 ± 42[d]	693 ± 44[d]	.92
TT	12.37 ± 2.25	12.34 ± 2.25	.82	6.97 ± 2.56	6.93 ± 2.72	.78	662 ± 52	674 ± 47	.72
MTHFR A1298C									
AA	9.0 ± 2.71[e]	9.35 ± 2.80[e]	.44	8.23 ± 2.73[e]	8.23 ± 2.84[e]	.89	739 ± 53[e,f]	745 ± 49[e]	.72
AC	10.83 ± 2.73[f]	10.71 ± 2.53[f]	.63	7.64 ± 2.34[f]	7.42 ± 2.74[f]	.67	695 ± 44[f]	691 ± 45[f]	.83
CC	11.90 ± 2.65	12.74 ± 2.58	.23	6.22 ± 2.24	6.14 ± 2.32	.61	649 ± 45	637 ± 49	.64
MTHFR G1793A									
GG	10.53 ± 2.13[g]	10.32 ± 2.12[g]	.65	7.27 ± 2.55[g]	7.53 ± 2.15[g]	.72	698 ± 45[g]	713 ± 50[g]	.64
GA	9.55 ± 1.71	8.83 ± 2.32	.27	7.93 ± 2.37	7.93 ± 2.16	.67	747 ± 46	760 ± 48	.53
AA	NA								
MTHFR haplotypes									
677C-1298C-1793G	11.07 ± 2.22[h,i]	11.47 ± 2.24	.51	8.62 ± 2.32[j]	8.94 ± 2.16	.37	782 ± 45[j]	796 ± 48	.41
677T-1298C-1793G	10.60 ± 2.62	10.36 ± 2.54	.50	7.53 ± 2.18	7.70 ± 2.15	.45	702 ± 37	718 ± 47	.42
677C-1298A-1793G	10.61 ± 2.44	10.14 ± 2.32	.41	7.16 ± 2.25	7.51 ± 2.72	.41	671 ± 43	688 ± 46	.28
677C-1298C-1793A	8.91 ± 2.28	9.42 ± 2.34	.35	8.43 ± 2.74	7.84 ± 2.14	.34	751 ± 41	768 ± 48	.39
677T-1298A-1793G	9.63 ± 2.64	9.92 ± 2.32	.47	7.65 ± 2.32	8.21 ± 2.24	.22	698 ± 45	726 ± 45	.54
677C-1298A-1793A	8.70 ± 2.27	8.86 ± 2.67	.74	8.32 ± 2.26	8.53 ± 2.25	.74	722 ± 47	742 ± 49	.55
677T-1298C-1793A	NA	NA		NA	NA		NA	NA	
677T-1298A-1793A	NA	NA		NA	NA		NA	NA	

Abbreviations: MTHFR, methylenetetrahydrofolate reductase.
[a] Values are shown as mean ± SD.
[b] P value controls vs. patients.
[c] 677TT vs.= 677CC, *P* value <.001.
[d] 677TT vs 677CT, *P* value < .01.
[e] 1298CC vs 1298AA, *P* value < .001.
[f] 1298CC vs 1298AC, *P* value < .01.
[g] 1793GG vs 1793GA, *P* value < .01.
[h] 677C-1298C-1793G vs 677C-1298A-1793A, *P* value < .001.
[i] 677C-1298C-1793G vs 677C-1298C-1793A and 677C-1298A-1793G, *P* value < .01.
[j] 677C-1298C-1793G vs 677C-1298A-1793G; *P* value < .01.

methylation and greater serum homocysteine levels.[33,34] However, the current study suggests that adverse effects of the 677TT genotype on fertility status are not influenced by serum folate levels. We determined a positive correlation between serum folate levels and the sperm density, percentage of progressive motile sperm, and percentage of sperm with normal morphology in both groups. Homocysteine levels are inversely correlated to folate levels by virtue of MTHFR activity. In the current study, serum tHcy concentrations was inversely correlated with the sperm density, percentage of progressive motile sperm, and percentage of sperm with normal morphology. There are few published reports on the association between the *MTHFR* polymorphisms and the risk of unexplained male infertility. To our knowledge up to date, 7 studies have addressed the possible association between *MTHFR* C677T polymorphism and male infertility, but mainly because of nonhomogenous population selection, different collecting and genotyping methods, and different sample sizes and ethnic groups, the results are conflicting. Our findings regarding association between *MTHFR* C677T mutation and idiopathic male factor infertility are in line with studies from Germany,[35] India,[23] Korea,[22] and China,[36] whereas studies from the Netherlands[37] and Italy[38,39] could not demonstrate such association. However, none of above-mentioned studies examined the role of the *MTHFR* G1793A polymorphism. The novel MTHFR 1793A variant is rare in Caucasians, and the allele frequency in our study population was consistent with that demonstrated by Rady et al,[7] nevertheless we did not find evidence for an association between this 1793A variant genotype and risk of idiopathic male infertility. Because our study is the first to examine the association between this polymorphism and male infertility, no comparison among published studies could be made currently.

Although genetic factors play a role in the etiology of idiopathic male infertility, the biochemical abnormalities underlying the predisposition to infertility and the exact pathophysiology of impaired spermatogenesis remain to be elucidated. It has been shown that, a high level of homocysteine can induce the auto-oxidation that might cause DNA damage. In addition to the damage to germ line DNA, oxidative stress could also damage the cell membranes.[40] Hyperhomocysteinemia can result from decreased *MTHFR* gene activity, owing to

Table 8. Serum Homocysteine, Folate, and Vitamin B12 Levels of Combined MTHFR C677T/A1298C and MTHFR C677T/G1793A Genotypes in Participants[a]

MTHFR Genotypes	Biochemical variables								
	Homocysteine (μmol/L)			Folate (ng/mL)			Vitamin B12 (pg/mL)		
	Patients	Controls	*P* Value[b]	Patients	Controls	*P* Value[b]	Patients	Controls	*P* Value[b]
C677T/C1298A									
CC + AA	8.66 ± 2.44[c]	9.17 ± 2.55[c]	.46	8.33 ± 2.32[c]	8.23 ± 2.33[c]	.75	735 ± 45[c]	744 ± 48[c]	.44
CC + AC	10.07 ± 2.72[d]	9.69 ± 2.68[d]	.55	7.94 ± 2.43[d]	8.0 ± 2.35[d]	.85	710 ± 45[d]	722 ± 47[d]	.62
CC + CC	9.54 ± 2.52[e]	9.45 ± 2.76[e]	.65	7.8 ± 2.43[e]	7.07 ± 2.43[e]	0.08	706 ± 43[e]	728 ± 47[e]	.62
CT + AA	10.43 ± 2.32[f]	10.12 ± 2.47[f]	.43	7.45 ± 2.43[f]	7.84 ± 2.53[f]	.64	711 ± 56[f]	722 ± 49[f]	.48
CT + AC	11.06 ± 2.36[g]	11.0 ± 2.67[g]	.63	7.49 ± 2.43[g]	7.36 ± 2.46[g]	.75	693 ± 46[g]	693 ± 50[g]	.91
CT + CC	11.44 ± 2.41[h]	11.46 ± 2.72[h]	.89	7.08 ± 2.46[h]	7.10 ± 2.52[h]	.92	683 ± 49[h]	685 ± 51[h]	.91
TT + AA	9.97 ± 2.45[i]	9.75 ± 2.44[i]	.55	7.85 ± 2.65[i]	8.03 ± 2.45[i]	.64	716 ± 49[i]	733 ± 52[i]	.72
TT + AC	NA	11.02 ± 2.52[j]	NA	NA	7.32 ± 2.47[j]	NA	NA	688 ± 46[j]	NA
TT + CC	NA			NA			NA		
C677T/G1793A									
CC + GG	9.98 ± 2.41[k,l]	9.93 ± 2.55[k,l]	.64	7.61 ± 2.55[k,l]	7.76 ± 2.42[i,k]	.74	707 ± 53[k,l]	723 ± 53[k,l]	.44
CC + GA	8.74 ± 2.62[m]	8.94 ± 2.55[m]	.75	8.34 ± 2.42[n]	8.17 ± 2.51[n]	.54	733 ± 42[n]	745 ± 53[e,n]	.41
CC + AA	NA			NA			NA		
CT + GG	10.91 ± 2.37[°]	10.57 ± 2.41[°]	.31	7.31 ± 2.56	7.47 ± 2.35	.67	695 ± 42	708 ± 44	.41
CT + GA	11.06 ± 2.61[P]	10.92 ± 2.64[P]	.62	7.42 ± 2.47	7.41 ± 2.65	.92	697 ± 51	703 ± 46	.71
CT + AA	NA			NA			NA		
TT + GG	10.77 ± 2.54	10.48 ± 2.46[q]	.44	7.24 ± 2.66	7.46 ± 2.47	.62	693 ± 49	710 ± 49	.70
TT + GA	NA	10.67 ± 2.39	NA	NA	7.34 ± 2.58	NA	NA	712 ± 51	NA
TT + AA	NA			NA			NA		

[a] Values are shown as mean ± SD.
[b] P value controls vs. patients.
[c] 677CC + 1298AA vs. 677TT + 1298CC, *P* value < .001.
[d] 677CC + 1298AC vs. 677TT + 1298CC, *P* value < .001.
[e] 677CC + 1298CC vs. 677TT + 1298CC, *P* value < .001.
[f] 677CT + 1298AA vs. 677TT + 1298CC, *P* value < .001.
[g] 677CT + 1298AC vs. 677TT + 1298CC, *P* value < .01.
[h] 677CT + 1298CC vs. 677TT + 1298CC, *P* value < .01.
[i] 677TT + 1298AA vs. 677TT + 1298CC, *P* value < .001.
[j] 677TT + 1298AC vs. 677TT + 1298CC, *P* value < .01.
[k] 677CC + 1793GG vs. 677CT + 1793GA, *P* value < .01.
[l] 677CC + 1793GG vs. 677TT + 1793GG, and 677CC + 1793GG vs. 677TT + 1793GA, *P* value < .01.
[m] 677CC + 1793GA vs. 677CT + 1793GA, *P* value < .001.
[n] 677CC + 1793GA vs. 677CT + 1793GA, *P* value < .01.
[°] 677CT + 1793GG vs. 677CT + 1793GA, *P* value < .01.
[P] 677CT + 1793GA vs. 677TT + 1793GG, and 677CT + 1793GA vs. 677TT + 1793GA, *P* value < .01.
[q] 677TT + 1793GG vs. 677TT + 1793GA, *P* value < .01.

genetic polymorphisms and/or inadequate folate and vitamin B12 intake. Inadequate serum levels of folate and vitamin B12 are responsible for approximately two thirds of the hyperhomocysteinemia cases.[41] The amount of folate and vitamin B12 daily taken by participants is an important determinant of serum tHcy levels. We have no idea about the amount of folate and vitamin B12 in food daily taken by our participants. However, an advantage of the current study is that, folate nutrition status was evaluated by direct measurement of serum folate level rather than by dietary recall or records. High-serum tHcy levels may also be accompanied by environmental factors including smoking, excessive coffee consumption, and low level of physical activity.[42] Life style factors such as coffee consumption, tobacco smoking, and level of physical activity were not recorded in participants, which is one of the limitations of our study. In this study, the *MTHFR* A1298C and G1793A polymorphisms were not associated with infertility risk. This finding was unexpected, because serum tHcy and folate levels with these 2 polymorphisms were similar to those

Table 9. Correlation Between Serum Total Homocysteine and Folate Levels With Semen Parameters

Variables	tHcy		Folate	
	r	*P* Value	*r*	*P* Value
Total sperm/ejaculate	−.76	.001	.78	.001
Sperm density	−.71	.001	.74	.001
Sperm motility	−.64	.001	.68	.001
Sperm morphology	−.72	.001	.72	.001

Abbreviation: tHcy, total homocysteine.

we noted in *MTHFR* C677T polymorphism. Another important conflicting issue is that, the serum tHcy and folate concentrations were similar in infertile patients and fertile controls. As such, the biochemical mechanism by which *MTHFR* C677T mutation would influence fertility risk remains unclear. Perhaps, variation in dietary intake or other relevant environmental factors might obscure *MTHFR* A1298C and G1793A polymorphism-related effects on infertility. In addition, it has been shown that downstream alterations in MTHFR activity appear less pronounced for A1298C than for C677T.[27] Although A1298C is transmitted in disequilibrium with C677T, the r^2 value for the 2 polymorphisms is somewhat low, consistent with their differing clinical effects. However, it should be noted that, although *MTHFR* A1298C and G1793A polymorphisms were not associated with infertility risk, but the relationships between these 2 polymorphisms and semen parameters were similar to those we observed with *MTHFR* C677T polymorphisms. Participants carrying *MTHFR* genotypes that are associated with lower serum folate levels and higher serum tHcy levels exhibited impaired semen parameters. The correlations between serum tHcy and folate levels and semen parameters were linear.

Although our sample size is relatively small for genetic analysis, we had an 80% power at a .05 or smaller significant level to detect an OR of 1.5 or greater. There are several limitations of the current study. First, similar to other hospital-based case–control investigations, the control participants in our study may not be representative of the general population. Another limitation is the recruitment of cases through tertiary infertility clinic. Although this clinic sees many of the infertility cases occurring within this geographic region, the study is not formally population based.

Conclusion

Our study made a contribution to the assessment of all 3 *MTHFR* polymorphisms and haplotype influence on male infertility, serum folate, and tHcy levels in an Iranian population. These findings suggested that the TT genotype of *MTHFR* C677T is associated with the highest susceptibility to developing unexplained infertility, followed by heterozygous CT carriers. Neither *MTHFR* A1298C polymorphism nor *MTHFR* G1793A contributes to fertility status. These findings also suggested that the variant genotypes of *MTHFR*, which can cause a predisposition to increased serum tHcy levels, may deteriorate semen parameters. Further studies performed on different ethnic groups with statistically significant sample sizes are required to establish the exact role of *MTHFR* gene in male factor infertility.

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