Original Research Article

Study of Association between MTHFR Polymorphism and Recurrent pregnancy loss

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Abstract:

Introduction: Recurrent pregnancy loss (RPL) is defined as two or more failed pregnancies. It is a multifactorial disorder like genetic disorders, endocrine dysfunctions, uterine pathologies, autoimmune diseases, acquired and inherited thrombophilia as well as environmental factors are major concern in gynecology. Mutations of MTHFR are most extensively stated thrombophilic mutants when evaluating patients suffering from RPL. The occurrence of mutations of MTHFR 677 was found to be with higher significant frequency in the women presented with RPL. **Materials & Methods:** A total of 50 women with RPL were selected as study group and 50 women were included as the control group. Blood samples were collected and DNA extraction was done and is subjected to PCR amplification. Statistical analysis was done using IBM SPSS Statistics 20 package. p-value of <0.05 is considered as statistically significant. **Results:** Compared with the MTHFR 677CC genotype, the MTHFR 677TT genotype was associated with a greater likelihood of RPL. Similar finding was observed with regard to the frequencies of the 2 genotypes and alleles of MTHFR A1298C also between the patient group and the control group. **Conclusion:** Overall, the present study shows that RPL is associated with MTHFR A1298C, C677T polymorphisms, but the relationship requires further in-depth analysis with inclusion of wide range of parameters.

Keywords: A1298C, C677T, MTHFR, recurrent pregnancy loss (RPL), single nucleotide polymorphism (SNP)

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Introduction:

Recurrent pregnancy loss (RPL) is defined as two or more failed pregnancies.[1] In general, it is the incidence of babies who die before 28 weeks of pregnancy. And babies who die at or after 28 weeks are stillbirths. It is a multifactorial disorder like genetic disorders, endocrine dysfunctions, uterine pathologies, autoimmune diseases, acquired and inherited thrombophilia as well as environmental factors are major concern in gynecology.[2-6] Pregnancy loss is among the most common obstetric complications and affects up to 15% of pregnancies. Recurrent pregnancy loss affects 4.2% of pregnancies and results in high medical, emotional, social, and economic burdens.[7-10] Many factors like lifestyle and environmental can also be considered for RPL occurrence. Gene polymorphisms may predispose to an increased risk of RPL without consideration of the effects of environmental factors.[11]

Mutations of MTHFR are most extensively stated thrombophilic mutants when evaluating patients suffering from RPL. The occurrence of mutations of MTHFR 677 was found to be with higher significant frequency in the women presented with RPL. MTHFR a key enzyme in folate and homocysteine metabolism catalyzes the biologically irreversible reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which provides the methyl group for the remethylation of homocysteine to methionine.[12] Several single nucleotide polymorphisms (SNPs) are observed in MTHFR gene such as the two most frequently observed C677T and A1298C, which is observed to affect folate and total homocysteine (tHcy) status. MTHFR C677T means substitution of thymine (T) in place of cytosine (C) at position 677, which results in change of alanine to a valine.

The C677T increases thermolability of MTHFR and causes impaired folate binding and reduced activity of the MTHFR enzyme. MTHFR C677T is associated with decreased concentrations of folate in serum, plasma, and red blood cells, and mildly increased plasma tHcy concentration.[13] With respect to the biological actions, MTHFR C677T can be considered as a candidate gene with a role for RPL. The objective of this study was to evaluate the risk of RPL according to maternal MTHFR polymorphisms (677 C>T and 1298 A>C) and other lifestyle risk factors.

Materials & Methods:

A total of 50 women with RPL who received perinatal care at the Department of Obstetrics & Gynecology were selected as study group and 50 women were included as the control group after obtaining informed consent. The study has got Institutional ethical clearance. Blood sample has been collected from the participants who have given consent as per the inclusion and exclusion criteria.

The criteria for inclusion of the control group were women with no history of spontaneous miscarriage and with at least one child with no birth complications. The RPL group comprised women who had a history of two or more spontaneous miscarriages or still births with a diagnosis of recurrent pregnancy loss.[1]

The exclusion criteria for the selection of the subjects were women with genital anomalies; couples found to be having any chromosomal defects; women under any teratogenic drugs therapy during early pregnancy; women having history of any pathological microbial infections of the genital tract and medical disorders like thyroid dysfunction, diabetes and Anti-phospholipid antibody syndrome which can be associated with pregnancy loss. None of the study and control group were given any folate supplementation as it may result in supply of folic acid which may alter the outcome of the pregnancy and may such interfere in the result findings.

The blood sample was collected from the cubital vein of each participant. It is centrifuged and the serum obtained was separated and stored for further analysis. All samples were analyzed at Central Laboratory, Dr.Patnam Mahender Reddy Institute of Medical Sciences. Total genomic DNA was extracted from blood sample by using Qiagen QIAcube Connect with QIAamp DNA mini QIAcube kit (QIAGEN India Pvt. Ltd.) as per the manufacturer's protocol.

The MTHFR C677T polymorphism was analyzed by Light Cycler® 96 polymerase chain reaction (PCR) (Roche Diagnostics India Pvt Ltd). Specific primer pairs were selected according to the related reference sequence (5'-TGA AGG AGA AGG TGT CTG CGG GA-3' as the upstream primer and 5'-AGG ACG GTG CGG TGA GAG TG-3' as the downstream primer for MTHFR C677T; 5'-CTT TGG GGA GCT GAA GGA CTA CTA C-3' as the upstream primer and

5'-CAC TTT GTG ACC ATT CCG GTT TG-3' as the downstream primer for MTHFR A1298C.[14,21`]

PCR reactions were performed in a final volume of 10µl containing 1 Light Cycler master mix hybridization probes from Roche Diagnostics, magnesium chloride (3.5 mmol/L), 677-primer mix (677F/677R, 0.5µmol/L of each), 1298-primer mix (1298F/1298R, 0.5µmol/L of each), 677-probe mix (677-HP1/677HP-2, 0.2µmol/L each), and 1298-probe mix (1298-HP1/1298-HP2, 0.2µmol/L each). Water (2µl; Roche) was added to make up the volume to 8µl per assay.

Template DNA (2µl, concentration as eluted from the extraction columns) was pipetted into Light Cycler capillaries (Roche) containing 8 µl of master mix. Each test run was done with a reagent blank, in which DNA template was replaced with PCR-grade water. Capillaries were capped, centrifuged briefly at 1000g and placed into the Light Cycler instrument.

The amplification protocol was as follows: one cycle of 30 seconds at 95°C followed by 45 cycles consisting of denaturation at 95°C for 4 minutes, annealing at 52°C for 10 seconds, and extension at 72°C for 10 seconds at a transition rate of 20°C/second. The fluorescence emitted was noted at the end of the annealing phase in each cycle.

All analyses were done with color compensation using Roche reagents. The test results from the Light Cycler PCR for both polymorphisms were confirmed by RFLP/PCR using restriction enzymes *Hin*fI and *Mbo*II (both from New England BioLabs, Boston, MA) as described previously.[14,15]

The digestion products were evaluated on an ethidium bromide-stained agarose gel (3% for MTHFR C677T, 4% for MTHFR A1298C) and then the genotype was determined. All the genotypes observed by electrophoresis method were evaluated for verification by DNA sequencing. Data were analyzed using the SPSS statistical package, version 20 (SPSS, Inc., Chicago, IL, USA).

A chi-square test was used to compare genotype and allele frequencies, with a P value <0.05 considered to be statistically significant. Odds ratios and 95% confidence intervals were calculated.

Results:

The genotype distributions of MTHFR C677T and MTHFR A1298C in all study subjects were tested according to the Hardy-Weinberg law of genetic equilibrium (P > 0.05). The association between the MTHFR C677T genetic polymorphism and RPL is observed in this study. The frequency of the MTHFR 677T allele was higher in the patient group than in the control group.

Compared with the MTHFR 677CC genotype, the MTHFR 677TT genotype was associated with a greater likelihood of RPL. Similar finding was observed with regard to the frequencies of the 2 genotypes and alleles of MTHFR A1298C also between the patient group and the control group (Table-1).

The frequency of the CT polymorphisms of the MTHFR C677T locus was statistically significant in the RPL group than in the control group (OR 3.64, 1.44–9.20; P=0.0064), and similar significant relation was found with TT genotypes also compared with the control group (OR 7.80, 2.03–29.98; P=0.0028).

The frequency of the AC polymorphism of the MTHFR A1298C locus in RPL patients was statistically significantly higher than that in the control group (OR 2.38,1.01–5.59; P=0.0459), which is similar to the finding of the CC genotypes in the RPL group were statistically significantly increased (OR 8.15, 1.56–42.44; P=0.0126).

Table 1: Genotype and allele distribution of MTHFR SNPs in RPL and normal women

SNP	Genotype	All Subjects (N=100)	Patients with RPL (N=50)	Control (N=50)	P value	OR (95%CI)
C677T	CC	36	10 (20%)	26 (52%)		
	CT	48	28 (56%)	20 (40%)	0.0064	3.64 (1.44–9.20)
	TT	16	12 (24%)	04 (8%)	0.0028	7.80 (2.03–29.98)
	C allele	120	48 (48%)	72 (72%)		
	T allele	80	52 (52%)	28 (28%)	0.366	1.08 (0.84–1.40)
A1298C	AA	45	16 (32%)	29 (58%)		
	AC	44	25 (50%)	19 (38%)	0.0459	2.38 (1.01–5.59)
	CC	11	09 (18%)	02 (4%)	0.0126	8.15 (1.56–42.44)
	A allele	134	57 (57%)	77 (77%)		
	C allele	66	43 (43%)	23 (23%)	0.0030	2.5256 (1.37–4.65)

Discussion:

The reason for recurrent pregnancy loss is multifactorial having possibilities of many reasons. Several studies across the globe have noted increased maternal age, obesity and change in healthy lifestyle habits such as smoking, alcohol use, sedentary life style are high risk factors for RPL.[16-19] Changes in genetic background of different ethnic groups is also found to be associated with occurrences of different allele frequencies, especially in the presence of disease conditions.[20] Furthermore, different studies have reported the occurrence of RPL is significantly related to occurrence of polymorphisms in MTHFR C677T and A1298C positions of the gene.

Currently, even though there are many studies which have studied the association between MTHFR C677T polymorphism and RPL, the observations remain controversial.[21-28] A study in 206 women with RPL and healthy women reported higher risk for RPL in individuals having the MTHFR 677T allele and the homozygous genotype 677TT.[28] In our study, upon the analysis of samples it has been observed that the frequency of the CT& TT alleles of the MTHFR 677 locus in the RPL group was also statistically significantly higher when compared to the control group, indicating that the MTHFR 677T allele is a risk factor for RPL, which is similar to the other studies.[28]

Contrary to this, some of the studies have observed the risk of RPL in women with genotype TT, CT was not significantly related to the control group.[23] This finding may be as a result of

insufficient sample size or may be due to some other factors such as supplementation of folic acid during the period of pregnancy. Therefore, the association of MTHFRC677T, A1298C and RPL cannot be confirmed and deserves a more in-depth studies with higher sample size. It has also been observed that there was no association between MTHFR A1298C polymorphism and RPL [26,27], other studies have reported that MTHFR 1298C allele is a risk factor for RPL.[23,25-27, 29-31] When analyzed genetic polymorphisms in women with RPL was analysed, it was found that the risk of developing RPL in women carrying the homozygous mutant genotype(CC) was increased.[30]

It is also observed that women with the mutated gene have an increased risk of miscarriage and other pregnancy complications.[31] It is also reported that the MTHFR C677T andA1298C mutant genotypes were present in healthy women, and not all carriers experienced recurrent pregnancy loss, indicating that the synergistic effect of multiple hereditary and nonhereditary thrombotic disorders maximizes the risk in women.[30] A study has showed that the estimated risk for RPL in patients carrying the compound heterozygous (677CT/1298AC) genotype increased4.86-fold compared with individuals with the wild-type (677CC/1298AA) genotype.[28]

Conclusion:

Overall, the present study shows that RPL is associated with MTHFR A1298C, C677T polymorphisms, but the relationship requires further in-depth analysis with inclusion of wide range of parameters. The chance of RPL in women having MTHFR677CT/1298AC compound genotype is observed to increase, and the incidence of RPL was highest in individuals carrying two MTHFR mutant genes, indicating that this gene can be used as a biological indicator to predict the occurrence of RPL and to provide guidelines for folic acid supplementation and other corresponding measures among women carrying relevant mutations to prevent pregnancy loss. In future gene-related research should include both women with RPL women and their partners as well as the spontaneously aborted embryos for a clearer and wide information.

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Conflict of interest: Nil

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