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COMBINED EFFECT OF GSTM1 GENE DELETION, GSTT1 GENE DELETION AND MTHFR C677T MUTATION IN MALE INFERTILITY

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Abstract - The aim of the study was to investigate the association between the GSTM1 and GSTT1 gene deletion and MTHFR C677T mutation and male infertility. The study has encompassed 52 infertile and 56 fertile males. Genotype distributions of GSTM1 and GSTT1 gene deletions and the MTHFR C677T mutation did not differ significantly among the analyzed groups, however, a difference in distribution of certain genotype combinations was observed. The obtained results indicate that carriers of double GSTM1/GSTT1 deletion and the MTHFR 677CC genotype are at higher risk of infertility than carriers of any other combination of genotypes (OR 3.5, 95%CI 0.68-18.30).

Keywords: Gene mutation, GSTM1, GSTT1, male infertility, MTHFR

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INTRODUCTION

Male infertility is a heterogeneous disorder, with various genetic and environmental factors that contribute to the impairment of spermatogenesis. In spite of the attention that has been given to the research on mechanisms underlying male infertility, molecular events important for the onset and development of this disorder are still poorly understood. The best characterized genetic causes of male infertility still remain microdeletions in the AZF (azoospermia factors) region of the Y chromosome and some mutations in the CFTR gene (Sertic et al., 2001, Poongothai et al., 2009). Over the past couple of decades molecular genetics has discovered a large number of genes for which it has been proposed that, if mutated or deleted, can cause pathological changes in male reproductive system (Vogt et al, 2004, Tüttelmann et al., 2007).

One of the most important mechanisms involved in the homeostasis of male reproductive tissue is protection from oxidative stress, which male germ cells are highly susceptible to (Rao and Shaha, 2000, Schuppe et al., 2000, Baker and Aitken, 2004). Glutathione S-transferases (GSTs) constitute a superfamily of ubiquitous, multifunctional enzymes which play a key role in phase II cellular detoxification and are generally considered to be "antioxidant" enzymes (Rushmore and Pickett, 1993). Several epidemiological studies have reported that the Glutathione S-transferases M1 and T1 null genotypes (GSTM1 and GSTT1) that result in a lack of functional protein are correlated with an increased susceptibility to diseases associated with oxidative stress (Parl, 2005, Bolt and Their, 2006, Bohanec et al., 2009). The possible role in male infertility has been already suggested for these two gene variants, but published data are inconsistent (Chen et al., 2002, Aydos SE et al., 2009, Aydemir et al., 2007).

The metabolism of folate is essential for proper cellular function and is also an important part of reproductive tissue homeostasis. Within the folate pathway, one of the most important enzymes is methylenetetrahydrofolate reductase (MTHFR). C677T mutation in the MTHFR gene affects the

functionality of the MTHFR protein, subsequently leading to hyperhomocysteinemia. It has been shown that severe MTHFR deficiency in male mice results in abnormal spermatogenesis and infertility (Kelly et al., 2005). These findings, in conjunction with clinical evidence, suggest an important role for MTHFR in spermatogenesis. However, the role of MTHFR C677T as a genetic risk factor for infertility remains unclear (Park et al., 2005, Stuppia et al., 2003, Bezold et al., 2001, Ravel et al., 2009).

Compound genetic factors as a cause of male infertility were first observed in isolated cases (Black et al., 2000). The xenobiotic metabolism pathways in which MTHFR and glutathione S-transferases are involved are closely functionally related and mutations in the genes encoding these enzymes were suggested to have a combined effect.

The aim of the current study was to evaluate the association between the GSTM1 and GSTT1 deletion and MTHFR C677T mutation and male infertility.

PATIENTS AND METHODS

Subjects

This study has encompassed 52 unrelated men presented with infertility associated with reduced or absent sperm counts. Written informed consent was obtained from all study patients and the investigation was approved by the hospital's ethical committee. A spermogram was performed in order to determine the sperm density, and the diagnosis of azoospermia and severe oligozoospermia (<1x106 spermatozoa/mL) was made according to WHO guidelines (WHO, 1999). Previously performed cytogenetic analysis has shown normal karyotypes in all patients. The control group consisted of 56 age-matched men who had fathered at least one child which was confirmed through paternity testing.

Genetic Analysis

Genomic DNA was isolated from blood taken with sodium citrate by GFX Genomic Blood Purification Kit (GE Healthcare, Fairfield, USA) according to the manufacturer's instructions.

Detection of GSTM1 and GSTT1 gene deletions was performed by the multiplex PCR method (Arand et al, 1996). The following primer pairs were used: 5'-GAACTCCCTGAAAAGCTAAAGC-3' and 5'-GTTGGGCTCAAATATACGGTGG-3' for GSTM1 and 5'-TTCCTTACTGGTCCTCACATCTC-3' and 5'-CAGCTGCATTTGGAAGTGCTC-3' for GSTT1. Part of the albumin gene was used as an internal amplification control and was amplified with the following primers: 5'-CAACTTCATCCACGTTCA CC-3' and 5'-GAAGAGCCAAGGACAGGTAC-3'. The amplification was performed in the following mixture (total volume 30 μL): 50 mM Tris-HCl pH 9, 0.1% Triton X-100, 5 mM $MgCl_2$, 0.2 mM each dNTP (dATP, dTTP, dGTP and dCTP), 10 pmol of each primer for GSTM1 and albumin, 12 pmol of each primer for GSTT1 and 1 U of Taq polymerase (Applied Biosystems, Foster City, USA) with approximately 800 ng of isolated DNA. The reaction mixtures were first subjected to initial denaturation at 94° C for 10 min; 35 cycles consisting of denaturation at 94° C for 1 min, primer annealing at 58° C for 30 s and DNA extension at 72° C for 45 s; the final DNA extension was at 72° C for 10 min. The PCR reactions were performed in a Mastercycler gradient apparatus (Eppendorf). The products of the multiplex PCR (GSTM1-215bp, GSTT1-480 bp and albumine-350 bp) were separated by horizontal electrophoresis on 2% agarose gels and visualized by ethidium-bromide. Both GSTM1 and GSTT1 products were categorized as having either a non-null or null (homozygous deletion) genotype.

Detection of the MTHFR C677T mutation was performed by PCR-RFLP analysis (Djordjevic et al, 2005). We used the following primer pairs: 5'- TGA AGG AGA AGG TGT TG CGG GA -3' and 5'-AGG ACG GTG CGG TGA GAG TG -3' (Frost et al, 1995). The amplification was performed in a mixture (25 μL final volume) containing 50 mM KCl, 100mM Tris-HCl, pH 9.0, 0.1% Triton X-100, each dNTP (dATP, dTTP, dGTP and dCTP), 5 mM $MgCl_2$, 10 pmol of forward and reverse primers and 1 μL of whole blood. The thermal cycle profile was

Table 1. Distribution of MTHFR, GSTM1 and GSTT1 genotypes in patients and controls

MTHFR C677T	GSTM1/ GSTT1	No. of patients	%	No. of controls	%	OR	p	
N / N	+ / +	8	15.4	10	17.7	0.8	0.80	0.60-2.31
	+ / -	1	1.9	3	5.4	0.3	0.62	0.04-3.44
	- / +	7	13.5	8	14.4	0.9	1.00	0.31-2.78
	- / -	6	11.5	2	3.6	3.5	0.15	0.68-18.30
M / N	+ / +	5	9.6	10	17.9	0.4	0.27	0.16-1.54
	+ / -	5	9.6	4	7.1	1.4	0.74	0.35-5.46
	- / +	9	17.3	9	16.1	1.1	1.00	0.40-3.01
	- / -	5	9.6	3	5.4	1.9	0.48	0.43-8.29
M / M	+ / +	2	3.8	0			0.23	
	+ / -	2	3.8	2	3.6	1.1	1.00	0.15-7.96
	- / +	1	1.9	4	7.1	0.3	0.36	0.03-2.36
	- / -	1	1.9	1	1.8	1.4	1.00	0.07-17.70

N-wild type allele, **M**-mutant allele, **No**-number, **-**null, +present

5 cycles (98°C for 3 min and 55°C for 3 min), to assure complete denaturation of the DNA. One unit Taq polymerase (AppliedBiosystems, Foster City, USA) was added at 50°C and after 5 min, 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 61°C for 30 s and polymerization at 72°C for 30 s were applied. The final extension of PCR products was performed at 72°C for 10 min. The PCR reactions were performed in a Mastercycler gradient apparatus (Eppendorf). Polymorphic 677C and 677T alleles were identified by restriction enzyme cleavage of the PCR product using the restriction enzyme HinfI (New England, BioLabs). Digestion products were analyzed by electrophoresis on a 10% polyacrylamide gel, followed by silver staining of the gels.

Statistical analysis

Statistical analysis was performed using SPSS 10.0. For the calculated frequencies of each allele, genotype and combination of genotypes two statistical parameters were determined – p value and OR value, with 95%CI. Values $p \leq 0.05$ were considered statistically significant.

RESULTS

Detection of GSTM1 deletion, GSTT1 deletion and MTHFR C677T mutation was performed in 52 infertile men and 56 fertile men as control group.

Deletion of the GSTM1 gene was detected in 29 patients (56%) and in 27 controls (48%), while the GSTT1 gene was deleted in 20 patients (38%) and in 15 controls (27%). (Table 1). Both deletions were present in 12 patients (23%) and in 6 controls (11%).

Mutation MTHFR C677T was present with a frequency of 34.6% (36 out of 104 analyzed chromosomes) in infertile men and a frequency of 35.7% (40 out of 112 analyzed chromosomes) in controls. Among the 52 patients, 22 were homozygous for normal allele (42%), 24 were heterozygous (46%) and 6 were homozygous for the mutated allele (12%). Among the 56 controls, 23 were homozygous for the normal allele (41%), 26 were heterozygous (46%) and 7 were homozygous for the mutated allele (13%) (Table 1).

Regarding the presence of combined genotypes, deletion of the GSTM1 gene was present in 2 (3.8%) carriers of the MTHFR 677TT genotype in patients, and in 5 individuals (8.9%) in the control group. In combination with the 677CT genotype, this deletion was present in 14 (26.9%) patients and 12 (21.4%) control individuals. A wild-type MTHFR genotype and GSTM1 gene deletion were found in 13 (25%) patients and 10 (17.8%) subjects in the control group. The GSTT1deletion/MTHFR677TT haplotype was present in three patients (5.8%) *vs.* three controls (5.4%); the GSTT1deletion/MTHFR677CT haplotype was observed in 10 patients (19.3%) *vs.* 7 controls (12.5%), and the GSTT1deletion/MTHFR677CC haplotype in 7 patients (13.5%) *vs.* 5 control subjects (8.9%). Among 12 patient carriers of both GST deletions we found in 1.9% of MTHFR677TT, 9.6% of MTHFR677CT and 11.5% of MTHFR677CC genotypes. This was the case in 1.8%, 5.4% and 3.6% of control subjects, respectively (Table 1). No significant differences were observed for any genotype combination.

DISCUSSION

Male infertility is a heterogeneous disorder, with various genetic and environmental factors contributing to the impairment of spermatogenesis. In this study we analyzed three different genes (GSTM1, GSTT1 and MTHFR) with important roles in pathways of reactive oxygene species. We compared the distributions of GSTM1 and GSTT1 gene deletion and MTHFR C677T mutation among 52 men diagnosed with a severe spermatogenic defect and 56 controls whose fertility was confirmed through paternity testing.

Deletions of GSTM1 and GSTT1 genes probably cause an imbalance of oxidants and antioxidants in the reproductive tissue that could play a role in the etiology of male infertility. In our patients, the prevalence of GSTM1 and GSTT1 deletions did not considerably differ when compared to the control group (15.4% and 32.7% vs.16.1% and 37.5%, respectively). Increased risk of infertility was observed in carriers of both GST deletions (OR value 2.5) in comparison to carriers of GSTM1 (OR value 0.8) and GSTT1 deletion (OR value 0.9). Although the double GSTM1/GSTT1 deletion was two times higher in patients than in the controls (23% *vs.* 11%), this difference was not statistically significant (p=0.12). Our results are in agreement with the study of Schuppe who observed the GSTM1/T1 deletion as the most frequent genotype in individuals with an abnormal spermogram (Schuppe et al., 2000). On the other hand, results obtained in the study of Polonikov reported the association of normal GSTT1 gene genotype with the increased risk of idiopathic male infertility (Polonikov et al., 2010).

The involvement of MTHFR in spermatogenesis is believed to be through the regulation of DNA methylation (Friso et al., 2002, Khazamipour et al., 2009). It also affects the oxidative status of the reproductive tissue, since homocysteine can mediate DNA damage in spermatozoa (Forges et al., 2007). In our study, the distribution of the MTHFR C677T polymorphism among patients and controls was similar (Table 1). This is consistent with previous findings that do not support an association between the MTHFR 677T allele and male infertility (Stuppia et al., 2003, Ravel et al., 2009). However, meta-analyses of Tüttelmann and coworkers revealed significant associations between C677T polymorphism and male infertility (OR 1.39, 95%CI 1.15-2.69, P=0.0006) (Tüttelmann et al., 2007).

A recent study which has indicated the interaction between MTHFR and GSTM1 showed that subjects who are carriers of a 677CT or 677TT genotype of the MTHFR gene and who carry a functional copy of GSTM1 have a significantly higher risk of infertility than subjects who carry the wild-type genotype for both genes (Paracchini et al., 2006). We also found that interaction between MTHFR and GSTs played a role in the etiology of infertility, but in the opposite way. We observed the highest risk of infertility (OR=3.5, CI95% 0.68-18.29) in patients homozygous for MTHFR normal allele (677CC genotype) who at the same time carried both GSTM1 and GSTT1 deletions. In addition, the risk was lower in the presence of one mutated MTHFR allele (OR=1.9, CI95% 0.43-8.29).

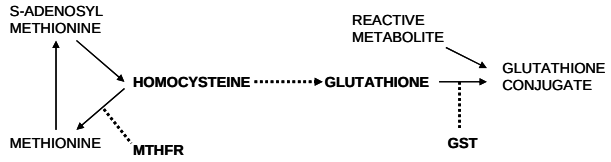

Fig. 1. Schematic representation of metabolic pathways involving GST and MTHFR

The possible explanation for the combined effect of mutations in GST genes and the MTHFR gene could be that the enzymes encoded by these genes are closely related in the metabolic pathways involved in protection from oxidative stress (Figure 1). Homocysteine can serve not only as a precursor of S-adenosylmethionine (SAM) in the methylation pathway, but also as a precursor of glutathione required for various detoxification processes (Hoey et al., 2009).

It has been speculated that in mutant MTHFR C677T homozygotes who carry the GSTM1 deletion, higher glutathione levels that have a positive effect on SAM synthetase activity could lead to an increase in methylation, thus compensating for the lack of methylation due to the decrease in methionine synthesis (Castagna et al., 1995, Paracchini et al., 2005). Our findings that the homozygote carriers of mutant MTHFR C677T and double GSTM1/GSTT1 deletion are not at risk of infertility could be explained by this mechanism. In such a combination of genotypes, excessive homocysteine levels could induce the production of higher amounts of glutathione, which can compensate for the absence of the GSTM1 and GSTT1 detoxification function by taking part in other detoxification processes in cell.

To summarize, our study has shown that the combination of GSTM1 and GSTT1 deletion and MTHFR C677T could represent a complex genetic risk factor predisposing male infertility. We speculate that the GSTM1/GSTT1 gene deletion represents an initial risk factor. On the other hand, the MTHFR C677T mutation can act as a protective factor if GSTM1 and GSTT1 are deleted. Although the results of the present study are preliminary and should be verified in larger groups of infertile males, they point to the possible role of the combined effect of mutations in GSTM1, GSTT1 and MTHFR genes in the etiology of male infertility.

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