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Association of Plasminogen Activator Inhibitor-1 4G/5G and Angiotensin-Converting Enzyme I/D Polymorphisms with Recurrent Pregnancy Loss in Sudanese Women: A Case-Control study

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Abstract

The aim of our study was to investigate the relationship between the *ACE* I/D and *PAI-1* 4G/5G polymorphisms and recurrent pregnancy loss (RPL) in Sudanese women.

Methods and Results: A total of 232 people participated in this case-control study, including 119 women who had been diagnosed with RPL (Case group) and 113 healthy women (Control group). The case group (RPL) consisted of Sudanese women (mean age of 31.3+5.9 years) who had at least three unfavorable pregnancy outcomes. Women in the control group (Control) were matched by age (mean age of 30.3+5.4 years), had at least two healthy pregnancies, and had no history of unfavorable pregnancy outcomes or recurrent losses. Genomic DNA samples were isolated from the whole blood by using the GF-1 Blood DNA Extraction Kit (Vivantis Technologies Sdn. Bhd., Malaysia). The status of the *PAI-1* 4G/5G and *ACE* I/D polymorphism was determined by PCR.

Analysis of the multiplicative and additive models for the *ACE* I/D polymorphism showed a significant risk of RPL with the carriage of the D allele (OR=2.07, 95% CI: 1.28-3.35, *P*=0.003) and the homozygous DD genotype (OR=2.40, 95% CI: 1.34-4.29, *P*=0.008). The multiplicative and additive models for the *PAI-1* 4G/5G polymorphism showed a significant risk of RPL with the carriage of the 4G allele (OR=3.11, 95% CI: 2.12-4.58, *P*=0.000) and the homozygous 4G/4G genotype (OR=3.09, 95% CI: 1.77-5.39, *P*=0.000). However, the carriage of risk-polymorphic markers, the *ACE* I/D and *PAI-1* 4G/5G polymorphisms, was not associated with the number of RPL. The combined carriage of the homozygous DD genotype and heterozygous ID genotype of the *ACE* I/D polymorphism with the homozygous 4G/4G genotype of the *PAI-1* 4G/5G polymorphism occurs significantly more often in RPL women than healthy women (*P*=0.000 and *P*=0.019, respectively). Carriage of the *PAI-1* 5G/5G genotype in healthy women was not associated with the *ACE* I/D polymorphism.

Conclusion: Testing for the *ACE* I/D and *PAI-1* 4G/5G polymorphisms should be part of the standard examination for patients with RPL.(**International Journal of Biomedicine. 2023;13(1):127-133.**)

Keywords: angiotensin-converting enzyme • plasminogen activator inhibitor-1 • recurrent pregnancy loss

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Abbreviations

ACE, angiotensin-converting enzyme; **AT-II**, angiotensin II; **PAI-1**, plasminogen activator inhibitor-1; **PCR**, polymerase chain reaction; **RPL**, recurrent pregnancy loss; **RAS**, renin-angiotensin system.

Introduction

According to conventional wisdom, three or more consecutive pregnancy losses constitute recurrent pregnancy loss (RPL). The American Society for Reproductive Medicine (ASRM) has described RPL as two or more miscarriages. A clinically confirmed pregnancy loss, which affects 2%-5% of fertile women, occurs when the pregnancy spontaneously terminates before 20 weeks.[1] RPL is a complex disorder with a poorly known pathogenesis.(2) Multiple factors—including chromosomal anomalies, anatomical conditions, and endocrine, immunological, and infectious diseases—are thought to affect RPL.[3-5] The identifiable reasons for RPL include genetic abnormalities, structural abnormalities, infection, endocrine abnormalities, immune dysfunction, and thrombophilic disorders.[6]

The renin-angiotensin system (RAS), a hormone-signaling cascade, plays an important role in regulating blood pressure levels and fluid balance. Circulating angiotensin II (AT-II) is the main effector of the RAS. The angiotensin-converting enzyme (ACE) is a key enzyme (a zinc metallopeptidase) that plays a role in generating AT-II by catalyzing the extracellular conversion of the decapeptide angiotensin I. The ACE hydrolyzes a number of other substrates, but probably the most important is the potent vasodilator bradykinin. Endocrine secretions from the decidua, placenta, and ovary affect RAS throughout gestation. For example, estrogen increases angiotensinogen synthesis by the liver, leading to increased serum AT-II.[7] The uteroplacental unit is where RAS components are expressed, highlighting the significance of its local role.[8] The uteroplacental RAS plays a crucial role in regenerating the endometrium after shedding, decidualization, implantation, and placentation.[9] In addition, local RAS participates in the production of prostaglandin, the release of estradiol, and the control of blood flow to the placenta and uterus.[10]

The *ACE* gene is known to contain a polymorphism consisting of either the insertion (I) or deletion (D) of a 287bp Alu repetitive sequence inside intron 16. Notably, the D allele and the DD genotype are associated with elevated levels of ACE.[11-13] Homozygotes for the I allele may display as low as half of the plasma ACE level, compared to the homozygotes for the D allele, whereas the ID heterozygotes display an intermediate level.[14]

PAI-1, a single-chain glycoprotein member of the superfamily of serine-protease inhibitors, is one of the most important inhibitors of plasma fibrinolytic activity. PAI-1 is the principal inhibitor of the tissue-type plasminogen activator (t-PA) and the urinary-type plasminogen activator (u-PA). The increased expression of PAI-1 in vivo suppresses fibrinolysis, consequently leading to pathological fibrin deposition and tissue damage.[15,16]

A number of studies have reported that *PAI-1* gene polymorphism is possibly associated with hypofibrinolysis and thrombotic complications.[17] An association between the promoter −675 4G/5G polymorphism of the *PAI-1* gene and plasma PAI-1 concentrations has been suggested.[18,19] Homozygosity for the deletion genotype (4G/4G) has been associated with PAI-1 concentrations higher than those associated with the insertion genotype (5G/5G), causing reduced fibrinolytic activity, thereby increasing the risk of venous thromboembolism.[20,21] The 5G homozygotes have the lowest PAI-1 concentrations.[21-23] However, such a relationship is still under study and, for some aspects, controversial.[24-27]

A number of previous studies have investigated the association of the *PAI-1* 4G/5G and *ACE* I/D polymorphisms with infertility, recurrent miscarriage, and major pregnancy complications.[17,28-32]

The aim of our study was to investigate the relationship between the *ACE* I/D and *PAI-1* 4G/5G polymorphisms and RPL in Sudanese women.

Materials and Methods

Due to restricted resources, a total of 232 people participated in this case-control study, including 119 women who had been diagnosed with RPL (Case group) and 113 healthy women (Control group). The study was conducted from February 2019 to February 2020 at Omdurman Medical Hospital in Sudan. The case group (RPL) consisted of Sudanese women (mean age of 31.3±5.9 years) who had at least three unfavorable pregnancy outcomes. Women in the control group (Control) were matched by age (mean age of 30.3±5.4 years), had at least two healthy pregnancies, and had no history of unfavorable pregnancy outcomes or recurrent losses. The inclusion criteria for the Case group were three or more RPLs in a row without a known reason for the abortion. Exclusion criteria included a history of vascular thrombotic disease, fetal congenital malformations, fetal chromosomal anomalies, uterine abnormalities, or a known reason for the abortion.

A structured questionnaire was used to collect data regarding the age, medical history, family history, and obstetric history of the cases and controls.

After interviewing each participant and obtaining their verbal and written agreement, five ml of venous blood was taken from each and placed into a specific container. Patient name, medical record number, collection date, and time were written on the labels of the samples.

DNA extraction and quantification

Genomic DNA samples were isolated from the whole blood by using the GF-1 Blood DNA Extraction Kit (Vivantis Technologies Sdn. Bhd., Malaysia) according to the manufacturer`s protocol. Until PCR analysis, DNA was stored in a -20°C freezer. The DNA concentration was determined at a wavelength of 260 nm using a GeneQuant spectrophotometer (Amersham Biosciences, UK).

Detection of the *PAI-1* 4G/5G polymorphism

The *PAI-1* 4G/5G polymorphism was tested by the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) technique,[33] using an upstream control primer (5′-AAGCTTTTACCATGGTAACCCCTGGT-3′), a 4G or 5G allele-specific primer (5′-AGAGTCTGGACACGTGGG GA-3′ and 5′-AGAGTCTGGACACGTGGGGG-3′, respectively), and a common downstream primer (5′-TGCAGCCAGCCACGTGATTGTCTAG-3′). 138 and

139-bp fragments for 4G and 5G alleles, respectively, at an annealing temperature of 55°C, and a 257-bp fragment for positive control were obtained from amplification by these primers. The conditions for the PCR reaction were denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 10 s, and extension at 72°C for 20 s, followed by a final extension at 72°C for 3 min. The PCR products were fractionated by 2% agarose gel electrophoresis and visualized under UV light.

Detection of the *ACE* I/D polymorphism

For the *ACE* I/D polymorphism, the following primers (F:5′-CTGGAGACCACTCCCATCCTTTCT-3′ and R: 5′-GATGTGGCCATCACATTCGTCAGAT-3′) were used to amplify the region of Alu insertion (intron 16). In order to avoid the mistyping of ID genotype as DD due to preferential amplification of the shorter D allele, a separate PCR was carried out in all the DD samples. [34] All PCR products were visualized after electrophoresis on a 2% agarose gel and ethidium bromide staining. Two alleles were identified: a 490-bp fragment I (with the insertion) and a 190-bp fragment D (without the insertion). Two bands (490 and 190 bp) were detected in heterozygous samples.

Statistical analysis was performed using statistical software package SPSS version 24.0 (Armonk, NY: IBM Corp.). The frequency distribution of genotypes for the studied polymorphic loci was checked for compliance with the Hardy–Weinberg equilibrium (HWE). Differences in the allele and genotype distribution between the groups were assessed by Chisquare test or Chisquare test with the Yates' correction, when appropriate. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Two genetic models were analyzed: the additive inheritance model (Cochran-Armitage Linear Trend Test) and the multiplicative inheritance model.

Results

In Case group women, 30(25.2%) had ≥4 RPL and 89(74.8%) had <4 RPL (Table 1). The frequency distribution of alleles of the *ACE* I/D polymorphism showed that the carriage of the D allele prevailed in RPL patients more than in controls (86.6% vs. 75.7%; $\chi2$=9.025, *P*=0.0027). The frequency distribution of alleles of the *PAI-1* 4G/5G polymorphism showed that the carriage of the 4G allele was greater in RPL patients than in controls (72.3% vs. 45.6%; $\chi2$=34.216, *P*=0.0000) (Table 2).

Table 1.

Baseline characteristics of the study groups.

Variable		RPL (n=119)	Control (n=113)	P-value
Age, years		31.3±5.9	30.3±5.4	0.180
RPL number, n (%)	<4	89 (74.8%)	-	-
	≥4	30 (25.2%)	-	-

Table 2.

The frequency distribution of alleles and genotypes of the ACE I/D and PAI-1 4G/5G polymorphisms in the study groups.

Gene	Genotype Allele	RPL (n=119)	Control (n=113)	Statistics
ACE Genotype	II ID DD	7(5.9%) 18(15.1%) 94(79.0%)	1(9.7%) 33(29.2%) 69(61.1%)	$\chi2$=8.986 *P*=0.0112
Allele	I D	32(13.4%) 206(86.6%)	55(24.3%) 171(75.7%)	$\chi2$=9.025 *P*=0.0027
PIA-1 Genotype	5G/5G 4G/5G 4G/4G	7(5.9%) 52(43.7) 60(50.4%)	38(33.6%) 47(41.6%) 28(24.8%)	$\chi2$=33.111 *P*=0.0000
Allele	5G 4G	66(27.7%) 172(72.3%)	123(54.4%) 103((45.6%)	$\chi2$=34.216 *P*=0.0000

The distribution of polymorphic markers of the *ACE* I/D and *PAI-1* 4G/5G polymorphisms in controls was in HWE (Table 3).

Analysis of the multiplicative and additive models for the *ACE* I/D polymorphism showed a significant risk of RPL with the carriage of the D allele (OR=2.07, 95% CI: 1.28-3.35, *P*=0.003) and the homozygous DD genotype (OR=2.40, 95% CI: 1.34-4.29, *P*=0.008) (Tables 4 and 5).

The multiplicative and additive models for the *PAI-1* 4G/5G polymorphism showed a significant risk of RPL with the carriage of the 4G allele (OR=3.11, 95% CI: 2.12-4.58, *P*=0.000) and the homozygous 4G/4G genotype (OR=3.09, 95% CI: 1.77-5.39, *P*=0.000) (Tables 4 and 5).

However, the carriage of risk-polymorphic markers, the *ACE* I/D and *PAI-1* 4G/5G polymorphisms, was not associated with the number of RPL (Table 6).

The combined carriage of the homozygous DD genotype and heterozygous ID genotype of the *ACE* I/D polymorphism with the homozygous 4G/4G genotype of the *PAI-1* 4G/5G polymorphism occurs significantly more often in RPL women than healthy women (*P*=0.000 and *P*=0.019, respectively). Carriage of the *PAI-1* 5G/5G genotype in healthy women was not associated with the *ACE* I/D polymorphism (Table 7).

Discussion

Pregnancy complications can affect the mother's health, the baby's health, or both. Two or more consecutive pregnancy losses constitute RPL, the causes of which are unidentifiable in 40%-50% of pregnancy losses.[35] However, thrombophilic disorders and hypofibrinolysis were demonstrated to be risk factors in a majority of women with RPL. PAI-1 plasma levels in RPL patients are increased, compared to women with healthy pregnancies.[36]

The most common polymorphisms studied for association with RPL are thrombophilic gene polymorphisms. [37-39] Among all the thrombophilic genes, functional *PAI-1*-6754G/5G polymorphism is one of the most frequently analyzed *PAI-1* genetic variants. However, the contribution of *PAI-1*-6754G/5G to unexplained RPL has remained controversial.[40]

Table 3.

The distribution of polymorphic markers of the ACE I/D and PAI-1 4G/5G polymorphisms in RPL patients and controls.

Gene	Polymorphism	Genotype	RPL (n=119)	HWE (n=113)	χ^2	*P*	Control	HWE	χ^2	*P*	Allele	Frequency of alleles	
												RPL	Control
ACE	I/D	DD	0.790	0.749	5.09	0.025	0.611	0.572	2.08	0.15	D	0.866	0.757
		ID	0.151	0.233			0.292	0.368			I	0.134	0.243
		II	0.059	0.018			0.097	0.059					
PAI-1	4G/5G	4G/4G	0.504	0.522	0.44	0.51	0.248	0.208	1.62	0.2	4G	0.723	0.456
		4G/5G	0.437	0.401			0.416	0.496			5G	0.277	0.544
		5G/5G	0.059	0.077			0.336	0.296					

Table 4.

Genetic predisposition to RPL (the multiplicative inheritance model)

Gene	Polymorphism	Allele	Frequency of alleles		χ^2	*P*	OR (95%CI)
			RPL (n=119)	Control (n=113)			
ACE	I/D	I	0.134	0.243	9.03	0.003	0.48 (0.30-0.78)
		D	0.866	0.757			2.07 (1.28-3.35)
PAI-1	4G/5G	4G	0.723	0.456	34.22	0.000	3.11 (2.12.-4.58)
		5G	0.277	0.544			0.32 (0.22-0.47)

Table 5.

Genetic predisposition to RPL (the additive inheritance model [CATT])

Gene	Polymorphism	Genotype	RPL (n=119)	Control (n=113)	χ^2	*P*	OR (95%CI)
ACE	I/D	DD	0.790	0.611	7.06	0.008	2.40 (1.34-4.29)
		ID	0.151	0.292			0.43(0.23-0.82)
		II	0.059	0.097			0.58 (0.22-1.55)
PIA-1	4G/5G	4G/4G	0.504	0.248	30.65	0.000	3.09 (1.77-5.39)
		4G/5G	0.437	0.416			1.09 (0.65-1.83)
		5G/5G	0.059	0.336			0.12 (0.05-0.29)

Table 6.

Polymorphic markers the ACE I/D and PAI-1 4G/5G polymorphisms and the number of RPL

Gene	Polymorphism	RPL number		*P*	χ2
		<4 (n=98)	≥4 (n=21)		
ACE	D/D	77(78.6)	17(81.0)	0.947	0.109*
	D/I	15(15.3)	3(14.3)		
	I/I	6(6.1)	1(4.8)		
PAI-1	5G/5G	4(4.1)	3(14.3)	0.141	3.92
	4G/5G	42(42.9)	10(47.6)		
	4G/4G	52(53.1)	8(38.1)		

**Yates' chi-square*

Table 7.

The combined carriage of the ACE I/D and PAI-1 4G/5G polymorphism genotypes in the study groups.

ACE		*PlA-1*			*P*	χ2
		5G/5G	4G/5G	4G/4G		
D/D	RPL (n=94)	6(6.4%)	44(46.8%)	44(46.8%)	0.000	19.93
	Control (n=69)	21(30.4%)	32(46.4%)	16(23.2%)		
I/D	RPL (n=18)	0	6(33.3%)	12(66.7%)	0.019	7.933*
	Control (n=33)	12(36.4%)	11(33.3%)	10(30.3%)		
I/I	RPL (n=7)	1(14.3%)	2(28.6%)	4(57.1%)	0.482	1.461*
	RPL (n=11)	5(45.5%)	4(36.4%)	2(18.2%)		

**Yates' chi-square*

A meta-analysis by Li et al.[41] that included 22 studies with 4306 cases and 3076 controls showed that *PAI-1* 4G/5G polymorphism is associated with an increased RPL risk (P=0.0003), especially in the Caucasian subgroup (P<0.001). Other studies suggest that *PAI-1*-6754G/5G alone is not responsible for RPL.[42,43]

Many studies have indicated that ACE affects hemostasis through different mechanisms, including platelet aggregation, blood clotting, and fibrinolysis.[44-46] Endothelial PAI-1 synthesis is induced by AT-II, which is generated by ACE.

Results of a study performed by Buchholz et al.[28] showed that homozygosity for the D allele of the *ACE* gene, which results in elevated PAI-1 concentrations and hypofibrinolysis, is associated with an elevated risk of recurrent spontaneous miscarriages, and the combination of the DD genotype with 4G/4G genotype of the *PAI-1* promoter, which further increases PAI-1 plasma levels, is significantly more frequent in patients with recurrent spontaneous miscarriages than in controls.

In a study by Fazelnia et al.,[44] there was a significant association between the DD genotype of the *ACE* I/D polymorphism and RPL in women from the north of Iran (OR=2.04; 95% CI=0.94-4.44; P=0.036) with unexplained RPL. The D allele of the *ACE* I/D polymorphism was also significantly associated with the RPL (OR=1.59; 95% CI=1.05-2.41; P=0.013).

A systematic review and meta-analysis by Su et al.[42] were conducted to investigate the association between the *PAI-1* 4G/5G and *ACE* I/D polymorphisms with idiopathic RPL. Case-control studies comprising a total of 2820 RPL patients and 3009 controls were analyzed. Meta-analyses showed a significant association between *ACE* I/D polymorphism and idiopathic RPL [OR=1.29, 95% CI: 1.02-1.62)]. There were no associations between *PAI-1* 4G/5G polymorphism and RPL in studies including more than two or three recurrent abortions.

A study performed by Aarabi et al.[43] investigated the *PAI-1* 4G/5G and *ACE* I/D polymorphisms in association with RPL in Iranian patients and normal healthy controls. Patients with the homozygote 4G/4G genotype were significantly more prone to RPL than others (OR=11.0, 95% CI: 2.3-52.4). For the *ACE* I/D polymorphism, no such association was found. A study performed by Shakarami et al.[47] also showed that patients with a homozygote 4G mutation were significantly more prone to RPL than the control group (OR: 4.63, % 95 CI: 1.55-13.84); at the same time, there were no significant associations between the *ACE* D allele or DD genotype and RPL.

Coulam et al.[37] found that women with a history of implantation failure after IVF-embryo transfer displayed a higher prevalence of the *PAI-1* 4G/5G mutations than controls (P=0.007). In a study by Goodman et al.,[48] a total of 550 women with a history of RPL were examined for the association of specific inherited thrombophilias and RPL. It was found that the *PAI-1* 4G/5G polymorphism correlated significantly with RPL, compared with controls (P=0.009). In contrast, Wolf et al.[30] did not find an association between the *PAI-1* 4G/4M polymorphism and RPL.

In our study, the combined carriage of the homozygous DD genotype and heterozygous ID genotype of the *ACE* I/D polymorphism with the homozygous 4G/4G genotype of the *PAI-1* 4G/5G polymorphism occurs significantly more often in RPL women than in healthy women (P=0.000 and P=0.006, respectively). In contrast, Goodman et al.[48] showed that homozygosity for the D allele of the *ACE* gene and the combination of the D/D genotype with two 4G alleles of the *PAI-1* promoter gene were not associated with a significant increase in the risk of recurrent miscarriage.

Conclusion

Our results showed a significant risk of RPL development with the carriage of the D allele and the homozygous DD genotype of the *ACE* I/D polymorphism and of the 4G allele and the homozygous 4G/4G genotype of the *PAI-1* 4G/5G polymorphism in Sudanese women. The combined carriage of the homozygous DD genotype and heterozygous ID genotype of the *ACE* I/D polymorphism with the homozygous 4G/4G genotype of the *PAI-1* 4G/5G polymorphism occurs significantly more often in RPL women than healthy Sudanese women. Testing for the *ACE* I/D and *PAI-1* 4G/5G polymorphisms should be part of the standard examination for patients with RPL.

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Competing Interests

The authors declare that they have no competing interests.

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