

LACK OF ASSOCIATION BETWEEN ANGIOTENSIN CONVERTING ENZYME I/D POLYMORPHISM AND UNEXPLAINED RECURRENT MISCARRIAGE IN SAUDI ARABIA

NEPOSTOJANJE POVEZANOSTI IZMEĐU I/D POLIMORFIZMA ANGIOTENZIN-KONVERTUJUĆEG ENZIMA I NEOBJAŠNJENIH VIŠESTRUKIH SPONTANIH POBAČAJA U SAUDIJSKOJ ARABIJI

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Summary

Background: An insertion/deletion (I/D) polymorphism in the angiotensin converting enzyme (ACE) gene has been associated with recurrent miscarriage (RM) in several populations. We initiated this study to determine the association, if any, between the I/D polymorphism of ACE gene and RM in Saudi females.

Method: This study was conducted on 61 Saudi females suffering from RM (mean age: 34.1±6.2 years; range 15–45) attending clinics at King Khalid University Hospital, and 59 age matched females who had at least 2 children, as controls. Blood samples were drawn in EDTA tubes by venipuncture. DNA was extracted using the Puregene DNA purification kits. Insertion/Deletion (I/D) polymorphism of ACE gene was investigated by amplifying the genomic DNA by PCR using gene-specific primers. A single 190 bp or 490 bp band was obtained in the homozygous cases for the D allele or I allele, respectively, while the presence of both 190 and 490 bp bands indicated heterozygosity (ID).

Statistical analysis: Deviation from Hardy-Weinberg equilibrium was determined (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). A standard chi-square (χ^2) test was used for comparing the genotype and allele frequencies in the two

Kratik sadržaj

Uvod: Insercioni/delecijski polimorfizam (I/D) u genu za AKE (angiotenzin-konvertujući enzim) doveden je u vezu sa višestrukim spontanim pobačajima (VSP) u nekoliko populacija. Ovu studiju smo sproveli kako bismo utvrdili da li postoji ili ne postoji povezanost između I/D polimorfizma gena za AKE i VSP kod žena u Saudijskoj Arabiji.

Metod: Studija je obuhvatila 65 žena saudijske nacionalnosti sa VSP (prosek godina: 34,1±6,2 godina; raspon 15–45) koje su se lečile na klinikama Univerzitetske bolnice kralj Halid i 65 žena iste starosne dobi koje su imale najmanje dvoje dece, kao kontrolnu grupu. Uzorci krvi sakupljeni su u EDTA epruvete venepunkcijom. DNK je ekstrahovana pomoću Puregene DNA purification kitova. Insercioni/delecijski (I/D) polimorfizam gena za AKE je ispitivan putem amplifikacije genomske DNK pomoću PCR uz korišćenje prajmera specifičnih za gene. U slučaju homozigota za D alel ili I alel dobijana je po jedna traka duga 190 bp ili 490 bp, dok je prisustvo obe trake od 190 i 490 bp značilo heterozigotnost (ID).

Statistička analiza: Procenjeno je odstupanje od Hardi-Vajnerbergove ravnoteže. Za poređenje učestalosti genotipova i alela u dve grupe korišćen je standardni hi-kvadratni test (χ^2), dok su za poređenje vrednosti između dve grupe pri-

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Abbreviations: ACE: Angiotensin converting enzyme; CI: confidence intervals; EDTA: ethylene diamine tetra acetate; DNA: deoxyribonucleic acid; bp: base pair; dNTPs: deoxyribonucleotide triphosphates; HLA: human leukocyte antigen; HWE: Hardy-Weinberg equilibrium; IRB: Institutional Review Board; KKHU: King Khalid University Hospital; PCR: polymerase chain reaction; RM: recurrent miscarriage; RPL: recurrent pregnancy loss; RAS: renin-angiotensin system; SNP: single nucleotide polymorphism.

groups and Students' *t* test and χ^2 test were employed to compare values between the two groups. $P < 0.05$ was considered statistically significant.

Results: The frequencies of DD, ID, and II genotypes were 56.7%, 29.5% and 4.9%, respectively, in females with RM and 54.2%, 42.3% and 3.3% respectively in the control group, but the difference was not statistically significant.

Conclusion: In some populations, meta-analyses showed an association between I/D polymorphism and RM risk, and the D allele was implicated as an increased risk factor for RM. However, this association was not apparent in the Saudi females.

Keywords: ACE polymorphism, renin-angiotensin system, recurrent miscarriage, habitual abortion, recurrent pregnancy loss

Introduction

Recurrent miscarriage (RM), habitual abortion or recurrent pregnancy loss (RPL) is defined as three or more consecutive pregnancy losses before the 20th week of gestation (1). Several potentially causative factors have been implicated in the etiology of RM, including genetic and environmental factors and epidemiological and genetic studies suggest multifactorial inheritance (2). These different factors include: anatomical conditions such as uterine malformation and cervical incompetence; chromosomal disorders including translocations and aneuploidy; endocrine disorders, including hypothyroidism, poorly treated diabetes mellitus, polycystic ovary syndrome and toxoplasmosis (3–6). Several immune factors e.g. presence of autoantibodies, increased uterine natural killer cells and parental HLA sharing have also been implicated as causative factors for RM. In addition, thrombophilia i.e. tendency for blood clots formation, due to genetic or non-genetic causes has been shown to play a role in the development of RM (7). Despite all these causes, there remain a high percentage of patients with idiopathic causes of RM.

Genome analyses carried out during the last 1–2 decades have implicated several genes that are aberrantly expressed in females with RM. However, the genetic etiologies of RM still remain largely unknown. Studies have identified various candidate genes involved in the regulation of high blood pressure in pregnancy and preeclampsia that may lead to miscarriage or abortion (8). Special attention was paid to the study of genes of the renin-angiotensin system (RAS) because of its involvement in the synthesis of angiotensin II (9–11). Angiotensin converting enzyme (ACE; EC 3.4.15.1) is an important member of RAS. It is a dipeptidyl carboxypeptidase which is encoded by the ACE gene, located on chromosome 17q23 and contains 26 exons and 25 introns (12, 13). The ACE plays an important role in blood pressure regulation and electrolyte balance by hydrolyzing angiotensin I into angiotensin II, a potent vasopressor and aldosterone-stimulating peptide, and consequently participates in the regulation of vascular tone (13, 14). Changes in

menjeni studentov T test i χ^2 test. $P < 0,05$ smatrano je statistički značajnim.

Rezultati: Učestalosti DD, ID i II genotipova bile su 56,7%, 29,5% i 4,9% kod žena sa VSP, odnosno 54,2%, 42,3% i 3,3% u kontrolnoj grupi, ali razlika nije bila statistički značajna.

Zaključak: U nekim populacijama metaanalize su pokazale povezanost između I/D polimorfizma i rizika za VSP, ali D alel je bio umešan kao faktor povećanog rizika za VSP. Međutim, ova povezanost nije bila ispoljena kod saudijskih žena.

Ključne reči: polimorfizam ACE, renin-angiotenzin sistem, višestruki spontani pobačaji

vascular metabolites affect the functions of the fetoplacental complex and may induce abnormalities of blood circulation in the placenta (14, 15), resulting in RM.

A polymorphism was defined in the intron 16 of the ACE gene by Rigat et al. (16, 17), as the presence (insertion, I) or absence (deletion, D) of a 287 bp fragment, producing three genotypes: DD, ID, and II. This polymorphism was later classified as SNP rs4646994, and was shown to have a considerable influence on the level of ACE in plasma, where the D allele was associated with an elevated level (16, 18). For over two decades considerable interest had been directed to the study of I/D polymorphism in association with different disease states (19, 20). Several diseases were related to the frequency of I and D alleles and II, ID or DD genotypes of the ACE gene. This was the case particularly with hypertension and cardiovascular disease susceptibility. However, the reports presented contradictory findings, where associations between I/D polymorphism and various diseases were reported in some populations, but not in others (21–23). Several studies were also conducted to investigate the association between ACE gene polymorphism and the frequency of RM (10, 24–26). Again, the studies reported contradictory results, where an association was reported in some studies and not in others.

In Saudi females, RM occurs frequently (ZB. personal observation) and several of the abovementioned factors play some role in causing RM. These include toxoplasmosis, chromosomal abnormalities and polycystic ovarian syndrome. However, a study reported that HLA sharing among couples appears to be unrelated to idiopathic recurrent fetal loss in the Saudi females (27). During the last few years, attention had been directed towards single nucleotide polymorphisms (SNPs) in association with unexplained RM and a recent study from our group reported a significant association between the -308 G/A polymorphism in the TNF-alpha gene promoter and the occurrence of unexplained RM in Saudi females (28). A survey of literature however showed clearly

that more studies need to be conducted on different polymorphisms to identify their role in RM.

We conducted this study on Saudi females suffering from RM in an attempt to identify any association between the I/D polymorphism of ACE gene and RM in Saudi females.

Materials and Methods

Study population

A case control study was conducted on females referred to the Recurrent Abortion Clinic, King Khalid University Hospital (KKUH), Riyadh, Saudi Arabia. The study was approved by the Institutional Review Board (IRB), College of Medicine, King Saud University. The Ethics Committee of King Khalid University Hospital and the Ethical Committee at King Saud University, Riyadh, Saudi Arabia, approved the protocol. All females included in the study were briefed and they signed the informed consent form prior to inclusion in the study.

The study group comprised 61 females with recurrent RM (cases) (mean age: 34.1 ± 6.2 years; range 15–45), while the reference population (controls) consisted of 59 age matched females who had at least 2 children, and did not have a history of pregnancy loss or any known medical illness. They were attending the prenatal clinics of the clinical co-investigator at KKUH for regular checkup. History was taken on predesigned forms and age, height (m) and weight (kg) were recorded. Body Mass index was calculated as kg/m^2 for each female (29). Routine analyses were performed at the Central Laboratory at the KKUH, to exclude any known causes of abortion. These included: parental karyotyping, toxoplasmosis, cytomegalovirus, rubella, and antiphospholipid antibodies. In addition, Protein C, Protein S, hormone levels and blood glucose levels were estimated using the procedures standardized at the KKUH Lab. Hysteroscopy, hysterosalpingography and serial ultrasound were conducted, if needed. The criteria for inclusion as »cases« were: females having unexplained recurrent miscarriage, after the tests mentioned above gave normal results.

Genotyping

Blood samples (approximately 3 mL) were drawn from RM cases and controls in ethylenediaminetetra acetic acid (EDTA) tubes by venipuncture, and were used to extract DNA using the DNA Puregene purification kit following the manufacturer's instructions. After extraction and purification, the DNA was quantitated on a NanoDrop 8000, to determine the concentration, and purity was examined using standard A260/A280 and A260/A230 ratios (NanoDrop 8000). To analyze the I/D polymorphism in intron 16

of the ACE gene, PCR using gene-specific primers, i.e. forward primer: 5'-CTG GAG ACC ACT CC ATC CTT TCT-3' and reverse primer: 5'-GAT GTG GCC ATC ACA TTC GTC AGA T-3' (Integrated DNA Technologies), following the method published earlier (30, 31). Polymerase chain reaction (PCR) amplification was performed for each sample using 50 μL reaction mixture, which contained 5 μL Tris-Cl, KCl, $(\text{NH}_4)_2\text{SO}_4$, 15 mmol/L MgCl_2 , pH 8.7, 200 $\mu\text{mol}/\text{L}$ of each dNTP, 0.2 $\mu\text{mol}/\text{L}$ of each forward and reverse primer, ~100 ng of high molecular weight DNA, and 2.5 unit/reaction Taq DNA Polymerase (Hotstar PCR, Qiagen). The PCR involved an initial 15 minute at 95° , followed by denaturation at 94°C for 20s, annealing and amplification in 40 cycles of 55°C for 30s and final extension at 72°C for 1 min. After amplification, the PCR products were separated on a 2% agarose gel, and DNA was visualized by ethidium bromide staining. The ACE I/D genotype was characterized by the length of the PCR product, where a 190 bp was obtained in the homozygous cases of the deletion (D) and 490 bp in the homozygous cases for the insertion (I) and both 190 and 490 bp bands in the heterozygotes (I/D).

The genotypes were counted manually in the cases and controls. Genotype and allele frequencies were calculated and checked for deviation from Hardy-Weinberg equilibrium (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). A standard chi-square (χ^2) test was used for comparing the genotype and allele frequencies. The odds ratio (OR) and confidence intervals (CIs) were calculated at the 95% level by Fisher's exact test (two-tailed), to measure the strength of association between the genotype or the allele and RM. Students't' test, carried out using Statistical Package for Social Sciences (SPSS) version 18 for Microsoft Window (SPSS Inc, Chicago, IL, USA) was used to compare the values of the different parameters in the two groups. A p value of <0.05 was considered as statistically significant.

Results

The demographic and clinical characteristics of unexplained RM patients and control group are presented in *Table I*. Patients and controls matched in their age and BMI ($p > 0.05$). The patient group had a higher number of pregnancies compared to the controls, but had fewer children due to a higher frequency of abortions.

The electrophoretogram showing the different ACE I/D genotypes is presented as *Figure 1*. Each sample was assigned the genotype and the frequency of each genotype and allele was calculated in the patients and control group. The genotype and allele frequencies in the cases and controls were checked for deviation from Hardy-Weinberg equilibrium. No deviation was observed (*Table II*). The genotype and allele frequencies in the cases and controls, the odds

Table I Demographic clinical characteristics of Saudi patients suffering from RM compared to the control group.

	Patient (61) Mean ± SD	Control (59) Mean ± SD	p-value*
Age (Yrs)	34.1±0.77	34.6±0.97	0.6
Body weight (kg)	75.15±18.41	72.4±15.0	0.126
BMI (kg/m ²)	30.66±1.01	28.28±0.62	0.047*
Number of pregnancies	6.5±0.38	3.9±0.22	0.0001*
Number of abortions	4.51±1.73	Nil	
Number of children	2.1±0.27	3.8±0.22	0.04*

*Student 't' was used to compare the age, body weight and BMI in patients and controls, while χ^2 analysis was used to compare the other parameters. P < 0.05 is considered statistically significant.

Table II Distribution of Genotypes of ACE follows Hardy-Weinberg equilibrium (HWE) among Saudi female patients with RM and controls.

ACE genotype	RM Cases	HWE p value	Controls	HWE p value
DD	40	0.604	32	0.272
ID	18		25	
II	3		2	

P value >0.05 indicates that the studied population was in HWE.

ratio (OR), confidence interval (CI), χ^2 and p value are presented in Table III.

The DD genotype was more prevalent in females with RM (56.7%) compared to the controls 54.2%, although the difference between the frequency was not statistically significant, whilst OR=1.61 (95% CI, 0.77–3.37), indicating a higher risk of RM in females carrying a DD genotype.

The frequencies of DD, ID, and II genotypes were 56.7%, 29.5% and 4.9% respectively in females with RM, and 54.2%, 42.3% and 3.3% respectively in healthy pregnant females. In the females with RM, the frequency of DD+ID (dominant model) was 95.0% and for II+ID (recessive model) it was 34.4%, while in the healthy pregnant females for DD+ID (dominant model) it was 96.6% and for II+ID (recessive model) 45.7%. The frequency of D allele was 0.803 in females with RM compared to 0.75 in the control group.

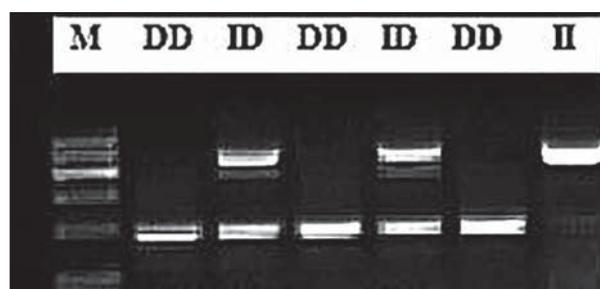


Figure 1 2% agarose gel electrophoresis, ethidium bromide staining. Lane M: DNA marker. Lane 1,3,5: 190 bp Band of genotype DD. Lane 2,4: 490 bp and 190 bp Bands of genotype I/D. Lane 6: 490 bp Band of genotype II.

Table III Genotype and allele frequencies of I/D polymorphism of the ACE gene in RM cases and controls.

	Abortion N=61	Control N=59	Odds ratio	CI	χ^2	P value
Genotype frequency						
DD, n (%)	40 (56.7)	32 (54.2)	1.61	0.77–3.37	1.606	0.205
ID, n (%)	18 (29.5)	25 (42.3)	0.57	0.27–1.21	2.159	0.141
II, n (%)	3 (4.9)	2 (3.3)	1.47	0.24–9.5	0.175	0.675
ID+DD, n (%)	58 (95.0)	57 (96.6)	1.31	0.66–2.58	0.606	0.4363
ID+ II, n (%)	21 (34.4)	27 (45.7)	0.76	0.39–1.50		
Allele frequency						
D, n (%)	98 (80.3)	89 (75.4)	1.33	0.72–2.45	0.838	0.3598
I, n (%)	24 (19.6)	29 (24.5)	0.75	0.41–1.39		

n = Number; CI = 95% confidence Interval; χ^2 = chi square.

Table IV Association studies between ACE I/D polymorphism and RM in different populations.

Ethnicity	No. investigated	Genotype frequencies			Associations (main results)	Ref. No.
		DD n (%)	ID n (%)	II n (%)		
German	RM case=184 Control=127	59 (32.1) 30 (23.6)	83 (45.1) 71 (55.9)	42 (22.8) 26 (20.5)	Significant association	34
American	RM case=120 Control=48	34 (28.3) 28 (33.3)	55 (45.8) 34 (40.8)	31 (25.8) 22 (26.2)	No significant association	35
Palestinian	RM case=100 Control=100	49 (49.0) 54 (54.0)	42 (42.0) 34 (34.0)	9 (9.0) 12 (12.0)	No significant association	36
Chinese	RM case=127 Control=132	21 (16.5) 8 (6.1)	49 (38.6) 34 (25.8)	57 (44.9) 90 (68.2)	Strong association	37
Italian	RM cases=48	25 (52)	20 (42)	3 (6)	Significant association	38
South Indian	RM case=104 Control=120	23 (22) 27 (23)	39 (38) 38 (31)	42 (40) 55 (46)	No significant association	39
Iranian Azeri Turkish	RM cases=50 Control=63	17 (34) 24 (38.1)	26 (52) 27 (42.9)	7 (14) 12 (19)	No significant association	40
Korean	RM case=251 Control=126	44 (17.5) 41 (32.5)	130 (51.8) 50 (39.7)	77 (30.7) 35 (27.8)	Significant association	41

Table IV presents the summary of ACE gene polymorphism results in different populations.

Discussion

The imbalance between fibrinolysis and coagulation pathways is included among the different factors implicated as causative factors for RM, and it may play a role in the outcome of conception. The ACE D allele was shown to be associated with an elevated level of ACE in plasma, which enhanced the production of angiotensin II from angiotensin I, thus increasing the risk of thrombotic events. In addition, the D allele also resulted in an elevated expression of plasminogen activator inhibitor-1 (PAI-1) which reduced fibrinolysis (32). It was suggested that excess fibrin accumulations in spiral arteries and within the intervillous spaces may impede perfusion and prevent normal development of the pregnancy that might lead to abortion. Thus, the D allele of ACE I/D polymorphism was considered as a hypofibrinolytic factor. Several studies showed an association between this ACE polymorphism and RM, though contradictions were not infrequent.

With this background, we investigated the ACE gene polymorphisms in Saudi females. The results obtained in the RM cases compared to the controls showed that Saudi patients with abortion had a slight-

ly increased prevalence of the ACE DD genotype compared with the controls (56.7% versus 54.2%, $P=0.205$, $OR=1.61$), but the difference was not statistically significant. Similarly, the D allele occurred more frequently in women with abortion compared to the controls (80.3% versus 75.4%, $P=0.359$, $OR=1.33$). The relative risk of abortion was 1.61-fold in pregnant women with a D allele compared to the group without a D allele. However, our results did not show a significant association between the ACE I/D polymorphism and RM in the Saudi females.

Two recently conducted meta-analyses of the available data published in 2012 and 2013 showed an association between ACE I/D polymorphism and RM risk, and the ACE polymorphic D allele was implicated as an increased risk factor for RM (24–26). Fatini et al. (33) reported a similar association of the DD genotype with first trimester miscarriages. However, contradictory results were frequent in literature and Table IV lists a few of these studies which showed differences reported in different ethnic groups (35–41). Studies on South Indian (38), Iranian Azeri Turkish (39), Italian (37) and Korean females (40), failed to show any association between I/D polymorphism and RM. In this regard, our results were in agreement with these reports in South Indian, Iranian Azeri Turkish, Italian and Korean women.

The association of DD homozygosity with other pregnancy complications such as pregnancy-induced

hypertension (41, 42) and preeclampsia, which may lead to abortion (37), have also been reported. Since the homozygosity for I allele could decrease ACE concentration in plasma, and thus decrease the rate of bradykinin inactivation in the placenta, it may be protective against preeclampsia. However, there are several controversial reports. Zhou et al. (43) and Zhu et al. (44) reported the association of ACE I/D polymorphism and preeclampsia in Chinese women, and other studies reported an association of ACE genotypes with preeclampsia in Turkish and Korean females (43, 44). Velloso et al. (45) suggested that the ACE DD genotype might be used as a marker for susceptibility to preeclampsia in Brazilian women. Li et al. (45) reported from China, that ACE gene I/D polymorphism were associated with the severe proteinuria and renal dysfunction seen in preeclampsia and preeclamptic patients carrying the D allele might be susceptible to renal dysfunction. In contrast, several studies in different countries did not support the hypothesis that ACE I/D polymorphism was associated with preeclampsia. These included studies of Nalowska-Glosnicka et al. (47) in Poland, Heiskanen et al. (48) in Iran, Galao et al. (49) in Brazil, Kim et al. (50) in Korea, Roberts et al. (51) in South Africa and Kobashi et al. (52) in Japan, who showed no association between ACE polymorphism and preeclampsia and/or abortion.

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Based on the results obtained from the unexplained RM cases used in this study, we found that the frequency of D allele was significantly higher in the Saudis compared to the other population. No evidence of association between the I/D polymorphism of ACE and RM in Saudi Arabian females was observed in this study and this might be related to the high frequency of D allele occurring even in the normal healthy Saudis.

It is obvious that there are significant population differences in the frequency of I/D polymorphism in the ACE gene, and this finding highlights the fact that each and every population must establish its own set of frequencies for the different SNPs expected to act as markers of a disease. Hence, larger studies are warranted in different regions of Saudi Arabia and on different ethnic groups.

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Disclosure of competing interests

Authors declare that they do not have any competing interests with any group.

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