Original article

Investigation of the relationship between the risk of spontaneous abortion and C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase gene

Investigarea relaţiei dintre riscul de avort spontan şi polimorfismele C677T şi A1298C ale genei metilentetrahidrofolat reductazei

Daniela Neagoş1*, Ruxandra Creţu1, Roxana C. Sfetea2, Dana Mierla3, Laurentiu C. Bohiltea1

1. Carol Davila University of Medicine and Pharmacy, Department of Genetics, Bucharest, Romania
2. Carol Davila University of Medicine and Pharmacy, Department of Modern Languages, Bucharest, Romania
3. Life Memorial Hospital, Department of Genetics, Bucharest, Romania

Abstract

Pathogenesis in spontaneous abortion is complex and involves the interaction of multiple genetic and environmental factors. C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase (MTHFR) gene have implications in the fetal viability. This paper aims to establish the association between these polymorphisms and the risk of miscarriage. In this study we determined the allelic and genotypic frequencies of the MTHFR gene polymorphisms in a sample of 250 women from Romania, 100 with 1-3 miscarriages and 150 without history of miscarriages. The genomic DNA was extracted from the peripheral blood collected on EDTA and the kit used was the pegGOLD blood DNA mini kit (ATP Biotech). The methods used to study polymorphisms were PCR and RFLP. The results of this study showed that women with spontaneous abortion have a higher proportion of the 677TT genotype compared with the control group and the distribution of allelic and genotypic frequencies for polymorphism A1298C, does not present significant differences between the study group and control. The study also found that the frequency of the combined 677TT/1298 AA genotypes is higher in the study group compared with control being associated with the risk of miscarriage. In conclusion, the study revealed significant differences between the results obtained from the two groups.

Keywords: spontaneous abortion, folate, methylenetetrahydrofolate reductase, polymorphisms.

Rezumat

Patogenia în avortul spontan este complexă și implică interacțiunea mai multor factori genetici și de mediu. Polimorfismele C677T și A1298C ale genei metilentetrahidrofolat reductaza (MTHFR) au implicații în viabilitatea fătului. Lacrarea de față și-a propus ca obiectiv stabilirea asociării dintre aceste polimorfisme și ris-

*Corresponding author: Daniela Neagos: “Carol Davila” University of Medicine and Pharmacy, Department of Genetics, Bucharest, Romania, Street Dimitrie Gerota, nr. 19-21, District 2; E-mail: delia_neagos05@yahoo.com
culturii de producere a avortului spontan. În acest studiu am determinat frecvențele alelice și genotipice ale polimorfismelor genei MTHFR la un șantion de 250 femei din România, dintre care 100 cu 1-3 avorturi spontane în antecedente și 150 fără avorturi spontane. ADN-ul genomic a fost extras din sânge periferic recoltat pe EDTA, iar kitul utilizat a fost pegGOLD blood DNA mini kit (ATP Biotech). Metodele utilizate pentru studiul polimorfismelor au fost PCR și RFLP. Rezultatele acestui studiu au arătat o proporție mai mare a genotipurii 677TT comparativ cu cele din grupul control iar distribuția frecvențelor alelice și genotipice, pentru polimorfismul A1298C, nu prezintă diferențe semnificative între lotul de studiu și cel control. De asemenea, studiul a mai evidențiat că frecvența genotipurilor combinate 677TT/1298 AA este mai mare în lotul de studiu comparativ cu cel control și asociat cu riscul de avort spontan. În concluzie, studiul a evidențiat diferențe semnificative între rezultatele obținute la cele două loturi.

Cuvinte-cheie: avortul spontan, folat, metilenetetrahidrofolat reductază, polimorfisme.

Received: 20th June 2012; Accepted: 06th November 2012; Published: 24th November 2012.

Introduction

Miscarriage occurs in 15-20% of pregnancies (1). The genetic defects are the most common causes of miscarriage in the first trimester of pregnancy. Pregnancy can lead to folate deficiency, therefore an increased intake of folic acid during pregnancy is recommended (2).

The normal concentration of folic acid is essential in embryogenesis and the embryonic growth. This statement was supported by several authors (3, 4), who found that the low folate level is linked to the spontaneous abortions and could be an embryotoxic factor in the serum of women who had abortions. George et al. reported that women with low folate level (<4.9 nmol/L) in plasma had a higher risk of miscarriage than women with high levels of folate, especially when fetal chromosomal abnormalities were present (5).

Folates are involved in a series of reactions involving the transfer of a carbon atom, holding important roles in two processes: DNA synthesis and DNA methylation (6).

The main enzymes involved in the folate metabolism are methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), methionine synthase reductase (MTRR), methylenetetrahydrofolate dehydrogenase (MTHFD) and cystathionine β-synthase (CBS) (7).

The methylenetetrahydrofolate reductase (MTHFR) is the essential enzyme for the conversion of 5,10-methylenetetrahydrofolate (5,10-methylene-THF) to 5-methyltetrahydrofolate (5-methyl-THF). This conversion allows the balance of the quantities of 5,10-methylene-THF and 5-methyl-THF present in the cell, a vital process because 5,10-methylene-THF is involved in the pyrimidine synthesis (DUMP → dTMP) used in the synthesis and DNA repair and the 5-methyl-THF is involved in the transformation of homocysteine to methionine (the reaction being catalyzed by MTR) (8).

The gene which codes for MTHFR has a length of 20.3 kb and is located on the short arm of chromosome 1 in region 1p36.3 (9).

The mutations in the MTHFR gene are present in individuals with hyperhomocysteinemia, representing a risk factor for NTD (10-14), miscarriages in the first trimester of pregnancy (15), diseases of the vascular system (16-18), cancer (19,20), Down syndrome (21,22,23), arteriosclerosis (24,26) and psychiatric diseases (27, 28).

Hyperhomocysteinemia caused by the mutations in the MTHFR gene are associated with placental abruptio or infarction (29), pre-eclampsia (30) and pregnancy-associated hypertension (31).

In literature, at the level of the MTHFR gene, two polymorphisms have been frequently described: C677T and A1298C. These polymorphisms have been associated with the termolabile form of the MTHFR gene that causes the accumulation of homocysteine in the circulation (hyperhomocysteinemia) and decrease in folic acid (8).

The C677T polymorphism (Ala222Val) consists in the substitution of C with T in the
exon 4 of the gene, resulting in the substitution of alanine with valine and low activity of the enzyme. The heterozygotes with CT genotype have a reduced activity by 35% and the homozygotes with TT genotype have a decreased activity by 70% and increased levels of homocysteine (8).

The A1298C polymorphism (Glu429Ala) consists in the substitution of A with C in the exon 7, resulting in the substitution of glutamate with alanine. This mutation is located in the region coding for the regulator domain of the protein (11, 32). The A1298 polymorphism determines the decrease of the enzyme activity to a lesser extent than C677T (11, 32, 33).

The purpose of this study is to establish the association between C677T and A1298C polymorphisms of the MTHFR gene and the risk of spontaneous abortion.

Material and methods

The present study includes 250 women (100 cases with unexplained spontaneous abortion and 150 healthy voluntarily control group). The cases had a history of at least three consecutive fetal losses before 20 weeks of gestation with the same partner. The control group consisted of fertile patients from the general population who had at least one uncomplicated pregnancy and no history of abortion. The women included in our study come from the same geographical area. Peripheral blood samples (5 ml/sample) were collected from both groups on EDTA. Genomic DNA was isolated from whole blood, using the perGOLD blood DNA mini kit (ATP Biotech) according to the manufacturer’s instructions. The C677T and A1298C mutations of the MTHFR gene were analyzed using the following techniques: PCR (polymerase chain reaction) and RFLP (restriction fragment length polymorphism).

For the C677T polymorphism, the primers were: forward 5’-TGA AGG AGA AGG TGT CTT CGG GA-3’, reverse 5’-AGG ACG GTG CGG TGA GAG TG-3’, amplifying a fragment of 198 bp. PCR conditions were 40 cycles of 30 sec at 94°C, 30 sec at 62°C, and 30 sec at 72°C, preceded by an initial denaturation of 2 min at 94°C and followed by a final extension of 7 min at 72°C. The presence of the T-allele generated a HinfI site, produced a 175 bp fragment upon restriction in standard conditions, versus the 198 bp fragment of the C allele. The primers for the PCR reaction to analyze the A1298C polymorphism were: forward 5’-CTT TGG GGA GCT GAA GGA CTA CTA C-3’, reverse 5’-CAC TTT GTG ACC ATT CCG GTT TG-3’. PCR conditions were 38 cycles of 1 min at 92°C, 1 min at 60°C, and 30 sec at 72°C, preceded by an initial denaturation of 2 min at 92°C, and followed by a final extension of 7 min at 72°C. The amplified fragment is 163 bp; in the presence of cytosine, a MboII site is modified. Thus, while the wild type allele is restricted into five fragments of 56, 31, 30, 28, and 18 bp, the mutated allele is digested only into four fragments of 84, 31, 30 and 18 bp (11). Digestion products were visualized after electrophoresis on a 3% agarose gel with ethidium bromide.

Statistical analysis

The MTHFR allele frequencies were determined for the study and control groups and compared by a Chi squared test. For all statistical analysis, the χ² and p value, odds ratio (OR) and 95% confidence interval (CI) were calculated by SPSS v.16.0 and Microsoft Excel 2003. Statistical significance was defined as p< 0.05.

Results

The investigation of the MTHFR 677 and 1298 polymorphisms was performed using the PCR amplification of genomic DNA, using the primers described in the Material and methods section, followed by the enzymatic digestion with the corresponding endonucleases. The results for a few representative cases obtained from the analysis of the two polymorphisms are presented in Figure 1.
As observed in Figure 1a, cases 3, 6, 8 and 27 have the homozygous 677CC genotype, cases 4 and 12– homozygous 677TT genotype and cases 5, 7 and 14 – heterozygous 677CT genotype.

For the polymorphic site at 1298 (Figure 1b), cases 8, 14 and 27 have the homozygous AA genotype, case 3 and 7 – homozygous CC genotype and cases 4, 5, 6 and 12 – heterozygous AC genotype.

The allele frequencies of MTHFR C677T and A1298C for the cases with spontaneous abortion and controls are listed in Table 1. The frequencies of C and T alleles were 0.56 and 0.44 for the cases of spontaneous abortion group and 0.65 and 0.35 for healthy control group, respectively. The frequencies of A and C alleles were 0.65 and 0.35 for the spontaneous abortion group and 0.70 and 0.30 in healthy control, respectively. The allele frequencies were not significantly different between the groups (Table 1).

The genotype frequencies we obtained were consistent with the Hardy-Weinberg equilibrium. The genotypes (CC, CT and TT) for the MTHFR C677T polymorphism in the patients with spontaneous abortions had the following frequencies: 35% CC, 43% CT and 22% TT.

The corresponding frequencies among controls were 41.3, 46.6 and 12%. The comparative analysis of the allelic frequencies observed in the two groups revealed statistically significant differences (p = 0.034) (Table 2).

The frequencies of MTHFR A1298C genotypes (AA, AC, and CC) among case women were 37, 56, and 7%, respectively. The corresponding frequencies among controls were 46.7, 45.3, and 8%. There were no significant differences in genotype frequencies between the two groups (Table 2).

We compare also the genotype frequencies between MTHFR 677CC, CT, TT and MTHFR 1298AA, AC, CC between women with spontaneous abortion and control group. The results are presented in Table 3.

Our study revealed that only 2 of 100 cases of women with spontaneous abortion had the wild type combined MTHFR genotype (677CC/1298AA) as compared to the control group in which 18 of 150 individuals had 677CC/1298AA genotypes (P=0.0043) (Table 2). Analysis of combined MTHFR genotypes revealed an increased prevalence of 677CC/1298AC genotypes in the spontaneous abortion group compared with the control group (p=0.018).

All possible combined MTHFR genotypes were represented in the control group. In the cases of women with spontaneous abortion: the MTHFR 677CT/1298CC, the 677TT/1298AC and 677TT/1298CC genotypes were absent.

The frequency of the 677TT/1298AA combined genotype was higher in women with spontaneous abortions (22%) compared to the frequency observed in women in the control group (8.66%), which proves the correlation with the risk of miscarriage (p = 0.002). The combinations between the 677CT/1298CC and
677TT/1298CC genotypes were not noticed in the patients with spontaneous abortions, but they were noticed in the control group, although with a low frequency (p=0.154) (Table 3).

**Discussions**

Early pregnancy loss is one of the fundamental problems of modern obstetrics. Miscarriage is the spontaneous termination of pregnancy before 24 weeks of gestation.

Miscarriages occur in approximately 20% of pregnancies (1). Fetal demise can occur either during the embryonic development (up to 12 weeks of gestation) or in the fetal period (second and third trimester of pregnancy) Miscarriages in the first trimester and the beginning of the second trimester, Occurs in 15-25% of all clinically diagnosed pregnancies. The risk of miscarriage in the first pregnancy is estimated to be 10%, while for the second pregnancy 24%, for the third pregnancy 26% and for the fourth pregnancy 32% (34,35). The first trimester of pregnancy has highest rate of spontaneous abortions (36,37). The exact mechanisms involved in the cause of spontaneous abortion can not always be specified. In the first trimester of pregnancy the main factor responsible for early pregnancy loss is the genetic one followed by anatomic, immunologic factors and coagulation defects, and endocrine and infectious factors (38,39).

In recent years many studies have examined the association between MTHFR gene polymorphisms and the risk of predisposition to spontaneous abortions (15,40 - 51). Some of them have reported an association of these polymorphisms with spontaneous abortions (15,40- 45) and others have found no correlation (46-51). In this study the distribution of allelic and genotypic frequencies for C677T polymorphism in the 2 groups, case and

<table>
<thead>
<tr>
<th>MTHFR allele</th>
<th>Spontaneous abortion group alleles (%)</th>
<th>Control group alleles (%)</th>
<th>Odds ratio (95% CI)</th>
<th>χ²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>677C</td>
<td>113 (56.5)</td>
<td>194 (64.66)</td>
<td>0.71 (0.492 -1.024)</td>
<td>3.377</td>
<td>0.066</td>
</tr>
<tr>
<td>677T</td>
<td>87 (43.5)</td>
<td>106 (35.34)</td>
<td>1.41 (0.977 -2.032)</td>
<td>3.377</td>
<td>0.066</td>
</tr>
<tr>
<td>1298A</td>
<td>130 (65)</td>
<td>208(69.33)</td>
<td>0.82 (0.562 - 1.202)</td>
<td>1.029</td>
<td>0.310</td>
</tr>
<tr>
<td>1298C</td>
<td>70 (35)</td>
<td>92 (30.66)</td>
<td>1.22 (0.832 - 1.781)</td>
<td>1.029</td>
<td>0.310</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MTHFR genotype</th>
<th>Spontaneous abortion group (%)</th>
<th>Control group (%)</th>
<th>Odds ratio (95% CI)</th>
<th>χ²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>677CC</td>
<td>35 (35)</td>
<td>62 (41.3)</td>
<td>0.76 (0.453 -1.291)</td>
<td>1.014</td>
<td>0.314</td>
</tr>
<tr>
<td>677CT</td>
<td>43 (43)</td>
<td>70 (46.6)</td>
<td>0.86 (0.518 -1.435)</td>
<td>0.326</td>
<td>0.568</td>
</tr>
<tr>
<td>677TT</td>
<td>22 (22)</td>
<td>18 (12)</td>
<td>2.07 (1.045 -4.095)</td>
<td>4.464</td>
<td>0.034</td>
</tr>
<tr>
<td>1298AA</td>
<td>37 (37)</td>
<td>70 (46.7)</td>
<td>0.67 (0.4 - 1,126)</td>
<td>2.290</td>
<td>0.130</td>
</tr>
<tr>
<td>1298AC</td>
<td>56 (56)</td>
<td>68 (45.3)</td>
<td>1.53 (0.922 - 2.554)</td>
<td>2.731</td>
<td>0.098</td>
</tr>
<tr>
<td>1298CC</td>
<td>7 (7)</td>
<td>12 (8)</td>
<td>0.87 (0.329- 2.28)</td>
<td>0.085</td>
<td>0.770</td>
</tr>
</tbody>
</table>
control, are similar to those reported by Zetterberg (52). The present study demonstrates that women with spontaneous abortion have a higher proportion of the 677TT genotype compared with control group. Bae et al (53) have shown that the 677CC genotype have a risk of miscarriage that does not coincide with those determined by Zetterberg (52) and with the one obtained in the present study. These conflicting results may be due to different technical methods, distribution of alleles in different populations or different sizes of the control and study groups.

The difference may also be due to the contribution of the environmental factors, such as diet.

The decreased fetal associated with mutations in the MTHFR gene was detected by Nelen who demonstrated that maternal homozigosity for 677T mutation increases up to 2-3 times the risk of recurrent spontaneous abortion (15).

As for the A1298C polymorphism, the distribution of allelic and genotypic frequencies do not differ significantly between the study and control group, except the 1298AC heterozygous genotype that had a higher frequency in the group of women with spontaneous abortion compared to the control group.

The examination of the combined genotypes showed that all possible combinations of allele MTHFR gene have been found in the control group. In the study group, the 677CT/1298CC, 677TT/1298AC and 677TT/1298CC genotypes were absent, while Zetteberg and Bae did not identify these polymorphisms in either group (control and study) (52,53). These genotypes had a low frequency in populations and cause an increased risk of miscarriage (54).

The frequency of the combined 677CC/1298 AC genotypes is higher in the study group compared to the control and the 677CC/1298AA combined genotype was observed in 2 of the 100 samples from women with miscarriages compared with 18 of the 150 control group. Similar results were obtained also by Zetterberg et al who examined the distribution of C677T and A1298C polymorphisms in 80 samples of fetal tissue, from miscarriages between 6-20 gestational weeks. These samples were compared with 125 samples taken from healthy subjects. Only one of the 80

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Spontaneous abortion group (%)</th>
<th>Control group (%)</th>
<th>Odds ratio (95% CI)</th>
<th>( \chi^2 )</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC/AA</td>
<td>2 (2)</td>
<td>18 (12)</td>
<td>0.15 (0.034 -0.66)</td>
<td>8.152</td>
<td>0.004</td>
</tr>
<tr>
<td>CC/AC</td>
<td>26 (26)</td>
<td>24 (16)</td>
<td>1.84 (0.988 -3.445)</td>
<td>5.538</td>
<td>0.018</td>
</tr>
<tr>
<td>CC/CC</td>
<td>7 (7)</td>
<td>20 (13.33)</td>
<td>0.49 (0.199 -1.204)</td>
<td>2.498</td>
<td>0.114</td>
</tr>
<tr>
<td>CT/AA</td>
<td>28 (28)</td>
<td>28 (18.66)</td>
<td>1.69 (0.931 -3.086)</td>
<td>3.007</td>
<td>0.082</td>
</tr>
<tr>
<td>CT/AC</td>
<td>17 (17)</td>
<td>39 (26)</td>
<td>0.78 (0.404-1.521)</td>
<td>2.796</td>
<td>0.094</td>
</tr>
<tr>
<td>CT/CC</td>
<td>0</td>
<td>3 (2)</td>
<td>-</td>
<td>2.024</td>
<td>0.154</td>
</tr>
<tr>
<td>TT/AA</td>
<td>22 (22)</td>
<td>13 (8.66)</td>
<td>2.97 (1.418 -6.229)</td>
<td>8.859</td>
<td>0.002</td>
</tr>
<tr>
<td>TT/AC</td>
<td>0</td>
<td>2 (1.33)</td>
<td>-</td>
<td>1.344</td>
<td>0.246</td>
</tr>
<tr>
<td>TT/CC</td>
<td>0</td>
<td>3 (2)</td>
<td>-</td>
<td>2.024</td>
<td>0.154</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
samples from the spontaneous abortions group presented 677CC/1298AA normal genotype, compared with 19 of the 125 people in the control group. The conclusion was that the presence of one or more mutant alleles in the MTHFR gene may affect the process of embryogenesis when the folate concentration is low (52).

The genotype composed of all four mutations is considered nonviable. The present study showed an association between the 677CT/1298CC or 677TT/1298CC combination and the risk of miscarriage. Isotalo et al. obtained also from fetal samples a high percentage of genotypes that had three or four mutations (55). Volcik et al. presented a series of data supporting the hypothesis that the 677CT/1298CC combination does not affect the fetal viability (56). These inconsistencies justify the need for additional studies to prove the association of MTHFR gene polymorphisms with the risk of miscarriage.

In conclusion, our findings indicate that the polymorphisms of the MTHFR gene, involved in the metabolic pathway of the folate may be a cause of the complications appeared in cases of miscarriage. The effect can be explained by the potential of the polymorphisms to change the homocysteine status that affects the hemostatic balance.

Acknowledgements

The authors state that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Abbreviations

PCR- Polymerase Chain Reaction
RFLP- Restriction Fragment Length Polymorphism
MTHFR- Methylene tetrahydrofolate Reductase
MTHFD1- Methylene tetrahydrofolate Dehydrogenase
MTR- Methionine Synthase
MTRR- Methionine Synthase Reductase
RFC1- Reduced Folate Carrier
CBS- Cystathionine β-Synthase

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