Association of methionine synthase *A2756G* SNP, methionine synthase reductase *A66G* and male infertility

Asocierea între mutația *A2756G* a methionin sintazei, *A66G* a methionin sintaz reductazei şi infertilitatea masculină

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Abstract

The folate metabolic pathway consists of an important chain of reactions which lead to nucleic acid production and methylation of various substrates. Within this pathway MS, and MTRR, the enzyme which is responsible for the regeneration of MS, catalyzes the conversion of homocysteine to methionine. The MS A2756G and MTRR A66G variants are associated with reduced enzyme activity, increased homocysteine and reduced availability of SAM. We analyzed the distribution of these single nucleotide polymorphisms in the two genes in a case group of 65 infertile Romanian patients with idiopathic azoospermia or severe oligozoospermia and a control group of 67 Romanian men, to explore the possible association of the MS A2756G and MTRR A66G polymorphisms and male infertility. Using the polymerase chain reaction - restriction fragment length polymorphism technique (PCR-RFLP), the allele and genotype distribution of the two SNPs were investigated in both patients and controls. The frequencies of these polymorphisms in infertile patients were not significantly higher than those in controls. Our findings suggest that there is no significant association of SNP A2756G in the MS gene or SNP A66G in the MTRR gene with azoospermia or severe oligozoospermia, indicating that these polymorphisms would not be genetic risk factors for male infertility in our Romanian population group.

Key words: *infertility, homocysteine, folate, azoospermia, methylation*

Rezumat

Calea metabolica a folatilor este compusa dintr-un lant important de reactii care contribuie la biosinteza acizilor nucleici si la metilarea diferitelor substraturi. In cadrul acestei cai MS, si MTRR, enzima responsabila pentru regenerarea MS, catalizeaza conversia homocisteinei in metionina. Variantele polimorfice MS A2756G si MTRR A66G sunt asociate cu activitate enzimatica redusa, nivel crescut al homocisteinei si disponibilitate redusa a SAM.

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Am analizat distributia acestor polimorfisme in cele doua gene intr-un lot de 65 de pacienti romani infertili cu azoospermie idiopatica sau oligozoospermie severa si un lot control de 67 de barbati romani fertili, pentru a explora posibila asociere a polimorfismelor MS A2756G si MTRR A66G cu infertilitatea masculina. Utilizand tehnica polymerase chain reaction – restriction fragment lenght polymorphism (PCR-RFLP), distributia alelica si a genotipurilor a celor doua polimorfisme a fost investigata in ambele loturi. Frecventele acestor polimorfisme in lotul de pacienti infertili nu au fost semnificativ mai mari decat cele din lotul de control. Rezultatele noastre sugereaza faptul ca nu exista o asociere semnificativa a SNP A1756G in gena MS sau SNP A66G in gena MTRR cu azoospermia idiopatica sau oligozoospermia severa, indicand astfel ca cele doua polimorfisme nu sunt factori de risc genetici pentru infertilitatea masculina in lotul de barbati romani studiat.

Cuvinte cheie: *infertilitate, homocisteina, folat, azoospermie, metilare*

Introduction

The World Health Organization declared couple infertility a global health problem and acknowledged that approximately one couple in seven is affected by fertility or subfertility problems (1). Male infertility in humans has been declared as the cause of couple's inability to have children in 20-50% of total cases (2) and a significant proportion of infertile men are affected either by oligozoospermia (reduced sperm count) or azoospermia (lack of any sperm in the ejaculate).

There are several most common known non-genetic causes of male infertility such as: hypogonadism, testicular maldescence, structural abnormalities of the male genital tract, genital infections, previous scrotal or inguinal surgery, varicoceles, chronic illness, medication and exposure to chemicals. However in about 50% of cases infertility could not be attributed to any known cause (3), hence the idea that a high number of idiopathic male infertility cases could be attributed to genetic factors. Genetic abnormalities were identified in men with unexplained oligozoospermia and azoospemia, including numerical and structural chromosomal abnormalities (4, 5), deletions of the azoospermia factor region (AZF) of the Y chromosome or translocations between the Y chromosome and other chromosomes (3, 6, 7), mutations in the cystic fibrosis conductance regulator (*CFTR*) gene, commonly associated with congenital vas deferens abnormalities (8, 9) and also other genetic factors (10). It was observed that some abnormalities associated with infertility such as reciprocal and Robertsonian translocations and *CFTR* mutations (11) are inherited, while the majority of numerical chromosome abnormalities and AZF deletions are de novo events in the parental germ cells.

Folates are a group of inter-convertible co-enzymes, differing by their oxidation state, number of glutamic acid moieties and one carbon substitutions. They are involved in amino acid metabolism, purine and pyrimidine synthesis and methylation of a large number of proteins, lipids, and nucleic acids as well. The relation between folate metabolism and the methionine/homocysteine pathway is particularly important. Homocysteine, a sulfhydryl-containing amino acid that is not used in protein synthesis, originates exclusively from the one-carbon metabolism of methionine and it is remethylated into methionine with folates acting as methyl donors (12). In the last decade increased plasmatic levels of homocysteine have been found to be associated with an increased risk for several diseases, such as atherosclerotic, thromboembolic and neurodegenerative disorders, and also with early pathological events of life (13, 14). The latter category of disorders includes the following: neural tube defects, late pregnancy complications such as pre-eclampsia, abruptio placentae, intrauterine growth retardation, preterm birth and intrauterine fetal death (15-19). However, despite the recent progress in understanding the physiopathology of hyper-

homocysteinemia-induced health events, there is little information on the role of folates/homocysteine on male reproduction.

Within the folate metabolic pathway methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase) catalyzes the remethylation of homocysteine to methionine in a reaction in which methylcobalamin serves as an intermediate methyl carrier. This occurs by transfer of the methyl group of 5-methyltetrahydrofolate to the enzyme-bound cob(I)alamin to form methylcobalamin with the subsequent transfer of the methyl group to homocysteine to form methionine. Over time, cob(I)alamin may become oxidized to cob(II)alamin rendering the enzyme inactive. Regeneration of the functional enzyme requires a process of reductive methylation performed by MTRR (methionine synthase reductase) in which S-adenosyl methionine is used as a methyl donor. Wilson et al. (20) identified the *MTRR A66G* SNP, a missense mutation resulting in an amino-acid substitution of an isoleucine to a methionine residue at codone 22. Several studies identified this polymorphism as a risk factor for high plasma homocysteine levels and the development of vascular disease, spina bifida and Down syndrome (20-22), while the *MTR A2756G* SNP was identified by Leclerc et al. (23) and consists of an aspartic acid to glycine residue conversion. This polymorphism was associated with spina bifida (24), Down syndrome (22) and orofacial clefts (25). Both mutations by affecting the activity of the corresponding enzymes could lead to elevated levels of plasma homocysteine.

Materials and methods

In order to determine if there is an association between the *MS A2756G* SNP or *MTRR A66G* SNP and male infertility we conducted a case-control study comprising a total number of 132 men. The case group (group number 1) consisted of 65 men suffering from idiopathic azoospermia (53 cases) or severe oligozoospermia (12 cases), while the control group (group number 2) consisted of 67 healthy men who had at least one child. In order to exclude other conditions that would bias our study, patients from the first group with a history of congenital abnormalities, varicocele, undescended testes or urogenital infections were excluded after investigations performed by a specialist. Also, after performing chromosomal and molecular analysis patients with chromosomal abnormalities and microdeletions in the AZF region of the Y chromosome were excluded from the first study group of cases.

The study was conducted in accordance with The World Medical Association Declaration of Helsinki statements and written informed consent was obtained from each participant.

For genetic testing, 3ml of peripheral blood was extracted on EDTA to prevent blood clotting. Genomic DNA was extracted from blood leucocytes contained in a volume of 300µl using a commercially available extraction kit (Wizzard Genomic DNA Purification Kit, Promega®). For genotyping we used the PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) technique as previously by Leclerc et al. (23) for the *MS A2756G* SNP and Wilson et al. (20) for the *MTRR A66G* SNP, with both protocols being performed with some modifications. For both polymorphisms the PCR reaction was set-up in a volume of 25µl reaction mix containing: 12.5 2xPCR Master Mix - Taq DNA-polymerase 0.05U/µl, MgCl2 4mM, dNTPmix 0.4mM each (Fermentas MBI®, Vilnius, Lithuania); 10p-moles of each forward and reverse primers (Eurogentec®, Seraing , Belgium), approximately 50-100ng genomic DNA; and nuclease-free water to complete the 25µl volume. For the *MS A2756G* SNP the conditions for amplification were: an initial denaturation at 95°C for 5 min, followed by 35 cycles consisting of: 50 s denaturation at 94°C, 50 s annealing at 52°C and 1 min elongation at 72°C, and a final elong-

Table I. *MS A2756G* and *MTRR A66G* genotypes and alleles frequencies

Genotypes MS	Group 1 (cases) n (%)	Group 2 (controls) n (%)	OR (95%CI)	p value
Total no. of subjects	65 (100)	67 (100)		
AA	40 (61.5)	42 (62,7)		
AG	23 (35.4)	24 (35.8)	1.006 (0.491-2.062)	1
GG	2 (3)	1 (1.5)	2.1 (0.1831-24.089)	0.616
AG+GG	25 (38.4)	25 (37.3)	1.05 (0.5195-2.122)	1
Allele MS	**Allele frequencies**			
Total no. of alleles	130	134		
A allele	0.39	0.41		
G allele	0.102	0.098	1.089 (0.5961-1.989)	0.878

Genotypes MTRR	Group 1 (cases) n (%)	Group 2 (controls) n (%)	OR (95%CI)	p value
Total no. of subjects	65	67		
AA	13 (20)	18 (26.9)		
AG	46 (70.8)	42 (62.7)	1.516 (0.6631-3.468)	0.4044
GG	6 (9.2)	7 (10.4)	1.187 (0.3224-4.369)	1
AG+GG	52 (80)	49 (73.1)	1.469 (0.6515-3.314)	0.4139
Allele MTRR	**Allele frequencies**			
Total no. of alleles	130	134		
A allele	0.273	0.295		
G allele	0.22	0.212		

ation of 5 min at 72°C. The primers used for amplification were: forward primer 5'-CATGCAAGAATATGAAGATATTAGAC-3' and reverse primer 5'-GAACTAGAAGACAGAAATTCTCTA-3'. For the *MTRR A66G* SNP the conditions for the amplification were: and initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of: denaturation at 94°C for 30s, annealing at 55°C for 30s, elongation at 72°C for 30s, and a final elongation at 72°C for 5 min. The primers used were: forward primer 5'-GCAAAGGCCATCGCAGAAGACAT-3' and reverse primer 5'-GTGAAGATCTGCAGAAAATCCATGTA-3'. The reaction was set up on a MastercyclerGradient thermal cycler (Eppendorf®, Hamburg, Germany). A 12.5µl aliquot of the amplicon solution was consequently digested overnight with HaeIII restriction enzyme and NdeI restriction enzyme (Fermentas MBI®, Vilnius, Lithuania) for the *MS A2756G* SNP and *MTRR A66G* SNP, respectively. The digested fragments were resolved in a 3% MetaPhor agarose gel (Lonza®, Basel, Switzerland) stained with ethidium bromide and then visualized on a UV transilluminator (VilberLourmat®, Marne-la-Vallée, France). After enzyme digestion the *MS* A allele presented a 189bp fragment, the *MS* G allele resulted in two fragments of 150 and 39bp, while the *MTRR* A allele resulted in a 66bp fragment and the G allele in two fragments of 44 and 22bp.

The observed alleles and genotypes frequencies were calculated for both groups and the Chi-square test for deviation was performed in order to establish if the genotypes distribution was in accordance with the Hardy-Weinberg equilibrium. A comparison of the results

between the study group and control group was made and the differences were tested for significance through Fischer test using GraphPad InStat version 3 statistical software (GraphPad InStat 3, San Diego, California, USA).

Results

The genotype and allelic frequencies obtained for are presented in table I. The observed genotypes frequencies among the study groups were in agreement with the Hardy-Weinberg equilibrium for the *MS A2756G* SNP ($\chi 2=1.584$, $p=0.208$), while for the *MTRR A66G* the observed genotypes were not consistent with Hardy-Weinberg equilibrium ($\chi 2=16.974$, $p<0.00004$). Hardy-Weinberg could have been violated in our study due to population stratification, although there are other possible factors that could have led to this result. Hardy-Weinberg equilibrium may be violated because of genotyping errors, chance, inbreeding, non-random mating, differential survival of marker carriers, genetic drift, population stratification, or combination of this reasons. Unfortunately, most gene-disease association studies report very limited information to provide any detailed insight into these potential problems. Thus, also at a meta-analysis level, typically there is no way to decipher for which of the above reasons the violation has occurred, except perhaps for population stratification under special conditions (26). Our results, after applying the statistical Fischer test, indicate that the two study groups had a similar genotype and allelic distribution for both polymorphisms; all the p-values obtained were above 0.05, while the odds ratio indicate that the two polymorphism are not risk factors for the studied pathology.

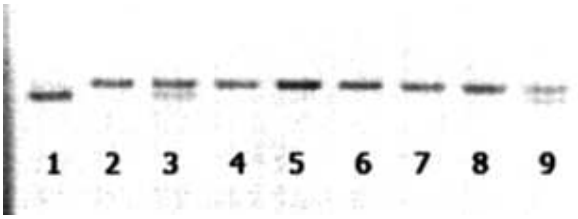

Figure 1. Electrophoresis of *MS A2756G* SNP in 3% MetaPhor agarose
1 - GG homnozygous
3, 9 heterozygous
2, 4, 5, 6, 7, 8 - AA homozygous

Figure 2. Electrophoresis of *MTRR A66G* SNP in 3% MetaPhor agarose
1, 2 - AA homozygous
5, 6, 7, 8 - heterozygous
3, 4, 9, 10 - GG homozygous
11 - Undigested
12 DNA ladder 50bp

We obtained for the G variant of the *methionine synthase* gene a frequency of 20%, while for the other studied polymorphism *MTRR A66G*; the G variant had a frequency of 43.2% in our Romanian population group.

Discussion

The metabolic pathway of folate which may be impaired by an unbalanced diet or by genetic risk factors is thought to influence DNA stability in two different ways (27, 28). The first is related to the role of folate in one carbon unit transfer during de novo synthesis of nucleotides. Low levels of 5,10 methylenetetrahydrofolate, the cofactor of thymidylate synthase, depress thymidylate synthesis, leading to an increased dUMP/dTMP ratio and increased dUTP misincorporation in DNA. The removal of dUTP by DNA-glycosylase may lead to single and double strand breaks; furthermore, the unbalanced nucleotide pool resulting from inefficient thymidylate synthase activity can increase DNA misrepair, contributing to the overall level of DNA damage in the cell, an important etiolo-

gic factor for the rapidly dividing spermatozoa. The second way in which folate metabolism may affect DNA maintenance involves the production of S-adenosyl methionine, the methyl donor of most methylation processes, including CpG methylation, low levels of SAM could eventually lead to DNA undermethylation, a cause of abnormal gene expression (29) and chromosome segregation (30). *MTR* or *MTRR* single nucleotide polymorphisms could determine, through the reduced activity of the corresponding enzymes, an altered folate status, determining epigenetic alterations in DNA by undermethylation of the CpG rich regions, the highly conserved sequences in promoter regions of first exons of genes (31). The process of DNA methylation is a fundamental as well as potentially reversible mechanism for epigenetic control of gene expression.

Until this date there are a few previous published studies that have investigated the possible association of several *MTHFR* gene polymorphisms with idiopathic male infertility in patients from Germany, The Netherlands, Italy, India, South Korea and China (32-38). Five of them (32, 35-38) reported an association between the *MTHFR C677T* polymorphism in the *MTHFR* gene and male infertility. The *MTHFR A1298C* SNP was also studied; Varinderpal et al. (2) and Lee et al. (37) reported no association between the *A1298C* SNP and male infertility in an Indian study group, respectively a Chinese study group. The same Chinese study (37) showed that there is an association between the *MTRR A66G* and the unexplained cases of infertility; even if *MS A2756G* wasn't associated with all the cases of infertility, this polymorphism was found to be a genetic risk factor for the subgroup of patients with severe oligozoospermia.

It was shown that folate deficiency is a factor that determines a reduced proliferation of various cell types (39) and also that folate intake is very important for male fertility, hence future studies will need to focus on the relation between idiopathic cases of infertility, genetic risk factors and the nutritional status of subjects; dietary habits which are particular in the country were the study is conducted influence plasmatic levels of homocysteine and folates. Taken together the impact of nutrient factors such as vitamin B12 and folates seem more important in determining the overall effect of plasma homocysteine and folate levels, however this is evident in the United States where a fortification of enriched grain products was implemented starting from the mid-1997, a program which aimed at reducing the number of children with neural tube birth defects. A similar study must be undertaken on a Romanian population group in order to investigate the effect of vitamin B12 and folic acid supplementation and the evolution of plasmatic levels of homocysteine and folate in respect to genetic modifying factors, markers of DNA damage and spermatozoa status. It has already been shown that sperm concentration is increased by folic acid and zinc sulphate treatment (33).

The authors of this study would also like to emphasize that compared to other types of pathologies (vascular, neurodegenerative), wherein wide genome association studies are being used to determine possible risk factors, until this date there is only one study of this type published by Aston et al. (40) on male infertility, which investigated 370.000 SNPs and found 20 SNPs significantly associated with idiopathic forms of male infertility. However this pilot study indicates the fact that without proper financial support genome wide association studies are not feasible and that the candidate gene approach is still required if we are to uncover the molecular mechanisms of male infertility.

Conclusion

In the present study we evaluated for the first time the possibility of an association between the *MS A2756G* SNP and *MTRR A66G* SNP male infertility in a Romanian population group. The genotype and allelic distribution of

the *MS A2756G* and *MTRR A66G* polymorphisms observed in our study were similar in the two groups; hence these polymorphisms are not genetic risk factors for male infertility in our Romanian population group.

Although our study had some limitations, like the impossibility to measure plasma levels of homocysteine and folate and the small number of subjects, as well as the potential bias indicated by the Hardy-Weinberg equilibrium, our work provides for the first time data in a Romanian population group regarding risk factors for male infertility possibly attributed to abnormal folate and homocysteine status.

Future studies should be focused on identifying new genes as well as studying other polymorphisms in genes encoding crucial enzymes of the folate metabolic pathway in order to obtain a better understanding of the complex relation between the etiology of male infertility and abnormal folate and homocysteine plasmatic levels. New findings regarding gene-to-gene and gene-nutrient interactions could eventually lead to new and improved techniques of assisted reproduction.

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