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Original Article

Association between the A1298C Polymorphism of the Methylenetetrahydrofolate Reductase Gene and Recurrent Spontaneous Abortion

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ABSTRACT

Introduction: A factor known to cause thrombophilia in women with recurrent pregnancy loss (RPL) is the A1298C polymorphism of methylenetetrahydrofolate reductase gene (MTHFR). This study aimed to determine the association between RPL and this polymorphism in Iranian patients.

Methods: In this case-control study, 30 patients with a previous history of two or more consecutive unexplained abortions and 10 women with at least two live births without a miscarriage, who referred to Baqiyatallah Hospital and Avicenna Infertility Clinic, were analyzed for MTHFR A1298C polymorphism using PCR-RFLP method. The results obtained via estimating the genotype of each polymorphism were analyzed using SPSS *v*16.

Results: Thirteen subjects (43.3 %) with RPL and 6 women (20 %) in the control group were heterozygous for MTHFR A1298C polymorphism. C allele frequency in the patient group was higher than the control group (41.7% and 40% for the patient and control groups, respectively).

Conclusion: The prevalence of MTHFR A1298C polymorphism was slightly higher in RPL patients compared to the controls. This finding failed to support the relationship between this polymorphism and the increasing risk of RPL in the evaluated Iranian women.

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

Introduction

In medical terminology, spontaneous abortion or miscarriage is defined as the involuntary end of pregnancy before 20 weeks of gestation. Recurrent pregnancy loss (RPL), also known as recurrent miscarriages, is defined by the consecutive loss of two or more pregnancies with the same partner, and having no more than one living child (1).

Miscarriages may occur for many reasons, not all of which can be identified. Thrombophilia disorders are among the few recognized risk factors and potential causes of recurrent miscarriages. During pregnancy, the maternal body adapts to the needs of the fetus, since maternal blood provides all the nutrients and oxygen the baby needs throughout the pregnancy (Figure 1). In patients with thrombophilia, placental damage happens mostly due to blood clot formation in the maternal as well as the fetal vessels (2, 3).

Single-nucleotide polymorphisms (SNPs) in metabolic pathways, which regulate enzymes such as MTHFR, are considered to be risk factors for thrombo-philia. MTHFR is the key enzyme in folate, methionine, and homocysteine metabolism (4). The disturbances in MTHFR activity could be the cause of increased serum level of homocysteine (a potentially toxic amino acid).

Hyperhomocysteinemia is a risk factor for changes in coagulation cascade through direct cytotoxic influence on endothelium, atherogenesis, activation of coagulation factor V and VII, increased level of thrombin, platelet aggregation, and a tendency toward venous thrombosis (4, 5).

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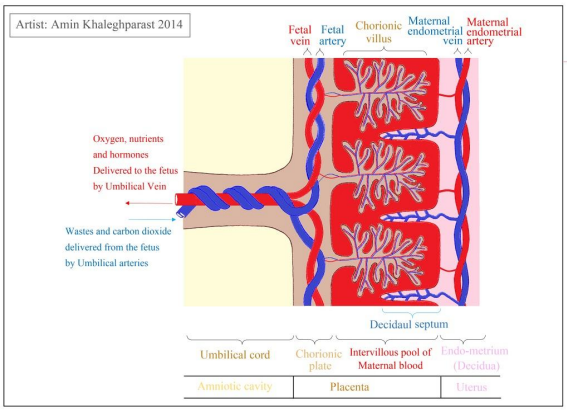

Figure 1. Diagram of the circulation of blood within the human placenta

MTHFR irreversibly reduces 5,10-methyl-enetetrahydrofolate (substrate) to 5-methyltetra-hydrofolate (product). *5*-Methyltetrahydrofolate is the dominant *folate form* in the circulating human plasma, and is used to convert homocysteine to methionine via methionine synthase (5-7). Decreased MTHFR activity hamstrings the methylation cycle, resulting in increasing levels of homocysteine (7). The enzyme is coded by the gene with the symbol *MTHFR* on chromosome 1, location p36.3 in humans, and is composed of 11 exons (8).A common polymorphism in the gene for the enzyme MTHFR is known as the A1298C MTHFR polymorphism, which leads to an altered amino acid sequence; this polymorphism has been associated with a decreased enzyme activity. The adenine (A) to cytosine (C) substitution at nucleotide 1298 in exon 7 of MTHFR gene leads to the substitution of glutamic acid by alanine residue at position 429 in the regulatory domain of the MTHFR enzyme, and therefore, reduces its enzymatic activity, which is more pronounced in the homozygous than the heterozygous state.

Reduced activity can lead to elevated levels of homocysteine (also called hyperhomocys-teinemia), especially when folate levels are low. A1298C polymorphism is known to have a less significant effect on reducing enzyme activity, compared to the 677 mutation (9, 10).

According to previous research, although individuals who are homozygous for the 1298C allele have about a 40% reduced enzyme activity *in vitro*, they do not appear to have higher plasma homocysteine levels compared to the controls. However, individuals who are compound heterozygous for the 677T and 1298C alleles (which produce a 677CT/1298AC genotype) have a 40-50% reduced MTHFR activity *in vitro* and a biochemical profile similar to that seen among 677T homozygotes with increased homocysteine levels and decreased folate levels.

The A1298C polymorphism by itself may have important clinical effects on folate metabolism under conditions such as low folate intake or elevated folate requirement during pregnancy and embryogenesis (11). Considering the effects of MTHFR A1298C polymorphism on enzyme activity and the increased risk of venous thrombosis, as well as the association between inherited thrombophilia and RPL, this study aimed to investigate the association between A1298C polymorphism of the MTHFR gene as a genetic risk factor for idiopathic recurrent miscarriage.

Materials and Methods

Study population

The present case-control prevalence study included 30 women with a previous history of two or more unexplained RPLs, who attended Avicenna Infertility Clinic (AIC), Tehran, Iran. In addition, 10 fertile women with at least two live births, attending Baqiyatallah Hospital, Tehran, were selected as the control group between August 2009 and March 2009.

Mutation analysis

Genomic DNA was extracted from an 80 µl aliquot of whole blood, collected in EDTA, using the standard salting-out method; the extracted DNA was stored at −20°C until analysis. To detect the presence or absence of the C677T polymorphism,

Table 1. Primer sequence, amplified product, and restriction enzyme for the detection of A1298C polymorphism in MTHFR gene

Polymorphism	PCR primers	PCR Product (bp)	Restriction enzyme (for RFLP)	RFLP products (bp)
MTHFR A1298C	F:5'-CTTCTACCTGAAGAGCAAGTC-3' R: 5'-CATGTCCACAGCATGGAG-3'	256	MboII	(176, 30, 28, 22)* (204, 30, 22)**

Note: * Normal allele ** Mutant allele

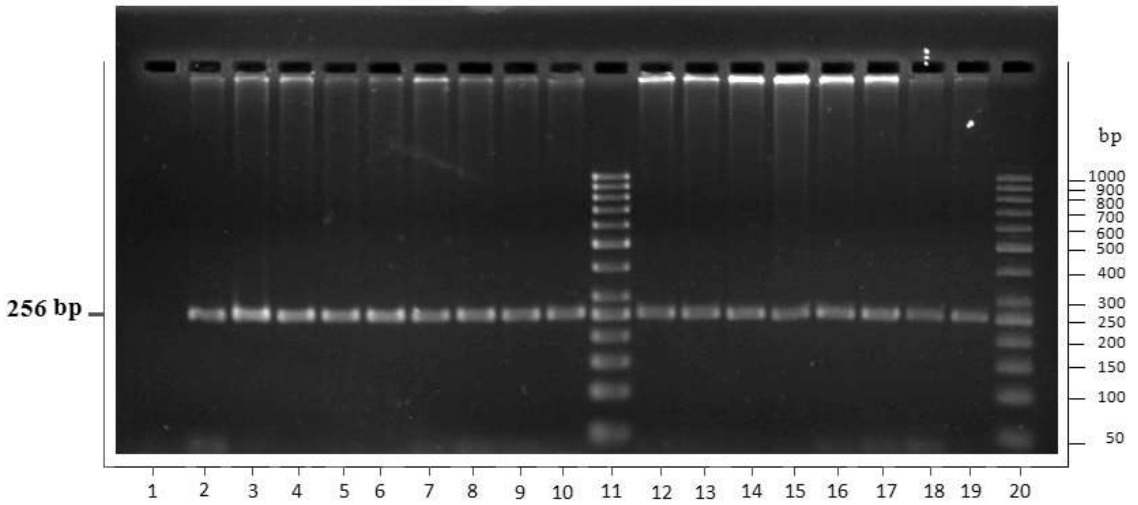

Figure 2. The electrophoresis image of the PCR product for a 256-bp fragment of the *MTHFR* gene on agarose gel; the size of PCR product (256 bp) was determined in comparison with 50 bp molecular weight marker (in bp) on lines 20 and 11 of the image

we selectively amplified a 256-bp fragment of the *MTHFR* gene by polymerase chain reaction (PCR) method (Table 1). PCRs were performed with 40 ng of genomic DNA, 1 U/µl of Taq DNA polymerase, 3.5 µL of 10X PCR buffer [500 mM KCl and Tris-HCl (pH 8.4)], 2.0 mM $MgCl_2$, 0.25 mM of all four deoxynucleoside triphosphates, and 0.4 mM of each primer, in a volume of 20 µL.

After denaturation at 95°C for 5 min, the temperature was cycled 30 times (95°C for 1 min, 63°C for 1 min, and 72°C for 2 min), followed by extension at 72 °C for 7 min to amplify the target DNA.

MTHFR A1298C polymorphism was assayed by polymerase PCR amplification, using primers and under conditions previously described (Figure 2). Ten µl of PCR product was digested overnight at 37°C with 1 unit of MboII restriction enzyme in a final volume of 15 µl, according to the manufacturers' instructions (New England Biolabs). This reaction yielded fragments of 176, 30, 28, and 22 base pairs in the presence of the A allele, and 204, 30, and 22 base pairs in the presence of the C allele.

A heterozygous individual gives five fragments of lengths 204, 176, 30, 28, and 22 bp. Digestion results were submitted to electrophoresis in a 3% agarose gel, and the bands were visualized using ethidium bromide staining on a ultraviolet trans illuminator. Control on each gel included a known homozygous.

Statistical analysis

The genotypes and allele frequencies of MTHFR A1298C variations in both groups of patients were compared with the controls using the χ^2 test. P-value less than 0.05 was considered statistically significant. SPSS version 16 was used for the statistical analysis.

Results

According to the results demonstrated in the electrophoresis image, the genotype RFLP product per patient was established for each polymorphism (Figure 3). Thirteen women (43.3 %) with RPL and 6 women (20 %) in the control group were heterozygous for MTHFR A1298C polymorphism. No homozygous individual was found among patients with RPL or the control group. C allele frequency in the patient group was higher than the control group (41.7% and 40% for the patient and control groups, respectively, $P<0.05$) (Table 2).

Discussion

Studies on different human populations indicate different results regarding the association between MTHFR A1298C polymorphism and spontaneous abortion. Some studies show that the scarcity of folic acid during pregnancy, hyperhomocysteinemia, and homozygosity for MTHFR polymorphisms are associated with an increased risk for spontaneous abortion and the destruction of placenta, whereas others reported no such association (12-14).

Wang *et al* from China reported that the prevalence of MTHFR A1298C, associated with 3 genotypes and A/C alleles, in the group with unexplained recurrent spontaneous abortion (URSA) did not differ significantly from the controls; however, the frequency of 677CC/1298AA

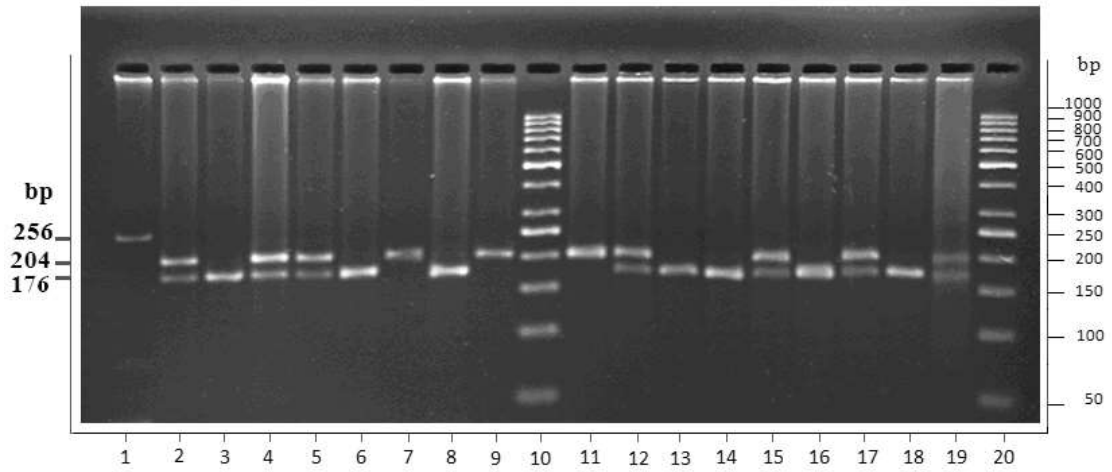

Figure 3. Agarose gel electrophoretic analysis of the MTHFR A1298C polymorphism; lines 10 and 20 represent 50 bp molecular weight marker; line 1 represents the 256 bp fragment of PCR product before RFLP; lines 7, 9, and 11 represent the 204 bp fragment corresponding to the genotype CC after digestion with MboII as a positive control; lines 2, 4, 5, 12, 15, 17, and 19 represent the 176 bp fragment corresponding to the genotype AA after digestion with MboII; lines 2, 4, 5, 12, 15, 17, and 19 represent fragments corresponding to the genotype AC after digestion with MboII (204, 176 bp)

Table 2. The prevalence and allelic frequencies of MTHFR A1298C polymorphism among RPL patients and the control group

Study groups	Genotype, n (%)				Allelic frequency, n (%)	
	CC	AC	AA	CC + AC	C	A
Patients (n=30)	6 (20)	13 (43/3)	11 (36/7)	19 (63/3)	35 (41/7)	25 (48/3)
Controls (n=10)	3 (30)	2 (20)	5 (50)	5 (50)	8 (40)	12 (60)

Note: No significant difference was observed in terms of genotypes between the two groups

in the URSA group was significantly lower compared with the control group (15).

Mtiraoui *et al* in Tunisia also analyzed female patients with RPL and control women using PCR-RFLP analysis. The frequency of the CC genotypes of A1298C SNP was significantly higher in the patients. A comparable frequency of double heterozygosity (677C/T and 1298A/C) was seen in 14 patients vs. 5 control subjects (*P*=0.10) (16).

Studies in other regions have indicated completely different results from the current study. In a study in Poland, 104 Polish women with a previous history of 3 or more unexplained recurrent miscarriages in the first pregnancy trimester (6-13 weeks of gestation) and 169 women (without obstetrical complications, history of miscarriage, and with at least one live birth in anamnesis) as the control group were observed. Regarding C677T and A1298C polymorphisms of MTHFR gene, their study showed no significant association between these polymorphisms and recurrent miscarriages (17).

Settin *et al* in Egypt focused on the association between maternal MTHFR polymorphisms and URSA in 70 Egyptian women with a previous history of two or more events of URSA and 136 controls with a good obstetric history. They reported that the prevalence of the homozygous mutant MTHFR 677 TT, 1298 CC genotypes, and the mutant haplotype 677T/1298C was higher in women with RPLs in comparison with the control population, though this difference was not statistically significant (18).

In a study in Austria, 145 women with a history of three or more consecutive pregnancy losses before 20 weeks' gestation and 101 healthy postmenopausal women with at least two live births and no history of pregnancy loss were observed. The results demonstrated that allele and genotype frequencies of single MTHFR (MTHFR C677T, MTHFR A1298C) polymorphisms were not significantly different among patients and the control group (19). In the present study, although the prevalence of MTHFR A1298C polymorphism was slightly higher in RPL patients compared to the controls, there was not a significant difference.

The discrepancies among these studies may be due to the variations in the definition of abortion, sample selection criteria, geographical distribution, or even different ethnicities in Iran. It should be noted that the A1298C mutation is located within the C-terminal regulatory domain of the MTHFR gene, while the C677T mutation is located within the gene catalytic domain; subjects

with A1298C mutation have reduced MTHFR enzyme activity, but to a lesser extent compared to those with the C677T mutation (20).

Conclusion

In this study, although the prevalence of MTHFR A1298C polymorphism was slightly higher in RPL patients compared to the controls, there was not a significant difference. The findings failed to support the relationship between this polymorphism and the increasing risk of RPL in the evaluated Iranian women.

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