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Original Research Article

Association of ACE Insertion/Deletion Polymorphism with Recurrent Miscarriage among Iraqi Women: A Preliminary Case-Control Study

Rashad Imad Al-Zamili[1*], Mohanad Jawad[2], Rahma Majid Al-Allawi[2]
[1]College of Science, University of Al-Qadisiyah, Iraq
[2]College of Biotechnology, University of Al-Qadisiyah, Iraq

***Corresponding Author:** Rashad Imad Al-Zamili
College of Science, University of Al-Qadisiyah, Iraq

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Abstract: ***Background***: Recurrent Miscarriage (RM) or Recurrent Pregnancy Loss is a complex reproductive issue that can be influenced by multiple risk factors including; Genetic, Anatomical, Endocrine, Immune System Thrombotic and Environmental factors. As such, the Angiotensin-Converting Enzyme (ACE) Insertion/Deletion (I/D) Polymorphism in Intron 16 has been considered as one of the possible genetic risk factors for RM because the D allele is associated with increased ACE Activity and could affect blood vessel constriction, Placenta Blood Flow and Pathways related to Thrombosis. ***Objective***: To assess if there is an association between the ACE I/D genetic polymorphism and RM in a group of Iraqi women. ***Methods***: Samples of EDTA blood were collected from women who had experienced RM but had been sent to have their thrombophilias assessed and from women who did not have a history of RM. Peripheral blood genomic DNA was isolated. Following DNA quality assessments, we genotyped for the ACE I/D genetic polymorphism by PCR using 14 RM samples and 8 control samples. The D allele was identified by a 190 bp product and the I allele by a 490 bp product. The Chi-Square/Fisher Exact test was used to compare genotype and allele frequencies. ***Results:*** The DD, ID and II genotypes were observed in 9/14 (64.3%), 5/14 (35.7%) and 0/14 (0.0%) RM cases, respectively, compared with 2/8 (25.0%), 4/8 (50.0%) and 2/8 (25.0%) controls. The genotype distribution showed a trend toward difference between groups (chi-square = 5.33, $p = 0.070$). In an exploratory dominant-risk comparison, the DD genotype was more frequent in RM cases than controls (OR = 5.40, 95% CI = 0.78-37.50; Fisher exact $p = 0.183$). The D allele frequency was higher in RM cases than controls (82.1% vs. 50.0%; OR = 4.60, 95% CI = 1.16-18.23; Fisher exact $p = 0.040$). ***Conclusion***: The ACE D allele and DD genotype were more frequent among Iraqi women with RM in this preliminary dataset. However, because the effective genotyped sample was small, these findings should be interpreted cautiously and validated in a larger, clinically well-characterized Iraqi cohort.

Keywords: ACE Gene, Insertion/Deletion Polymorphism, Recurrent Miscarriage, Recurrent Pregnancy Loss, Iraqi Women, PCR Genotyping.

INTRODUCTION

Recurrent Miscarriage (RM), also referred to as Recurrent Pregnancy Loss (RPL), is an important reproductive disorder that can have significant emotional and physical effects on individuals experiencing this condition. Definitions of RM/RPL are somewhat variable across different organizations; The European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) both use the definition of two or more losses in pregnancy to define RPL, whereas the Royal College of Obstetricians and Gynaecologists (RCOG) has historically defined recurrent miscarriage as three or more early miscarriages. However, the RCOG does encourage clinicians to utilize their own medical judgment regarding whether additional testing should be performed if there are only two losses where pathology may exist [1-3]. This variability is important because it affects participant selection, comparability between studies and interpretation of reported genetic associations.

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Pregnancy loss is common, and most sporadic first-trimester miscarriages are related to embryonic chromosomal abnormalities, especially numerical chromosome errors. In contrast, RM is a heterogeneous condition that may involve parental chromosomal rearrangements, uterine malformations, endocrine disorders, antiphospholipid syndrome, inherited or acquired thrombophilia, immunological dysregulation, maternal age, body mass index, smoking, excessive caffeine or alcohol exposure, and psychological stress [1-8]. Despite clinical evaluation, a substantial proportion of RM cases remain unexplained, indicating a need to investigate population-specific genetic susceptibility factors [4-7].

The renin-angiotensin-aldosterone system (RAAS), is an important regulatory mechanism for regulating both arterial pressure and vascular tone, fluid-electrolyte homeostasis and endothelial function. Angiotensin converting enzyme (ACE; Kininase II), converts Angiotensin I into the potent vasoconstrictor angiotensin II and also has a role in breaking down bradykinin. Beyond its cardiovascular role, ACE and other RAAS components have been implicated in reproductive physiology, placentation, trophoblast function and pregnancy complications [9-11]. Alteration of vascular tone, local uteroplacental blood flow, inflammation, fibrinolysis and coagulation may therefore provide biologically plausible links between ACE variation and RM [10, 11].

The human ACE gene is found at chromosome location 17q23 and it has 26 exons, and 25 introns. A well-studied and often used ACE insertion/deletion (I/D) polymorphism, commonly referred to as rs4646994/rs1799752, exists due to either the presence (I allele) or absence (D allele) of a 287 base-pair Alu repeat within intron 16. PCR amplifies three genotypes based on the two alleles I and D (II, ID, DD). The D allele is linked to elevated circulating ACE levels; the majority of individual variability in serum ACE activity is due to the I/D polymorphism [12, 13].

Previous investigations of ACE I/D and RM have produced inconsistent findings across ethnic groups. There are several systematic reviews and meta-analyses which state that the ACE D allele and DD genotype are associated with an increased RMP/PL risk however the magnitude of this association can vary based upon population studied and how studies defined RMP/PL [14-16]. Other case-control studies have found no significant association in some populations, including a Saudi cohort (17). These discrepancies may reflect small sample sizes, population stratification, differing RM definitions, clinical heterogeneity, and differences in genotyping quality control.

The purpose of this current study was to find out if the ACE I/D gene polymorphism has an association with RM in Iraqi women using PCR genotyping on their samples.

MATERIALS AND METHODS

Study Design and Participants

This laboratory-based preliminary case-control study evaluated the ACE I/D polymorphism in women with RM and in control women without a history of RM. Fifty EDTA-anticoagulated blood samples from women with a clinical history of RM were referred to Al-Sultan Medical Laboratories for thrombophilia-related analysis. Eight EDTA blood samples were obtained from apparently healthy control women without a history of RM after informed consent.

After genomic DNA extraction and quality assessment, only 14 of the 50 RM DNA samples had sufficient concentration and purity for ACE genotyping. These 14 RM samples and the 8 control samples were included in the final genotype analysis. This sample-quality attrition was retained in the revised manuscript because it materially affects interpretation of the results and study power.

Ethically Considered; Participants provided informed consent according to the initial version. Authors should insert the name of the approving ethics committee and approval number before submission, if available.

DNA Extraction and Quality Assessment

Blood genomic DNA was isolated using the Zymo Quick-G-DNA Kit (USA) according to the manufacturers instructions. The concentration and quality of the DNA were measured by Spectrophotometer (NanoDrop). Integrity of DNA was also tested by Agarose Gel Electrophoresis Samples with acceptable DNA quality were selected for downstream PCR genotyping.

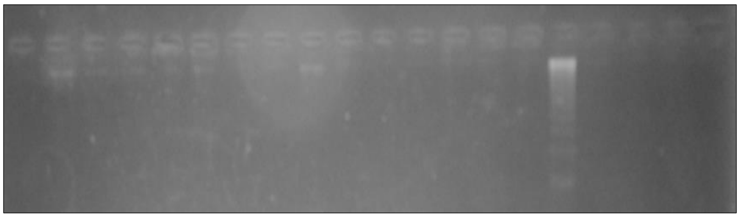

Figure 1: Agarose gel electrophoresis showing extracted genomic DNA from representative RM samples.

Table 1: Representative DNA concentration and purity values for recurrent miscarriage samples

Sample	DNA concentration (ug/ml)	Purity (A260/280)
372	2.45	1.30
721	4.75	1.10
924	3.71	1.00
67	4.15	1.80
470	16.10	1.30
282	7.76	1.16
827	5.10	1.15
674	2.90	0.90
176	11.20	1.30
168	25.60	1.50

PCR Amplification of ACE I/D Polymorphism

ACE I/D genotyping was performed using gene-specific primers previously used for ACE I/D detection (18): forward primer 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3' and reverse primer.

5'-GAT GTG GCC ATC ACA TTC GTC AGA T-3'. The expected PCR products were 190 bp for the D allele and 490 bp for the I allele. Thus, DD genotype was identified by a 190 bp band, II genotype by a 490 bp band, and ID genotype by both bands.

Table 2: PCR reaction mixture for ACE I/D genotyping

Component	Volume
Genomic DNA	1 ul
Forward primer (10 pmol)	1 ul
Reverse primer (10 pmol)	1 ul
PCR master mix (1x)	10 ul
Nuclease-free water	7 ul
Total reaction volume	20 ul

The PCR reaction included the following cycling parameters: a 15-minute initial denaturation activation step at 95°C was performed; then 40 cycles that contained 20 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C and an extension period of one minute at 72°C. The PCR products were run in a 2% agarose gel and stained using Red Safe stain for visualization. In future studies confirming whether or not there is a duplication (DD) based on the first set of data, it would be best to perform insertion specific PCR to reduce the likelihood of misclassifying due to preferential amplification of the smaller D allele.

Statistical Analysis

Genotype and allele frequencies were calculated as numbers and percentages. Because of the small effective sample size and low expected cell counts, Fisher exact tests were prioritized for two-by-two comparisons, and chi-square analysis was used descriptively for the overall three-genotype comparison. Exploratory, unadjusted odds ratios (ORs) with 95% confidence intervals (CI) were generated for descriptive purposes only. Hardy-Weinberg equilibrium was used only to describe. Reporting was revised to align more closely with STROBE and STREGA principles for observational genetic association studies [18-20].

RESULTS

DNA Quality and PCR Genotyping

Of the 50 DNA samples obtained from women with RM, 14 met the concentration and purity requirements for PCR genotyping. The control group consisted of 8 samples. Genomic DNA quality assessment is shown in Figure 1, while PCR amplification patterns for control and patient samples are shown in Figures 2 and 3. PCR genotyping identified the ACE I/D polymorphism according to the expected 190 bp (D allele) and 490 bp (I allele) bands.

Figure 2: PCR results for control samples. The 490 base pair (bp) bands indicate the insertion allele (I). The 190 bp bands are indicative of the deletion allele (D).

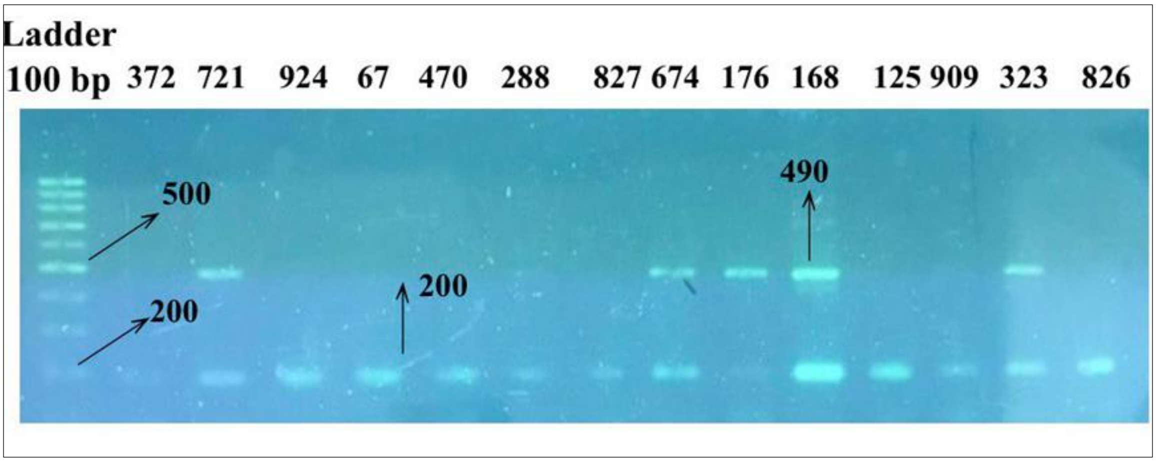

Figure 3: PCR results for RM samples. Genotypes were interpreted as DD (190 bp), II (490 bp) and ID (both 190 and 490 bp).

Genotype and Allele Frequencies

The ACE DD genotype was identified in 9 of the 14 cases of (RM) (64.3%) and 2 of 8 controls (25.0%). The ID genotype was found in 5 of 14 RM cases (35.7%) and 4 of 8 controls (50.0%). The II genotype was not observed among RM cases but was present in 2 of 8 controls (25.0%). The overall genotype distribution showed a trend toward difference between groups (chi-square = 5.33, df = 2, p = 0.070).

In an exploratory comparison of DD versus non-DD genotypes, the DD genotype was more frequent in RM cases than controls (OR = 5.40, 95% CI = 0.78-37.50; Fisher exact p = 0.183). The D allele frequency was 82.1% among RM cases and 50.0% among controls; allele-level analysis suggested a higher frequency of the D allele in RM cases (OR = 4.60, 95% CI = 1.16-18.23; Fisher exact p = 0.040). The control genotype distribution was consistent with Hardy-Weinberg expectations, while the RM group had no observed II genotypes; however, formal equilibrium testing is limited by the small sample size.

Table 3: ACE I/D genotype and allele distribution in RM cases and controls

Variable	RM cases (n = 14)	Controls (n = 8)	Exploratory comparison
DD genotype	9 (64.3%)	2 (25.0%)	DD vs non-DD: OR = 5.40; 95% CI = 0.78-37.50; p = 0.183
ID genotype	5 (35.7%)	4 (50.0%)	Overall genotype comparison: chi-square = 5.33; p = 0.070
II genotype	0 (0.0%)	2 (25.0%)	
D allele	23/28 (82.1%)	8/16 (50.0%)	D vs I allele: OR = 4.60; 95% CI = 1.16-18.23; p = 0.040
I allele	5/28 (17.9%)	8/16 (50.0%)	

DISCUSSION

The preliminary results of this case control study, conducted in Iraq, show a higher incidence of the DD genotype (and therefore the D allele) among RM patients as opposed to controls. While 64.3% of RM patients had the DD genotype, only 25.0% of controls did; while 82.1% of all RM cases had at least one copy of the D allele, only 50.0% of controls did. These findings support the theory that the D allele of the ACE gene could be a risk factor for developing RM; however, these data provide very little useful information because of the small number of subjects used.

The biological plausible relationship of this is demonstrated by the well-known functional impact of the ACE I/D genetic variation. The D allele is related to greater serum ACE concentrations; as a result of its enzymatic activity, ACE participates in the production of angiotensin II and degrades bradykinin [12, 13]. Increased ACE activity may promote vasoconstriction, endothelial dysfunction, inflammatory activation and prothrombotic imbalance. In pregnancy, such mechanisms could theoretically impair uteroplacental perfusion and trophoblast-endometrial interactions, especially in women with additional thrombophilic or vascular risk factors [10, 11].

The present results are also consistent with previous meta-analyses. Wang *et al*., reported that DD and ID genotypes were associated with increased recurrent miscarriage risk compared with II genotype, and concluded that the D allele contributed to increased risk [14]. Yang *et al*., a subsequent study, conducted by another group, also observed that there was a correlation between ACE I/D and recurrent pregnancy loss in a meta-analysis [15]. In addition, a later meta-analysis of 26 case-control studies of Aslbahar *et al*., discovered statistically significant correlations for allele, homozygote and recessive models and proposed that the association may vary among different populations; in particular they noted strong associations were seen within Caucasian and West Asian populations [16]. These published findings support the relevance of studying ACE I/D in Middle Eastern populations, including Iraqi women.

However, there are inconsistencies in the research. Al-Mukaynizi *et al*., found no statistically significant relationship between the ACE I/D polymorphism and unexplained RM among female Saudis; however, positive associations have been reported in some other populations [17]. Such inconsistency may result from differences in ethnicity, genetic background, definition of RM, inclusion and exclusion criteria, age matching, history of live birth, number and gestational age of losses, concurrent thrombophilia, environmental exposures and genotyping protocols. Thus, it can be thought that ACE I/D polymorphism may serve as an additional or indirect susceptibility marker for RM as opposed to being a causative agent for RM.

The statistical version of this interpretation is even less optimistic than the first draft. Despite an increased frequency of DDs in RM pregnancies, there was no statistically significant difference as measured by Fisher's Exact Test due to a very large confidence interval. The allele-level result was statistically significant in exploratory analysis, but allele-based tests can overestimate precision because two alleles from the same individual are not fully independent. Therefore, the main conclusion should emphasize an observed frequency difference and the need for confirmation rather than a definitive causal relationship.

This study has several limitations. First, only 14 of the 50 RM samples were genotyped after DNA quality assessment, reducing statistical power and potentially introducing selection bias. Second, the control group was small (n = 8), and demographic and clinical matching variables were not reported. Third, Age, Body Mass Index, Number of Miscarriages, Gestation Length at Loss, Presence of Live Births, Antiphospholipid Antibody Results, Inherited Thrombophilias Results, Uterine Anatomy, Endocrine Factors, and Parental Karyotype Data were unavailable in the Draft Fourth, the study did not report confirmatory insertion-specific PCR for samples typed as DD, which is recommended in ACE I/D studies to reduce potential misclassification. Finally, no multivariable analysis could be performed because of the small sample size.

CONCLUSION

The ACE D allele and DD genotype were more frequent among Iraqi women with RM than among controls in this preliminary study. The finding supports further investigation of ACE I/D as a potential genetic susceptibility marker for RM in Iraqi women. Because the effective sample size was small and the DD genotype comparison was not statistically definitive, the results should not be interpreted as proof that ACE DD is a direct cause of RM. Larger, well-controlled Iraqi studies are required to validate this association and evaluate interactions with thrombophilia, antiphospholipid syndrome and other clinical risk factors.

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