

Study the Correlation Between Polymorphism of MTHFR Thrombophilic Genes and Pregnancy Loss in Ahvaz City

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Occurrence of various pregnancy losses is one of the most common problems associated with gynecology and obstetrics. It is usually known as a multi-factoral and complicated disease. One of the danger factors diagnosed so far is thrombophilic disorder. The present study seeks to investigate the correlation between polymorphism of MTHFR thrombophilic genes and repetitive pregnancy losses in Ahvaz city. This study was conducted on 114 women afflicted with autonomous pregnancy loss resorting to Nour and Narges laboratories. One-hundred and fourteen women without any past pregnancy losses were selected for control group. DNA was extracted and presence or absence of polymorphism of MTHFR thrombophilic genes (A1298C and C677T) were studied. The results of the study showed no significant difference between people with TT/677CT genotypes in women who had experienced several pregnancy losses and those in the control group ($P = 0.069$). However, a significant difference was observed between women with several pregnancy losses and control group in terms of CC/1298AC genotypes ($P = 0.000$). The present study has found a significant relationship between MTHFR gene polymorphism (A1298) and several pregnancy losses, but no significant relationship was observed between MTHFR gene polymorphism (C677T) and repetitive pregnancy losses.

Keywords: MTHFR thrombophilic genes, Polymorphism, Pregnancy loss, Ahvaz, Iran.

The most common phenomenon during the first and second two months of pregnancy is pregnancy loss¹. Pregnancy loss is defined as the end of pregnancy before the 20th week. Women who have experienced consecutive pregnancy loss more than twice are referred to as suffering from recurrent pregnancy loss (RPL)². Recurrent

pregnancy loss is one of the most common problems associated with pregnancy and roughly 2% of women suffer from it³. It is usually known as a multi-factoral and complicated disease and various danger factors such as genetics, infections, anatomical, hormonal and immune problems are involved in it⁴. Although the cause of this disease may remain unknown in some cases, about 55% of the patients are report to be afflicted with thrombophilic disorders⁵.

The correlation between recurrent pregnancy loss and thrombophilia is a challenging

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issue about which various studies have been conducted with contradictory results about its confirmation or rejection. Various studies have been conducted over recent years about the mutation of thrombophilic genes in women afflicted with RPL.

Some of these studies have investigated the relationship between mutation of thrombophilic genes and RPL. Although some studies have drawn a link between these two, others have rejected any such relationship. The different results achieved in these studies might be due to the different definitions of recurrent pregnancy loss and also different criteria for choosing the sample and geographic distribution.

MTHFR enzyme plays a major role in the metabolism of Folate-Methionine and Homocysteine. Plasma Homocysteine is a potentially poisonous amino acid. Her poisonous effects will be neutralized when it turns into Methionine amino acid¹⁰. Due to its negative pathologic effects on the Endothelium of Angiotensin vessels and V and VII coagulation factors, high levels of Homocysteine result in the rise of thrombin level, accumulation of Platelets and, consequently, venous thrombosis¹¹⁻¹². Reduction of MTHFR enzyme activity lowers the substrate for methionine synthetase. Consequently, production of methionine stops and we will have a rise in the level of homocysteine¹³. Polymorphisms (such as A1298C and C677T) of individual nucleotides in MTHFR encoding gene is considered to be one of the major factors which cause thrombophilia¹². Considering the results achieved thereby, we may say that the mutation of the above said thrombophilic genes may play a major role in raising the danger of recurrent pregnancy loss among the Iranian women. The present research was conducted to study the influence of polymorphism of MTHFR thrombophilic genes on the potential of RPL among women in Ahvaz city.

The present research was carried out in order to evaluate the prevalence of thrombophilic genes mutation among the women with RPL in Ahvaz as well as compare the relationship between A1298C and C677T with recurrent pregnancy loss using PCR-RFLP method in the case and witness group.

MATERIALS AND METHODS

Samples collection and criteria of study

The samples in this study were 114 women with RPL and the average age of 30.77 ± 5.33 with 2 or more abortions before the 20th week. They didn't have any womb anatomical problems, cytogenetic problems, hormone problems, and sperm disorders. The number of the patients was determined using Allele frequency and other researches conducted in this field. All the samples had resorted to Nour and Narges medical-genetic laboratories from 2014 to 2015. On the other hand, 114 women with at least one successful delivery and no records of abortion were chosen as witnesses with the average age of 30.935.70. After giving them the necessary information and obtaining 5 ml blood from the samples and witnesses and storing them in pipes containing EDTA, blood samples were sent to lab for molecular tests.

Entrance to the study

Patient group: 2 or more than 2 times of recurrent pregnancy loss.

Witness group: with at least one successful pregnancy.

Leaving the study

Patient group: records of underlying disease (diabetes, thyroid disease, hyperprolactinemia, antiphospholipid syndrome), womb anatomic disorders (congenital or acquired), ovarian surgery, spouse's sperm disorders.

Witness group: records of abortion.

Genetic analysis

Through the saturated salt standard method, genomic DNA was extracted from blood leukocytes. The following steps were taken in the saturated salt method:

- 1- Rinsing the blood twice with sterilized water for lysis of red blood cells
- 2- Rinsing with solution 1 (tris, HCl, sucrose, MgCl₂, triton 100) in order to eliminate the red blood cells
- 3- Using solution 2+SDS+ Proteinase K for the lysis of white blood cells
- 4- Using saturated salt for sedimentation of DNA
- 5- Watering DNA with 70% and 90% ethanol

Then, PCR reaction was conducted according to the following schedule (table 1) an in

accordance with AB-ANALITIKA kit protocol with a volume of 24 l comprised of 20 l PREMIX, 2.5 l OLIGOMIX and 1 l genomic DNA. It must also be remembered that PREMIX and OLIGOMIX were also available in the kit. After the PCR reaction was over, STRIP painting was carried out according to table 2.

After final rinse stage, the strips were dried and placed on the special paper of the kit which marks mutations. The mutations in each patient were determined based on a comparison with the mutations mentioned in the protocol.

Statistical analysis

The observations were fed to SPSS (ver. 22) software. Distribution of the genotypes of each mutation and the Homozygote and Heterozygote frequency in both groups was studied using Pearson's chi-square. The significance level was set to the P-Value of less than 0.05. P-Value and odd's ratio for people with RPL were measured.

RESULTS AND DISCUSSION

The present research studied the frequency of C677T and A1298C genes polymorphism in 114 women with RPL. We also studied RPL and its relative frequency among 114 women without any abortion who had at least one successful delivery. The results of studying C677T are represented in table 3.

The following ratios were recorded for the group with RPL and the witness group respectively:

57.9% vs. 70.2% for CC genotype, 32.5% vs. 26.3% for CT genotype, and 9.6% vs. 3.5% for TT genotype. No significant relationship was observed between the case and witness groups in terms of the polymorphism of this gene and recurrent pregnancy loss (P=0.069).

The results of studying A1298C are represented in table 4. The following ratios were recorded for the group with RPL and the witness group respectively: 26.3% vs. 50.9% for AA genotype, 42.1% vs. 0.0% for AC genotype, and 31.6% vs. 49.1% for CC genotype. A significant relationship was observed between the case and witness groups in terms of the polymorphism of this gene and recurrent pregnancy loss. The frequency of CC and AC genotypes in group with RPL is significantly more than what is observed in the witness group (P=0.000).

Several studies have been conducted in recent years in order to study the mutation of

Table 1. PCR conditions and steps used for amplification of gene targets

Number of cycles	Temperature	Duration
1	95	5 min
10	95 61	20 s 2 min
25	95	25 s
	58	40 s
	72	40 s
1	72	5 min
hold	10	

Table 2. Conditions used for STRIP painting

Step	Required reagents	Condition of incubation	Incubation time
1-Denaturation of amplification product	10 µL amplification product + 20 DEN	Room temperature	5 min
2-Hybridization	HYB-2 (prewarmed to 46)	Shaking at 46	30 min
3-Stringent washing	CON-D2 (prewarmed to 46)	Shaking at 46	10 min
4-Incubation with Streptavidine-AP conjugate	Diluted CON (0.5 CON + 1 ml prewarmed (46)	Shaking at 46	30 min
4-Rinse	RIN	Shaking at room temperature	2 min
5-Rinse	RIN	Shaking at room temperature	2 min
6-Staining	STAINING SOLUTION (dissolve 1 NBT/BCIP tablet in 10 mL distilled)	Shaking IN DARK at Room temperature	10-12 min
7-Staining stop	STOP	Shaking at Room temperature	2 min
8-Final rinse	Dstilled	Shaking at Room temperature	2 min

Thrombophilic genes among women afflicted with RPL. Some of these studies have pointed out to the relationship between the mutation of Thrombophilic genes and RPL, while no relationship was proved in the other studies. The various results achieved in these studies may be due to different definitions of RPL and differences in the criteria for choosing the sample and geographic distribution. Studying this phenomenon and linking it with RPL is still one of the attractive issues for the researchers. Several studies have been conducted about the polymorphism effect of MTHFR enzyme on the activity of enzyme and the relationship between it (as a factor of danger) with RPL.

In the present research, the frequency of the mutated genotypes (CT and TT) of C677T polymorphism (table 1) showed no significant relationship with RPL. The result of this study on C677T was in line with the researches conducted by Zenouri³³ in the north-western parts of Iran, but they are not in line with the researches conducted by Torabi¹⁴, Eskandari³¹ and Behjati³². In a study conducted by Torabi *et al.* (2012), the relationship between MTHFR 1298AC and RPL was investigated and the end results were similar to the results we achieved. Abu-Asab *et al.* (2011) found no direct and specific relationship between the mutations and RPL. This is similar to the results of the present study about MTHFR C677T²⁵. The study conducted by Habibovic *et al.* (2011) found

no significant relationships in terms of MTHFR C677¹⁸. Farah Idali *et al.* (2010) arrived at the conclusion that a significant relationship was observed between 1298 polymorphism, MTHFR 677 and RPL among the Iranian women²⁶. The study conducted by Gonca (2009) found a significant relationship between MTHFR C677 and RPL. However, no significant relationship was observed in the case of A1298C whose result is not in line with the present research³. A significant relationship was observed in the studies of Goodman (2006) and Gertrud Unfried (2002)²⁷, while Foka (2000) found no explicit relationship about this issue¹⁷. A specific relationship between these polymorphisms and RPL was rejected in the studies conducted by Vettrisevi (southern India, 2008), Makino (Japan, 2006), and Wiwanit kit (meta-analysis in Thailand, 2005)³¹.

These contrasting results suggest that the role of A1298C and C677T in RPL is probably influenced by the absorption of Folate in the diet and geographic and racial factors and the final phenotype is the result of the interaction between these factors. This mutation is probably tolerated in people who are rich in Folate. However, in people with a low absorption level of Folate, this mutation may result in clinical or biochemical phenotypes. Thus, the discrepancy in the level of Folate in the diet can explain the discrepancy due to the influence of MTHFR polymorphism on RPL in various societies. As the studies indicate, low

Table 3. Genotype frequency and studying the level of significance for C677T polymorphism in the case and witness group

Genotype	Genotype frequency in the witness group	Genotype frequency in the patient group	Genotype frequency in all those studied	P-Value
A/A	50.9% (58)	26.3% (30)	38.6% (88)	0.000
A/C	49.1% (56)	42.1% (48)	40.6% (104)	0.000
C/C	0.0% (0)	31.6% (36)	15.8% (36)	0.000

Table 4. Genotype frequency and studying the level of significance for A1298C polymorphism in the case and witness groups

Genotype	Genotype frequency in the witness group	Genotype frequency in the patient group	Genotype frequency in all those studied	P-Value
A/A	50.9% (58)	26.3% (30)	38.6% (88)	0.000
A/C	49.1% (56)	42.1% (48)	40.6% (104)	0.000
C/C	0.0% (0)	31.6% (36)	15.8% (36)	0.000

levels of Folate in the foods of the Indians and African communities compared to western and European societies make the former prone to some problems such as nervous pipe disorders during the fetus development and compilation of Homocysteine and failure in the synthesis and recovery of DNA. Folic acid supplements may solve the problem for women. The other effective factor in describing the contradictory results in various communities or even the results of various studies in a population include (but are not limited to) the size and composition and geographic distribution and the race of the population studied. The results of the present study indicate that MTHFR C677T polymorphism in the population studied has no significant relationship with RPL, however, this is not the case with MTHFR A1298C polymorphism.

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