Evaluation of homocysteine, folic acid and methylenetetrahydrofolate reductase C677T gene polymorphism associated with recurrent spontaneous miscarriage

Abdul-Hussein Moyet M. Al-Faisal, *Saad Mohamed Nada, **Rasha Abdull-Hussein Mahood
Institute of Genetic Engineering/ Baghdad University
*Biotechnology Research Center/ AL-Nahrian University
**College of Science/Baghdad University

Abstract
This study was aimed to assess the association between evaluation of homocysteine level, folic acid and methylenetetrahydrofolate reductase MTHFR C677T gene polymorphism and recurrent pregnancy loss RSA. Sixty four women diagnosed with RSA and 41 normal fertile female, who had at least one or more child, 21 of them were pregnant women while 20 were non-pregnant. The level of homocysteine was high in women with RSA (11.41±3.75) (p<0.05) compared to normal fertile women either pregnant (7.02±2.13) or non-pregnant (6.84±2.08), low levels of folic acid was observed (6.35±2.09) in women with RSA (p<0.05) compared to fertile women either pregnant(10.08±3.08) or non-pregnant(11.27±3.15). No statistically significant difference was observed in the distribution of genotypes between cases and controls for MTHFR polymorphism. In conclusion, the present study reveals to lack of association of MTHFR C677T polymorphism in RSA women.

Introduction
Recurrent Spontaneous Abortion RSA represents a clinically challenging problem in obstetric practice and affects up to 2% of women in the reproductive age group. Though several etiological factors have been proposed, in approximately 50% of these cases, the underlying Pathophysiological mechanisms remain undetermined (1). Pregnancies require an even balance of coagulation and fibrinolysis in order to
avoid excess fibrin accumulation in placental vessels and inter villous spaces as well as to secure fibrin polymerization and stabilization of the placental basal plate (2). Deficiency in the homocysteine metabolism pathway resulting in an elevation of homocysteine level in plasma (hyperhomocysteinemia) has been regarded as a cause of Thrombophilia (3). Methylene tetrahydrofolate reductase MTHFR is one of the main regulatory enzymes in the metabolism of homocysteine that catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (4). Mutations in MTHFR gene lead to decrease activity of enzyme and hyperhomocysteinemia, which induces platelet aggregation through promotion of endothelial oxidative damage (5). Although several mutations within the MTHFR gene were described, C677T and A1298C mutations are the two most common mutations (6). C677T transition is a missense mutation in the exon 4 of this gene, which converts an alanine to a valine codon (at codon 222) in the N-terminal catalytic domain of the protein leading to a thermostable protein, with decrease enzymatic activity (7). A strong association between MTHFR genetic polymorphisms with RSA was confirmed by some researchers, whereas others denied this association (6, 8, 9). In this study, we attempt to establish an association between homocysteine, folic acid levels and the C677T polymorphism in MTHFR gene with the occurrence of RSA in Iraqi population.

Materials and methods

Study subjects

Patients

The study was carried out in the laboratories of biotechnology research center/AL-Nahrain University. Sixty four women who had previous history of recurrent spontaneous abortion attending some private clinics of gynecology in Baghdad, defined as three or more consecutive miscarriages, were selected in this study. The women had experienced three to five miscarriages, all miscarriages had taken place during the first trimester (weeks 1-12 of pregnancy). Any patients with past or present medical disorder associated or not associated with pregnancy were excluded from the study.

Control

A positive control group includes 21 normal pregnant women, who had at least one or more child. A negative control consist of 20 fertile non pregnant with normal menstrual cycle and were not affected by pre-existing clinical conditions.

Blood sampling

Five milliliters of blood were collected by vein puncture from all patients and control groups during the period between Januarys to December 2014. Each collected blood sample was dispensed into EDTA tubes for molecular studies and whole blood to obtained serum for homocysteine and folic acid measurement.

Determination of homocysteine and folic acid by ELISA kit

Assessment of homocysteine based on enzyme linked immunosorbent assay (ELISA) technology (Abnova kits®, Taiwan). Folic acid assay
based on the same technology using another kit (Cusabio/Germany).

**Isolation of genomic DNA**

Genomic DNA was isolated from blood cells under aseptic condition according to the protocol described by iNtRON Biotechnology: Company for wizard genomic DNA purification kit. The estimation of DNA concentration was done using Nanodrop software. The nucleic acid concentration and purity ratios were automatically calculated by the software.

**Detection of MTHFR C677T gene polymorphism by using ARMS-PCR**

**ARMS analysis**

ARMS-PCR (Amplification Refractory Mutation System) allows the characterization of point mutations directly by the presence or absence of amplification using allele specific primers. For the diagnosis of specific point mutation, a pair of allele-specific primers one of which has its 3’ terminal nucleotide complementary to the point mutation (Mt ARMS primer) and other to the normal DNA sequence (N ARMS primer) was used as shown in (Table 1)

**Table (1): Primer pairs used for screening of MTHFR C677T mutation by ARMS-PCR**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer sequence</th>
<th>Size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR C677T</td>
<td>CommonF:CCCAGCCACTCACTGTTTTAGTTCCAGGC</td>
<td>407</td>
</tr>
<tr>
<td></td>
<td>CommonR:GGTGAGAGTGGGGTGAGGAGGAGCTTAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C: CAAAGAAAAGCTGCTGATGATGAAATAGG</td>
<td>273</td>
</tr>
<tr>
<td></td>
<td>T: TTGAAGGAGAAGGTGTCTGCGGCGGT</td>
<td>190</td>
</tr>
</tbody>
</table>

**Primer-PCR program**

The optimization of amplification was performed under the conditions listed in (Table 2).

**Table (2): Optimization of PCR conditions for MTHFR gene**

<table>
<thead>
<tr>
<th>Name of cycle</th>
<th>Temperature °C</th>
<th>Time</th>
<th>No. of cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>5 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>25 sec</td>
<td>10 cycle</td>
</tr>
<tr>
<td>Annealing</td>
<td>60.4</td>
<td>30 sec</td>
<td>33 cycle</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>25 sec</td>
<td>35 cycle</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>10 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Soak</td>
<td>4</td>
<td>&amp;</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

The PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide.
**Statistical analysis:** Statistical analysis was performed using SPSS version 13. The genotype distributions of mutation, the frequency of heterozygous and homozygous were compared between patients and controls using Pearson’s Chi-square test. The $P$ value of <0.05 was regarded as significant.

**Results and discussion**

**Determination of homocysteine and folic acid concentration**

In the current study, the level of homocysteine was high in women with RSA (11.41±3.75) with significant differences compared to normal fertile women either pregnant (7.02±2.13) or non-pregnant (6.84±2.08). Low levels of folic acid was observed (6.35±2.09) in women with RSA with statistically significant difference compared to fertile women either pregnant(10.08±3.08) or non-pregnant(11.27±3.15) as shown in (Table 3).

**Table (3): levels of homocysteine and folic acid in women with RSA and controls**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Homocysteine(µmol/L) (Mean±SD)</th>
<th>Folic acid (ng/ml) (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSA</td>
<td>A 11.41±3.75</td>
<td>A 6.35±2.09</td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>B 6.84±2.08</td>
<td>B 11.27±3.15</td>
</tr>
<tr>
<td>Normal pregnant</td>
<td>B 7.02±2.13</td>
<td>B 10.08±3.08</td>
</tr>
<tr>
<td>LSD</td>
<td>2.62</td>
<td>2.70</td>
</tr>
</tbody>
</table>

Hyperhomocysteinemia HHCY has been underlined as an emerging risk factor for several diseases such as arterial and venous thrombosis(10), adverse pregnancy outcome (11), congenital malformations (12) and vascular dementia (13). Inherited and acquired conditions have been involved to explain pathophysiology of HHCY such as gene polymorphisms as cystationin beta synthase CBS or methylenetetrahydrofolate reductase (MTHFR) (14) folate and vitamin B6/B12 deficiencies due to disregulation of their normal metabolism and low dietary intake (15). Maristella et al. (16) were found that women with RSA showed HHCY compared to control group total plasma homocysteine concentration was 19.2 ± 6.14 µM for patients with recurrent pregnancy loss, while was 21.05 ± 8.78 µM for patients with unexplained sterility and 7.85 ± 3.31 µM of control group (p < 0.05).In another study also, it has been found that the mean homocysteine level was 10.8±4 Umol/L in patients with primary unexplained first trimester repeated pregnancy loss who were admitted for termination of pregnancy due to early pregnancy failure, while homocysteine level in healthy pregnant women in the first
trimester with no history of bad obstetric outcome and had at least one living newborn was 7.9 ± 4.3 Umol/L with statistical significant difference between the 2 groups (p < 0.01). It suggests that high level of HHCY may contribute for the etiology of first trimester unexplained recurrent early pregnancy loss (17). In this study, serum folic acid levels were low in women with RSA compared to normal non-pregnant and pregnant women with no statistical differences between them. Data from a large-scale folic-acid intervention study conducted in China provided strong evidence that periconceptional folic acid use does not increase miscarriage rates (18). The study showed that, the miscarriage rate was 9.0% for women who took folic acid alone (400g) and 9.3% for women who did not take folic acid during early pregnancy, the strengths of this study include the fact that the investigation was larger than all of the previous studies combined (19, 20). The association between folate status and the occurrence of miscarriage was further evaluated in a large study in Sweden (21). Cases were women who had spontaneously aborted a fetus with a gestational age of 6–12 weeks and controls were women matched for gestational age of the fetus. Women with low plasma folate concentrations (4.9 nmol/L) were more likely to have had a miscarriage than women with plasma folate concentrations between 5.0 and 8.9 nmol/L. The occurrence of miscarriage was not increased in women with higher plasma folate concentrations (14.0 nmol/L) relative to women with plasma folate concentrations between 5.0 and 8.9 nmol/L. Other studies reported that the mean folic acid was 8.7 ± 2.1 ng/ml in patients with primary unexplained first trimester repeated pregnancy loss who were admitted for termination of pregnancy due to early pregnancy failure and 10.8 ± 2.2 ng/ml in healthy pregnant women in the first trimester with no history of bad obstetric outcome and had at least one living newborn, with statistical significant difference between both groups (p < 0.01) they concluded that high level of HHCY and low level of folate and vitamin B12 may contribute for the etiology of first trimester unexplained recurrent early pregnancy loss (17).

Detection of MTHFR C677T gene polymorphism by ARMS-PCR

The primers used as described in two studies by Lajin et al. (22) and Elik et al. (23). Concerning the MTHFR gene polymorphism, we found no significant differences in genotype distribution for the MTHFR gene mutation between patients with RSA and healthy controls (Table 4 and Figure 1).
Table (4): MTHFR genotype frequencies with RSA and normal fertile women

<table>
<thead>
<tr>
<th>MTHFR C677T gene polymorphism</th>
<th>Genotype frequencies</th>
<th>Women with RSA n=64</th>
<th>Normal fertile women n=41</th>
<th>$X^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/C Wild-type</td>
<td></td>
<td>51(79.68%)</td>
<td>33(80.40%)</td>
<td>0.010</td>
<td>0.920</td>
</tr>
<tr>
<td>C/T Heterozygous</td>
<td></td>
<td>11(17.18%)</td>
<td>8(19.51%)</td>
<td>0.091</td>
<td>0.763</td>
</tr>
<tr>
<td>T/T Homozygous</td>
<td></td>
<td>2(3.12%)</td>
<td>0.0</td>
<td>1.306</td>
<td>-</td>
</tr>
</tbody>
</table>

MTHFR is one of the most frequently studied thrombophilic genes controversially suspected to be associated with RSA (24). The frequency of the MTHFR gene mutation differs among ethnic populations. C677T polymorphism is a missense mutation in the exon 4 of MTHFR gene, which converts an alanine to a valine codon (at codon 222) in the N-terminal catalytic domain of the protein leading to a thermolabile protein, with decreased enzymatic activity (25).

Figure (1): Agarose gel electrophoretograms showing MTHFR C677T SNP genotyping by ARMS-PCR, lanes (1, 2, 4, 5 and 6) showing normal (wild type) genotype CC, lane 3 showing heterozygous mutant CT.
For the MTHFR C677T polymorphism of the patients analyzed 79.68% had C/C genotype, 17.18% were C/T and 3.12% were T/T. Among the controls 80.40% were C/C.19.51% were C/T and 0% were T/T. There is no significant difference in the prevalence of 677T/T genotype among women with RSA and healthy controls. Some studies reported that there is no statistically significant differences in the frequency of MTHFR gene mutations between the patients with recurrent pregnancy loss and normal healthy controls (26-32). Other studies reported no association between MTHFR polymorphisms and RPL include those done among Jewish women (33, 34), Austrian women (35), German women (36), Lebanese women (37), Spanish women (38), and Indian women (39). There are a number of retrospective and case-control studies demonstrating a higher frequency of MTHFR mutations in patients with recurrent pregnancy loss (40-42). We conclude that there is no association between MTHFR C677T mutation and the occurrence of recurrent miscarriage.

References


