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Polymorphisms in Folate-Related Enzyme Genes in Idiopathic Infertile Brazilian Men

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Abstract

The aim of the study was to analyze the distribution of the methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase reductase (*MTRR*), and methionine synthase (*MTR*) polymorphisms in idiopathic infertile Brazilian men and fertile men. Case–control study comprising 133 idiopathic infertile Brazilian men with nonobstructive azoospermia ([NOA] n = 55) or severe oligozoospermia ([SO] n = 78) and 173 fertile men as controls. *MTHFR* C677T, A1298C, and G1793A; *MTRR* A66G; and *MTR* A2756G polymorphisms were studied by quantitative polymerase chain reaction (qPCR). The results were analyzed statistically and a *P* value <.05 was considered significant. Single-marker analysis revealed a significant association among *MTHFR* C677T polymorphism and both NOA group ($P = .018$) and SO group ($P < .001$). Considering the *MTHFR* A1298C, *MTHFR* G1793A, and *MTRR* A66G polymorphisms, no difference was found between NOA group and SO group. Regarding the *MTR* A2756G polymorphism, a significant difference was found between NOA and controls, $P = .017$. However, statistical analysis revealed no association between SO group and controls. Combined genotypes of 3 *MTHFR* polymorphisms did not identify a haplotype associated with idiopathic infertility. The combinatory analysis of the 3 polymorphisms *MTHFR*, *MTRR*, and *MTR* did not show difference between cases and controls. The findings suggest the *MTHFR* C677T and *MTR* A2756G polymorphisms could be an important genetic factor predisposing to idiopathic infertility in Brazilian men.

Keywords

male infertility, folate, homocysteine, *MTHFR* gene, *MTRR* gene, *MTR* gene

Introduction

Infertility is a very common health problem that affects approximately 15% to 20% of couples who attempt pregnancy.[1] In almost 50% of infertile couples, the problem is related to the male and in about 15% of these cases genetic abnormalities could be present, including chromosomal aberrations and single-gene mutations.[1,2]

Folate participates in amino acid metabolism, purine and pyrimidine synthesis, and methylation of nucleic acids, proteins, and lipids. Dietary or genetically determined folate deficiency may impair the function of these metabolic pathways and lead to homocysteine (Hcy) accumulation.[3] Homocysteine, a thiol-containing amino acid, originates from the 1-carbon-donating metabolism of methionine and is remethylated to methionine, with folate acting as methyl donors.[4]

Methylenetetrahydrofolate reductase (MTHFR) is a key regulatory enzyme involved in folate metabolism. Methionine, the precursor for the universal methyl donor (*S*-adenosylmethionine) is produced through the irreversible transfer of a methyl group from 5-methyltetrahydrofolate. This reaction is regulated by 2 enzymes, methionine synthase (MTR) and methionine synthase reductase (MTRR).[5] Disturbances in the catalytic activity of MTRR could lead to higher levels of Hcy. MTHFR, MTR, and MTRR play an important role in folate metabolism, and Hcy levels could affect DNA synthesis and methylation, leading to an increased oxidative stress[6] and disturbed methylation reactions.[7] Such processes are involved in male infertility.[8,9]

The *MTHFR* gene, located on the short arm of chromosome 1 (1p36.3), presents 3 common polymorphisms involving nucleotides C677T, A1298C, and G1793A. The change of C for T at position 677 causes the substitution of alanine for valine in the MTHFR protein and the consequent reduction

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in enzyme activity. The specific activity of the MTHFR enzyme is reduced by 35% in the presence of heterozygosis, genotype C/T, compared to the normal genotype C/C, and by 70% in homozygosis, genotype T/T. A reduction in the enzyme activity that is more effective when in homozygosis considering the A1298C and G1793A polymorphisms.[7,10] *MTR* is polymorphic at nucleotide 2756 A/G and has been associated with decreased plasma Hcy levels.[11] *MTRR* is polymorphic at nucleotide 66 A/G and the variant has a lower affinity for MTR[12] and is inconsistently associated with Hcy level.[7,10]

Besides, Wu et al[13] examined methylation patterns of the promoter of *MTHFR* in sperm DNA obtained from 94 idiopathic infertile men and 54 normal fertile controls. Overall, 45% (41 of 94) of idiopathic infertile males had *MTHFR* hypermethylation, compared with 15% of fertile controls ($P < .05$). The authors concluded that hypermethylation of the promoter of *MTHFR* gene in sperms is associated with idiopathic male infertility.

Thus, the objective of the present study was to determine the distribution of the polymorphisms in folate-related enzyme genes (*MTHFR* C677T, A1298C, and G1793A; *MTRR* A66G; and *MTR* A2756G) in idiopathic infertile Brazilian patients and controls to explore the possible association of these polymorphisms to idiopathic male infertility.

Materials and Methods

Patients

Among the patients of the Andrology Outpatient Clinic of the Human Reproduction and Genetics Center of Faculdade de Medicina do ABC, 133 idiopathic infertile men were studied (mean age 36.6 $\pm$ 5.6 years). Only idiopathic infertile men with severe oligozoospermia ([SO] n = 78) and nonobstructive azoospermia ([NOA] n = 55), with at least 1 year of infertility were included in this study. Individuals with known causes of infertility including genetic factors (chromosome anomalies, azoospermia factor [AZF] microdeletions), lifestyle factors (eg, smoking, alcoholism, and occupation), clinical factors (varicocele and cryptorchidism) and men whose partner had factors involved in infertility were excluded from this study. To compose the control group, 173 fertile men (mean age 58.9 $\pm$ 3.2 years) who have at least 1 child by direct survey and who lacked any history of requiring assisted reproduction technology, were selected from the Family Planning Outpatient Clinic of the Faculdade de Medicina do ABC.

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 Faculdade de Medicina do ABC (No. 237/2008).

Methods

Semen Analysis. Semen analysis was performed strictly according to the guidelines of World Health Organization (WHO).[14] The diagnosis of azoospermia was made on the basis of 2 semen analyses. Nonobstructive azoospermia was determined after physical examination, sperm analysis (including assessment of sperm volume, pH, and evaluation of fructose concentration), endocrine profile (FSH, follicle-stimulating hormone; LH, luteinizing hormone; testosterone; and androstenedione), ultrasound testicular volume measurement, and seminal vesicle evaluation. Classification of SO was done if the spermatozoa count was less than 5 million/mL, according to WHO criteria.[14]

Genotyping. Peripheral blood was collected from each patient and control in an EDTA-containing tube. Genomic DNA was extracted from lymphocytes of peripheral blood according to standard protocols.[15]

Detection of *MTHFR* C677T (rs1801133), A1298C (rs1801131), and G1793A (rs2274976); *MTRR* A66G (rs1801394); and *MTR* A2756G (rs1805087) polymorphisms was performed using real-time polymerase chain reaction (PCR). TaqMan primers and probes were commercially available and provided by Applied Biosystems (Foster City, California). Assays were performed with TaqMan Universal Master Mix with 50 ng of DNA extract per reaction. Polymerase chain reaction conditions were provided by the manufacturer: 40 cycles of 95°C denaturation (15 seconds) and 60°C anneal/extension (1 minute).

Statistical Analysis

Statistical analyses were carried out using SPSS for Windows 11.0 (SPSS, Inc, Chicago, Illinois). The chi-square was used to compare allele and genotype frequencies between groups and also to calculate Hardy-Weinberg equilibrium. Genetic Power Calculator[16] was used to estimate the statistical power of the results concerning the less significant results and showed 80% of power to detect the genetic effects regarding the association with idiopathic infertility for the allele frequencies and sample size in the present study. The odds ratio (OR) and range with 95% confidence interval (CI) were calculated for the presence of the reference genotype using a logistic regression model. The association between the combined genotypes of *MTHFR* polymorphisms and the risk of idiopathic male infertility were also assessed by the study of haplotypes using Haploview software version 4.1 (http://www.hapmap.org). An association study was used to compare combined genotypes of *MTHFR, MTRR*, and *MTR* polymorphisms in order to investigate whether the presence of more than 1 polymorphism in the same patient increases the odds of idiopathic male infertility. All *P* values were 2-tailed, 95% CIs were calculated, and a *P* value <.05 was considered statistically significant.

Results

The results of statistical analysis of the *MTHFR* C677T, A1298C, and G1793A; *MTRR* A66G; and *MTR* A2756G polymorphisms for the idiopathic infertile male and controls were

Table 1. Statistical Analysis of the *MTHFR* C677T, A1298C, and G1793A; *MTRR* A66G; and *MTR* A2756G Polymorphisms for the Brazilian Idiopathic Infertile Men and Controls

			Cases				Controls					
			NOA		SO				*P* Value		Odds Ratio (95% CI)	
Gene	SNP	Genotype	n	%	n	%	n	%	NOA	SO	NOA	SO
MTHFR	C677T	CC	35	63.6	31	39.7	136	78.6				
		CT	14	25.5	37	47.4	27	15.6				
		TT	6	10.9	10	12.8	10	5.8	.080	<.001		
		TT + CT	20	36.4	47	60.2	37	21.4	.039	<.001	2.10 (1.09-4.06)	5.57 (3.12-9.96)
		T frequency	26	26.6	57	36.5	47	13.6	.018	<.001	1.97 (1.15-3.37)	3.66 (2.34-5.73)
	A1298C	AA	14	25.5	16	20.5	18	10.4				
		AC	31	56.4	36	46.2	121	70.0				
		CC	10	18.2	26	33.3	34	19.6	<.001	<.001		
		CC + AC	41	74.5	62	79.5	155	89.6	.01	.049	0.34 (0.16-0.74)	0.45 (0.22-0.94)
		C frequency	51	46.4	88	56.4	189	54.6	.161	.783	0.72 (0.47-.1.10)	1.08 (0.73-1.57)
	G1793A	GG	51	92.7	75	96.2	161	93.0				
		GA	3	5.5	3	3.8	12	7.0				
		AA	1	1.8	0	0.0	0	0.0	.193	.914		
		AA + GA	4	7.3	3	3.8	12	6.9	.827	.504	1.05 (0.33-3.41)	0.54 (0.15-1.96)
		A frequency	5	4.5	0	0.0	12	3.5	.817	.510	1.33 (0.46-3.85)	0.55 (0.15-1.96)
MTRR	A66G	AA	17	30.9	20	25.6	59	34.1				
		AG	26	47.3	36	46.2	84	48.6				
		GG	12	21.8	22	28.2	30	17.3	.742	.114		
		GG + AG	38	69.1	58	74.3	114	65.9	.784	.234	1.16 (0.60-2.22)	1.50 (0.83-2.73)
		G frequency	50	45.5	80	51.3	144	41.6	.549	.055	1.17 (0.76-1.80)	1.48 (1.01-2.16)
MTR	A2756G	AA	31	56.4	47	60.3	109	63.0				
		AG	9	16.4	14	17.9	47	27.2				
		GG	15	27.3	17	21.8	17	9.8	.003	.022		
		GG + AG	24	43.6	31	39.7	64	37.0	.470	.783	1.32 (0.71-2.44)	1.12 (0.65-1.94)
		G frequency	39	35.5	48	30.8	81	23.4	.017	.102	1.80 (1.13-2.86)	1.45 (0.95-2.22)

Abbreviations: SNP, single-nucleotide polymorphism; NOA, nonobstructive azoospermia; SO, severe oligozoospermia; CI, confidence interval; *MTHFR*, methylenetetrahydrofolate reductase; *MTRR*, methionine synthase reductase; *MTR*, methionine synthase.

summarized in Table 1. Information of single-nucleotide polymorphisms (SNPs) and summary of single-marker association analysis, considering Hardy-Weinberg equilibrium and minor allelic frequencies, in NOA and SO groups are shown in Table 2.

Single-marker analysis revealed a significant association among *MTHFR* C677T polymorphism and both NOA ($P = .018$, OR = 1.97, 95% CI = 1.15-3.37) and SO groups ($P < .001$, OR = 3.66, 95% CI = 2.34-5.73). Considering the *MTHFR* A1298C polymorphism, no difference was found in NOA group ($P = .161$, OR = 0.72, 95% CI = 0.47-1.10) or in SO group ($P = .783$, OR = 1.08, 95% CI = 0.73-1.57). Similar results were found in *MTHFR* G1793A polymorphism. No difference was found between NOA group and controls ($P = .817$, OR = 1.33, 95% CI = 0.46-3.85) or between SO group and controls ($P = .510$, OR = 0.55, 95% CI = 0.15-1.96).

Regarding the *MTRR* A66G polymorphism no association was found between NOA and SO groups compared to controls, $P = .549$, OR = 1.17, 95% CI = 0.76-1.80 and $P = .055$, OR = 1.48, 95% CI = 1.01-2.16, respectively.

Considering the *MTR* A2756G polymorphism, a significant difference was found between NOA group and controls ($P = .017$, OR = 1.80, 95% CI = 1.13-2.86). However, statistical analysis revealed no association between SO group and controls ($P = .102$, OR = 1.45, 95% CI = 0.95-2.22).

Haplotype analysis of 3 *MTHFR* polymorphisms, C677T, A1298C, and G1793A, did not identify a haplotype associated with idiopathic infertility; even when they were separated in NOA or SO group.

The combinatory analysis of the 3 *MTHFR*, *MTRR*, and *MTR* polymorphisms to NOA, SO, and control groups showed no statistical difference to any combination.

Discussion

In the present study, we concomitantly evaluated the association of polymorphisms in *MTHFR* (C677T, A1298C, and G1793A), *MTRR* (A66G), and *MTR* (A2756G) genes involved in folate metabolism and their possible risk of infertility. The presence of allele T of the *MTHFR* C677T polymorphism seems to be associated with both NOA and SO groups, whereas the allele G of the *MTR* A2756G was associated only with NOA group. The *MTHFR* A1298C, *MTHFR* G1793A, and *MTRR* A66G polymorphisms were not associated with idiopathic male infertility. Statistical analysis did not show a haplotype associated with idiopathic male infertility. Our findings

Table 2. Information of SNPs and Summary of Single-Marker Association Analysis in Nonobstructive Azoospermia and Severe Oligozoospermia Group

Population	SNP	HWE	MAF	Alleles	*P* Value
NOA	rs1801131	<0.001	0.474	C:A	.603
	rs1801133	<0.001	0.16	C:T	.130
	rs2274976	0.5324	0.037	C:T	.012
SO	rs1801131	<0.001	0.448	C:A	.709
	rs1801133	0.0012	0.207	C:T	1.0
	rs2274976	1.0	0.030	C:T	.356

Abbreviations: SNP, single nucleotide polymorphism; HWE, Hardy-Weinberg equilibrium; MAF, minor allelic frequencies; NOA, nonobstructive azoospermia; SO, severe oligozoospermia.

demonstrate relevance of *MTHFR* C677T and *MTR* 2756G polymorphisms of folate metabolism in susceptibility to idiopathic infertility among Brazilian male population.

Some authors have supposed the association of folate metabolism genes polymorphisms and infertility. Recently, Lee et al[17] evaluated polymorphisms of 3 folate metabolism enzymes (*MTHFR* C677T and A1298C, *MTR* A2756G, and *MTRR* A66G) and the association of nonobstructive Korean male infertility. The results showed genetic evidence that *MTHFR* C677T, *MTR* A2756G, and *MTRR* A66G genotypes were independently associated with male infertility. On the other hand, Ravel et al[18] performed an association study among 3 variants in *MTHFR* (G203A, C665T, and A1286C), 2 variants in *MTRR* (A66G and C524T), a mutation in the *CBS* gene (G919A), and reduced sperm counts in infertile individuals of French ethnic origin. The authors failed to detect an association between any of these variants and unexplained reduced sperm counts leading to male infertility.

Similarly, Montjean et al[19] investigated the relationship between *MTHFR* (A1286C, C665T, and G203A) and *MTRR* (A66G and C524T) genetic variants with respect to both blood plasma Hcy concentration and sperm counts in a mixed ethnic origin men from French origin. No association was found between *MTHFR* and *MTRR* genetic variants and sperm counts. Nevertheless, Safarinejad et al[20] determined the associations among 3 *MTHFR* gene polymorphisms (C677T, A1298C, and G1793A), serum folate, and total Hcy levels, with male fertility status and semen parameters in Iranian population. The data showed a significant difference in genotype frequency distribution of *MTHFR* C677T polymorphism between infertile patients and controls. The 677T allele carriers had a significantly increased risk of infertility compared with the CC homozygotes.

One possible explanation to reconcile the conflicting data between studies could be ethnic and geographic variation in the distribution of the polymorphisms in folate-related enzyme genes. Moreover gene-nutrient/environmental and gene racial/ethnic interactions have been shown to affect the impact of these genetic variants.[12] Besides, Chan et al[21] investigated the effects of MTHFR deficiency on early germ cell development in both BALB/c mice and C57BL/6 mice and assessed whether MTHFR deficiency results in DNA methylation abnormalities in sperm. The authors found different reproductive phenotype between the 2 strains: BALB/c mice showed an early postnatal loss of germ cell number and proliferation resulting in infertile BALB/c MTHFR-deficient mice with hypo- and hypermethylation in the sperm of the imprinted genes, whereas the C57BL/6 mice had decreased sperm numbers and altered testicular histology but showed normal fertility and unaffected imprinting pattern. The findings also may help to explain population differences in infertility among men with common *MTHFR* polymorphisms.

In humans, spermatozoa generate reactive oxygen species (ROS) which are known to affect hyperactivation of spermatozoa, the acrosome reaction, and the attachment of spermatozoa to oocytes thereby contributing to the fertilization of oocytes.[22–24] Thiols, like Hcy, are scavenging ROS and are, therefore, suggested to be important in sperm function and fertilization as well.[23] The DNA in the spermatozoa head is intensely compacted as a result of disulfide bridges between oxidized Hcy residues in protamine molecules, which are important during the maturation of spermatozoa in the epididymis. The oxidation of thiols is also important for the stabilization of the tail structure, sperm motility, and the protection of sperm DNA against physical or chemical damage. After fertilization of the oocyte, the compacted sperm nucleus is decondensed to form the male pronucleus (PN). The decondensation depends on the presence of a small amount of free Hcy capable of initiating a thiol-disulfide exchange.[25]

Besides the beneficial effects of ROS, an excess of ROS is detrimental to spermatozoa and leads to damage of the DNA and plasma membrane through lipid peroxidation.[26] Because spermatozoa have discarded most of their cytoplasm during the final stages of spermatogenesis, the availability of cytoplasmic defensive enzymes is limited and, therefore, these cells in particular are susceptible to ROS.[27,28] Increased lipid peroxidation of spermatozoa plasma membranes may lead to altered membrane fluidity, which can render sperm dysfunctional through impaired metabolism, acrosome reaction reactivity, and ability of the spermatozoa to fuse with the oocyte.[27] This may result in abnormal sperm concentrations, loss of motility, and abnormal morphology of the spermatozoa, leading to loss of fertility.[29,30]

A major limitation of our study is that levels of folate and Hcy in men with idiopathic infertility were not measured. However, Brazilian studies showed that allele T of *MTHFR* C677T polymorphism is relatively common in Brazilian population, and it was previously associated with folate and Hcy levels. Aléssio et al[31] determined transcobalamin II (*TCII*) C776G polymorphism, Hcy, folate, and vitamin B12 levels and analyzed the interactive effect with the *MTHFR* (C677T and A1298C) and *MTRR* (A66G) polymorphisms in 207 healthy Brazilian children (age range of 1-8 years, median age 4.8 years). Folate levels were significantly decreased in the carriers of the *TCII* 776CG and *MTHFR* 677CT genotypes. A multivariate analysis was performed considering Hcy levels as variable response using a linear regression model and demonstrated that the *TCII* C776G (CC/CG vs GG), *MTHFR* C677T (CC/CT

vs TT), folate, gender, and age presented statistical significance in the Hcy level ($P = .0005$).

Additionally, Tavares et al[32] studied 90 Brazilian Parkatêjê Indians (aged ≥20 years). Rates of mutated allele 677T and TT genotype were 40.7% and 14.0%, respectively. Total Hcy was higher among TT genotype ($P < .001$). The multiple linear regression model, containing variables for sex, folic acid, TT genotype, and triglycerides, explained 50.0% of the variation in Hcy.

In summary, the findings suggest the *MTHFR* C677T and *MTR* A2756G polymorphisms could be an important genetic factor predisposing to idiopathic infertility in Brazilian men.

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Declaration of Conflicting Interests

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