ORIGINAL ARTICLE

The Possible Role of rs2227631 and rs1799889 Polymorphisms of *SERPINE1* in Idiopathic Recurrent Miscarriage

Baharak Bagheri[1)], Nastaran Asghari Moghaddam[1,2)], Sina Mirzaahmadi[3)]

ABSTRACT

Plasminogen activator inhibitor-1 (PAI-1) is crucial in the fibrinolysis process; also, rs2227631 and rs1799889 are the common promoter variants of *SERPINE1*influencing the plasma levels of PAI-1.

Objective: **Despite numerous evidences supporting the association of altered PAI-1 level with the idiopathic recurrent miscarriage (iRM), the relation of mentioned variants to iRM remains ambiguous. In the current study, the contribution of rs2227631 and rs1799889 with RM risk was investigated.**

Methods: **This was a case-control study. 100 women diagnosed with iRM and 100 healthy women were involved. Sanger sequencing method was used to designate rs2227631 and rs1799889 genotypes.**

Results: **Both polymorphisms were significantly in association to the risk of iRM (rs1799889: P = 0.0001, OR: 9.421, 95%CI: 5.542-16.01; rs2227631: P = 0.0001, OR: 5.914, 95%CI: 5.542-16.01). the distribution of 4G4G/AA combined genotype significantly increased iRM risk (P = 0.0001, OR: 39.00, 95%CI: 8.657-175.6).**

Conclusions: **The results replicated rs1799889 association to iRM and suggest rs2227631 contribution to elevated risk of iRM. Collectively, it can be suggested that these two variants are the likely attribution of *SERPINE1* to iRM.**

KEY WORDS

idiopathic recurrent miscarriage, plasminogen activator inhibitor-1, *SERPINE1*, polymorphism, variant

INTRODUCTION

According to World Health Organization (WHO), miscarriage is a relevant medical and social problem despite effective modern methods of diagnosis and treatment. Recurrent miscarriage (RM), classically defined as two or more spontaneous miscarriages before the gestational age of 20th weeks for at least two or more consecutive pregnancies, is a heterogenous disorder affecting 1-3% of fertile couples in reproductive period[1)]. Although numerous scientific studies have introduced possible causes of RM, such as adverse environmental factors, pernicious habits, infectious diseases, fetal and parental chromosomal abnormalities, endocrine disorders, maternal reproductive organ disorders, immunological dysfunction, thrombophilic disorders, the etiology of RM (around 50%) remains uncertain[2,3)]. These cases of RM have no explicable etiology and effective therapy, require comprehensive study of their etiopathogenesis and are considered idiopathic RM (iRM). Excluding known clinical and environmental risk factors, there is cumulative evidence of genetic multifactorial etiology of iRM. The absence of large-scale genome-wide association studies (GWAS) on iRM is due to a number of objective reasons, e.g., the lack of clear definition and classification of iRM, difficulty in recruiting and small sample size, and the lack of replication studies in ethnically homogeneous populations[4)].

It should be noted that published systematic reviews did not confirm clear association of polymorphisms in hereditary thrombophilia, cytokines, angiogenesis and placental function genes with iRM[5,6)].

Several studies suggested the significant role of hematological disorders, particularly thrombophilic defects, in RM predisposition. Due to thrombocytosis, increased platelet aggregation, level of blood coagulation factors activity raised by fibrinolytic inhibitors; however, these associations are very controversial[4,5,7)]. Coagulation and fibrinolysis play a crucial role in stable pregnancy since fetal growth is directly related to the maternal circulation. It seems that any thrombosis in the placental capillaries causes abortion due to the material exchange disruption between mother and fetus[8)]. As possible genes predisposing to iRM is *SERPINE1* which encoded plasminogen activator inhibitor-1 (PAI-1), a vital player in the fibrinolysis process. This protein belongs to the serine protease inhibitor (serpin) family, and inhibits the urokinase-type plasminogen activator (uPA) and tissue-plasminogen activator (tPA), which are mainly responsible for plasminogen conversion to plasmin. *SERPINE1* gene is located on chromosome 7q22.1, possessing nine exons[9)].

Although several mutations within the given gene were reported, which affects the plasma concentration of *PAI-1*[9,10)], the results of associative genotypic studies of iRM in different ethnic population are insufficient and controversial[11)]. One of the most investigated *PAI-1* gene polymorphisms is rs2227631 [-844 G > A] in the 5' - flanking region. Another polymorphism is rs1799889 [-675 4G/5G] in the promoter region. Several studies indicated the association between 4G allele and elevated *SERPINE1* gene expression. Indeed, this deletion is well-established to increase the promoter activity. Although the association of rs1799889 and RM has been widely investigated, the results were controversial[10,12)]. rs2227631 is a tag polymorphism of *SERPINE1* and was introduced as an influential factor on plasma PAI-1 levels[13)]; however, other study suggest that G/A polymorphism has not significantly altered

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1) Department of Biology, Electronic Campus, Islamic Azad University Tehran, Iran
2) Department of Biology, Central Tehran Branch, Islamic Azad University Tehran, Iran.
3) Department of Molecular Genetics, Islamic Azad University, Zanjan Branch Zanjan, Iran
Correspondence to: Nastaran Asghari Moghaddam
(e-mail: nas.asgharimoghaddam@iauctb.ac.ir)

ORCID ID:
Baharak Bagheri: 0000-0002-6041-0269
Nastaran Asghari Moghaddam: 0000-0002-6553-7875
Sina Mirzaahmadi: 0000-0003-1811-3188

Table 1: Genotype and allele distribution of *PAI-1* rs1799889 and rs2227631 polymorphisms in patients with RPL and healthy controls

SNP	Genotypes	Cases %	Controls %	OR (95% CI)	*P* value
rs1799889					0.0001
Co-dominant	5G5G	33	84	1.000	-
	5G4G	29	11	6.710 (3.008 - 14.97)	0.0001
	4G4G	38	5	19.34 (7.006 - 53.41)	0.0001
Dominant	(5G5G vs. 5G4G + 4G4G)	-	-	10.65 (5.411 - 20.99)	0.0001
Recessive	(5G5G + 5G4G vs. 4G4G)	-	-	11.64 (4.345 - 31.20)	0.0001
4G allele		52.50	10.50	9.421 (5.542 - 16.01)	0.0001
HWE P value		0.0001	0.0001	-	-
rs2227631					0.0001
Co-dominant	GG	34	78	1.000	-
	GA	36	17	4.858 (2.404 - 9.917)	0.0001
	AA	30	5	13.76 (4.919 - 38.51)	0.0001
Dominant	(GG vs. GA + AA)	-	-	6.882 (3.670 - 12.90)	0.0001
Recessive	(GG + GA vs. AA)	-	-	8.142 (3.008 - 22.04)	0.0001
A allele		48.00	13.50	5.914 (3.618 - 9.667)	0.0001
HWE *P* value		0.0214	0.0246	-	-

Abbreviation: RPL, recurrent pregnancy loss; OR, odds ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium.

P value were calculated by Chi-square test.

Bold values indicate statistically significant differences ($P < 0.05$).

Table 2: Distribution of *PAI-1* rs1799889 and rs2227631 polymorphisms combined genotypes in patients with RPL and healthy controls

Combined genotypes	Cases (n = 100) %	Controls (n = 100) %	OR (95% CI)	*P* value
rs1799889/rs2227631				
5G5G/GG	26	78	1.000	-
5G5G/GA	6	3	6.000 (1.400 - 25.71)	**0.014**
5G5G/AA	1	3	1.000 (0.099 - 10.03)	1.000
5G4G/GG	3	0	ND	ND
5G4G/GA	23	11	6.272 (2.695 - 14.59)	**0.0001**
5G4G/AA	3	0	ND	ND
4G4G/GG	5	0	ND	ND
4G4G/GA	7	3	7.000 (1.686 - 29.06)	**0.006**
4G4G/AA	26	2	39.00 (8.657 - 175.6)	**0.0001**

Abbreviation: OR, odds ratio; CI, confidence interval; ND, not determined.

P value calculated by Chi-square test.

Bold values indicate statistically significant differences (P < 0.05).

PAI-1 concentration. Besides, reports have not consistently been supportive of its role in iRM[14,15]. The different associations of rs1799889 and rs2227631 are likely related to the genetic variation in divergent populations.

OBJECTIVE

This study sought to assess the contribution of rs1799889 and rs2227631 *SERPINE1* polymorphisms to the risk of iRM in Iranian Azeri ethnic group iRM patients and fertile controls. This investigation has been done at allele, genotype, and haplotype levels.

METHODS

Subjects

This case-control study was performed from June 2018 to June 2019 in Massoud Molecular Laboratory, Tehran, Iran. Patients comprised 100 women with idiopathic RM; according to the Practice Committee of the American Society for Reproductive Medicine (ASRM), RM is defined as two or more consecutive pregnancy losses before 20 weeks of gestation[1]. Control subjects consist of 100 aged-matched healthy women with at least two live births and interviewed about clinical and family history of RM and iRM. All the participants (cases and controls) were chosen from Azeri ethnic group. None of the participants in both case and control groups consume alcohol or smoke cigarette. All the participants had regular menstrual cycle and normal 46, xx karyotype. The exclusion criteria were the first pregnancy at age ≥ 40 years, structural and numerical chromosomal abnormalities in both parents (by traditional karyotyping or fluorescent in situ hybridization), endocrine disorders (diabetes mellitus, luteal failure, thyroid disorders, and hyperprolactinemia), genitourinary tract anomalies (submucosal leiomyoma, uterine septum, arcuate and bicornuate uteri, and endometrial polyps), infections (rubella, toxoplasmosis, HIV, HBV, HCV, HCMV and chronic genitourinary infection (mycoplasma, chlamydia, GBS, and bacterial vaginosis)), chronic diseases (anti-phospholipid antibodies (e.g. anti-cardiolipin antibodies, lupus anticoagulant, and anti-β2-glycoprotein 1 antibodies), antinuclear antibodies, Rh incompatibility), inherited thrombophilia (Factor V- Leiden and Factor II G20210A) and autoimmune diseases. The exclusion criteria were evaluated according to Jeon *et al*.[9]. In addition, participants' spouses had normal volumes and semen criteria according to WHO reference values. This experimental study was designed and performed according to the Helsinki declaration and relevant guidelines. All participants were comprehensively informed about the investigation protocol and signed the written consent before being enrolled in the study. The study was fully explained to the participants and they were given informal consent.

Genotyping

5 ml blood of all participants were collected in Ethylenediaminetetraacetic acid (EDTA)-anticoagulant tubes. Samples were stored at -20°C. Genomic DNA extraction was performed by DNA extraction Kit (BIORON, Germany) as the protocol provided. DNA quality and quantity were evaluated by gel electrophoresis and spectrophotometry (Berthold nanodrop). For the amplification of the given region of *SERPINE1* gene, one set of primer was designed by the Gene

Table 3: Pairwise LD of *PAI-1* rs1799889 and rs2227631 polymorphisms in patients with RPL and healthy controls

	Cases		Controls	
Locus pair	D'	r^2	D'	r^2
rs1799889/rs2227631	0.001	0.001	0.001	0.001

Abbreviation: LD, linkage disequilibrium.

r^2 reflects statistical power to detect LD.

Table 4: Haplotype distribution of *PAI-1* rs1799889 and rs2227631 polymorphisms in patients with RPL and healthy controls

Haplotype		Frequency		OR (95% CI)	*P* value
rs1799889	rs2227631	Cases	Controls		
4G	G	0.776	0.700	1.487 (0.347 - 6.380)	0.591
4G	A	0.224	0.300	0.672 (0.157 - 2.884)	0.592

Abbreviation: OR, odds ratio; CI, confidence interval.

P value were calculated using Pearson's chi-square test.

Bold values indicate statistically significant differences ($P < 0.05$).

Runner Software (v. 3.05) (Hastings Software Inc, USA). Then, to confirm the specificity of primers NCBI Primer-BLAST site (*https://www.ncbi.nlm.nih.gov/tools/primer-blast/*) was used. The primer sequences were as follow: 5'- CACACCCTGCAAACCTGCCATGAATTGA-3'(forward) and 5'- GGACTTGGGCCCAACAGAGGACTCTTGG-3 ' (reverse) with the product size of 373 base- pair (bp). The amplification of the given fragment was performed by Bio-Rad T^{100} Cycler (USA). The polymerase chain reaction (PCR) condition contained the following steps: initial denaturation at 95℃ for 5 minutes, denaturation at 94℃ for 1 minute, annealing at 63℃ for 1 minute, extension at 72℃ for 1 minute. The process was repeated for 32 cycles, and a final extension was done at 72℃ for 5 minutes. The 25-µL reaction mixture contained 2 µl of template DNA, 1 µl of both forward and reverse primers, 12.5 µl of the Ampliqon Taq 2X master mix (Denmark), and 8.5 µl double distilled water. The specificity and quality of amplified PCR products were verified on 1% agarose gel. Afterward, the amplified fragments were sequenced by Sanger sequencing method in Massoud Laboratory. To ultimately evaluate the accuracy of the study, the sequence data were analyzed by MEGA 7.

Statistics

All statistical analysis was calculated in SPSS software (v.24) (SPSS Inc, USA). The parametrical variables were displayed as mean ± standard deviation (SD). Normal distribution was calculated by the Kolmogorov- Smirnov test, Shapiro- Wilk test, and post hoc Mann-Whitney test. Categorical variables were presented as the percent of the total. Allele, genotype, and haplotype frequencies among the groups were analyzed for Hardy-Weinberg equilibrium by Pearson's and Fishers' chi-square, respectively. The association between genotype frequencies and RM, and LD statistics were calculated using the odds ratio (OR), and 95% confidence intervals (CIs) using SNPAnalyzer. D' and r^2 between polymorphisms pair were evaluated by SHEsis software. P-values less than 0.05 were considered statistically significant.

RESULTS

In the current study, 100 women diagnosed with iRM and 100 normal controls were enrolled to investigate two polymorphisms of *SERPIN1* gene promoter. The average age of patients was 28.82 ± 4.77 years and the average age of controls was 28.55 ± 5.47 years (P- value = 0.886). The body mass index (BMI) of cases and controls were 24.2 ± 4.3 and 23.8 ± 4.4, respectively P = 0.36).

The number of miscarriages among iRM cases was 3.48 ± 0.81. Minor allele frequency (MAF) of rs1799889 (P = 0.0001) and rs2227631(P = 0.0001) was significantly higher amid women suffering from iRM; Thus, suggesting a potential role for both variants in iRM susceptibility. Besides, the distribution of rs1799889 (P < 0.0001) and rs2227631 (P < 0.05) polymorphisms in the studying population were not in Hardy-Weinberg equilibrium either (Table 1). The prevalence of 4G allele and A allele were significantly different between patients and healthy groups (P < 0.001) (Table 1).

By combining the effects of rs1799889 and rs2227631, it was found that the combined genotype of 4G4G/AA significantly more important in case group (OR = 39.00, 95% CI, 8.657-175.6; P = 0.0001) (Table 2). The obtained data suggested that the distribution of 4G4G/AA combined genotype was significantly different among patients and healthy controls (P < 0.0001) (Table 2).

Pairwise linkage disequilibrium (LD) was measured for rs1799889 and rs2227631 loci. The D was used to calculate LD between alleles (Table 3). A week LD was observed between loci rs1799889and rs2227631 (D' = 0.001). To evaluate the combined influence of rs1799889 and rs2227631 polymorphisms on iRM susceptibility, haplotype analysis was done between iRM patients and healthy controls (Table 4). Haplotype analysis did not mark a LD between these two variants (P > 0.05)

DISCUSSION

A balance between fibrinolysis and coagulation is necessary to support a successful pregnancy. The PAI-1 is a substantial component of the fibrinolysis cascade. It has been indicated that PAI-1 plasma level is associated with the RM[5]. Polymorphisms in *SERPINE1* promoter region alter its expression level in plasma. rs1799889 both 4G and 5G alleles provide a binding site for a transcription activator. Furthermore, the 5G allele bears a transcription repressor site. Therefore, the carriers of 4G/4G genotype possess higher plasma PAI-1 levels[16]. For G > A rs2227631 polymorphism, the nucleotide substitution alters the sequence affinity to corresponding factors[17]. Considering the potential role of rs1799889 and rs2227631 in iRM and the controversial results from various studies, we investigated the association of rs1799889 and rs2227631 with iRM in Iranian Azeri women. The results indicated that both polymorphisms were associated to the iRM. The haplotype frequency of rs1799889 and rs2227631 were analyzed. There was a significant association between 4G/4G/AA haplotype and iRM (P < 0.0001; OR: 39.00, 95% CI, 8.657 - 175.6); however, there was no LD between them.

Most of the previous studies were focused only on the relation between the 4G/5G variant and RM. Chen *et al*. concluded in a meta-analysis that the rs1799889 is of a potential role in the development of RM; while the rs2227631 has not a significant association with RM[10].

Several conducted studies were supportive of rs1799889 role in the pathogenesis of iRM. Li *et al*. suggested that in Caucasians, rs1799889 may be related to developing RM[18]. In Europe, the 4G allele frequency is relatively high (55%)[19,20]. The highest frequency of 4G allele in the women with RM was in Bulgarian (61.8%) and Czech (61.5%) patients[21,22]. A study from Germany reported 60.2% for 4G allele frequency[19]. There was no association of the 4G allele with RM among Bosnian women[20], which is opposing the other researches. Elmahgoub *et al*. showed that the coexistence of 4G allele and *FXIII* Val34Leu polymorphism significantly increased the risk of iRM and the relative risk of 4G allele compared to the 5G was statistically significant[23]. In Turkey, a report indicated the role of 4G allele in RM, although it considered the influence of other thrombophilic gene mutations[24]. The data from India were controversial. Parveen *et al*. could not found any association between 4G allele and disease[25], but Patil *et al*. found the 4G allele susceptible to RM[26]. These two opposing results may be reconciled by the differences in the criteria of selecting participants. For instance, the case group of the latter study consisted of women suffering from thrombophilia.

Previously, various studies were done in Iran by which the existence of rs1799889 and increased risk of RM was investigated. In a systematic review, Kamali *et al*. concluded that rs1799889 polymorphism plays a role in the risk of recurrent abortion[27]. Our results were in agreement with some Iranian studies[28]. Jeddi-Tehrani *et al*., and Aarabi *et al*. showed only the homozygous 4G/4G genotype is linked to RM[11,29]. Our finding indicated the association of both homozygous and heterozygous genotypes with iRM.

Our finding was in disagreement with the study of Khosravi *et al*., in which they did not find any significant difference in the frequency of 4G allele between cases and controls[30].

There was no report of the presence of rs2227631 and the significant risk of iRM. Two studies analyzed it with rs1799889 and other polymorphisms of *PAI-1*[9,31].

Jeon *et al*. proposed that in Korean women, rs1799889 and rs2227631 polymorphisms could be considered the markers of iRM. In their study, in addition to two given polymorphisms, the relation of 43G > A, 9785G > A, and 11053T > G and iRM were analyzed. The haplotype calculated data revealed the protective role of A-5G-G-G-G haplotype[9]. In another research conducted by Maghdoud *et al*., the effect of rs2227631 and rs1799889 variants on recurrent miscarriage were investigated in Tunisian women. Their results indicated that only rs1799889 was associated to RM[31], which is in apparent disagreement with our finding.

Our result suggested *SERPINE1* rs2227631 and rs1799889 act as risk factors for iRM. Further studies should be done to evaluate a larger population and concerning the different ethnic groups to better determine the contribution of given variants in the pathogenesis of recurrent pregnancy loss. Moreover, it is better to consider other mutations in the thrombophilic genes to designate the influence of genetic causes of the recurrent miscarriage.

CONCLUSION

To conclude, this study's finding proposed the importance of rs2227631 and rs1799889 of SERPINE1 gene in raising the risk of recurrent pregnancy loss in women.

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ETHICAL STATEMENT

The study was approved by the ethical committee of Electronic Branch, Islamic Azad University (900950190640).

INFORMED CONSENT

All participants were comprehensively informed about the investigation protocol and signed the written consent before being enrolled in the study.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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