

Evaluation of Thrombophilic Genes in Recurrent Pregnancy Loss: A Case-control Study in Iranian Women

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ABSTRACT Although thrombophilia is associated with the etiology of recurrent miscarriage, the evaluation of specific inherited thrombophilic genes in women experiencing recurrent pregnancy loss remains controversial. This study compared the prevalence of four thrombophilic gene mutations among recurrent miscarriage and fertile control women. PCR-RFLP was performed to genotype four single nucleotide polymorphisms, *MTHFR* C677T, *MTHFR* A1298C, *FV* G1691A, and *FII* G20210A (causes of inherited thrombophilias) in both, case and control groups. About genotypes, there were statistically significant differences for mutant homozygote *MTHFR* A1298C genotype ($P=0.01$) between two cases and control groups, but other genotypes did not show any difference. About total allelic mutations, women experiencing recurrent pregnancy loss did not demonstrate more total mutations than control women ($P=0.644$). In conclusion, no association was observed between homozygous or heterozygous *MTHFR* C677T, *FV* G1691A, and *FII* G20210A mutants and pregnancy loss in Iranian women. However, the role of the homozygous *MTHFR* A1298C polymorphism should be investigated further.

INTRODUCTION

The American Society of Reproductive Medicine has defined RPL (Recurrent Pregnancy Loss) as ≥ 2 failed pregnancies (Medicine 2013). Some cases of recurrent miscarriage have a thrombotic basis (Coulam et al. 2006; Goodman et al. 2006; Coulam et al. 2008).

Thrombophilia is an important predisposition to blood clot formation, which can further result in abnormal implantation and spontaneous pregnancy loss (Ebrahimzadeh-Vesal et al. 2014). Of the most prevalent gene mutations causing inherited thrombophilia, mutation in genes encoding methylene tetrahydrofolate reductase (*MTHFR*), factor V (*FV*), and factor II (prothrombin) are important (Khan and Dickerman 2006; Miljanovi et al. 2010). *MTHFR* catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (to active folate) (Kolte et al. 2010). Deficiency of *MTHFR*

induces platelet aggregation by promoting endothelial oxidative damage (Liew and Gupta 2015). C677T transition, a missense mutation in the gene encoding *MTHFR*, at codon 222 converts alanine to valine in the N-terminal catalytic domain of the protein and decreases its enzymatic activity. A1298C transversion, another point mutation in the gene encoding *MTHFR*, is characterized by the substitution of glutamate by alanine at codon 429 in the C-terminal regulatory domain of the protein (Liew and Gupta 2015; Narayanan et al. 2004; Weisberg et al. 1998).

FV engages in co-agulation and is controlled by activated protein C (APC) (Pirhoushiaran et al. 2014). FV Leiden (FVL) is an autosomal dominant form of thrombophilia. It is characterized by the replacement of guanine by adenine at nucleotide 1691, which results in the replacement of glutamine by arginine at the cleavage site (Kujovich 2011). This single amino acid substitution makes FV resistant to APC. However, the risk of obstetric complications in homozygous carriers of FVL is unclear.

FII, a proenzyme of thrombin, is a vitamin K dependent glycoprotein that alters fibrinogen into fibrin. A common genetic mutation G20210A in the 3' untranslated region of the gene encoding FII increases plasma prothrombin levels (Pirhoushiaran et al. 2014). This autosomal re-

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cessive disorder can manifest as hypoprothrombinemia, which is characterized by a decrease in the overall synthesis of prothrombin (Lancellotti et al. 2013). Comparison of the prevalence of polymorphisms or mutations in genes encoding MTHFR, FV and prothrombin between women experiencing RPL and fertile women did not show obvious differences (Coulam et al. 2006).

Objectives

The objectives of this study was to analyze the occurrence of combination of multiple mutations in the 4 thrombophilic gene mutations among women with a history of two or more recurrent miscarriages, in comparison with the women who have had at least one normal pregnancy as controls to investigate any correlation between these mutations and pregnancy failure in the subpopulation of Iran.

MATERIAL AND METHODS

Patients and Controls

Peripheral blood samples were obtained from 210 women as cases and 160 women as control groups. Inclusion criteria for patients were as follows, experienced ≥ 2 miscarriages, age less than 40 years, and absence of identifiable causes, including anatomic, endocrinologic, cytogenetic, and autoimmune causes, for their history of recurrent miscarriage (Ford 2009).

DNA Isolation

Genomic DNA was extracted from the peripheral blood samples by using the salting-out procedure (Miller et al. 1988).

Genotyping

PCR-RFLP was performed to detect the four mutations. Analysis was performed by using the following conditions of initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 60 seconds, annealing at 63°C (MTHFR C677T)/59°C (MTHFR A1298C)/58°C (FV)/56°C (FII) for 60s, and extension at 72°C for 60 seconds, and final extension at 72°C for 5 minutes. PCR was performed using a 20 μ l reaction mixture containing 100 ng genomic DNA, 10 pM of each primer, 1.5 U Taq DNA polymerase, 2 μ l 10 $\times$ buffer, 1.5mM MgCl₂, and 0.2 mM dNTPs. After digestion with restriction enzymes, the PCR products were separated by electrophoresis on three percent agarose gels and were visualized under ultraviolet light (Table 1).

Statistical Analysis

Statistical analysis was performed using SPSS version 16. Genotype distribution of each mutation and the frequency of heterozygous and homozygous mutations were compared between cases and controls by using Pearson's Chi-square test. A *P* value of less than 0.05 was considered statistically significant. Homozygous and heterozygous genotypes in each group were unified into a new group, and odds ratios with ninety-five percent confidence intervals were calculated.

RESULTS

Frequencies of different genotypes, *P* values, and odds ratios of women experiencing RPL and normal controls are shown in Table 2. The

Table 1: Sequences of primers, sizes of PCR products, restriction enzymes used, and sizes of digested fragments

Mutation Sequence of primers	PCR product (bp)	Restriction enzyme	Wild type	Heterozygote	Mutant
MTHFR F- TGAAGGAGAAGGTGTCTGCGGGA C677T R-AGGACGGTGCGGTGAGAGTG	198	HinfI	198	198/175/23	175/23
MTHFR F-CTTTGGGGAGCTGAAGGACTACTAC A1298C R-CACTTTGTGACCATTCGGGTTTG	163	MboII	56/31/30/ 28/18	84/56/31 30/28/18	84/31/ 30/18
FV F-ACCCACAGAAAATGATGCCCAG G1691A R-TGCCCCATTATTTAGCCAGGAG	223	MnII	104/82/37	104/141/82	141/82
FII G20 F-TCTAGAAACAGTTGCCTGGC 210A R-ATAGCACTGGGAGCATTGAAGC	345	HindIII	345		322/23

FII: prothrombin; FV: factor V; MTHFR: methylenetetrahydrofolatereductase.

Table 2: The frequency of genotype in the cases and controls

	Genotype	Cases (%)		Controls (%)		P value	OR (95% CI)	
<i>MTHFR C677T</i>	CC(WT)	110	(52.38%)	81	(50.62%)	0.7	1	(0.7–1.7)
	CT	90	(42.85%)	67	(41.87%)	0.9	1	(0.6–1.6)
	TT	10	(4.76%)	12	(7.50%)	0.3	0.6	(0.2–1.4)
<i>Total</i>		210		160				
<i>MTHFR A1298C</i>	AA(WT)	72	(34.28%)	54	(33.75%)	1	1	(0.6–1.5)
	CA	119	(56.66%)	105	(65.62%)	0.8	0.7	(0.4–1)
	CC	19	(9.04%)	1	(0.62%)	<0.01*	15.8	(2–119)
<i>Total</i>		210		160				
<i>FV G1691A</i>	GG(WT)	202	(96.19%)	158	(98.75%)	0.2	0.3	(0.06–1.5)
	GA	8	(3.80%)	2	(1.25%)	0.2	3	(0.6–15)
	AA	0		0		ND		
<i>Total</i>		210		160				
<i>FII G20210A</i>	GG(WT)	210	(100%)	160	(100%)	ND		
	GA	0		0		ND		
	AA	0		0		ND		
<i>Total</i>		210		160				

95% CI: Cornfield's 95% confidence interval for OR; FII: prothrombin; FV: factor V; MTHFR: methylenetetrahydrofolatereductase; ND: not determined (0% frequency); OR: odds ratio; SNP: single nucleotide polymorphism; WT: wild type

*Bold: significant.

results for the MTHFR C677T mutation showed no statistically significant differences in all the three genotypes between the two groups, with the risk in the case group not higher than that in the control group.

The results for the MTHFR A1298C mutation showed no statistically significant differences in wild-type homozygous and heterozygous genotypes between the two groups. However, the mutant homozygous genotype showed a statistically significant difference ($P < 0.01$), with a higher risk in the case group than in the control group.

The results for the FV G1691A mutation showed no statistically significant differences in wild-type homozygous and heterozygous genotypes between the two groups. The mutant homozygous genotype was not found in any of the group.

Both patients and controls had the wild-type homozygous genotype and mutant homozygous. Heterozygous and heterozygous FII G20210A genotypes were not found in any of these groups.

When the total number of mutations was compared by counting a heterozygous mutation as one mutation and a homozygous mutation as two mutations, the number of mutations in women experiencing RPL was not higher than that of control women (16.3% versus 15.62%, $P = 0.644$) (Table 3).

DISCUSSION

The risk of RPL is determined by the interaction of many genetic and environmental factors. However, only a few of these factors have been identified thus far. Awareness regarding the aspects of hereditary thrombophilia can help determine the requirement for prophylactic anti-

Table 3: Total allele frequency

Total allele frequency	Groups				OR (95% CI)	P-value
	Case(%)		Control(%)			
Wild Type	1405	(83.63%)	1080	(84.37%)	0.95 (0.78–1.16)	0.644
Mutant Type	275	(16.36%)	200	(15.62%)		
Total	1680	(100%)	1280	(100%)		

Total number of mutations (by counting a heterozygous mutation as 1 mutation and a homozygous mutation as 2 mutations) in 210 women experiencing RPL compared with that in 160 fertile control women. The number of mutations in women with a history of recurrent miscarriage was not higher than that in control women ($P = 0.644$).

thrombotic factors for special disorders and risks during pregnancy. Comparison of the prevalence of mutations in genes encoding MTHFR, FV and prothrombin between women experiencing RPL and fertile women has yielded inconsistent results (Coulam et al. 2006). Therefore, the researchers performed the present study in this population to identify whether evaluation of common thrombophilic gene mutations in women experiencing RPL is associated with any clinical benefit. This data showed that none of the thrombophilic gene mutations examined were a risk factor for recurrent miscarriage, except homozygous MTHFR A1298C whose prevalence was significantly higher in women experiencing RPL. Further, comparison of the total number of allelic mutations between women experiencing RPL and fertile women showed no significant difference ($P=0.644$).

Although most studies confirmed the association of thrombophilic gene mutations with recurrent miscarriage (Younis et al. 2000; Sarig et al. 2002; Finan et al. 2002; Reznikoff-Etievant et al. 2001; Rey et al. 2003; Martinelli et al. 2000). This association was not reported by other studies (Rai et al. 2001; Alonso et al. 2002; Pickering et al. 2001; Clark et al. 2008). Despite the negative association reported in these studies, a possible association between these mutations and recurrent miscarriage cannot be entirely ruled out. The researchers' results suggested that the difference might become significant if more patients and controls are enrolled and if more genes involved in hereditary thrombophilia are evaluated. Some studies that evaluated more than one mutation showed an increased risk of RPL when combinations of thrombophilic polymorphisms were examined (Sarig et al. 2002; Tranquilli et al. 2004).

These inconsistent results may be because the frequency of MTHFR C677T, MTHFR A1298C, FV G1691A, and FII G20210A polymorphisms varies among different regions and ethnic populations. Because not all women with hereditary thrombophilia experience adverse effects, such as venous thromboembolism or abortion during pregnancy, it can be suggested that additional but undetermined environmental factors are involved. Therefore, the risk of RPL depends on the type of thrombophilia and the presence of additional risk factors (Kupferminc 2003). Furthermore, other enzymes involved in coagu-

lation may trigger alternate pathways leading to adverse effects.

CONCLUSION

The mutant homozygous MTHFR A1298C genotype was significantly associated with RPL in the study population. However, the other genotypes, that is, mutant homozygous or heterozygous MTHFR C677T, FV G1691A and FII G20210A genotypes were not significantly associated with pregnancy loss in the population.

RECOMMENDATIONS

Based on these observations, it can be explicated that each thrombophilic genes as a single factor have a very modest effect with respect to the risk of recurrent miscarriage. Further studies addressing the other mutation sites in these genes or related genes are required.

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