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Methylenetetrahydrofolate Reductase Polymorphisms Are Related to Male Infertility in Brazilian Men

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Objective: The objective of this study was to analyze the distribution of the methylenetetrahydrofolate reductase (*MTHFR*) C677T and A1298C polymorphisms in idiopathic infertile Brazilian patients with nonobstructive azoospermia (NOA) or severe oligozoospermia and fertile Brazilian men as controls to explore the possible association of these polymorphisms and male infertility. *Methods:* A case–control study was carried out, including 156 idiopathic infertile Brazilian patients with NOA ($n = 49$) or severe oligozoospermia ($n = 107$) and 233 fertile men as controls. Polymorphisms C677T and A1298C were studied by quantitative polymerase chain reaction and the results were statistically analyzed. *Results:* The frequency of genotypes *MTHFR* 677CC, 677CT, and 677TT in idiopathic infertile men with NOA were 55.1%, 30.6%, and 14.3% ($p = 0.0305$); 50.6%, 42.0%, and 7.5% ($p = 0.0006$) regarding the severe oligozoospermic men; and 71.7%, 53.0%, and 5.6% in the control group. As for polymorphism A1298C, regarding the NOA group, the frequencies of the 1298AA, 1298AC, and 1298CC genotypes were 53.0%, 28.6%, and 18.4% ($p = 0.0132$); 42.0%, 44.9%, and 13.1% ($p = 0.0188$) among the severe oligozoospermic group; and 55.8%, 38.2%, and 6.0% (14/233) in the control group. *Conclusion:* The data suggest that *MTHFR* C677T and A1298C could be important genetic factors predisposing to infertility in Brazilian infertile men.

Introduction

Infertility is a very common health problem that affects ~15%–20% of couples who attempt pregnancy (Oliva *et al.*, 2001). In almost 50% of infertile couples, the problem is related to the male, and in about 15% of male infertile subjects, genetic abnormalities could be present, including chromosomal aberrations and single gene mutations (Ferlin *et al.*, 2006; Pieri *et al.*, 2002).

Folate is essential for DNA synthesis and methylation reactions and for protein synthesis (Fang and Xiao, 2003). Methylenetetrahydrofolate reductase (*MTHFR*) is a key regulatory enzyme involved in folate metabolism, DNA synthesis, and remethylation reactions. The metabolic pathways of folate can be modified by polymorphisms in relevant genes such as *MTHFR* or by the action of carcinogenic elements, for example, alcohol or tobacco (Lee *et al.*, 2006).

The *MTHFR* gene, located on the short arm of chromosome 1 (1p36.3), presents two common polymorphisms involving nucleotides C677T and A1298C. The change of C for T at position 677 causes the substitution of alanine for valine in the MTHFR protein and a consequent reduction in enzyme activity. The specific activity of the MTHFR enzyme is reduced by 35% in the presence of heterozygosis, genotype C/T, compared with the normal genotype C/C, and by 70% in homozygosis, genotype T/T. Polymorphism A1298C brings about the substitution of a glutamate for a valine, causing a reduction in the enzyme activity that is more effective when in homozygosis (Fross *et al.*, 1995; van der Put *et al.*, 1998).

Low folate coupled with *MTHF*R polymorphisms can alter RNA/DNA synthesis and has the potential to be linked with infertility (Stern *et al.*, 2000). Animal model studies suggest that *MTHFR* plays a critical role in spermatogenesis because of exceptionally higher activity in adult testis than other organs (Chen *et al.*, 2001).

Thus, the objective of the present study was to determine the distribution of the *MTHFR* C677T and A1298C polymorphisms in idiopathic infertile Brazilian patients and controls to explore the possible association of these polymorphisms to male infertility.

Material and Methods

Patients

Among the patients of the Andrology Outpatient Clinic of the Division of Pathological Gynecology and Human

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Reproduction of ABC School of Medicine, 156 idiopathic infertility men were studied (age ranged between 25 and 52 years; mean: 36.6 ± 5.6 years). Only infertile men with severe oligozoospermia ($n = 107$) and nonobstructive azoospermia (NOA) ($n = 49$), with no chromosome abnormalities or Y chromosome microdeletions, with at least 1 year of infertility were included in this study. To compose the control group, 233 fertile men (mean: 56.7 + 3.2 years) who have at least one child by direct survey and who lacked any history of requiring assisted reproduction technology were selected from the Family Planning Outpatient Clinic of the ABC School of Medicine.

Clinical data and peripheral blood samples were collected only after explaining the objectives of the study and obtaining a signed informed consent form, as approved by the Research Ethics Committee of the ABC School of Medicine.

Methods

Semen analysis. Semen analysis was performed strictly according to World Health Organization (WHO, 1999) guidelines. The diagnosis of azoospermia was made on the basis of two semen analyses performed according to the WHO-recommended procedure. NOA was determined after historical and physical examination, sperm analysis (including assessment of sperm volume, pH, and evaluation of fructose concentration), endocrine profile (follicle-stimulating hormone [FSH], luteinizing hormone [LH], testosterone, androstenedione), ultrasound testicular volume measurement, and seminal vesicle evaluation. Classification of severe oligozoospermia was done if spermatozoa numbered <5 million/mL, according to WHO criteria (WHO, 1999).

***MTHFR* genotyping.** For molecular study, genomic DNA was extracted from lymphocytes, using the Illustra™ Blood GenomicPrep Mini Spin Kit (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom), according to the manufacturer's instructions.

Detection of *MTHFR* polymorphism for C677T (rs1801133) and A1298C (rs1801131) were performed using Taq Man real-time polymerase chain reaction using Rotor-Gene Q 6 plex Platform (Qiagen, Valencia, CA). Taqman primers and probes for C677T and A1298C were commercially available and provided by Applied Biosystems (Foster City, CA) (*MTHFR* C677T: C___1202883_20 and *MTHFR* A1298C: C____850486_20). Assays were performed with Taqman Universal Master Mix (Applied Biosystems®, Foster City, CA), with 50 ng of DNA extract being used per reaction. Polymerase chain reaction conditions were provided by the manufacturer: 40 cycles of 95°C denaturation (15 s), 60°C annealing/extension (1 min). Samples were run with negative and positive controls for both C677T and A1298C polymorphisms.

Statistical analysis. The chi-square test was used to compare allele and genotype frequencies between groups. Statistical tests of significance and χ^2 analysis were carried out using SPSS for Windows 8.0 (SPSS, Chicago, IL). All *p*-values were two tailed, and 95% confidence intervals were calculated. A *p*-value of <0.05 was considered statistically significant.

Results

The distribution of genotypes *MTHFR* 677CC, 677CT, and 677TT and of genotypes 1298AA, 1298AC, and 1298CC in idiopathic infertile males and in the controls is shown in Tables 1 and 2. Regarding the infertile patients, 68.6% (107/156) had severe oligozoospermia and 31.4% (49/156) NOA.

The frequency of genotypes *MTHFR* 677CC, 677CT, and 677TT in idiopathic infertile men with NOA were 55.1% (27/49), 30.6% (15/49), and 14.3% (7/49), respectively ($p = 0.0305$). Regarding the severe oligozoospermic men, the genotypes 677CC, 677CT, and 677TT were presented in 50.6% (548/107), 42.0% (45/107), and 7.5% (8/107), respectively ($p = 0.0006$). Among the control group, genotypes 677CC, 677CT, and 677TT were found at the following frequencies: 71.7% (167/233), 53.0% (53/233), and 5.6% (13/233), respectively.

For the polymorphism A1298C, regarding the NOA group, the frequencies of 1298AA, 1298AC, and 1298CC were 53.0% (26/49), 28.6% (14/49), and 18.4% (9/49), respectively ($p = 0.0132$). Among the severe oligozoospermic group, 42.0% (45/107) presented normal homozygous genotype 1298AA, 44.9% (48/107) presented heterozygous genotype 1298AC, and 13.1% (14/107) presented mutated homozygous genotype 1298CC ($p = 0.0188$). In the control group, genotypes 1298AA, 1298AC, and 1298CC were present in 55.8% (130/233), 38.2% (89/233), and 6.0% (14/233), respectively.

Considering the alleles, the allele C of the *MTHFR* C677T polymorphism was present in 70.4% of the NOA men, 71.5% of the severe oligozoospermic men, and 83.0% of control group, whereas the allele T was present in 29.6% of the NOA men ($p = 0.006$), 28.5% of the severe oligozoospermic men ($p = 0.0008$), and 17.0% of control group.

Regarding the *MTHFR* A1298C polymorphism, the allele A was present in 67.3%, 64.5%, and 74.9%, respectively, in NOA men, severe oligozoospermic men, and controls. The allele C was present in 32.7% of nonobstructive azoospermic men

Table 1. Genotype Frequencies of the Methylenetetrahydrofolate Reductase C677T Polymorphism in Infertile Brazilian Men and Controls

	Genotypes MTHFR *C677T*				*Alleles*			
	CC	*CT*	*TT*		*C*	*T*		
Population studied	n (%)	n (%)	n (%)	p-*Value*	n (%)	n (%)	p-*Value*	*OR (95% CI)*
NOA ($n = 49$)	27 (55.1)	15 (30.6)	7 (14.3)	0.0305	69 (70.4)	29 (29.6)	0.006	2.06 (1.25–3.38)
Severe oligozoospermia ($n = 107$)	54 (50.6)	45 (42.0)	8 (7.5)	0.0006	153 (71.5)	61 (28.5)	0.0008	1.95 (1.33–2.86)
Controls ($n = 233$)	167 (71.7)	53 (22.7)	13 (5.6)		387 (83.0)	79 (17.0)		

OR, odds ratio; CI, confidence interval; NOA, nonobstructive azoospermia; *MTHFR*, methylenetetrahydrofolate reductase.

Table 2. Genotype Frequencies of the Methylenetetrahydrofolate Reductase A1298C Polymorphism in Infertile Brazilian Men and Controls

	Genotypes MTHFR *A1298C*				*Alleles*			
	AA	*AC*	*CC*		*A*	*C*		
Population studied	n *(%)*	n *(%)*	n *(%)*	p-*Value*	n *(%)*	n *(%)*	p-*Value*	*OR (95% CI)*
NOA ($n = 49$)	26 (53.0)	14 (28.6)	9 (18.4)	0.0132	66 (67.3)	32 (32.7)	0.1573	1.45 (0.90–2.32)
Severe oligozoospermia ($n = 107$)	45 (42.0)	48 (44.9)	14 (13.1)	0.0188	138 (64.5)	76 (35.5)	0.0069	1.64 (1.116–2.33)
Controls ($n = 233$)	130 (55.8)	89 (38.2)	14 (6.0)		349 (74.9)	117 (25.1)		

($p = 0.1573$), 35.5% of the severe oligozoospermic men ($p = 0.0069$), and 25.1% of control group.

Discussion

In the present study, we concomitantly evaluated the associations of common polymorphisms in *MTHFR* (C677T and A1298C) gene, which is involved in folate metabolism, and their associated risk for infertility. The presence of allele T of the *MTHFR* C677T polymorphism seems to be associated with both severe oligozoospermia and NOA, whereas the allele C of the *MTHFR* A1298C polymorphism seems to be associated especially with severe oligozoospermia. Our findings demonstrate relevance of folate metabolism in susceptibility to infertility among the Brazilian male population.

A few previous studies have evaluated the association of *MTHFR* polymorphisms in infertile patients with conflicting results (Ebisch *et al.*, 2003; Stuppia *et al.*, 2003; Park *et al.*, 2005; Singh *et al.*, 2005; Lee *et al.*, 2006; A *et al.*, 2007; Dhillon *et al.*, 2007; Ravel *et al.*, 2009) (Table 3). The inconsistence between the studies may be explained on the basis of the subjects studied, ethnic or geographic factors, and dietary habits; folate level in human serum may differ in different countries. Moreover, gene–nutrient/environmental and

Table 3. Studies That Evaluated the Association of Methylenetetrahydrofolate Reductase C677T and A1298C Polymorphisms in Infertility Patients

Study	*Population*	*Genes*	*Conclusion of the study*
Ebisch *et al.* (2003)	113 fertile and 77 subfertile males	*MTHFR* (C677T)	*MTHFR* C677T polymorphism is not a risk factor for male factor subfertility.
Stuppia *et al.* (2003)	93 Italian infertile patients and in 105 Italian fertile controls	*MTHFR* (C677T)	The results do not support an association between the *MTHFR* 677T allele and male infertility in Italy.
Singh *et al.* (2005)	151 cases of nonobstruction, idiopathic oligo-/azoospermia and 200 fertile males	*MTHFR* (C677T)	*MTHFR* C677T is clearly a risk factor for infertility in the Indian population
Park *et al.* (2005)	373 infertile and 396 healthy fertile men	*MTHFR* (C677T and A1298C)	The *MTHFR* 677TT genotype may be a genetic risk factor for male infertility, especially with severe OAT and NOA in unexplained infertile males.
Lee *et al.* (2006)	360 patients with nonobstructive infertility and 325 fertile men without any chromosomal abnormalities	*MTHFR* (C677T and A1298C), *MTRR* (A66G), and *MTR* (A2756G)	*MTHFR* C677T, *MTR* A2756G and *MTRR* A66G genotypes were independently associated with male infertility.
A *et al.* (2007)	355 infertile Chinese patients with idiopathic azoospermia or severe oligozoospermia and 252 fertile Chinese men as controls	*MTHFR* (C677T)	There is an association of SNP C677T in the MTHFR gene with male infertility.
Dhillon *et al.* (2007)	179 oligoasthenoteratozoospermia patients and 200 fertile men	*MTHFR* (C677T and A1298C) and *DNMT3b* (C46359T)	The *MTHFR* (C677T and A1298C) and *DNMT3b* (C46359T) frequencies did not differ significantly in two groups.
Ravel *et al.* (2009)	253 infertile French men and 114 controls	*MTHFR* (G203A, C677T, and A1298C), *MTRR* (I22M and S175L), and *CBS* (G307S)	No evidence for an association between reduced sperm counts and polymorphisms in enzymes involved in folate metabolism in the French population.
Present study	156 Brazilian patients with nonobstructive infertility and 233 Brazilian fertile men without any chromosomal abnormalities	*MTHFR* (C677T and A1298C)	The *MTHFR* C677T and A1298C genotypes might be a genetic risk factor for male infertility in Brazilian infertile man.

OAT, oligoasthenoteratozoospermia group; SNP, single-nucleotide polymorphism.

gene–racial/ethnic interactions have been shown to affect the impact of these *MTHFR* genetic variants (Toffoli and De Mattia, 2008).

Changes in folate status could affect spermatogenesis in two ways: (1) causing DNA hypomethylation and thereby disrupting gene expression, and (2) inducing uracil misincorporation during DNA synthesis, leading to errors in DNA repair, strand breakage, and chromosomal anomalies. Spermatogenesis is a complex process, involving numerous genes. One of the mechanisms regulating their expression is DNA methylation. As experimentally induced undermethylation of premeiotic germ cells in mouse has been shown to inhibit their differentiation into spermatocytes (Raman and Narayan, 1995; Tamara *et al.*, 2003), it is possible that *MTHFR* (C677T) mutation in man causes infertility by the same mechanism. This suggestion is strengthened by the fact that in humans the global genomic methylation in 677T is lower than in the 677C genotype (Stern *et al.*, 2000; Friso *et al.*, 2002). Another obvious effect of *MTHFR* mutation on cell physiology is auto-oxidation, leading to the production of toxic reactive oxygen metabolites, for example, hydrogen peroxide (Starkebaum and Harlan, 1993; Loscalzo, 1996). An increased production of reactive oxygen species results in homocysteine-mediated DNA damage (Huang *et al.*, 2000). Human spermatozoa are particularly susceptible to peroxidative damage, and antioxidants such as folate can overcome oxidative stress and maintain the integrity of sperm cells by preventing oxidative damage to sperm DNA. Thus, in addition to undermethylation, homocysteine-mediated DNA damage because of oxidative stress may be another plausible mechanism of male infertility in subjects with *MTHFR* polymorphisms (A *et al.*, 2007).

Besides *MTHFR* polymorphisms, many genes are also related to male factor infertility, such as follicle-stimulating hormone receptor (*FSHR*), estrogen receptor alpha (*ERα*), estrogen receptor beta (*ERβ*), and testis-specific serine kinase 6 (*TSSK6*) polymorphisms, mutations in deleted in azoospermia-like (*DAZL*), synaptonemal complex protein 3 (*SYCP3*), and ubiquitin-specific protease 26 (*USP26*). *USP26* was first identified by Wang *et al.* (2001), who confirmed expression of *USP26* RNA in mice. Preliminary data indicate increased number of mutations in the *USP26* gene in men with severe male factor infertility. Another gene possibly involved in male infertility is *TSSK6*, a member of the testis-specific serine/threonine kinase family. Male *Tssk6* knockout mice are infertile owing to spermatogenic impairment, including sperm count reduction, a decrease in motile sperm number and motility rates, and an increase in the number of sperm with abnormal morphology. Polymorphisms in this gene were associated with male infertility in a study performed by Su *et al.* (2010).

In summary, the present study found an association between the *MTHFR* C677T and A1298C polymorphisms and infertility in men with NOA and severe oligozoospermia, suggesting that these mutations might be a genetic risk factor for infertility in Brazilian men.

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Disclosure Statement

None of the authors has any conflict of interest to disclose.

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