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Research Article

THE INSERTION/DELETION (I/D) POLYMORPHISM IN THE *ANGIOTENSIN-CONVERTING* ENZYME GENE AND RECURRENT PREGNANCY LOSS

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ABSTRACT

The renin-angiotensin system (RAS) plays a crucial role in the well-being of mother and fetus. Similarly, placental RAS is involved in placental angiogenesis, proliferation and trophoblast invasion. An insertion/deletion polymorphism within the angiotensin-I converting enzyme gene (ACE-I/D) has shown to be reliably associated with differences in angiotensin-converting enzyme (ACE) activity. Previous studies on ACE and recurrent pregnancy loss (RPL) from various ethnic groups differed in their findings and there are not many studies available from India. Therefore, the present study was aimed to investigate the association of ACE I/D in predisposition of RPL in women from South India.

The ACE I/D genotyping were carried out in 230 patients and 234 controls. Our findings demonstrated a significant association of ACE I/D polymorphism with RPL. Further categorization of cases into groups (A = 2 abortions and B= >2 abortions) and analysis revealed an increased frequency of DD in a group A and II in group B. These results were indicative that high or low levels of angiotensin II interfere with normal development of fetus and cause frequent miscarriages in the studied population. Hence, ACE genotyping along with Ang II intravenous infusion or treatment with ACE inhibitors in gravid women is required in order to achieve successful pregnancy.

INTRODUCTION

Recurrent pregnancy loss (RPL) refers to the loss of three consecutive pregnancies that are clinically recognised, prior to 20 weeks from the last menstrual period. RPL affects approximately 1-5% of pregnancy cases [1]. This disorder is becoming one of the major issues being faced by today´s women, since every 1 in 300 pregnancy is a case of RPL. Various aetiologies have been attributed to RPL such as chromosomal rearrangements, antiphospholipid antibodies [2], heritable or acquired thrombophilia, untreated hypothyroidism, untreated diabetes mellitus, immunological abnormalities, uterine anatomic abnormalities, infections and environmental factors [3]. Approximately 9% of the RPL cases are due to genetic abnormalities [4] such as parental chromosomal rearrangements in which there is a balanced translocation or Robertsonian translocation. To date, several candidate genes such as VEGF, p53, TNF, IL's, eNOS etc., have been implicated in the aetiopathogenesis of RPL. There are several studies suggesting a remarkable degree of coordination between angiogenic factors (VEGF, ACE, p53, etc.,) and cells responsible for proper placental vasculature and circulation [5,6,7].Abnormal placental vasculature leads to various gestational pathologies such as pregnancy loss, intrauterine fetal death (IUFD), intrauterine growth restriction (IUGR), placental abruption and preeclampsia.

Angiotensin Converting Enzyme (ACE) is a vital component of Renin – angiotensin system (RAS) that plays an important role in physiological remodelling of spiral arteries during pregnancy [8]. ACE is expressed in multiple cells and tissues, endothelium (inner layer) of blood vessels in lungs and kidneys [9]. It is a dipeptidyl carboxypeptidase which catalyses the conversion of a biologically inactive decapeptideangiotensin 1 to the active octapeptideangiotensin 2 [10], and plays a major role in regulation of blood pressure. Till date more than seventy six polymorphisms in ACE gene have been identified, the most common is the presence (insertion I) or absence (deletion D) of

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a 287 bp sequence of DNA in intron 16 of the gene. The plasma levels of angiotensin 1, angiotensinogen and aldosterone are modulated by ACE and in turn plasma levels of ACE is associated with this I/D polymorphism [11].

Apart from regulating blood pressure and fluid balance, ACE and its products have been reported to play a crucial role in the embryo's viability in early pregnancy. The ACE gene increases the risk for other vascular complications, such as pre-eclampsia and abortion, reduces brinolysis and restricts bleeding during pregnancy. Women with RPL are at an increased risk of developing complications such as preeclampsia in later pregnancies [12], coronary artery disease/ cardiovascular complications, [13,14] and increased risk of ovarian cancer [15] and mortality for women with a history of 3 miscarriages [16]. Agachan *et al*., (2003) [17] suggested the involvement of high angiotensin II levels and low bradykinin causing subsequent pregnancy loss. Various studies conducted on RPL patients, were from various ethnic groups (Caucasians, Italians etc.,) [18] and differed in their reports. Additionally, there are not many studies available from Indian population pertaining to ACE I/D gene polymorphism in relation to RPL susceptibility and its clinical manifestations. Therefore, the present study was focused to investigate the role of ACE I/D polymorphism in predisposition of RPL among women from South India.

MATERIALS AND METHODS

A total of 464 women were considered for the study, out of which, 230 were patients and 234 were controls. These samples were collected from Institute of Genetics and Hospital for Genetic Diseases, Begumpet, Hyderabad, India. Informed consent prior to sampling and ethical clearance was obtained from local ethical committee (Institute of Genetics & Hospital for Genetic Diseases, Osmania University, Hyderabad, India). Detailed clinical information was collected through proforma. The inclusion criterion for patient was a woman with history of two or more than three abortions with no live births. Patients who had chronic hypertension, diabetes, polycystic ovary syndrome, having pregnancy with a multifetal gestation or conceived by assisted reproductive technology or with, premature preterm rupture of membranes or with, unexplained vaginal bleeding or the usage of antihypertensive drugs are excluded from the present study. Age matched healthy women with two or more normal children and no history of abortion were selected as controls.

Molecular analysis

Five millilitres of blood sample was collected from all the participants in EDTA coated tubes. Genomic DNA was extracted by standard protocol routinely used in our laboratory [19]. Polymerase chain reaction for ACE genotype was performed for each subject by using forward and reverse primers. PCR amplification was carried out in a total volume of 10 µl containing 1.00 µl template DNA, 0.2 µlof each primer (Bioserve, India) (Forward: 5′-CTG GAG ACC ACT CCC ATC CTT TCT-3′; Reverse: 5′-GAT GTG GCC ATC ACA TTC GTC AGA T-3′), 1.25 µl PCR buffer with 15mM $Mgcl_2$ and 3 U of Taq DNA polymerase (Bangalore Genei, India).

PCR was performed using a thermocycler (Biorad) with following conditions : an initial denaturation step for 5 min at 95 °C, then 30 cycles consisting of 30 s of denaturation at 94 °C, 45 s of annealing at 59 °C and a final extension for 5 min at 72 °C. The products were run on 1.5 % agarose gel analysed using gel documentation system (Biorad). A product of 490 bp indicates a genotype homozygous for insertion (II), 190 bp homozygous for deletion DD and the presence of 490 and 190 bp products specify heterozygous genotype (ID).

Statistical analysis

Genotypic, allelic frequencies and Hardy-Weinberg equilibrium were calculated using chi-square analysis. The association between genotypes and RPL risk was evaluated by calculating the odds ratios (OR) at 95 % confidence interval. A two-tailed value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Characteristics of the study group

Data analysis on a total cohort of 464 individuals revealed that the mean age of the patients and controls at the time of sample collection was 25.21 ± 3.76 years and 25.50 ± 4.38 years respectively. Nearly 63% of RPL women had a history of =2 while 47% had 3 or more than 3 miscarriages.

Molecular Analysis

The percentage distribution of DD, ID and II genotypes was 56, 8 and 36 in patients while it was 33, 38 and 29 in controls correspondingly. The genotype frequencies differed significantly between the groups ($p< 0.05$). Individuals with DD genotype predominated in patients and exhibited an OR of 2.82 while in controls, the ID genotype demonstrated an OR of 0.15. The frequencies of D and I alleles did not vary between the groups ($p>0.05$). The distribution of ACE genotypes was not in agreement with Hardy Weinberg equilibrium between the groups. Table 1 represents the distribution of ACE I/D genotypes in patients and controls.

Additionally, in order to see the association of ACE genotypes with number of abortions, the patients were further divided in two groups (Group A and Group B). The Group A were women with two abortions (=2 abortions) while Group B were women with three or more than three abortions (>3 abortions). The percentage distribution of DD, ID and II genotypes was 75, 8 and 17 in Group A while it was 20, 7 and 70 in Group B correspondingly. The genotype and allele frequencies differed significantly between the groups ($p < 0.05$).Women with DD genotype predominated in Group A and showed an OR of 9.9 while II genotype in Group B revealed an OR of 11.53. The distribution of ACE genotypes was not in agreement with Hardy Weinberg equilibrium in both the groups (Table 2, 3).

DISCUSSION

A normal pregnancy is dependent on adequate placental circulation and fetal vasculature that requires appropriate development of the placental vascular network, vasculogenesis, angiogenesis, and trophoblast mediated arterial remodeling [19]. The development and functioning of vascular network requires complicated cooperation between different cell types and various growth factors in the processes of implantation, embryo development and placentation [5,6,7]. Vasculogenesis occurs between 18 and 35 days after conception in humans

while angiogenesis, starts at 21 days after conception and continues throughout human gestation [20,21].

The renin-angiotensin system (RAS) plays a crucial role in the well-being of mother and fetus. Likewise placental RAS also plays a key role in placental angiogenesis, proliferation and trophoblast invasion. ACE, a component of RAS system is a key enzyme involved in the conversion of angiotensin I to angiotensin II and is expressed in multiple tissues including maternal decidua and placenta in gravid women. Apart from regulating blood pressure, water and electrolyte homeostasis, it is also implicated in several gynaecological diseases such as pre-eclampsia, recurrent miscarriages, intra uterine growth retardation etc. In view of the importance of angiogenesis and vascularisation in human pregnancy, the present study was carried out to establish the role of ACE I/D polymorphism with susceptibility to RPL.

Our findings, showed an increased prevalence of DD genotype in patients while the controls demonstrated a higher frequency of ID, suggesting the predisposing role of DD and protective role of ID genotypes respectively towards RPL. The DD genotype predominated in patients and revealed a relative risk of 3 fold towards miscarriage. These findings suggest a significant association of ACE I/D polymorphism with RPL in South Indian women. Till date, several studies had showed varied data on the involvement of ACE I/D polymorphism with RPL, based on ethnicity and maternity [22]. The homozygous D allele results in elevated PAI-1 concentration and hypofibrinolysis risk in Caucasians [18] however, the cause in nearly 50% of women remains unidentified.

Furthermore, there are several studies suggesting placental microvasculature thrombosis to be the prime cause for the idiopathic abortions. Abnormalities of placental vasculature may result in several gestational complications such as pregnancy loss, intrauterine fetal death, intrauterine growth restriction, preeclampsia, reduces fibrinolysis and restricts bleeding during pregnancy [23,6]. Studies on microarray by Choi *et al*., in 2003 [24] had shown a decreased expression pattern of angiogenesis-related genes in the chorionic villi of RPL patients (Choi *et al*., 2003).The transformation of spiral arteries to large vessels of low resistance by the ACE gene has been reported as a crucial event for the embryo's viability in early pregnancy.

Further analysis of our results revealed a significantly elevated frequency of DD genotype in Group A and II in Group B. The association of DD genotype with high ACE levels is indicative of higher conversion of angiotensin I to angiotensin II and less degradation of bradykinin [11] thereby causing several complications. Similar to our findings, Corbo *et al*., [25] demonstrated an increased prevalence of DD genotype among RPL women and showed an association with reduced reproductive issues. The most probable explanation could be the high angiotensin II levels primarily due to presence of DD genotype and other factor being the increased production of prostacyclins and progesterone during pregnancy. These in turn could hamper the interaction between angiotensin II and At1R that may further lead to alteration in vital cellular processes like cell growth, angiogenesis, vasculogenesis in developing fetus ultimately leading to inadequate blood flow for fetal oxygenation. Moreover, it was shown that the ACE D allele leads to overexpression of plasminogen activator inhibitor-1 (PAI-1), which can increase the risk of thrombotic events and enhance the production of angiotensin II. It is a well known fact that during pregnancy higher than normal levels of Angiotensin II are required for normal fetal growth. Women in group B showed increased prevalence of II genotype associated with low angiotensin II levels. These low levels might interfere with the normal development of fetus and hence lead to frequent miscarriages.

In conclusion, our findings suggest direct involvement of ACE I/D polymorphism in the genetic predisposition to recurrent miscarriage. An increased frequency of DD in a group A with 2 miscarriages and II with >2 miscarriages were indicative of high or low levels of angiotensin II that interferes with normal development of fetus and cause frequent miscarriages in the studied population. Hence, ACE genotyping along with Ang II intravenous infusion or treatment with ACE inhibitors in gravid women is mandate to achieve successful pregnancy.

Declaration of Interest

Conflicts of interest: None

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