Original Article

Association between thrombophilic gene polymorphisms and recurrent pregnancy loss in Iranian Women

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Abstract

Background: Nearly one in five pregnancies ends in abortion. The recurrent spontaneous abortion (RSA) occurs in about 2% of couples for various reasons. Thrombophilia is considered as a major risk factor in women with RSA. This study analyzed seven polymorphisms of five common genes in thrombophilia and their correlations with RSA in Iranian women. **Material and Methods:** This comparative, observational (case-control) study was conducted on 60 women with history of RSA and 60 healthy women with successful pregnancies who referred to Obstetrics and Gynecology Department of Eghbal Hospital (Tehran, Iran) within the 2018-2019 period. The amplification-refractory mutation system (ARMS) PCR technique was used to analyze polymorphisms of FV Leiden *(G1691A*), PAI-1 (-844 A/G), PAI-1 (-675 4G/5G), β-Fibrinogen (-455 G/A), MTHFR (C677T), MTHFR (A1298C), and Prothrombin (G20210A). **Results:** There was a significant difference between the two study groups in the genotypes of MTHFR (C677T) and PAI-1 (-844A/G) *(p value=0.03* and *0.00*, respectively). **Conclusion:** According to the research data, analysis of polymorphism of MTHFR (C677T) and PAI-1 (-844 A/G) can be used as a good marker to address the probable causes RSA and a significant prognostic factor for consecutive pregnancy losses.

Keywords: polymorphism, thrombophilia, pregnancy loss, MTHFR, PAI-1

Introduction

More than 15-20% of pregnancies end in miscarriage. Involving nearly 0.5-2% of couples, recurrent spontaneous abortion (RSA) is defined as two (or more) consecutive pregnancy losses before the 20th week of pregnancy without considering molar and ectopic pregnancies [1]. Various factors such as anatomical, hormonal, and immunological abnormalities, infections, environmental factors, and chromosomal abnormalities can cause this condition [2, 3]. In addition to all of these factors, the real causes of nearly half of RSAs have not yet been identified clearly and are still analyzed as idiopathic factors [4].

Research has shown that the subsequent live birth is lower in couples with karyotype abnormalities than in normal couples due to RSAs [5]. Therefore, genetic factors continue to play more significant roles in RSAs, in as much as scientific evidence indicates that genetic factors have affected the emergence of 50-60% of early spontaneous miscarriages [6]. In recent decades, thrombophilia has been identified as an important risk factor in women with RSA [7]. In fact, thrombophilia is classified as hereditary and acquired categories. According to other studies, inherited thrombophilia can cause RSAs at any gestational age. For instance, thrombotic events in placenta can cause fetal loss [8]. Moreover, pregnancy complications such as placental abruption, intrauterine growth restriction, intrauterine fetal death, preterm labor, and preeclampsia are closely correlated with inherited thrombophilia [9].

Therefore, thrombosis genes and their mutation, caused by different factors, were analyzed in RSAs. Hypercoagulable state, blood clots in small vessels in placenta, and declined oxygen delivery to fetus are pathophysiologies caused by thrombophilic gene mutations [10]. Mutations in FV Leiden *(G1691A*), gene promoters, prothrombin (G20210A), and C677T mutation homozygosity in the methylenetetrahydrofolate reductase (MTHFR) gene are among the thrombophilic variations analyzed in the Iranian population [11]. Although mutation is associated with the hypercoagulable state, it plays an arguable role in RSAs. The hypercoagulable state can also result from mutations that affect the factors of common pathway clotting cascades and the fibrinolytic system. These factors include the XIII factor, β-fibrinogen, and plasminogen activator inhibitor 1 (PAI-1) [12].

The amplification-refractory mutation system (ARMS) PCR is currently used to analyze single base change mutations and

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small deletions [13]. Given the thermal design of primers, all mutations were identified through a thermal program to save PCR time, reduce depreciation of laboratory devices, and prepare experimental results rapidly. The present study aimed to analyze polymorphisms of a few common genes in thrombophilia through ARMS-PCR and investigate their correlations with abortion in Iranian women with RSA.

Material and Methods

Study Population

This comparative (case-control) observational study was conducted on 60 women with history of consecutive miscarriages, who referred to Obstetrics and Gynecology Department of Eghbal Hospital (Tehran, Iran) within the 2018-2019 period. In addition, 60 healthy women with history of normal pregnancies (at least one successful pregnancy without complications) and no miscarriages were selected as the control group. All members of the case and control groups were non-smoking Iranian women. This study was approved by the Ethics and Human Rights Committee under the code IR.BMSU.REC.1398.054. Moreover, informed consent was obtained from all participants.

DNA Extraction

At the baseline, nearly 8-ml blood specimens with EDTA anticoagulants were taken from all the designated patients through venipuncture in order to isolate genomic DNA. A column-based technique was employed with a QIAmp DNA mini kit (Qiagen-Germany) for DNA extraction. The extracted DNA was then stored at -20°C. Furthermore, a spectrometer was utilized through agarose gel electrophoresis to check the quality and quantity of the extracted DNA.

Genotype Screening

The amplification refractory mutation system-real-time PCR (ARMS-real-time PCR) was used for the detection of mutations and variants in FV Leiden (G1691A), PAI-1 promoter polymorphisms (-844 A/G and -675 4G/5G), β-fibrinogen (-455 G/A), MTHFR (C677T and A1298C), and Prothrombin (G20210A). The ARMS-real-time PCRs were performed in a 20-µl solution containing the 5-µl genomic DNA (at least 50 ng) and a 15-µl PCR master mix (10 pmol of each primer, 10 nmol of each deoxyribonucleotide triphosphates, 1.5 mmolMg2+ and 1 U Taq polymerase with SYBR green color). Table 1 shows the real-time PCR condition. The PCR technique was employed through primers (shown in Table 1) purchased from BIONEER, EU for genotyping the −675 4G/5G polymorphism in the promoter region of the PAI-1 gene and the FXIII Val34Leu polymorphism. All reactions were performed in total volumes of 25 µL and 12.5 µL of ready-to-use PCR master mix, 1 µL of forward primer, 1 µL of reverse primers, and 5 µL of genomic extracted DNA. They were then completed by 5.5 µL of water nuclease-free. Initial denaturation was done at 94◦C for 4 min and followed by 34 cycles of denaturation at 95°C for 20 s while annealing at 66°C for 60 s (Table 2).

Statistical Analysis

All statistical calculations were performed in SPSS 15 (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) for Microsoft Windows. Pearson's chi-squared ($\chi 2$) and Fisher's exact tests were conducted to determine differences between case and control groups in genotype distributions of each mutation and frequencies of heterozygous, homozygous, and mutated alleles. The homozygote and heterozygote genotypes of each group were unified as a new group; odds ratios and 95 % confidence intervals were then calculated. P-values of <0.05 were considered statistically significant.

Exclusion Criteria

After medical history and clinical examinations, immunologic and endocrinologic tests were conducted along with other routine laboratory tests on all participants. Any clinical or laboratory findings referring to underlying conditions such as autoimmune diseases, endocrinologic dysfunction, liver function abnormalities, and anatomic abnormalities made the participants to be excluded from the study.

Table 1. Primer sequences

Gene Name	Primer Sequences	
FV Leiden	GAGCAGATCCCTGGACAGGCA	forward
(G1691A)	GACTACTTGACAATTACTGTTCTCTT	reverse
PAI-1	TCAGCCAGACAAGGTTGTTG	forward
(-844 A/G)	TTTTCCCCAGGGCTGGTCCCA	reverse
PAI-1	GTCTGGACACGTGGGGA	forward
(-675 4G/5G)	GTCTGGACACGTGGGGG	reverse
β-fibrinogen	ATATAACATTACTATTGATTTTAATA	forward
(-455G/A)	CTCAAAGAGAGATGTGTATCTTGTTTC	reverse
MTHFR	GAGAAGGTGTCTGCGGGAGT	forward
(C677T)	ACCTGGATGGGAAAGATCCCG	reverse
MTHFR	GGAGGAGCTGACCAGTGAAGC	forward
(A1298C)	CATGTCCACAGCATGGAGGGGAG	reverse
Prothrombin	CCAATAAAAGTGACTCTCAGCA	forward
(G20210A)	GCTCACTGCAGCCTCCACCTCCTG	reverse

Table 2. Real-time PCR thermal program

Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	15 minutes	1 cycle
Cycling	95 °C 66 °C	20 seconds 60 seconds	34 cycles

Results

Demographic Data

The case group included 60 women with the mean age of 32.42±5.09 years, whereas the control group consisted of 60 women with the mean age of 28.96±3.97 years. Therefore,

the mean age of participants was significantly higher in the case group (*P value=0.00*). The results also showed that 66% of patients in the case group were housewives and that 34% of them were employees. However, 55% of the participants were housewives in the control group where 45% were employees. There was no significant difference between the two groups in employment status (*P value=0.24*). Table 3 shows the quantities of abortions and of pregnancies in the study groups. Accordingly, the majority of patients had ≥2 abortions in the case group (86.7%).

Table 3. Results of number of abortions and number of pregnancies in study groups

Pregnancy status		Group	
		Case (%)	Control (%)
Number of abortions	0	0 (0 %)	60 (100%)
	1	8 (13.3%)	0 (0 %)
	2	39 (65%)	0 (0 %)
	3	13 (21.7%)	0 (0 %)
Total		60 (100%)	60 (100%)
Number of pregnancies	1	2 (3.3%)	28 (46.6%)
	2	35 (58.4%)	28 (46.6%)
	3	21 (35%)	4 (6.8%)
	4	2 (3.3%)	0 (0 %)
Total		60 (100%)	60 (100%)

Genotypic Frequencies of Gene Mutations

Table 4 shows different genotype frequencies of FV Leiden (G1691A), PAI-1 promoter polymorphisms (-844 A/G and -675 4G/5G), β-fibrinogen (-455 G/A), MTHFR (C677T and A1298C), and Prothrombin (G20210A) in the case and control groups. According to the results, there was a significant difference between the two groups (*P values=0.00 and 0.03*, respectively) in genotypes of PAI-1 (-844 A/G) and MTHFR (C677T) (Table 4-6).

Table 4. Genotypic Frequencies of the Gene Mutations

Mutation	Case (N=60)			Control (N=60)			*P Value*
	Normal (%)	Heterozygote (%)	Homozygote (%)	Normal (%)	Heterozygote (%)	Homozygote (%)	
FV Leiden (G1691A)	38 (63.4%)	14 (23.3%)	8 (13.3%)	48 (80%)	5 (8.3%)	7 (11.7%)	0.12
PAI-1 (-844 A/G)	21 (35%)	34 (56.7%)	5 (8.3%)	44 (73.4%)	14 (23.3%)	2 (3.3%)	0.00*
PAI-1 (-675 4G/5G)	34 (56.7%)	25 (41.7%)	1 (1.6%)	37 (61.7%)	23 (38.3%)	0 (0 %)	0.61
β-Fibrinogen (-455G/A)	35 (58.3%)	25 (41.7%)	0 (0 %)	41 (68.3%)	18 (30%)	1 (1.7%)	0.22
MTHFR (C677T)	49 (81.6%)	7 (11.6%)	4 (6.8%)	52 (86.6%)	0 (0 %)	8 (13.4%)	0.03*
MTHFR (A1298C)	43 (71.6%)	9 (15%)	8 (13.4%)	48 (80%)	7 (11.6%)	5 (8.4%)	0.41
Prothrombin (G20210A)	60 (100%)	0 (0 %)	0 (0 %)	60 (100%)	0 (0 %)	0 (0 %)	-

* P value ≤0.05 considered significant.

Table 5. Frequency of mutant allele and Odd ratio

Mutation	Frequency Of Mutant Allele		Odd Ratio		*P Value*
	Case (n=60)	Control (n=60)	Odd ratio	95 % confidence interval of an odds ratio	
FV Leiden (G1691A)	0.5	0.31	0.5526	0.268 to 1.141	0.12
PAI-1 (-844 A/G)	0.73	0.28	0.6555	0.3417 to 1.266	0.23
PAI-1 (-675 4G/5G)	0.45	0.37	0.8021	0.4123 to 1.616	0.60
β-Fibrinogen (-455G/A)	0.41	0.33	0.76	0.3735 to 1.518	0.48
MTHFR (C677T)	0.25	0.26	1.077	0.4634 to 2.338	0.99
MTHFR (A1298C)	0.41	0.24	0.5291	0.2346 to 1.155	0.13

* P value ≤0.05 considered significant.

Table 6. Sensitivity, Specificity, Positive Predictive Value and Negative Predictive Value.

Mutation	Parameter

	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
FV Leiden (G1691A)	54%	31%	75%	15%
PAI-1 (-844 A/G)	51%	38%	73%	19%
PAI-1 (-675 4G/5G)	56%	38%	77%	18%
β-Fibrinogen (-455 G/A)	55%	37%	79%	16%
MTHFR (C677T)	57%	44%	87%	13%
MTHFR (A1298C)	54%	30%	79%	12%

Discussion

Given that nearly one in five pregnancies ends in abortion and that this rate in Iran is similar to global statistics [14], it is necessary to analyze the relevant causes. Nowadays, thrombophilia is considered a risk factor in RSA [15]. In recent decades, many studies have addressed the correlation of genes involved in thrombophilia with RSA. This study analyzed the mutation of thrombophilia genes in 60 women with history of RSA in comparison with 60 healthy, fertile women. According to Table 4, mutations of PAI-1 (-844A/G) and MTHFR (C677T) were significantly higher in women with RSA (*P values=0.00 and 0.03*, respectively).

The PAI-1 gene is located on the seventh chromosome (7q21.3-22) and encodes the serine protease inhibitor protein (SERPINE-1). More than ten polymorphisms have been recorded for this gene so far. The most important polymorphisms are rs2227631 [-844 G/A], rs1799889 [-675 4G/5G], rs6092 [43 G/A], rs2227694 [9785 G/A], and rs7232 [11053 T/G] [16]. This protein plays its role in fibrinolysis through inhibiting tissue and urokinase plasminogen activators (tPA and uPA). The fibrinolytic activity has a major role in the early stages of placentation and separation of the placenta from the uterine wall after childbirth. Moreover, PAI-1 facilitates the trophoblast invasion on the uterine wall [17]. Increased levels of PAI-1 in blood can increase the number of platelets, decrease prothrombin time, and reduce the activated partial thromboplastin time. Therefore, PAI-1 affects fibrinolytic activities. Accordingly, the healthy and impeccable presence of this protein would be necessary for maintaining pregnancy, especially in early stages, and preventing miscarriage. Jeon et al. (2013) analyzed different polymorphisms of PAI-1 in 308 Korean women with RSA and concluded that there was a significant correlation between the mutation of PAI-1 -844G/A and RSA (*P value<0.05*) [18]. This finding has been backed by several studies on other races. Magdoud et al. (2013) studied 304 African women with RSA in Tunisia and found a significantly higher rate of PAI-1 -844G/A mutation in these women than those with successful pregnancies (*P value<0.05*) [19].

MTHFR is another gene correlated with RSA. This correlation has also been analyzed in recent decades. Two common polymorphism of MTHFR are MTHFR C677T (rs1801133) and MTHFR A1298C (rs1801131). Located on the first chromosome (1p36.22), its encoding gene generates and enzyme which has a key role in folate metabolism. Therefore, it has a close correlation with the processes of embryo development and cell division. Similar to PAI-1, MTHFR can be very important in the early stages of pregnancy. Zhu et al. (2018) analyzed the prevalence of MTHFR polymorphisms in 370 Chinese women with RSA and indicated that the mutation of MTHFR C677T was higher in these women than others. They also conducted another study on 151 other Chinese women with a history of repeated implantation failure and showed the higher prevalence of MTHFR mutation than others (*P value<0.05*) [20]. Zarfeshan Fard et al. (2019) studied 100 Iranian women and indicated that the higher prevalence of MTHFR C677T mutation in women with RSA could be considered a risk factor in pregnancy loss (*P value<0.05*) [21].

The β-fibrinogen mutation has been neglected in the studies into the correlation between thrombophilic genes and RSA. In the present study, both case and control groups were compared in terms of β-fibrinogen mutation (-455 G/A). According to the results, the mutation was not homozygote in the case group. Furthermore, it occurred in only 2.5% of the control group. In addition to odd ratio of 0.76, the findings indicated that β-fibrinogen mutation cannot be an appropriate marker for RSA risk factors, although some studies have shown contradictory results. Ticconi et al. (2011) analyzed the β-fibrinogen (-455 G/A) mutation in 176 Italian women and showed that the A/A genotype of β-fibrinogen was more prevalent in women with a history of RSA ($p<0.05$). They also introduced this mutation as an RSA risk factor [22]. However, Quintero-Ronderos et al. (2017) studied 22 novel genes and mutations affecting RSA. According to their results, mutation of fibrinogen α-chain can be also considered an important factor regarding the causes of RSA [23].

An important aspect of this study is the technique used for analyzing mutations of the introduced genes, i.e. the ARMS-real-time PCR. However, most studies utilize the restriction fragment length polymorphism (RFLP) technique to analyze thrombophilic genes [24-26] which is costlier and harder than real-time PCR. The temperatures of primers were designed to perform all mutations in a single PCR thermal program in order to save time, reduce depreciation of laboratory devices, and prepare laboratory results sooner for patients.

Conclusion

This study indicated that the polymorphisms PAI-1 (-844 A/G) and MTHFR (C677T) were significantly higher in Iranian women with RSA. This can be used as a genetic

marker and a significant prognostic factor for consecutive pregnancy losses. Due to the small number of participants, it is recommended to conduct future studies on a heterogeneous population and well-characterized women in order to confirm the research results and the role of these polymorphisms in the etiology of RSA.

Ethical Approval

This study was approved by our institutional review board.

Statement of Informed Consent

Even though there were no identifying details about the patient in this study, informed consent was obtained for inclusion in the study.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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