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Association study of four polymorphisms in three folate-related enzyme genes with non-obstructive male infertility

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BACKGROUND: Three typical folate metabolism enzymes—i.e. methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MS) and MS reductase (MTRR) in the folate cycle—play a critical role in DNA synthesis and methylation reactions. We evaluated whether polymorphisms of these three enzymes are associated with non-obstructive male infertility. METHOD: Three hundred and sixty patients with non-obstructive infertility and 325 fertile men without any chromosomal abnormalities were included in this study. The single-nucleotide polymorphism (SNP) analysis was performed by pyrosequencing and PCR-restriction fragment length polymorphism (RFLP) analysis RESULTS: The frequencies of MTHFR 677TT and MTRR 66GG genotypes were higher in non-obstructive infertile men compared with those in fertile men. By classifying 360 infertile patients into 174 azoospermia and 186 oligoasthenoteratozoospermia (OAT) subjects, the MTHFR 677TT and MS 2756GG types were significantly associated with the azoospermia group (P = 0.0227 and 0.0063, respectively). The frequency of MTRR 66GG was significant in the OAT group (P = 0.0014 versus fertile males). CONCLUSIONS: By analysis of a large number of subjects and a more specific patient selection, we showed the first genetic evidence that MTHFR C677T, MS A2756G and MTRR A66G genotypes were independently associated with male infertility. Each SNP of the three enzymes may have a different impact on the folate cycle during spermatogenesis.

Key words: azoospermia/folate metabolism/male infertility/oligoasthenoteratozoospermia/polymorphism

Introduction

Male infertility has been identified as the cause of a couple's inability to have children in half of the total cases. Several risk factors for male infertility have recently been identified such as chromosomal abnormalities (Dohle *et al*., 2002), Y-chromosome microdeletions, translocation (Foresta *et al*., 2001), cystic fibrosis transmembrane conductance regulator mutations (Anguiano *et al*., 1992) and other genetic factors (Lee *et al*., 2003).

Folate is essential for DNA synthesis and methylation reactions and for protein synthesis (Fang and Xiao, 2003). Homocysteine is a sulphur-containing amino acid and a metabolite of the essential amino acid methionine; it exists at a critical biochemical intersection in the folate cycle between *S*-adenosylmethionine and methionine (Figure 1).

Methylenetetrahydrofolate reductase (MTHFR) is one of the key enzymes in the remethylation and DNA synthesis pathway. MTHFR catalyses the reduction of methylenetetrahydrofolate (5,10-methyl THF) to methyltetrahydrofolate (5-methyl THF). Methionine synthase (MS) catalyses the transfer of a methyl group from 5-methyl THF to homocysteine, which generates methionine and THF. In this pathway, 5,10-methyl THF participates in DNA synthesis through the exchange of dUTP for dTTP. MS reductase (MTRR) catalyses the reductive methylation of MS using *S*-adenosylmethionine as the methyl donor, and MTRR may also participate in DNA methylation (Gaughan *et al*., 2001).

Folate deficiency is known to occur frequently, and the related hyperhomocysteinaemia is considered as a risk factor for various diseases, including infertility. The folate level and resultant methylation metabolism can lead to abnormal chromosome segregation. James *et al*. (1999) showed that a polymorphism of the MTHFR gene may be a direct genetic risk factor for meiotic non-disjunction such as Down's syndrome. Several single-nucleotide polymorphisms (SNPs) of folate metabolism-related genes have been identified, and these have

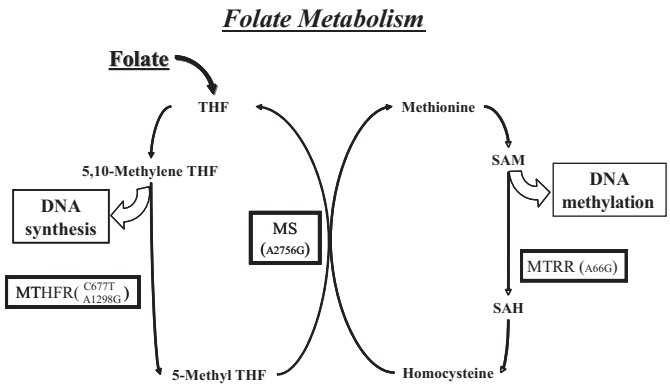

Figure 1. Methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MS) and methionine synthase reductase (MTRR) in the folate cycle. Three enzymes of folate metabolism play an essential role in both DNA synthesis and methylation processes. MS catalyses the transfer of the methyl group from methionine and 5-methyl-tetrahydrofolate (THF) to homocysteine, and this generates tetrahydrofolate; MTRR processes the remethylation with *S*-adenosylmethionine as a methyl donor. MTHFR has a role in reducing THF to 5-methyl THF.

been studied to discover their implications in the pathogenesis of cardiovascular disease, neural tube defects and colorectal cancer (Frosst *et al*., 1995; van der Put *et al*., 1995; Slattery *et al*., 1999; Ulvik and Ueland, 2001; Zhu *et al*., 2003; Singh *et al*., 2005). These SNPs include MTHFR C677T (Ueland *et al*., 2001), MTHFR A1298C (van der Put *et al*., 1998), MS A2756G (Chen *et al*., 1998; Ma *et al*., 1999) and MTRR A66G (Wilson *et al*., 1999). Thus, these folate metabolism-associated diseases are thought to be interrelated via increased homocysteine (tHcy) and *S*-adenosylhomocysteine and the subsequent hypomethylation of numerous substances, including DNA and proteins. MTHFR, MS and MTRR play an important role in folate metabolism, and tHcy levels could affect DNA synthesis and methylation.

DNA methylation may be important to spermatogenesis. The number of condensed spermatids and spermatozoa in the testes was decreased when 5-azacytidine blocked the DNA methylation during spermatogenesis (Raman and Narayan, 1995), which could result in asthenozoospermia and teratozoospermia in humans (Benchaib *et al*., 2003).

A previous report suggested that an SNP of MTHFR could be a risk factor for infertility, at least in the homozygous condition (Bezold *et al*., 2001). Because DNA synthesis and methylation is an integral part of spermatogenesis, folate metabolism is probably important to this process. Therefore, SNPs of the MTHFR, MS and MTRR genes in the folate cycle could be related to male infertility.

In this study, we analysed the association of four SNPs (MTHFR C677T, MTHFR A1298C, MS A2756G and MTRR A66G) with non-obstructive male infertility, and the subjects were classified into the azoospermia and oligoasthenoteratozoospermia (OAT) groups.

Materials and methods

Patients and controls

Three hundred and twenty-five fertile men between the ages of 26 and 72 years (mean ± SD: 42.8 ± 8.6 years) who had at least one child and who lacked any history of requiring assisted reproduction technology (ART) were included as the nation-wide control group—of which 250 men were consecutively enrolled from CHA General Hospital, the College of Medicine, Pochon CHA University (Seoul, Korea), and 100 men were from the Division of Genome Resources, the National Genome Research Institute, National Institute of Health, Korea. An informed consent was required from each subject to participate in this study.

From January 2000 to August 2003, by conducting physical examinations and hormone assays, 672 infertile men were classified as having non-obstructive infertility. The patients who were found to have cryptorchidism and varicocele via the physical examination and clinical tests were excluded from the study. Three hundred and sixty men who had no chromosome abnormality were selected for the SNP study by performing cytogenetic and Y-chromosomal microdeletion analyses (Simoni *et al*., 1999).

These 360 non-obstructive infertile patients were classified again by performing semen analysis. Semen analysis was strictly performed according to the World Health Organization (WHO) guidelines (WHO, 1999) and also according to the criteria of Kruger *et al*. (1986). The non-obstructive infertility patient group were classified into two subtypes: 174 in azoospermia group and 186 in OAT group (130 OAT, 46 severe OAT, 4 oligozoospermia and 6 teratozoospermia), according to a sperm count of $<20 \times 10^6$/ml (oligozoospermia), a progressive sperm motility of <50% (asthenozoospermia), a proportion of sperm <14% dependent on the grade with normal morphology (teratozoospermia), a disturbance of all the three variables (OAT), a sperm count of $<5 \times 10^6$/ml, a progressive motility of <10% and a proportion of <4% with normal morphology (severe OAT) and no spermatozoa (azoospermia). The diagnosis of azoospermia (no spermatozoa in the ejaculate) was made based on two semen samples. The experiments were performed at the CHA General Hospital.

Cytogenetic and Y-chromosome microdeletion assays

Cytogenetic analysis was performed on the metaphase spreads of cultured lymphocytes. Of the 672 non-obstructive infertile men, 312 men had abnormal chromosomes, which included Yq deletion (n = 102), inversion (n = 18), translocation (n = 26), derivate (n = 19), XXY (n = 129) and chromosomal mosaicism (n = 18); these patients were excluded from this SNP study (Lee *et al*., 2003; Manti *et al*., 2006). Only 360 infertile men who had a normal karyotype (46, XY) were finally included in this study.

For Yq deletion studies, DNA was extracted from the peripheral blood and amplified by a multi-PCR method with primers to 13 loci on the Y chromosome, including one SRY and 12 sequence-tagged sites (AZF-a region: sY 84 and sY86; AZF-b region: sY 134, sY 138 and MK5; AZF-c region: sY 152, sY 147, sY 254, sY 255, SPGY1, sY 269 and sY 158) (Simoni *et al*., 1999; Simoni, 2001).

The serum tHcy and folate measurement

Five millilitres of venous blood was collected from patients when they visited. The serum was promptly separated and stored by aliquots at –70°C before use. The serum tHcy levels were measured by performing chemiluminescent immunoassay. The folate levels were measured by radioassay. Serum was mixed with a pH 9.4 borate dithiothreitol buffer and radioactively labelled folate (^{125}I-pteroylglutamic acid) derivative. During incubation, the labelled and unlabelled (serum) folates competed for the binding sites of the folate-binding proteins based on their concentrations. The level of ^{125}I-labelled folate was measured by Gamma counter (Hewlett Packard, USA). The biochemical measurements were performed by Seoul Clinical Laboratory (SCL, Seoul, Korea). Forty-six samples were measured at the same time. The average value of serum folate and Hcy levels of Korean men was 4.9 mg/ml and 13.9 μmol/l, respectively (Min, 2001).

Pyrosequencing assay for screening of MTHFR, MS and MTRR

The PCR primers were designed using PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and Pyrosequencing Primer SNP Design Version 1.01 software. The PCR was carried out using 50 ng of genomic DNA and 10 pmol each of the forward and reverse oligonucleotide PCR primers (Integrated Bioneer, Daejeon, Korea). The biotinylated PCR product then underwent pyrosequencing (Biotage AB) analysis for direct analysis of the SNPs in the PCR product (Supplementary table) according to the method by Ahmadian *et al.* (Ahmadian *et al*., 2000; Park *et al*., 2005; Chung *et al*., 2006).

PCR–restriction fragment length polymorphism

PCR–restriction fragment length polymorphism (RFLP) analysis was performed by following the previously reported methods (Weisberg *et al*., 1998; Friedman *et al*., 1999). Regions containing the polymorphisms in each gene were amplified from the genomic DNA by PCR. The C677T, A1298C, A2756G and A66G variations created the HinfI, Fnu4HI, HaeIII and NdeI restriction enzyme recognition sequences, respectively; the digested fragments were separated by 3% agarose gel electrophoresis; and the gels were visualized under UV light. The restriction enzymes were purchased from the New England Biolabs (NEB, USA).

Statistical analysis

The statistical package used to estimate the odds ratio (OR) and 95% confidence intervals was SAS (r) Proprietary Software Release 8.1 (SAS Institute). The results for the enumeration of data (e.g. the number of individuals with the various genotypes) and the comparison of percentages between groups were evaluated with chi-squared and Fisher's exact tests (two-side). Allele frequencies were calculated by counting the alleles. ORs were calculated by the chi-squared test. P values <0.05 were deemed as being statistically significant.

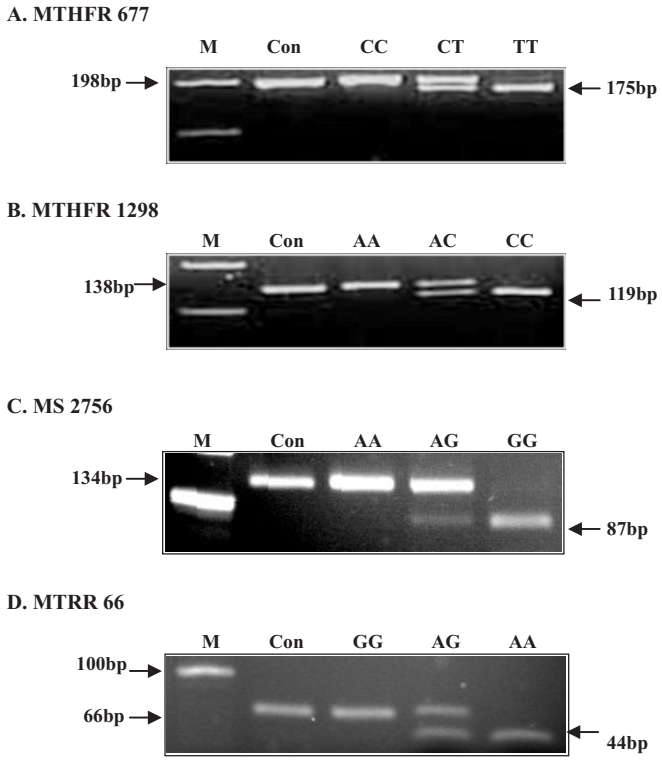

Figure 2. Representative restriction fragment length polymorphism (RFLP) results from the male controls and the infertile patients. PCR-RFLP analysis was carried out to confirm the pyrosequencing method. The RFLP results for MTHFR C677T (**A**), MTHFR A1298C (**B**), MS A2756G (**C**) and MTRR A66G (**D**) are shown. Each panel contains 'M' for 1 kb plus DNA ladder; 'Con' undigested PCR product as control and other single-nucleotide polymorphisms.

Results

We analysed the four polymorphisms of the MTHFR, MS and MTRR genes in 360 infertile and 325 fertile men by performing pyrosequencing and PCR-RFLP. Supplementary figure shows the results of the MTHFR C677T, MTHFR A1298C, MS A2765G and MTRR A66G analyses performed by the pyrosequencing method. Pyrosequencing is now widely used for many different types of DNA analysis. Using a four-enzyme mixture, this sequencing-by-synthesis method relies on the luminometric detection of pyrophosphate (PP_i) that is released on nucleotide incorporation (Ahmadian *et al*., 2000). To validate the MTHFR C677T, MTHFR A1298C, MS A2756G and MTRR A66G variations of the pyrosequencing data, we randomly selected 20 PCR samples and compared the pyrosequencing result with the PCR-RFLP genotypes. The PCR-RFLP results agreed with the pyrosequencing results for the fertile and infertile men (Figure 2).

The results of statistical analysis of the MTHFR C677T, MTHFR A1298C, MS A2756G and MTRR A66G for the fertile and non-obstructive infertile men are summarized in Table I. The frequencies of the homozygous MTHFR 677TT among the fertile and infertile men were 12.62 and 17.78% (P = 0.0481), respectively. The allelic frequency for the fertile men (12.62%) with the 677TT variation in this study was similar to the frequencies noted for healthy Koreans in other studies (12.0–14.7%) (Yoo *et al*., 2000; Hong *et al*., 2004; Kim *et al*., 2004).

The frequencies of homozygous MTRR 66GG differed significantly between the fertile (8.92%) and infertile men (12.78%) (P = 0.0469). However, the MTHFR A1298C and MS A2756G polymorphism data, according to the case–control analysis, showed no significant differences for the individuals with infertility (P = 0.5689 and 0.0941, respectively). The frequencies of homozygous MTHFR A1298C (CC) and MS A2756G (GG) among the fertile and infertile men were 4.31 and 5.00%, and 1.23 and 3.06%, respectively.

The analysed MTHFR C677T, MTHFR A1298C, MS A2756G and MTRR A66G variations in the azoospermia group are summarized in Table II. We observed statistical differences among these SNPs. The frequencies of MTHFR C677T of all types (CT, TT and CT + TT) and the homozygous form of MS A2756G (GG) in the infertile men with azoospermia had statistical significance (MTHFR C677T: CT, P = 0.0267; TT, P = 0.0227; CT + TT, 0.0122; and MS A2756G: GG; P = 0.0063). However, all the allelic types of MTRR A66G and MTHFR A1298C were not statistically significant.

The allelic frequency of the four SNPs in the OAT group is listed in Table III. Three SNPs (MTHFR C677T, MTHFR A1298C and MS A2756G) were not associated with the OAT group, but MTRR A66G was highly associated with the OAT group. The frequency of MTRR 66GG was statistically increased in the OAT group (P = 0.0014).

Table I. Distribution of the methylenetetrahydrofolate reductase (MTHFR) C677T, MTHFR A1298C, methionine synthase (MS) A2756G and methionine synthase reductase (MTRR) A66G genotypes in the infertile and fertile men

	Fertile men (n = 325)	Infertile men (n = 360)	OR (95% CI)	P
MTHFR 677				
677CC	36.31% (n = 118)	31.94 % (n = 115)	–	–
677CT	51.08% (n = 166)	50.28% (n = 181)	1.12 (0.80–1.56)	0.5076
677TT	12.62% (n = 41)	17.78% (n = 64)	1.60 (1.00–2.56)	0.0481[a]
CT + TT	63.69% (n = 207)	68.06% (n = 245)	1.21 (0.88–1.67)	0.2287
MTHFR 1298				
1298AA	65.54% (n = 213)	61.67% (n = 222)	–	–
1298AC	30.15% (n = 98)	33.33% (n = 120)	1.17 (0.85–1.63)	0.3331
1298CC	4.31% (n = 14)	5.00% (n = 18)	1.23 (0.60–2.54)	0.5689
AC + CC	34.46% (n = 112)	38.33% (n = 138)	1.18 (0.87–1.62)	0.2932
MS 2756				
2756AA	78.46% (n = 255)	75.00% (n = 270)	–	–
2756AG	20.31% (n = 66)	21.94% (n = 79)	1.13 (0.78–1.63)	0.5146
2756GG	1.23% (n = 4)	3.06% (n = 11)	2.60 (0.82–8.26)	0.0941
AG + GG	21.54% (n = 70)	25.00% (n = 90)	1.21 (0.85–1.73)	0.2850
MTRR 66				
66AA	22.15% (n = 72)	17.78% (n = 64)		
66AG	68.92% (n = 224)	69.44% (n = 250)	1.26 (0.86–1.84)	0.2424
66GG	8.92% (n = 29)	12.78% (n = 46)	1.78 (1.01–3.17)	0.0469[a]
AG + GG	77.75% (n = 253)	82.22% (n = 296)	1.32 (0.90–1.92)	0.1516

OR, odds ratio; CI, confidence interval.
[a]Significant P values were noted compared with those of the fertile male group ($P < 0.05$).

Table II. Genotype and allelic frequencies for MTHFR C677T, MTHFR A1298C, MS A2756G and MTRR A66G in the azoospermia group (n = 174)

Gene	Azoospermia	Allele type			
		677CC	677CT	677TT	CT +TT
MTHFR677	Frequency (%) (n = 174)	25.29 (n = 44)	57.47 (n = 100)	17.24 (n = 30)	74.71 (n = 130)
	OR (95% CI)	–	1.62 (1.06–2.47)	1.96 (1.09–3.52)	1.68 (1.12–2.54)
	P	–	0.0267[a]	0.0227[a]	0.0122[a]
	Fertile men (%) (n = 325)	36.31 (n = 118)	51.08% (n = 166)	12.62% (n = 41)	63.69% (n = 207)
		1298AA	1298AC	1298CC	AC + CC
MTHFR1298	Frequency (%) (n = 174)	62.64 (n = 109)	32.76 (n = 57)	4.60 (n = 8)	37.36 (n = 65)
	OR (95% CI)	–	1.14 (0.76–1.70)	1.12 (0.45–2.74)	1.13 (0.77–1.66)
	P	–	0.5302	0.8098	0.5195
	Fertile men (%) (n = 325)	65.54 (n = 213)	30.15 (n = 98)	4.31 (n = 14)	34.46 (n = 112)
		2756AA	2756AG	2756GG	AG + GG
MS2756	Frequency (%) (n = 174)	71.26 (n = 124)	23.56 (n = 41)	5.17 (n = 9)	28.74 (n = 50)
	OR (95% CI)	–	1.28 (0.82–2.00)	4.63 (1.40–15.31)	1.47 (0.96–2.24)
	P	–	0.2800	0.0063[a]	0.0730[b]
	Fertile men (%) (n = 325)	78.46 (n = 255)	20.31 (n = 66)	1.23 (n = 4)	21.54 (n = 70)
		1298AA	1298AG	1298GG	AG + GG
MTRR66	Frequency (%) (n = 174)	21.84 (n = 38)	69.54 (n = 121)	8.62 (n = 15)	78.16 (n = 136)
	OR (95% CI)	–	1.02 (0.65–1.61)	0.98 (0.47–2.05)	1.02 (0.65–1.59)
	P	–	0.9196	0.9572	0.9356
	Fertile men (%) (n = 325)	22.15 (n = 72)	68.92 (n = 224)	8.92 (n = 29)	77.85 (n = 253)

[a]Statistically significant P values were noted compared to the fertile male group ($P < 0.05$).
[b]Not significant ($P > 0.05$), but showed a statistical tendency.

We analysed the gene-to-gene correlation between three genes (MTHFR 677, MS and MTRR) in the azoospermia and OAT groups (Table IV). In the azoospermia group, the MS 2756GG type was significantly associated with the MTHFR 677CC type. When the genotype of MTHFR 677 was the CC homozygote, the frequency of the MS 2756GG genotype was significantly increased (P = 0.0184). This result demonstrated that both the MTHFR 677TT and MS 2756GG types independently contribute to azoospermia. However, in the OAT group, all the genotypes of MTHFR C677T and MS A2756G were not associated with the MTRR A66G genotypes.

The frequency of the MS A2756G genotypes, according to each of the MTHFR C677T genotypes, between the control group and the azoospermic group are shown in Figure 3. The fertile men with the MTHFR 677CC genotypes did not have the MS 2756GG types, and this was statistically significant (P = 0.0184); yet the azoospermia group with the MTHFR 677CC genotypes did have the MS 2756GG types (4.6%). In

Table III. Genotype and allele frequencies for MTHFR C677T, MTHFR A1298C, MS A2756G and MTRR A66G in the oligoasthenoteratozoospermia (OAT) group (n = 186)

Gene	OAT[a]	Allele type			
		677CC	677CT	677TT	CT + TT
MTHFR677	Frequency (%) (n = 186)	38.17 (n = 71)	43.55 (n = 81)	18.28 (n = 34)	61.83 (n = 115)
	OR (95% CI)	–	0.81 (0.55–2.21)	1.38 (0.80–2.37)	0.92 (0.64–1.34)
	P	–	0.3000	0.2449	0.6745
	Fertile men (%) (n = 325)	36.31 (n = 118)	51.08 (n = 166)	12.62 (n = 41)	63.69 (n = 207)
		1298AA	1298AC	1298CC	AC + CC
MTHFR1298	Frequency (%) (n = 186)	60.75 (n = 113)	33.87 (n = 63)	5.38 (n = 10)	39.25 (n = 73)
	OR (95% CI)	–	1.21 (0.82–1.79)	1.35 (0.58–3.13)	1.23 (0.85–1.78)
	P	–	0.3343	0.4879	0.2788
	Fertile men (%) (n = 325)	65.54 (n = 213)	30.15 (n = 98)	4.31 (n = 14)	34.46 (n = 112)
		2756AA	2756AG	2756GG	AG + GG
MS2756	Frequency (%) (n = 186)	78.49 (n = 146)	20.43 (n = 38)	1.08 (n = 2)	21.51 (n = 40)
	OR (95% CI)	–	1.01 (0.64–1.57)	0.87 (0.16–4.83)	1.00 (0.64–1.55)
	P	–	0.9805	0.8765	0.9930
	Fertile men (%) (n = 325)	78.46 (n = 255)	20.31 (n = 66)	1.23 (n = 4)	21.54 (n = 70)
		1298AA	1298AG	1298GG	AG + GG
MTRR66	Frequency (%) (n = 186)	13.98 (n = 26)	69.35 (n = 129)	16.67 (n = 31)	86.02 (n = 160)
	OR (95% CI)	–	1.59 (0.97–2.62)	2.96 (1.51–5.82)	1.75 (1.07–2.86)
	P	–	0.0648[c]	0.0014[b]	0.0239[b]
	Fertile men (%) (n = 325)	22.15 (n = 72)	68.92 (n = 224)	8.92 (n = 29)	77.85 (n = 253)

OR, odds ratio; CI, confidence interval.
[a]Including OAT, severe OAT, oligozoospermia and teratozoospermia.
[b]Statistically significant *P* values were noted compared with those of the fertile male group (P <0.05).
[c]Not significant (P > 0.05), but showed a tendency towards significance.

the MTHFR 677CT and TT types, the MS 2756GG homozygote was not significantly increased in azoospermia group, although those with the MTHFR 677CT genotype showed a trend towards increased MS 2756GG types (P = 0.0555).

To determine the effect of each SNP, we investigated the sperm quality according to the genotype of the three folate metabolism-related genes in the OAT subjects (n = 186). The sperm number, motility, vitality and morphology of the OAT group are summarized in Table V based on their genotypes. The number, motility and vitality of sperm were somewhat dependent on the MTHFR C677T and MTRR A66G genotypes, but there were no significant associations (P > 0.05).

We focused on investigating the genotypes of the folate-related enzymes, but the tHcy and folate levels of the patients were also important. Therefore, we tried to measure the average value of tHcy and folate levels to investigate any physiological association between the genotype of folate-related enzymes and serum level. Forty-six patients were selected according to MTHFR genotypes (18 with CC, 15 with CT and 13 with TT) for biochemical assays (Table VI). We measured tHcy and folate levels from the frozen sera at the same time. The tHcy level ranged from 9.14 to 13.22 μmol/l, and the folate level ranged from 4.06 to 8.98 ng/ml in all 46 patients, which were in the normal range. The folate and tHcy levels did not differ significantly with the genotypes of the folate metabolism enzymes by statistical analysis (Table VI).

Discussion

In this study, we analysed the association of the four SNPs of three genes in the folate cycle of infertile men, and the prevalence of three SNPs (MTHFR C677T, MS A2756G and MTRR A66G) was associated with non-obstructive male infertility.

Four previous studies (Bezold *et al*., 2001; Ebisch *et al*., 2003; Stuppia *et al*., 2003; Singh *et al*., 2005) have evaluated the association of MTHFR polymorphism in infertile patients of Western Europe, Italy and India. Bezold *et al*. and Singh *et al*. have reported that the frequency of the TT homozygote and the CT heterozygote of MTHFR C677T was significant in the infertile patients. Bezold *et al*. showed that the frequency of the TT homozygote in the infertile patient was increased by 9.3% comparing to that of control group, but the frequency of the CT heterozygote was decreased by 8% in Germany. However, in the article by Singh *et al*., the CT heterozygote and TT homozygote in the infertile patients were each increased by 8 and 4%, respectively. Ebish *et al*. concluded that the C677T polymorphism is not a risk factor for male factor subfertility and also indicated the importance of folates in sperm production. Stuppia *et al*. reported that there was no significant difference in the frequency of the CT heterozygote and the C and T homozygotes and also suggested that some gene(s) other than the MTHFR gene in the DNA methylation pathway could affect the male infertility.

Our study had many subjects in the control and patient groups (N = 685), and it gave more details of the inclusion and exclusion criteria for the patient selection. We selected the patients by performing cytogenetic and Yq deletion analyses and classified the patients without any chromosomal abnormalities into two groups by the existence or non-existence of spermatozoa. One group of azoospermia patients had no spermatozoa in the ejaculate, and the other group was OAT patients that included unexplained OAT, severe OAT and teratozoospermia.

Table IV. Gene-to-gene interaction analyses for MTHFR C677T, MS A2756G and MTRR A66G in the azoospermia group and the oligoasthenoteratozoospermia (OAT) group

Pateint group	SNP		Allele type											
Azoospermia group	MTHFR677		CC				CT				TT			
			C%[c] (*n*)	*P*%[d] (*n*)	*P*	OR (95%CI)	C% (*n*)	P% (*n*)	*P*	OR (95%CI)	C% (*n*)	P% (*n*)	*P*	OR (95%CI)
	MS 2756	AA	78.8 (93)	72.7 (32)	–	–	75.9 (126)	68.0 (68)	–	–	87.8 (36)	80.0 (24)	–	–
		AG	21.2 (25)	22.7 (10)	0.7240	1.16 (0.5–2.7)	21.7 (36)	25.0 (25)	0.4012	1.29 (0.7–2.3)	12.2 (5)	20.0 (6)	0.3683	1.8 (0.5–6.6)
		GG	0 (0)	4.6 (2)	0.0184[a]	–	2.4 (4)	7.0 (7)	0.0555[b]	3.24 (0.4–11.9)	0 (0)	0 (0)	–	–
		AG + GG	21.2 (25)	27.3 (12)	0.4118	1.4 (0.6–3.1)	24.1 (40)	32.0 (32)	0.1599	1.48 (0.9–2.6)	–	–	–	–
	MTRR 66	AA	23.7 (28)	27.3 (12)	–	–	21.7 (36)	21.0 (21)	–		19.5 (8)	16.7 (5)	–	
		AG	67.8 (80)	65.9 (29)	0.6810	0.85 (0.4–1.9)	68.7 (114)	71.0 (71)	0.8345	1.07 (0.6–2.0)	73.2 (30)	70.0 (21)	0.8588	1.12 (0.3–3.9)
		GG	8.5 (10)	6.8 (3)	0.6302	0.7 (0.2.–3.0)	9.6 (16)	8.0 (8)	0.7636	0.86 (0.3–2.3)	7.3 (3)	13.3 (4)	0.4231	2.13 (0.3–13.8)
		AG + GG	76.3 (90)	72.7 (32)	0.6417	0.83 (0.4–1.8)	78.3 (130)	79.0 (79)	0.8948	1.04 (0.6–1.9)	80.5 (33)	83.3 (25)	0.7594	1.21 (0.4–4.2)
OAT group	MTRR 66		AA				AG				GG			
			C%[c] (*n*)	P%[d] (*n*)	*P*	OR (95%CI)	C% (*n*)	P% (*n*)	*P*	OR (95%CI)	C% (*n*)	P% (*n*)	*P*	OR (95%CI)
	MTHFR 677	CC	38.9 (28)	50.0 (13)	–	–	35.7 (80)	36.4 (47)	–	–	44.5 (10)	35.5 (11)	–	–
		CT	50.0 (36)	46.2 (12)	0.4828	0.72 (0.3–1.8)	50.9 (114)	41.9 (54)	0.3832	0.81 (0.5–1.3)	55.2 (16)	48.4 (15)	0.7775	0.85 (0.3–2.6)
		TT	11.1 (8)	3.8 (1)	0.2127	0.27 (0.03–2.4)	13.4 (30)	21.7 (28)	0.1476	1.59 (0.8–3.0)	10.3 (3)	16.1 (5)	0.6243	1.52 (0.3–8.0)
		CT + TT	61.1 (44)	50.0 (13)	0.3249		64.3 (144)	63.6 (82)	0.8921	0.97 (0.6–1.5)	65.5 (19)	64.5 (20)	0.9352	0.96 (0.3–2.8)
	MS 2756	AA	75.0 (54)	76.9 (20)	–	–	79.0 (177)	79.8 (103)	–	–	82.8(24)	74.2(23)	–	–
		AG	25.0 (18)	23.1 (6)	0.8450	0.9 (0.3–2.6)	19.2 (43)	19.4 (25)	0.9974	1.0 (0.6–1.7)	17.2(5)	22.6(7)	0.5611	1.46(0.4–5.3)
		GG	0 (0)	0 (0)	–	–	1.8 (4)	0.8 (1)	0.4396	0.43 (0.05–3.9)	0 (0)	3.2(1)	0.3122	–
		AG + GG	–	–	–	–	21.0 (47)	20.2 (26)	0.8534	0.95 (0.6–1.6)	17.2(5)	25.8(8)	0.4210	1.67(0.5–5.9)

[a]Significant *P* values were noted compared with those of the fertile male group ($P < 0.05$).
[b]Not significant ($P > 0.05$) but showed a tendency towards significance.
[c]Percentage of the fertile men.
[d]Percentage of the infertile men.

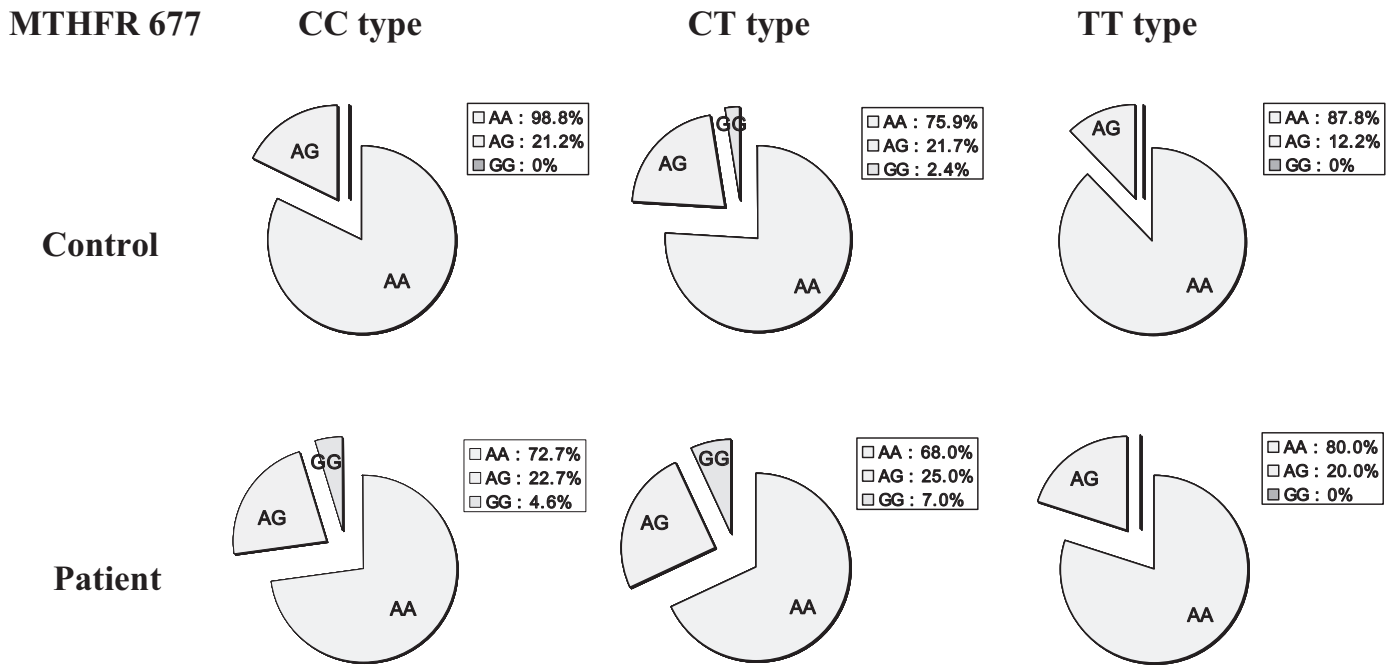

Figure 3. The frequency of the MS A2756G genotypes according to the MTHFR C677T genotypes for the control and the azoospermic groups. When the MTHFR C677T genotype was the CC homozygote, the MS 2756GG homozygote was significantly increased in the azoospermia group ($P = 0.0184$).

Table V. Sperm count, motility, vitality and morphology according to genotype of each folate metabolism-related gene in the oligoasthenoteratozoospermia (OAT) group ($n = 186$)

	Genotype	AA ($n = 26$)	AG ($n = 129$)	GG ($n = 31$)	*P* value
MTRR 66	Sperm count (10^6/ml)	40.29 (±39.5)	61.89 (±58.8)	42.97 (±35.6)	>0.05
	Motility (%)	32.56 (±14.6)	31.50 (±13.6)	26.47 (±14.4)	>0.05
	Vitality (%)	51.30 (±18.5)	51.12 (±15.3)	49.23 (±12.6)	>0.05
	Morphology (%)	2.67 (±2.2)	3.04 (±6.9)	3.60 (±4.3)	>0.05
MTHFR 677	Genotype	CC ($n = 71$)	CT ($n = 81$)	TT ($n = 34$)	
	Sperm count (10^6/ml)	60.71 (±54.5)	57.78 (±57.5)	47.93 (±49.0)	>0.05
	Motility (%)	32.12 (±13.9)	32.44 (±13.5)	27.62 (±14.8)	>0.05
	Vitality (%)	53.49 (±16.0)	49.92 (±14.6)	48.96 (±15.4)	>0.05
	Morphology (%)	3.93 (±9.7)	2.87 (±3.0)	2.08 (±2.2)	>0.05
MTHFR 1298	Genotype	TT ($n = 113$)	TG ($n = 63$)	GG ($n = 10$)	
	Sperm count (10^6/ml)	52.18 (±58.0)	69.86 (±47.8)	37.57 (±43.7)	NE
	Motility (%)	31.17 (±14.2)	32.73 (±13.6)	32 (±13.5)	NE
	Vitality (%)	50.11 (±15.6)	52.56 (±14.8)	51.14 (±15.9)	NE
	Morphology (%)	2.97 (±3.4)	3.54 (±9.6)	1.43 (±0.5)	NE
MS 2756	Genotype	AA ($n = 146$)	AG ($n = 38$)	GG ($n = 2$)	
	Sperm count (10^6/ml)	61.08 (±53.4)	34.46 (±32.8)	100.5 (±115.3)	NE
	Motility (%)	30.49 (±13.4)	36.96 (±15.3)	31.75 (±12.6)	NE
	Vitality (%)	50.27 (±15.7)	54.86 (±13.8)	47.25 (±13.0)	NE
	Morphology (%)	2.54 (±3.0)	5.35 (±12.6)	2.75 (±0.8)	NE

NE, not estimated.
The values in the parenthesis indicate the standard deviation.

Folate deficiency has been shown to reduce the proliferation of various cell types (Blount *et al*., 1997; Zhu and Melera, 2001). The activities of the folate metabolism-related enzymes are affected by the SNP genotype (Weisberg *et al*., 1998; Gaughan *et al*., 2001; Jacques *et al*., 2003). We conducted a case–control study to investigate the association of nutrient intake in the one-carbon pathway of folate for DNA synthesis and methylation reactions. The folate intake is important for male infertility, and this level in human serum may differ in different countries. The average of folate intake in Korea is about 347 μg/day, which is a similar to other world regions (http://europa.eu.int/comm/food/fs/sc/scf/index_en.html). Ebisch *et al.* (2003) have shown that the sperm concentration was significantly increased by folic acid and zinc sulphate treatment from the fertile and subfertile men of the MTHFR CC types but not from the MTHFR CT and TT types.

We investigated the tHcy and folate levels from the sera of 46 infertile patients, but those levels were not closely associated

Table VI. The serum homocysteine (tHcy) and folate levels of 46 selected patients in relation to the genotypes of folate metabolism enzymes

	MTHFR 677C→T			MS 2756A→G			MTRR 66A→G		
	CC	CT	TT	AA	AG	GG	AA	AG	GG
Number of individuals	18	15	13	31	5	10	11	15	20
Serum tHcy[a] (μmol/l)	10.23 (±2.6)	9.26 (±1.7)	10.87 (±3.1)	9.90 (±2.4)	13.22 (±2.9)	9.14 (±1.4)	9.52 (±2.6)	10.36 (±2.2)	10.21 (±2.7)
(95% confidence limits)	(9.01–11.44)	(8.42–10.10)	(9.14–12.60)	(9.06–10.73)	(10.65–15.79)	(8.28–10.00)	(7.96–11.08)	(9.20–11.52)	(9.04–11.37)
Serum folate[b] (ng/ml)	7.80 (±7.0)	6.60 (±4.1)	5.58 (±2.2)	6.44 (±2.8)	4.06 (±1.1)	8.98 (±9.4)	6.44 (±3.8)	6.01 (±2.4)	7.76 (±6.9)
(95% confidence limits)	(4.56–11.04)	(4.53–8.67)	(4.41–6.76)	(5.46–7.41)	(3.11–5.01)	(3.13–14.83)	(5.46–7.41)	(4.74–7.29)	(4.73–10.78)

[a]Standard serum tHcy level = 5.0–13.9 μmol/l.
[b]Standard serum folate level = 1.5–16.9 ng/ml.
The values in the parenthesis indicate the standard deviation.

with any genotype or subinfertile group (Table VI). These findings are somewhat inconsistent with those of other studies because we used sera from non-fasting patients. Serum tHcy and folate levels may also reflect differences in individual conditions such as the dietary intake and other genetic and environmental factors. Thus, further investigations are needed with long-term clinical information that would be adjusted for smoking, alcohol consumption, BMI and dietary fibre intake to determine the roles of the three enzymes and the effects that genetics and the folic acid status play in the aetiology of male infertility.

DNA methylation and DNA synthesis have dramatic effects on spermatogenesis (Tanaka *et al*., 1996; Doerksen *et al*., 2000; Kelly *et al*., 2003). Especially, inducing hypomethylation by 5-aza-deoxycytidine treatment inhibited differentiation of the spermatogonia to spermatocytes in a murine model (Raman and Narayan, 1995). Therefore, we also investigated sperm parameters in the OAT group (Table V). There is some correlation of the sperm count and motility according to the genotype of MTHFR C677T and MTRR A66G, but it did not reach the level of significance ($P > 0.05$). We need a more extended patient and normal group data of each genotype for statistical analysis, and we may need to repeat the semen analysis for each patient and control subject to get accurate data. The explanation of our data may lie in the effect that environmental factors and other genes have also on the semen quality.

Our result shows a clearer association of MTHFR and MTRR genotypes with non-obstructive male infertility as a whole. By classifying the infertile patients into the azoospermia group and the OAT patient group, the frequency of the MTHFR 677TT genotype was increased in the azoospermia group. Interestingly, MS 2756GG was also increased with the azoospermia group, but not in whole infertile group. The MTRR 66GG type was not only associated with the whole group but also with the OAT group (Table IV). Our association study showed that MTHFR C677T, MS A2756G and MTRR A66G genotypes were all important to male infertility. By performing a combined analysis for gene–gene interaction (Table IV), each SNP had independent effects on the subtype of male infertility. The three enzymes are in the same folate cycles but may have different roles in and impact on DNA synthesis and methylation reactions during spermatogenesis.

The SNP of each enzyme may alter the enzyme activity to different degrees depending on its position within the 3D structure and the amino acid substitution. Previous biochemical genetics studies showed that the MTRR 66GG genotype contributes more than the MTHFR 677TT and MS 2756GG genotypes for the regulation of tHcy levels (Gaughan *et al*., 2001; Jacques *et al*., 2003; Zijno *et al*., 2003). The association of the MTRR 66GG genotype with OAT in this study may have some link to these biochemical findings.

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Supplementary materials

Supplementary material is available online at http://humrep.oxfordjournals.org/.

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