Original Article

The Prevalence Of Specific Gene Polymorphisms Related To Thrombophilia In Egyptian Women With Recurrent Pregnancy Loss

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Abstract

Background: Despite the enhanced progress in identifying a number of leading causes to fetal miscarriage, still some women suffer from recurrent pregnancy loss (RPL) for unknown cause. A hidden genetic influence of coexisting hereditary thrombophilia was assumed to have a role. **Aim:** The aim was to investigate the association between unexplained RPL and thrombophilic gene variants of angiotensin I-converting enzyme (*ACE)* (rs4646994) and *β-fibrinogen* (rs1800790) genes. **Settings and Design:** The present case–control study was conducted on unexplained RPL in eighty women and eighty matched controls with no history of previous pregnancy loss. **Materials and Methods:** Analysis of extracted DNA was performed using polymerase chain reaction-restriction fragment length polymorphism method. **Statistical Analysis:** The frequency of genotypes and alleles was compared between groups using Chi-square test or Fisher's exact test. Risk assessment was made by odds ratio (OR) at a 95% confidence interval (CI). **Results:** Women with RPL group had higher frequency of DD than controls (47.5%, 31.25%, respectively, $P = 0.086$). D allele frequency was 0.67 and 0.54 in the control ($P = 0.022$). D allele carriers were at higher risk of RPL than the control as OR was 1.694 at 95% CI from 1.08 to 2.67. There was no association between the rs1800790 variant of *β-fibrinogen* gene and RPL. **Conclusion:** Females who are carriers for D allele of *ACE* I/D gene polymorphism are more liable to suffer from RPL. Screening for hereditary thrombophilia in females who are planning to conceive and have a history of RPL of unidentified cause is of great value to provide proper management and genetic counseling to high-risk couples.

Keywords: *β-fibrinogen gene, angiotensin I-converting enzyme gene, genetic polymorphisms, hereditary thrombophilia, recurrent pregnancy loss*

Introduction

Recurrent pregnancy loss (RPL) or recurrent miscarriage has been defined over decades as three or more miscarriages before 24 weeks of gestation.[1] This definition was revised by the American Society for Reproductive Medicine in 2013 and again by the European Society of Human Reproduction and Embryology (ESHRE) in 2018, as having two or more failed pregnancies before 24 weeks of gestation. It is believed that this new definition will facilitate research, shared decision-making, and psychological support to couples. Furthermore, testing for the most common treatable cause of RPL, anti-phospholipid syndrome (APS), can be valuable after two losses.[2,3]

RPL has an estimated worldwide prevalence of 1%–5% in married couples. Several causes are involved in the etiology, including endocrinal, infectious, immunological factors, uterine anatomical abnormalities, and parental

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chromosomal aberrations. However, in many cases, routine gynecological and laboratory investigations fail to identify the underlying cause.[1-3] Among these possible causes, great attention has been paid to the genetic basis of thrombophilia that may adversely affect the outcome of pregnancy. Hereditary thrombophilia may be caused by genetic mechanisms where polymorphisms of thrombophilic genes may lead to impaired placental circulation and subsequent fetal problems.

Rigat *et al*. discovered the angiotensin I-converting enzyme (*ACE*) gene in 1990. *ACE* gene is mapped to the long arm of chromosome 17 (17q23) and encodes the *ACE* enzyme, which plays a major role in the rennin-angiotensin system (RAS) and fibrinolysis regulation in the body through its conversion of angiotensin I to angiotensin II.[4]

It is well documented that RAS has a fundamental role in preserving normal pregnancy. Plasma levels of angiotensinogen and angiotensin II are constantly rising during the 1st week of gestation till the 20th week. Evidence based research proved that angiotensin II is an essential regulator for crucial biological processes, namely aldosterone secretion, fluid and electrolyte balance, and peripheral vascular resistance, in which these functions are mediated through its highly specialized receptors at target tissues.[5]

In addition, angiotensin II has angiogenic action in conjunction with other vascular endothelial growth factors to enhance endometrial angiogenesis and promote uteroplacental perfusion that is essential for successful intrauterine implantation and normal fetal growth.[6,7]

The well-studied *ACE* I/D gene polymorphism (rs4646994) is the consequence of the insertion-I or deletion-D of a 287 bp Alu repeat element at intron 16 in the *ACE* gene, which results in three genotypes: DD, ID, and II. *ACE* I/D polymorphism has its impact on ACE and subsequently angiotensin II levels in the blood. Individuals with DD genotype have higher plasma levels of ACE compared to individuals with ID and II genotype.[4]

The importance of RAS and blood homeostasis during pregnancy made this polymorphism and its effect on the pregnancy outcome a target for many scientists. Angiotensin II level is directly proportional to ACE enzyme activity. High levels of angiotensin II and ACE in early pregnancy may cause impaired implantation and fetoplacental microvasculature with subsequent adverse pregnancy outcomes resulting in recurrent miscarriage, restricted fetal growth or preeclampsia through its vasoconstrictor effect, enhanced production of free oxidative radicles, altered expression of endometrial angiotensin receptors and increased tendency to intravascular thrombosis.[8-12]

β-fibrinogen gene (FGB) is another thrombophilic gene located at 4q31.3 and encodes the β-chain of fibrinogen; coagulation factor 1. Fibrin and blood clot formation in response to tissue injury occurs as a response to thrombin cleavage of fibrinogen. Furthermore, it has a role in vasoconstriction and angiogenesis. Individuals who carry the common variant 455G/A (rs1800790) of the *β-fibrinogen* gene, which is caused by G/A substitution in the promoter region, may have 7%–10% higher plasma fibrinogen levels than others.[13,14]

For normal intrauterine implantation and fetal development, adequate fetoplacental circulation is a must, depending on the balance between coagulation and fibrinolysis cascades. Conflicting results were seen in finding the link between genetic issues and a tendency to thrombosis in women with unexplained RPL. The aim of the study was to investigate the common variants of *β-fibrinogen* (rs1800790) and angiotensin I-converting enzyme (*ACE*) (rs4646994) genes and their association with unexplained RPL.

Methods

The current case–control study included 160 women divided into two groups: Group 1 was comprised 80 females who had at least three RPLs at ≤24 weeks of gestation and were referred from the outpatient gynecology clinic at the Maternity University Hospital to the outpatient genetic clinic at the Medical Research Institute for genetic evaluation during the period from September 2016 to April 2017.

Group 2 was comprised 80 volunteered healthy women matched for age and ethnicity and with at least two live births as a control group.

A written, informed consent was obtained from all participants, and approval for conducting the study was obtained from the local ethical committees (10 GR 0008812). The study protocol was in agreement with the Declaration of Helsinki guidelines1975, as revised in 2000.

Inclusion and exclusion criteria

Complete history taking, full clinical and gynecological assessment in association with laboratory investigations and chromosome analysis to excluded other causes of recurrent abortion were done. Accordingly, participating women with unexplained RPL for more than three consecutive times before 24 weeks of gestation were selected depending on the following inclusion and exclusion criteria:

- No structural uterine abnormalities are found through transvaginal 3D ultrasound or hysteroscopy

- -Absence of infectious diseases by the high vaginal swab
- -Women with hypertension, diabetes mellitus, or thyroid gland disorders were excluded
- No hormonal disturbances in at least 6 months after last pregnancy loss
- APS, which exists in 15% of couples with recurrent miscarriage was excluded through screening by two positive tests at least 12 weeks apart for either lupus anticoagulants or anticardiolipin antibodies present in a medium or high titer over 40 g/L or >99th percentile
- All women with a past or family history of thrombosis were excluded from the study
- Screening for inherited thrombophilia was done to all women using Siemens commercial kits for antithrombin III, protein C, protein S and factor V Leiden by Sysmix 1500 to exclude activated protein C resistance (factor V Leiden), protein S deficiency and prothrombin gene mutations
- Chromosome analysis was performed on all enrolled women and their husbands to exclude any carriers of chromosomal aberration.

In addition, healthy women who had at least two normal children with no previous history of adverse pregnancy outcomes or abortion were included as control.

Genetic analysis

Blood samples were collected from both groups for genetic analysis, in which 2 ml of blood were collected in sodium heparin tubes from the women and their husbands to exclude chromosomal aberrations using trypsin G-banding technique with some modifications.[15]

While another 2 ml of blood were collected in EDTA tubes for DNA extraction using QIA Amp® DNA Blood Mini kits (QIAGEN Hilden, Germany) following the manufacturer's instructions and stored at −20°C for use. DNA concentration and purity were measured by a NanoDrop™ 1000 spectrophotometer. Genotyping was performed using the polymerase chain reaction-restriction fragment length polymorphism method using specific primers and restriction enzymes [Table 1]. PCR reaction was done in a total 25 µl volume for each SNP, 30 ng of genomic DNA were mixed with specific primers according to previous reports.[16,17]

PCR conditions were as following: for rs4646994, one cycle for initial denaturation at 96°C for 5 min, then amplification occurs by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s. and extension at 72°C for 1 min followed by one cycle of final extension at 72°C for 5 min.

For rs1800790, one cycle for initial denaturation at 95°C for 5 min, then amplification occurs by 35 cycles of denaturation at 94°C for 40 s, annealing at 53°C for 40 s and extension at 72°C for 40 s followed by one cycle of final Extension at 72°C for 7 min.

PCR products were digested with HaeIII restriction enzyme (Thermo Scientific Inc., USA®) for the *β-fibrinogen* gene and Hind III restriction enzyme (Thermo ScientificInc, USA®) for the *ACE* gene at 37°C for 16 h, then the cleaved bands were visualized on 2% agarose gel electrophoresis after staining with ethidium bromide under UV illumination. The results were confirmed after the repeat of some samples which were randomly selected.

Statistical analysis

The current study included all women with RPL referred at the assigned period and in the margin of the inclusion criteria. The number of enrolled participants met the requirements of minimal sample size in healthcare research to get normally distributed data (30 samples), the study of more than one common SNPs, and their frequency in comparison to previous reports. However, limits to increase the sample size were related to financial issues.

Statistical analysis was performed using the IBM SPSS software package version 20.0 (IBM Corp., Armonk, NY, USA). The age and body mass index of the studied groups were presented as mean ± standard deviation. The frequency of qualitative variables was addressed as percentage. The Kolmogorov–Smirnov test was used to verify the normality of the distribution of variables. Student's *t*-test was used to compare two groups for normally distributed quantitative variables while Mann–Whitney test was used to compare between two groups for not normally distributed quantitative variables. The Hardy–Weinberg equilibrium (HWE) equation was assessed in controls. Allele and genotype frequency was calculated by direct counting and compared between the groups by Chi-square test or Fisher exact test. The odds ratio (OR) at 95% confidence interval (CI) were estimated in order to assess the risk of association between RPL and the studied gene variants. *P* values less than 0.05 were considered statistically significant.

Results

One hundred sixty women were enrolled in the current study divided into two groups: Group 1 involved 80 of women with RPL; their mean age was 30 ± 5.07 years and Group 2 had 80 healthy women with mean age 31 ± 5.57 years. There was no difference in age and BMI between both groups (P = 0.237 and P = 0.417, respectively). Table 2 describes the demographic data of women enrolled in the study. Karyotype was normal in

Table 1: Primers and polymerase chain reaction products of studied *β-fibrinogen* and *ACE* gene polymorphisms

Gene	Primer sequence	Restriction enzymes	PCR products
FGB (rs1800790)	F: 5'-AGGGTCTTTCTGATGTGT-3'	Hae III	GG: 215 and 121 bp
	R: 5'-AAGTTAGGGCACTCCTCA-3'		AA: 336 bp
			AG: 336, 215 and 121 bp
ACE (rs4646994)	F: 5'-CTGGAGACCACTCCCATCCTTTCT 3'	Hind III	DD: 190 bp
	R: 5'- GATGTGGCCATCACATTCGTCAGAT 3'		II: 490 bp
			ID: 190 and 490 bp

PCR=Polymerase chain reaction

Table 2: Descriptive data of women with recurrent pregnancy loss and control women

	RPL (*n*=80)	Control (*n*=80)	*P*
Age (years), mean±SD	30±5.07	31±5.57	0.237
BMI (kg/m^2), mean±SD	24.5±5.4	23.8±5.5	0.417
Hypertension (%)	0 (0)	0 (0)	-
Number of live births, median (range)	1.5 (1-2)	4 (2-6)	<0.001*
Number of abortions, median (range)	6 (3-9)	0	<0.001*
Type of abortion (%)			
Primary RPL	54 (67.5)	0 (0)	<0.001*
Secondary RPL	26 (32.5)	0 (0)	<0.001*

*Statistically significant at P<0.05. BMI=Body mass index, SD=Standard deviation, RPL=Recurrent pregnancy loss

all females (46,XX), and no chromosomal abnormalities were detected in their husbands.

Genotype and allele frequency of *ACE* I/D (rs4646994) gene polymorphism

The frequency of *ACE* genotypes in the control group was in agreement with the HWE (χ2 = 0.37; P = 0.544).

DD genotype frequency (normal homozygous) was higher in females with RPL than the control group (47.5% vs. 31.25%) and the II (mutant homozygous) genotype was more frequent in the control than in the RPL group (22.5% vs. 13.7%). The frequency of the heterozygous ID genotype was higher in the control group than the RPL group (46.25% vs. 38.7%), although these observations were statistically nonsignificant (P = 0.086).

There was a statistically significant increase in D allele frequency in the RPL group than in the control group (0.67 vs. 0.54, P = 0.022). D allele carrier females were at risk to have RPL by 1.7-fold than the controls (OR 1.694; 95% CI: 1.08–2.67) [Table 3].

Genotype and allele frequency of the *β-fibrinogen* gene 455G/A polymorphism (rs1800790)

The genotype distribution of the *β-fibrinogen* gene in the control group was in agreement with the HWE (χ^2 = 0.016; P = 0.899). There were no statistically significant differences in genotype or allele frequency of *β-fibrinogen* gene polymorphism between the RPL and control groups (P = 0.810 and P = 0.520, respectively) [Table 4].

The association of different genetic models of the *β-fibrinogen* gene 455G/A (rs1800790) and the *ACE* I/D (rs4646994) gene variants with RPL were assessed by Odds and 95% CI. In the case of the *ACE* gene polymorphism, there was an increased risk of RPL in patients with the dominant model (OR: 1.82 [0.79–4.15], P = 0.151). A low risk of RPL was observed in the recessive model (OR: 0.502 [0.26–0.96], P = 0.035). No association was found between RPL and any genetic model of the *β-fibrinogen* gene variant [Tables 3 and 4].

Discussion

There are several physiological changes that occur during normal pregnancy to create a healthy environment for proper intrauterine fetal development. Hypercoagulability of pregnancy is a state, in which some coagulation factors increase in blood in harmony with a decreased blood level of others. These changes return back to the normal physiological state after labor and puerperium without causing any harm to the mother. However, the hypercoagulability state of pregnancy may be exacerbated by alterations in the coagulation/fibrinolysis cascades, together with associated vascular stasis and hormonal induced endothelial injury throughout the course of pregnancy.

Balanced coagulation/fibrinolysis mechanisms are under genetic control from several genetic loci. Gene mutations in such loci, whether inherited or acquired, may exacerbate hypercoagulability and predispose to placental vascular thrombosis and subsequent adverse pregnancy outcomes.[18]

Table 3: Distribution of *ACE* I/D gene polymorphism (rs4646994) genotypes in recurrent pregnancy loss and control groups

Genotypes/Allele	RPL (total 80), *n* (%)	Control (total 80), *n* (%)	OR (95% CI)	*P*
DD	38 (47.5)	25 (31.25)	Reference	0.086
ID	31 (38.7)	37 (46.25)	0.55 (0.28-1.10)	
II	11 (13.7)	18 (22.5)	0.40 (0.16-0.99)	
D	107 (67)	87 (54)	Reference	0.022*
I	53 (33)	73 (46)	1.694 (1.08-2.67)	
Dominant model (DD vs. II+ID)			1.82 (0.79-4.15)	0.151
Recessive model (DD+ID vs. II)			0.502 (0.26-0.96)	0.035*
HWE				0.544
MAF				0.46

*Statistically significant at $P<0.05$. HWE=Hardy-Weinberg equilibrium, MAF=Minor Allele frequency, OR=Odds ratio, CI=Confidence interval, RPL=Recurrent pregnancy loss

Table 4: Distribution of genotype frequency of *β-fibrinogen* gene polymorphism (rs1800790) in recurrent pregnancy loss and control groups

Genotypes/Allele	RPL (total 80), *n* (%)	Control (total 80), *n* (%)	OR (95% CI)	*P*
GG	47 (58.7)	43 (53.7)	Reference	0.810
GA	28 (35)	31 (38.7)	0.83 (0.43-1.59)	
AA	5 (6.25)	6 (7.5)	0.76 (0.22-2.68)	
G	122 (76)	117 (73)	Reference	0.520
A	38 (24)	43 (27)	1.180 (0.71-1.95)	
Dominant model (GG vs. GA+AA)			0.82 (0.44-1.5)	0.524
Recessive model (GG+GA vs. AA)			1.22 (0.356-4.159)	0.757
HWE				0.899
MAF				0.27

*Statistically significant at $P<0.05$. HWE=Hardy-Weinberg equilibrium, MAF=Minor Allele frequency, OR=Odds ratio, CI=Confidence interval, RPL=Recurrent pregnancy loss

An emerging interest in recent years is the endeavor to identify the possible role of thrombophilic as well as hypofibrinolytic genes on the susceptibility to RPL in different ethnic groups and populations.[19,20] This may guide against the prevalent use of therapeutic anticoagulants as a prophylactic regimen in cases of RPL of unidentified cause.

Based on these findings, we were encouraged to investigate the common polymorphisms of *ACE* I/D (rs4646994) and *β-fibrinogen* 455G/A (rs1800790) genes and their association with RPL.

In the current study, 80 females with RPL of unknown cause and 80 healthy females as a control were included. Regarding *ACE* I/D gene polymorphism, MAF was within the values found in Caucasians (0.42–0.608).[21] DD genotype frequency was higher in women with RPL than the control (47.5% vs. 31.25%, $P = 0.086$) with significant increase in D allele frequency in the RPL group than the control (0.67 vs. 0.54, $P = 0.022$). The risk of RPL in D allele carrier women was 1.7-fold higher than the I allele carriers. D allele frequency in our Egyptian sample was close to that reported among women withRPL in different studies from different ethnic groups.[8,9,22,29,31-34] In contrary, other studies did not find an association between *ACE* I/D gene polymorphism and RPL.[23-28,30] Table 5 shows the allele frequency of *ACE* I/D gene polymorphism of our studied sample in comparison with other studies from different countries.

Although the *ACE* I/D gene polymorphism (rs4646994) is well studied in different populations, its exact role in the predisposition to RPL is still unclear. This is due to contradictory results which may be attributed to ethnic differences and the prevalence of this polymorphism in different populations. Other factors that cannot be ignored are related to study design, the definition of repeated abortion- whether two times or three times, of pregnancy loss before the 24th week of gestation and criteria of selection of the cases studied. Other genetic factors and/or epigenetic modulations may have its impact.

In the current sample, the risk for RPL was significantly low with the recessive model of *ACE* I/D gene polymorphism ($P = 0.035$) and it was 1.82 fold higher with the dominant model, although it was statistically nonsignificant ($P = 0.151$). Overall, we cannot assume that the recessive model of *ACE* I/D polymorphism exerts a protective role against RPL unless these

Table 5: Comparison between allele frequency of *ACE* I/D gene polymorphism (rs4646994) in women with recurrent pregnancy loss and controls in different countries

Country	Sample size (case/control)	RPL time	Allele frequency				HWE	Reference
			Case		Control			
			D	I	D	I		
Current study (Egypt)	80/80	≥3	0.67	0.33	0.54	0.46	0.544	
Italy	59/70	≥3	0.65	0.03	0.59	0.50	0.231	Vettriselvi *et al*. (2008)[23]
India (South)	104/120	≥2	0.85	0.06	0.92	0.148	≤0.001	Goodmann *et al*. (2009)[24]
USA	120/84	≥2	0.51	0.025	0.44	0.56	0.001	Bucreeve *et al*. (2009)[8]
Germany	314/553	≥2	0.55	0.13	0.52	0.48	0.482	Corbo *et al*. (2011)[9]
Italy	18/74	≥2	0.83	0.11	0.39	0.60	0.001	Zhang *et al*. (2011)[25]
China	127/132	≥2	0.91	0.06	0.50	0.204	0.064	Kim *et al*. (2014)[26]
Korea	227/304	≥2	0.178	0.08	252	356	0.957	Kurzawinska *et al*. (2016)[27]
Poland	152/180	≥2	0.52	0.02	0.52	0.47	0.477	Pereza *et al*. (2016)[28]
Slovenia	149/149	≥3	0.54	0.02	0.57	0.42	0.071	Fazelnia *et al*. (2016)[29]
Iran	100/100	≥2	0.49	0.03	0.60	0.39	0.001	Al-Mukayannizi *et al*. (2016)[30]
Saudi Arabia	61/59	≥3	0.98	0.06	0.02	0.89	0.272	Hussian *et al*. (2016)[31]
Sudan	40/40	≥3	0.60	0.06	0.77	0.03	0.805	Lopez-Jimenez *et al*. (2016)[32]
Mexico	55/50	≥3	0.54	0.06	0.39	0.61	0.154	Chatzidimitriou *et al*. (2017)[33]
Greece	48/27	≥2	0.58	0.10	0.48	0.52	0.179	Gamus *et al*. (2018)[34]
Turkey	1007/169	≥2	0.58	0.42	0.50	0.49	0.430	Fatini *et al*. (2000)[22]

RPL=Recurrent pregnancy loss, HWE=Hardy-Weinberg equilibrium

observations are confirmed by a large, comprehensive study.

Lack of association between genotypes and alleles of *β-fibrinogen* 455G/A (rs1800790) gene polymorphism and RPL was noticed in the present work. By literature review, there was a paucity of studies concerning *β-fibrinogen* gene polymorphism and the risk of RPL, especially in Arab countries. Overall, most studies failed to find the meaningful association of different genetic models of *β-fibrinogen* 455G/A (rs1800790) polymorphism with RPL, especially in the Asian and Caucasian subgroups.[35-37] This could be attributed to gene-gene interaction, gene-environment interaction, or other *β-fibrinogen* polymorphisms that may mask *β-fibrinogen* gene function. These results were in contrary to other studies.[38,39]

Finally, the discrepancy between studies regarding the genetic predisposition of thrombophilia has made some clinicians satisfied with routine genetic screening for thrombophilia and prophylactic anticoagulant use in cases of RPL. However, the new ESHRE guidelines published in 2018[3] recommend that genetic screening should not be done routinely in any woman with RPL unless there is inherited thrombophilia in combination with other risk factors for thromboembolism. In such cases, antithrombotic prophylaxis (heparin and low-dose aspirin) may improve the live birth rate.

The limitation to our study may be related to the absence of measured ACE enzyme and *β-fibrinogen* levels in the blood of women with RPL and the control group, which rendered genotype-phenotype correlation inaccessible. We also propose to conduct a future study that investigates thrombophilic gene polymorphisms in a large number of affected females with RPL.

Conclusion

In the present study, there was a clear association between the D allele of *ACE* I/D gene polymorphism and RPL in the absence of evident association of *β-fibrinogen* 455G/A gene polymorphism. In view of the mentioned previous data, an expanded genetic screening panel of thrombophilia for women with unexplained RPL is needed to detect affected mothers and provide proper genetic counseling and management with anticoagulants (low dose aspirin and heparin). Indeed, more studies are required with genetic background to clarify the hidden role of inherited thrombophilia and its relation to antithrombotic drug response in women with RPL.

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Conflicts of interest

There are no conflicts of interest.

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