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METHYLENETETRAHYDROFOLATE REDUCTASE G1793A POLYMORPHISM AND MALE INFERTILITY IN A ROMANIAN POPULATION GROUP

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ABSTRACT. Folate metabolism is essential for proper cellular function, including DNA methylation. MTHFR provides methyl groups for numerous reactions including DNA methylation. Molecular analysis was performed in a case group of 66 infertile Romanian patients with idiopathic azoospermia or severe oligozoospermia and a control group of 67 Romanian men, to explore the possible association of the G1793A polymorphism and male infertility. Using the polymerase chain reaction - restriction fragment length polymorphism technique (PCR-RFLP), the allele and genotype distribution of SNP G1793A in the MTHFR gene were investigated in both patients and controls. The frequencies of the polymorphism in infertile patients were not significantly higher than those in controls, indicating that this polymorphism would not be a genetic risk factor for male infertility in our study group.

Keywords: homocysteine, folate, azoospermia, DNA methylation, infertility

INTRODUCTION

Couple infertility is a global health problem and according to the World Health Organization approximately one couple in seven is affected by fertility or subfertility problems (Randolph et al., 2005). Male infertility in humans has been acknowledged as the cause of couple's inability to have children in 20-50% of total cases. (Varinderpal et al., 2007)

The most common non-genetic causes of male infertility are: 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 40% of cases no cause was found related to infertility, hence launching 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 chromosomic abnormalities (Chandley et al., 1998, Dohle et al., 2002), deletions of the azoospermia factor region (AZF) of the Y chromosome or translocations between the Y chromosome and other chromosomes (Foresta et al., 2001, Reijo et al., 1995, Vogt et al., 1996), mutations in the cystic fibrosis conductance regulator (CFTR) gene, commonly associated with congenital vas deferens abnormalities (Jaffe et al., 1994; Dohle et al., 1999) and also other genetic factors (Lee et al., 2003). It was observed that some abnormalities associated with infertility are inherited, like reciprocal and Robertsonian translocations and CFTR mutations (Mak et al., 1996), 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 (Lucock, 2000). 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 (Herrman, 2001, Gueant et al., 2003). 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 (Eskes, 2000, Nelen, 2001, Hague, 2003, Steegers-Theunissen et al., 2004, Tamura et al., 2006). However, although the recent progress in understanding the physiopathology of hyperhomocysteinemia - induced health events, there is only little information on the role of folates/homocysteine on male reproduction.

Within the folate metabolic cycle the MTHFR gene encodes a key regulatory enzyme responsible for the reduction of 5, 10-methyltetrahyfrofolate, thus catalyzing the only reaction in the cell that ultimately

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generates 5-methyltetrahydrofolate, the biologically active folate derivative. The importance of folate metabolism is related to its function in providing one-carbon units for nucleic acids bases synthesis as well as for the synthesis of S-adenosylmethionine, the universal methyl donor for several biological methylation reactions.

Within the MTHFR gene several SNPs (single nucleotide polymorphisms) have been described. Martin et al. (2006) resequenced the MTHFR gene product, and found a total number of 65 polymorphisms, 11 of which were non-synonymous cSNPs. A transition from cytosine to thymine at the 677 position of the MTHFR gene causes enzyme thermolability and reduced activity (Frost et al., 1995), therefore, impairments of MTHFR function, such as those associated with the presence of the C677T polymorphic site, are critical for altering nucleic acid metabolic pathways. Van der Put et al. (1998) identified another polymorphism of the MTHFR gene, a 1298A-C mutation resulting in a glu-to-ala substitution in position 429. The C677T transition occurs within the predicted catalytic domain of the MTHFR enzyme while the A1298C transition is located in the presumed regulatory domain. Rady et al. identified in 2002 the G1793A SNP which results in Arg594Gln substitution. Until this date the functional relevance of this polymorphism has not been elucidated, although previous studies reported that the wild-type variant is associated with a higher level of plasma homocysteine in Swedish subjects (Bottiger et al., 2007).

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 test after examination by a specialist. Also after performing chromosomal and molecular analysis patients with chromosomal abnormalities, microdeletions in the AZF region of the Y chromosome were excluded from the study group. Informed consent regarding genetic testing was obtained from all study subjects. 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®). The presence of the MTHFR G1793A polymorphism was detected by means of molecular genetic techniques, respectively polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) under slightly modified conditions of a previous published protocol (Rady et al., 2002). The PCR amplification reaction was performed in a total volume of 25µl containing approximately 100ng of genomic DNA, 12.5µl PCR Master Mix (Fermentas MBI, Lituania®), 1µl BSA (Bovine Serum Albumine, 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 primer pairs: forward primer 5'-GGGACAGGAGTGGCTCCAACGCAGG-3' and reverse primer 5'-CTCTGTGTGTGTGTGCATGTGT GCC-3' under the following conditions: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 20 s, extension at 72°C for 20 s, and a final extension time of 5 min at 72°C. The amplified fragment of 310 bp was digested with BsrbI endonuclease (Fermentas MBI, Lituania®). The G1793A mutation abolishes a BsrbI restriction site. Digestion of the 310-bp fragment of the 1793 GG genotype gives two fragments of 233 and 77 bp, whereas the 1793AA genotype results in a single fragment of 310 bp. The digested fragments were resolved in a 2% agarose gel, stained with ethidium bromide and then visualized on a UV transilluminator VilberLourmat Imaging System®, Marne-la-Vallée, France). 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 genotype distribution in the studied population were in 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 the Fischer test of the statistical software GraphPad InStat version 3 statistical software (GraphPad InStat 3, San Diego, California, USA).

RESULTS AND DISCUSSIONS

The genotype and allelic frequencies obtained for the G1793A SNP are presented in table I. The observed genotypes frequencies among the study groups were in agreement with Hardy-Weinberg equilibrium ($\chi 2=0.351$, $p=0.553$) while the genotypes frequencies of the MTHFR G1793A polymorphism were 10.6% and 89.4% for the G/A and GG genotype respectively, among the idiopathic azoospermia and severe oligozoospermia (AZF) group and 9% and 91%, respectively among the control group.

After applying the statistical Fischer test to the observed genotypes the p value obtained was 0.778, considered not statistically significant. Also, the odds-ratios for all the association tests have been around the value of 1.2, revealing that the G1793A SNP in the MTHFR gene is distributed similarly in the two study groups. In our study we obtained an allelic frequency for the A allele of 4.9%, while previous studies which investigated the frequency of this polymorphism obtained the following results: 9.3% in Chinese population (Mao et al., 2008), 1.3% in Ashkenazi, 3.1% in African-Americans, 5.8% in Hispanics, 6.9%

in Caucasians (Rady et al., 2002) and non-Hispanics whites 3.6-7.3% (Neumann et al., 2005, Shi et al., 2005).

In the present study we evaluated for the first time the possibility of an association between the G1793A SNP in the MTHFR gene and male infertility in a Romanian population group.

Table 1

MTHFR G1793A genotype and allele frequencies

Genotypes	AZF group n (%)	Control group n (%)	OR (95%CI)	p value
Total no. of subjects	66 (100)	67 (100)		
GG	59 (89.4)	61 (91)		
GA	7 (10.6)	6 (9)	1.206 (0.3827-3.802)	0.778
Allele	Alleles frequencies			
Total no.of alleles	132	134		
G allele	0.947	0.955		
A allele	0.053	0.045	1.195 (0.3905-3.655)	0.784

A few previous studies have evaluated the association of MTHFR C677T polymorphism in infertile patients from Germany, The Netherlands, Italy, India, South Korea and China (Bezold et al. 2001, Ebisch et al. 2003, Stuppia et al., 2003, Singh et al. 2005, Park et al., 2005, Lee et al., 2006, A et al., 2007). Five of them (Bezold et al., 2001, Singh et al., 2005, Park et al., 2005, Lee et al., 2006, A et al., 2007) have reported an association between these polymorphism in the MTHFR gene and male infertility. The MTHFR A1298C SNP was also studied, Varinderpal et al. (2007) reported no association between the A1298C SNP and male infertility in an Indian study group; while another study done on Chinese infertile men also concluded that there is no association between this SNP and the idiopathic cases of male infertility. Until this date there is no published study on the MTHFR G1793A SNP and male infertility.

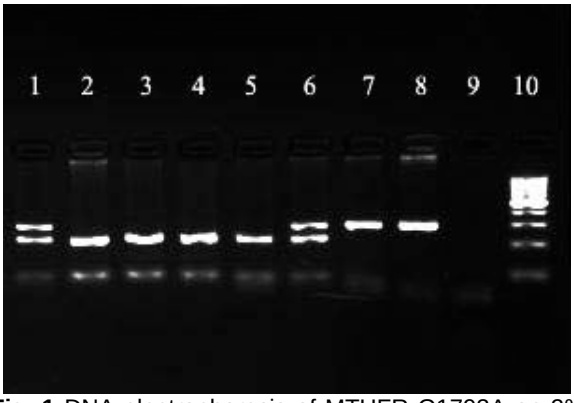

Fig. 1 DNA electrophoresis of MTHFR G1793A on 2% agarose gel: 1, 6 - heterozygous allele; 2, 3, 4, 5 - normal allele; 7, 8 – undigested; 9 – water; 10 - DNA ladder 100 bp

It has been found that whereas MTHFR genotypes do not seem to be directly linked to risk they may interact with folate in the development of endometrial cancer (Xu et al., 2007). Also the 1793A allele and 1793GA genotype has been found more frequent in female patients with unexplained infertility (Altmae et al., 2009). Another study which investigated the role of the MTHFR G1793A SNP on plasma homocysteine done on Swedish adolescents showed that the A allele was associated with a significantly lower level of plasma homocysteine (Bottiger et al., 2007)

Considering the fact that folate deficiency has been shown to reduce the proliferation of various cell types (Zhu et al., 2001) and also that it is already established that folate intake is very important for male infertility, future studies 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.

It has already been shown that sperm concentration is increased by folic acid and zinc sulphate treatment (Ebisch et al. 2003). Also in the cause of altered folate status due to reduced MTHFR enzyme activity, epigenetic alterations in DNA must be taken into account as important etiological factors. DNA methylation typically occurs in CpG dinucleotide rich regions, CpG islands, highly conserved sequences in promoter regions or first exons of genes (Robertson et al., 2000). Because of the strong correlation between DNA methylation in promoter regions and transcriptional repression (Robertson et al., 2000), DNA methylation appears to be a fundamental as well as potentially reversible mechanism for epigenetic control of gene expression. There is accumulating evidence that hypermethylation is involved in carcinogenesis since this phenomenon contribute to suppression of gene transcription (Siegfried et al., 1999).

Compared to other types of pathologies, vascular, neurodegenerative were 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. (2009) on male infertility, which investigated 370.000 SNPs and found 20 SNPs significantly associated with idiopathic forms of male infertility. However this pilot study emphasizes

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.

CONCLUSIONS

Despite our study had some limitations, like the impossibility to measure plasma levels of homocysteine and folates, our work provides data for the first time in a Romanian population group regarding risk factors for male infertility possibly attributed to abnormal folate status.

Considering the fact that the genotype and allelic distribution of the MTHFR G1793A polymorphism observed in our study was similar in the two groups we conclude that this polymorphism is not a genetic risk factor for male infertility in our Romanian population group.

To better understand the etiology of male infertility future studies will need to be conducted on more subjects to obtain a higher statistical significance and to focus on identifying and studying new candidate genes in order to obtain a deeper understanding of the complex gene-to-gene and gene-nutrient interactions which have a profound effect on the studied pathology.

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