Evaluation of the Association between the C677T Polymorphism of Methylenetetrahydrofolate Reductase Gene and Recurrent Spontaneous Abortion

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ABSTRACT

Introduction: One factor known to cause thrombophilia in women with unexplained recurrent spontaneous abortion (RSA) is C677T polymorphism of methylenetetrahydrofolate reductase gene. This study aimed to determine the association between RSA and MTHFR C677T polymorphism in Iranian patients.

Methods: In this case-control study, 30 patients with previous history of two or more consecutive unexplained abortions, and 10 women with at least two live births without a miscarriage were analyzed for MTHFR C677T polymorphism using PCR-RFLP (PCR-Restriction Fragment Length Polymorphism) method; the study was carried out on patients referring to Baqiyatallah Hospital and Avicenna Infertility Clinic. The results obtained via estimating the genotype of each polymorphism were analyzed by SPSS version 16.

Results: Seventeen women (56.6 %) with recurrent spontaneous abortions and 5 women (50 %) from the control group were heterozygous for MTHFR C677T polymorphism. T-allele frequency in the experiment group was higher than the control group (28.4 % and 25 % for the experiment and control group, respectively).

Conclusion: The prevalence of MTHFR C677T polymorphism was slightly higher in RSA patients compared with the controls. This finding failed to support the relationship between this polymorphism and the increasing risk of RSA in the evaluated Iranian women.

Keywords: Methylenetetrahydrofolate reductase (NADPH2), Polymorphism (genetics), Spontaneous abortion, Thrombophilia

Introduction

In medical terminology, spontaneous abortion or miscarriage, is defined as the involuntary termination of pregnancy before 20 weeks' gestation. Recurrent pregnancy loss (RPL), also known as recurrent miscarriage, is defined as the occurrence of three or more consecutive pregnancy loss with the same partner, and having no more than one live-born child (1).

Miscarriage may occur for many reasons, not all of which can be identified. Thrombophilic disorders are among the few recognized risk factors and potential causes of recurrent miscarriages. During pregnancy, mother’s body adapts to the needs of the fetus, since her blood provides all of the nutrients and oxygen the fetus needs throughout the pregnancy (2). The placenta is the primary site of nutrient and gas exchange between the fetus and the expectant mother. The perfusion of the intervillous space of placenta with maternal blood allows the transfer of nutrients and oxygen from the mother to the fetus and the transfer of waste products and carbon dioxide back from the fetus to the maternal blood supply. In patients with thrombophilia, damage of the placenta occurs mostly due to blood clot formation in the maternal as well as the fetal vessels. In addition, since the maternal blood comes out of the maternal vessels and flows very slowly between the branches of the fetal vessels (intervillous space), maternal blood is vulnerable to clot formation. If maternal blood clots in this space, the fetal vessels will be
Table 1. Primer sequence, amplified product, restriction enzyme for detection of C677T polymorphism in MTHFR gene

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>PCR primers</th>
<th>PCR Product (bp)</th>
<th>Restriction enzyme</th>
<th>RFLP Products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR C677T</td>
<td>F: 5' - TGAAGGAGAGGTTCTGCGGAGA -3'</td>
<td>198</td>
<td>Hinf I</td>
<td>(198)*</td>
</tr>
<tr>
<td></td>
<td>R: 5' - AGGAGCTGCGGTAGAGTG -3'</td>
<td></td>
<td></td>
<td>(175, 23)**</td>
</tr>
</tbody>
</table>

Note: * Normal allele  ** Mutant allele

destroyed, since nutrients and oxygen will not be provided for the vessels; this leads to placental degeneration and fetal vessel damage (3).

Single-nucleotide polymorphisms (SNPs) in metabolic pathways which regulate enzymes such as methylenetetrahydrofolate reductase (MTHFR) are regarded as risk factors for thrombophilia. MTHFR is the key enzyme in folate, methionine and homocysteine metabolism (4). Disturbances in MTHFR activity could be the cause of increased serum level of homocysteine (a potentially toxic amino acid). Hyperhomocysteinemia is considered a risk factor for changes in the coagulation cascade through direct cytotoxic effect on endothelium, atherogenesis, activation of coagulation factor V and VII, increased level of thrombin, platelet aggregation and a tendency toward venous thrombosis (4, 5).

MTHFR irreversibly reduces 5,10-methylenetetrahydrofolate (substrate) to 5-methyltetrahydrofolate (product). 5-Methyltetrahydrofolate is the dominant folate form in the circulating human plasma and is used to convert homocysteine to methionine through methionine synthase (5-7). Decreased MTHFR activityhamstrings the methylation cycle, resulting in increasing levels of homocysteine (7). In humans, the gene coding for MTHFR enzyme is located at chromosome 1.p36.3, and is composed of 11 exons (8).

A common polymorphism in the gene for the MTHFR enzyme is known as the C677T MTHFR polymorphism. It leads to an altered amino acid sequence, and is associated with a decreased enzyme activity. A thymine(T)-to-cytosine(C) transition at nucleotide 677 in the exon 4 of MTHFR gene, leads alanine to be substituted by valine residue at position 222 in the catalytic domain of the MTHFR enzyme. The C677T polymorphism of the MTHFR gene encodes a thermolabile variant of the enzyme with reduced activity; reduced activity can lead to elevated levels of homocysteine (also called hyperhomocysteinemia), especially when folate levels are low.

As a matter of fact, recent published studies suggest that the C677T polymorphism is likely to modify the enzyme stability rather than enzyme activity. Generally, it was revealed that individuals with homozygous (677TT) genotype have 30% of the MTHFR enzymatic activity of the wild variant, while the heterozygotes (677TT) have 65% of the enzymatic activity (9-11).

When folate intake is inadequate, individuals who are homozygous (T/T) for the abnormal gene have lower levels of the MTHFR enzyme and thus higher levels of homocysteine in their blood. Improved folate nutritional status appears to stabilize the MTHFR enzyme, resulting in improved enzyme levels and lower homocysteine levels (9, 10).

Considering the effects of MTHFR C677T polymorphism on hyperhomocysteinemia, the increased risk of venous thrombosis and also the association between inherited thrombophilia and recurrent pregnancy loss, the main objective of the present study is to investigate the relationship between the C677T polymorphism of MTHFR gene as a genetic risk factor for idiopathic recurrent miscarriage.

Materials and Methods

Study Population

This prevalence case–control study included 30 women with a history of two or more unexplained consecutive miscarriages, referring to Avicenna Infertility Clinic (AIC) in Tehran. Also, 10 fertile women with at least two live births who were referring to Baqiyatallah Hospital, Tehran, were included in this study, between August and March, 2009. The study on Avicenna patients was approved by the ethics committee of the clinic and informed consents were obtained from the study participants. Also, the institutional review board approval and informed consents were obtained from all participants in the control group. Women with a previous history of miscarriage, caused by chromosomal abnormality in the embryo, uterine anatomical anomaly, endocrine factors, and infections, were not included in this study. Blood samples were taken for testing.

Mutation analysis

Genomic DNA was extracted from an 80 μl aliquot of whole blood collected in EDTA (ethylene diaminetetraacetic acid) using salting-out method (as the standard method); the extracted DNA was stored at −20°C prior to analysis. To detect the presence or absence of the C677T polymorphism, we selectively amplified a 198-bp fragment of the MTHFR gene by polymerase chain reaction (PCR) (Table 1). PCRs
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Figure 1. The electrophoresis image of the PCR product for a 198-bp fragment of the MTHFR gene on agarose gel; the size of PCR product (198 bp) was determined in comparison with 50 bp molecular weight marker in bp on lines 19 and 10 of the image.

Figure 2. Agarose gel electrophoretic analysis of the MTHFR C677T polymorphism; lines 10 and 20 represent 50 bp molecular weight marker; line 1 represents the 198 bp fragment of PCR product before RFLP; lines 5 and 18 represent the 175 bp fragment corresponding to the genotype TT after digestion of HinfI as positive control; lines 3, 6, 11, 12, 16, 17, and 19 represent the 198 bp fragment corresponding to the genotype CC after digestion of HinfI; lines 2, 4, 7, 9, 13, 14, and 15 represent fragments corresponding to the genotype CT after digestion of HinfI (198, 175 bp).

were performed with 40 ng of genomic DNA, 1 U/µl Taq DNA polymerase, 3.5 µL of 10X PCR buffer [Tris-HCl (pH 8.4), 500 mM KCl], 2.0 mM MgCl2, 0.25 mM of all four deoxynucleotide triphosphates, and 0.4 µM of each primer, in a volume of 20 µL. After denaturation at 95°C for 5 min, the temperature was cycled 35 times (95°C for 1 min, 61°C for 1 min, and 72°C for 1 min), followed by an extension at 72°C for 7 min to amplify the target DNA.

MTHFR C677T polymorphism was assayed by polymerase chain reaction (PCR) amplification using primers, under the previously described conditions (Figure 1). Ten µl of PCR product was digested overnight at 37 °C with 1 unit of HinfI restriction enzyme in a final volume of 15 µl, according to the manufacturers instructions (New England Biolabs). This reaction yielded 198 base pair fragments in the presence of allele C, and 175 and 23 base pairs in the presence of allele T. A heterozygous individual will give three fragments—198, 175 and 23 bp in length.

Digestion results were submitted to electrophoresis in a 3% agarose gel and the bands were visualized using ethidium bromide staining on an ultraviolet transilluminator; the control on each gel included a known homozygous.

Statistical analysis

The genotypes and allele frequencies of MTHFR C677T variations in both groups of patients were compared with the controls, using the χ2 test. P<0.05 was considered statistically significant. SPSS version 16 was used for statistical analysis.
Results

According to the results observed in the electrophoresis, the RFLP (Restriction Fragment Length Polymorphism) genotyping of each polymorphism was established for each patient (Figure 2). Seventeen women (56.6 %) with recurrent spontaneous abortions and 5 women (50 %) among the controls were heterozygotes for MTHFR C677T polymorphism. No homozygous individual was found among patients with recurrent miscarriage or the control group. T-allele frequency in the experimental group was more than that of the control group (28.4 % and 25 % for the patients and the controls, respectively; P<0.05) (Table 2).

Table 2. The prevalence and allelic frequencies of MTHFR C677T polymorphism among recurrent miscarriage patients and control group

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Genotype, n (%)</th>
<th>Allelic frequency, n (%)</th>
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<tbody>
<tr>
<td>Patients (n=30)</td>
<td>CC 13 (43.3)</td>
<td>TT 17 (56.6)</td>
</tr>
<tr>
<td>Controls (n=10)</td>
<td>CT 17 (56.6)</td>
<td>TT + CT 17 (56.6)</td>
</tr>
</tbody>
</table>

Note: In compare with the 2 groups, no significant difference was observed among genotypes.

Discussion

Studies on different human populations reveal different results regarding the association between MTHFR C677T polymorphism and spontaneous abortion. Some studies show that scarcity of folic acid during pregnancy, hyperhomocysteinemia, and homozygosity for this MTHFR polymorphism are associated with an increased risk of spontaneous abortion and destruction of placenta, whereas others reported no such association (13, 14).

Wang et al from China reported that the distribution frequencies of C667T associated 3 genotypes with unexplained recurrent spontaneous abortion (URSA), and the control group showed statistically significant differences (P=0.012). The frequencies of C677T genotypes were: CC (33.3%), CT (53.1%), TT (13.6%) in URSA group and CC (52.4%), CT (51.5%), TT (6.1%) in the control group; also, the frequency of CC genotype in URSA group significantly decreased (P=0.005), while the frequency of T-allele in URSA had a significant increase (P<0.005) (15).

Mitraouki et al in Tunisia also analyzed RPL and normal subjects using PCR-RFLP analysis. It was concluded that the frequency of MTHFR 677TT genotype (30.0% and 7.0% in RPL and normal subjects, respectively) was significantly higher in RPL patients (16).

Studies in other regions of the world have shown completely different results from this study. Carp et al in 2002, focused on the association between maternal thrombophilia with recurrent miscarriage and reported that the prevalence of MTHFR C677T polymorphism was higher in women with RPL in comparison with the control group (13% and 8.5%, respectively); however, this difference was not statistically significant (17).

In 2009, Morales-Machin et al in Venezuela reported no significant difference of allele frequency between patients with recurrent miscarriage and the control group. The data presented in this study fail to support the relationship between MTHFR C677T polymorphism and the risks in women with recurrent abortion (18).

In a study in Austria, 145 women with a history of three or more consecutive miscarriages before 20 weeks’ gestation, and 101 healthy postmenopausal women with at least two live births (and no history of pregnancy loss) were observed. The result indicated that allele and genotype frequencies of all polymorphisms were not significantly different among the patients and the control group (19).

In the present study, although the prevalence of MTHFR C677T polymorphism was slightly higher in RSA patients compared with the controls, no significant difference was observed. The differences in the results of this study and the previous one may be due to variations in the definition of abortion, sample selection criteria or geographical distribution.

It should be noted that having high homocysteine levels may be caused by the C677T mutation in the MTHFR gene and also a decrease in co-factors in the homocysteine-methionine pathway such as folic acid, vitamin B12 and vitamin B6 (7). Individuals with the MTHFR 677 TT genotype consuming folic acid supplements and other B vitamins may be protected against hyperhomocysteinemia (10, 20).

Conclusion

In the present study, although the prevalence of MTHFR C677T polymorphism was slightly higher in RSA patients compared with the control group, no significant difference was observed. The obtained results failed to support the relationship between this polymorphism and the increasing...
risk of RSA in the evaluated Iranian women.

References