Original article

Association of betaine-homocysteine S-methyltransferase gene G742A SNP and male infertility

Asocierea polimorfismului genei betain-homocistein S-metiltransferazei G742A şi infertilitatea masculină

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Abstract

The folate metabolic pathway is a complex biochemical chain of reactions essential for cellular function. Within this pathway BHMT (betaine-homocysteine S-methyltransferase) catalyzes the conversion of homocysteine to methionine. The BHMT G742A SNP (single nucleotide polymorphism) is suspected to alter the enzyme's normal activity, hence increasing homocysteine and reducing folate plasma levels. We analyzed the distribution of this single nucleotide polymorphism in the BHMT gene in a case group of 66 infertile Romanian patients with idiopathic azoospermia or severe oligozoospermia and a control group of 67 fertile Romanian men, to explore the possible association of the G742A polymorphism and male infertility. Using the polymerase chain reaction – restriction fragment length polymorphism technique (PCR-RFLP), the allele and genotype distribution of SNP G742A in the BHMT gene was investigated in both patients and controls. The allelic frequencies of the variant A allele were significantly higher in the patients group compared to the controls. Our findings suggest that the BHMT G742A SNP is a genetic risk factor for idiopathic male infertility in our Romanian population group.

Keywords: *infertility, homocysteine, folate, azoospermia*

Rezumat

Calea metabolică a folaților este un lanț complex de reacții biochimice esențiale pentru funcția celulară. În cadrul acestei căi BHMT (betain-homocistein S-metiltranferaza) catalizează conversia homocisteinei în metionină. Varianta BHMT G742A (polimorfism mononucleotidic) este suspectată a modifica activitatea normală a enzimei, crescând astfel nivelele plasmatice ale homocisteinei şi scăzându-le pe cele ale folaților. Am analizat distribuția acestui polimorfism al genei BHMT într-un grup format din 66 de bărbați români diagnosticați cu azoospermie idiopatică sau oligozoospermie severă şi un grup de control format din 67 de bărbați români fertili, în vederea explorării posibilei asocieri a polimorfismului G742A cu infertilitatea masculină. Utilizând tehnica PCR-RFLP (polimorfismul lungimii fragmentelor de restricție), distribuția alelică şi a genotipurilor a fost inves-

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tigată pentru varianta G742A a genei BHMT în ambele loturi. Frecvența variantei alelice A este semnificativ mai ridicată în lotul de pacienți comparativ cu cel de control. Rezultatele noastre sugerează că polimorfismul BHMT G742A este un factor de risc genetic pentru infertilitatea masculină idiopatică în lotul de bărbați români studiat.

Cuvinte cheie: *infertilitate, homocisteină, folat, azoospermie*

Introduction

According to the World Health Organization, couple infertility has become a global health problem with one couple in seven being affected by fertility or subfertility problems (1). Male infertility represents an etiologic factor in 20-50% of the affected couples (2).

During last years, a lot of attention was paid to identifying the genetic factors in the etiology of idiopathic male infertility. Of particular interest is the folate metabolic cycle, which was demonstrated to be involved in numerous conditions. These range from early pathological events in life, such as birth defects and pregnancy complications (3), to cardiovascular disease (4), cancer (5) and neurodegenerative disorders (6).

Several genetic variants of key enzymes of the cycle have been found to confer an increased risk for idiopathic male infertility (7). MTHFR (methylenetetrahydrofolate reductase) participates in the conversion of 5,10-methyltetrahyfrofolate to 5-methyl tetrahydrofolate, while MS (methionine synthase) catalyzes the conversion of 5-methyl tetrahydrofolate and homocysteine to methionine, the precursor of S-adenosylmethionine (SAM). This latter effect is done with the participation of vitamin B12, and MTRR (methionin-synthase reductase). The importance of folate metabolism resides in providing one-carbon units for nucleic acids bases synthesis, reducing the plasmatic levels of homocysteine and synthesizing SAM, the universal methyl donor for several biological methylation reactions.

An alternative pathway of converting homocysteine to methionine is by means of betaine-homocysteine S-methyltransferase (BHMT) which catalyzes the transfer of a methyl group from betaine to homocysteine generating dimethylglycine and methionine. This enzyme is found mostly in the liver and kidney and it has been predicted to be responsible of about 50% of the remethylation capacity of the liver (8).

Within the *BHMT* gene a single nucleotide polymorphism (SNP) which may affect the folate pathway has been described. It is found in exon 6 of the *BHMT* gene at position 742 and determines an amino acid substitution of arginine by glutamine at position 239 in the corresponding protein (9). By altering the amino acid structure of the protein it has been hypothesized that the *BHMT* G742A polymorphism may modify the plasmatic levels of homocysteine. This variant was identified as a risk factor for placental abruption (3), but until now no studies were performed to determine what role it plays in male infertility. We investigated the distribution of this polymorphism to determine if it grants an increased risk for infertility in our Romanian population group.

Materials and methods

Our study was performed on a group of 66 infertile Romanian patients from which 54 were diagnosed with idiopathic azoospermia and 12 with severe oligozoospermia, and a control group of 67 Romanian men with at least 1 child. Patients with a history of varicocele, congenital abnormalities, urogenital infections and undescended testicles were excluded from the study after examination by a specialist. Also after performing chromosomal and molecular analysis patients with chromosomal abnormalities, microdeletions in the AZF (azoospermia factor) region of the Y chromosome were excluded from the study group. This study was approved by the Ethics Committee of the "Iuliu Hațieganu" University of Medicine and Phar-

macy, Cluj-Napoca, and was led in compliance with the Helsinki Declaration. Informed consent regarding genetic testing was obtained from all study subjects.

For genetic testing, 3 ml of peripheral blood was collected on EDTA (ethylenediaminetetraacetic acid) as anticoagulant. Genomic DNA was extracted using a commercially available extraction kit (Wizzard Genomic DNA Purification Kit, Promega) from blood leucocytes contained in a volume of 300µl. The presence of the *BHMT* G742A polymorphism was detected by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP), by modifying a previously described protocol (3). The PCR amplification reaction was performed in a total volume of 25µl containing approximately 100ng of genomic DNA, 12.5 µl PCR Master Mix (Fermentas MBI, Lituania), 1µl BSA (Bovine Serum Albumin) (Fermentas MBI, Lituania) solution 2 mg/ml, 8 pM of each primer, forward and reverse (Eurogentec, Belgium) and water free of nucleases to complete the 25µl volume. The PCR reactions were performed in a gradient thermocycler (MastercyclerGradient, Eppendorf), by using the following primers pair: Fw 5'-TGCTGGTTTCTG-GTGCATCCCTAA-3' and Rev 5'-AAGGGCT-GACTCATCAGGTGAGCT TTGAGT-3', under the following conditions: an initial step consisting in denaturation for 2 min at 94°C, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 64°C for 60 s, extension at 72°C for 2 min, and a final extension time of 6 min at 72°C. The amplified fragment of 171 bp was digested with HinfI endonuclease (Fermentas MBI, Lituania). Digestion of the 171-bp fragment corresponding to the 742 GG genotype gives three fragments, of 141, 19 and 11 bp, whereas the 742 AA genotype results in two fragments of 160 and 11 bp. The digested fragments were resolved in a 3% MetaPhor gel (Lonza, Basel, Switzerland), stained with ethidium bromide, and then visualized on a UV transilluminator (VilberLourmat Imaging System®, Marne-la-Vallée, France), *Figure 1*. The observed alleles and genotypes frequencies were calculated and the Chi-square test for deviation was performed to establish the consistency 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 using Fisher's exact test of the statistical software SPSS Statistics 17.0 (IBM Corporation, USA).

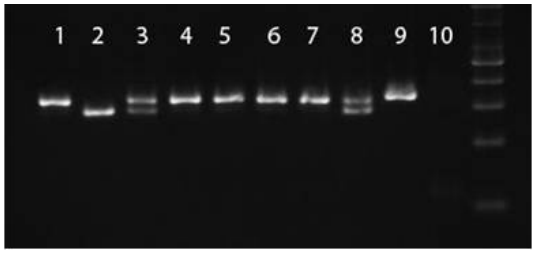

Figure 1. Electrophoresis of BHMT G742A in 3% MetaPhor agarose (50bp ladder)
1, 4, 5, 6, 7 and 9 – AA homozygous genotype
3 and 8 – heterozygous genotype
2 – GG homozygous genotype
10 – water

Results

The genotype and allelic frequencies obtained for the *BHMT* G742A SNP are presented in *Table 1*. We performed Fisher's exact test using two hypothetical models: the dominant model in which we hypothesise that an effect of the polymorphism could be manifested in both homozygous 742AA and heterozygous 742GA status and the recessive model, where the risk-associated genotype is considered the homozygous 742AA genotype alone (*Table 2*). The observed genotypes frequencies were in agreement with Hardy-Weinberg equilibrium for both the patients ($\chi 2$=2.1; p-value = 0.14) and control groups ($\chi 2$=1.4; p-value = 0.23). When we take the risk genotypes together (heterozygous and variant homozygous genotypes) we can observe a statistically significant difference between the two groups (OR 2.706; CI = 1.321-5.541; p- value of 0.008), which sug-

Table I. BHMT G742A genotypes and alleles frequencies

BHMT G742A genotypes	Group 1 (patients) n (%)	Group 2 (controls) n (%)	OR (95% CI)	p-value
Total no. of subjects	66 (100)	67 (100)		
GG	19 (28.8)	35 (52.2)		
GA	38 (57.6)	24 (35.8)	2.917 (1.368-6.219)	0.006
AA	9 (13.6)	8 (12)	2.072 (0.686-6.253)	0.257
BHMT G742A alleles	Allele frequencies (%)	Allele frequencies (%)		
G allele	76 (57.6)	94 (70.1)		
A allele	56 (42.4)	40 (29.9)	1.732 (1.044- 2.872)	0.04

Table II. The BHMT G742A polymorphism risk analysis for dominant and recessive models

Analysis model	Odds ratio	95% CI	p - value
GA+AA vs. GG	2.706	(1.321 - 5.541)	0.008
AA vs. GA+GG	1.164	(0.420 - 3.229)	0.800

gests that this gene variant is a risk factor for male infertility in our population. This difference is also observed when we analyze the distribution of alleles between the groups (OR 1.732; CI = 1.044-2.872; p-value of 0.04).

Discussion

The folate metabolic pathway is a highly tuned and precise metabolic mechanism, strictly correlated with the methionine and homocysteine metabolism and thus implicated in the process of DNA methylation, purine base synthesis, uracil incorporation into DNA and gene expression. Spermatogenesis is also a complex process involving highly regulated gene expression which requires correct DNA methylation (10).

The G742A polymorphism in the *BHMT* gene might result in hyperhomocysteinemia and low folate status (3). The toxic levels of homocysteine may subsequently generate the auto-oxidation of germline cells and as a result can cause DNA fragmentation and cell membrane damage (10). Furthermore folate deficiency has been previously shown to reduce the proliferation of various cell types (11); the altered function of the BHMT enzyme might lead to failure of differentiation also of germ cells into mature spermatozoa and to lower sperm counts.

In another published article (12), in which we investigated the same populations groups we found no difference between the distributions of two other genetic variants of key enzymes (MS and MTRR) of the metabolic pathway. These genes also participate in the conversion of homocysteine into methionine, but the precise way in which the BHMT enzyme related pathway interferes with them is currently unknown.

Because to date there is no association in the literature covering the possible effect of the *BHMT* G742A polymorphism on male infertility we compared our genotypes frequen-

cies with two other studies who investigated this variant in relation with other pathologies (3, 13). The frequencies obtained in our population are similar with the results of the other research groups. Our study had some limitations, like the relatively small number of patients and the impossibility to determine the plasmatic levels of homocysteine and folates. However the importance of this study resides in providing data concerning genetic risk factors for male infertility that can be found within the folate pathway. We could suppose that the alternative pathway of obtaining methionine which is regulated by the *BHMT* gene is critical for normal spermatogenesis and any modifying variant which might generate a rise of plasma homocysteine cannot be compensated in vivo by the other metabolizing pathways.

The folates intake and the plasma folate levels have been reported to play an important role in the detrimental effect caused by folate related gene variants. Several genetic risk variants might be tolerated in subjects with rich folate supply, whereas in individuals with insufficient folate intake the same polymorphisms may generate negative clinical and biochemical effects (14). Various levels of dietary folates uptake based on ethnic or geographic factors might explain the discrepancy of results obtained in different populations regarding the impact of folate-related genes polymorphisms.

Conclusions

In the present study we evaluated for the first time the possible association between the *BHMT* G742A polymorphism with male infertility in a Romanian population group. The genotype and allelic distributions of the studied polymorphism was statistically different in the patients group compared to the controls. Based solely on this results the *BHMT* G742A SNP is a genetic risk factor for idiopathic male infertility in our Romanian population group. Future genetic studies focused on gene-to-gene interactions and gene-environment interactions coupled with functional studies could unravel the complex relationship between folates and fertility.

Abbreviations

AZF = azoospermia factor
BHMT = betaine-homocysteine S-methyltransferase
EDTA = ethylene diaminetetraacetic
MS = methionine synthase
MTHFR = methylene tetrahydrofate reductase
MTRR = methionin-threonin reductase
PCR-RFLP = polymerase chain reaction – restriction fragment length polymorphism
SNP = single nucleotide polymorphism

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