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Original Article

Study of Relationship between Polymorphism in Estrogen Response Element (ERE) in Promoter Region of C3 Gene and Spontaneous Recurrent Abortion

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Abstract

Background: Miscarriage is one of the most common pregnancy complications for which various causes have been defined, such as genetic factors, infectious, metabolic, endocrine systemmal function and immune system undesired responses. The early development of embryo occurs in oviduct and uterine tube from which some factors such as growth factors, glyco-proteins and factors those stimulate development of embryo are secreted. The ETF3 embryotrophic factor which is a complex of C3 complements and its derivatives i.e., iC3b, enhances the development of trophectodermas a consequence of which expression of relevant genes are affected embryo. There are various response elements in C3 gene promoter region such as, estrogen response regions (ERE). Steroids such as estrogen and progesterone are secreted in early steps of embryonic period along with C3 secretion and cause increase in C3 expression through interaction with regulatory elements in promoter region of this gene. In this study the polymorphism in ERE regions of C3 gene promoter was investigated in women suffering from recurrent miscarriage.
Materials and methods: In this study, assuming that polymorphism in ERE regions is correlated with recurrent miscarriage during early months of pregnancy, 40 blood samples were collected from female patients admitted to an Infertility Clinic, Isfahan, Iran. DNA was extracted, amplification of regions harboring ERE with a pair of specific primer was done using Polymerase Chain Reaction-Single Strand Chain Polymorphism (PCR-SSCP) for studying possible polymorphisms in this region.
Results and conclusion: The results indicated a specific symptomless infertility among the women, however there was no correlation between the ERE polymorphism and symptoms in control and cases.

Keywords: Spontaneous; Recurrent Abortion; Complement 3;Estrogen Response Element.

Introduction

Miscarriage is one of the most common pregnancy complications (Rai and Regan, 2006). A couple of definitions are listed for spontaneous abortions, an example of which is as follows: "miscarriage is the expulsion or extraction of an embryo or fetus from mother while weighing 500 gram or less''(Garcıa-Enguıdanos et al. 2002). Miscarriage may be sporadic or recurrent. The rate of recurrent miscarriage is estimated to be approximately one percent, whereas, women who experience done or more sporadic miscarriages consist

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about 50 percent of all the cases.

Various factors may cause recurrent miscarriage, including genetic, environmental, infectious, metabolic, and endocrine effectors (Dhont, 2003;Rai and Regan, 2006).The etiology of approximately 40% of recurrent spontaneous abortions is unknown and the molecular bases involved is poorly understood (Lee et al., 2007). Human oviduct cells secrete growth factors and embryo-trophic materials that stimulate development of the embryo(Barmat, Worrilow and Paynton 1997, Lee et al. 2003). So far, three embryo-trophic fractions, namely; ETF-1, ETF-2 and ETF-3 with high molecular weight have been isolated from human oviduct by scholars(Xu et al. 2001, Liu et al. 1998).ETF-1 and ETF-2 stimulate the development of inner cell mass and ETF-3 enhances the development of trophectoderm, which leads to blastocysts having a larger diameter and a higher hatching rate. ETF-3is the mixtures of complement protein 3 (C3) and its derivative, i.e. C3b and iC3b (Lee et al. 2004). C3 gene promoter contains multiple elements which affect its expression. Among them, there are two sequences that share 85%homology with the estrogen responsive elements(Vik et al. 1991). Estrogenis a physiological regulator of the reproductive system, bone metabolism and supports the cardiovascular and nervous system (Sommer and Fuqua 2001;Couse and Korach 1999). Estrogen activates its receptor (ER-α, ER-β) after binding to it resulting in conformational changes in the molecule leading to its dissociation from chaperones, such as hsp70 and hsp90. In the next step, estrogen-receptor construct binds to ERE in the promoter region of target genes, which have this element in their promoter region, and initiates transcription (Sommer and Fuqua 2001). Both ERα and ERβ can bind to ERE with high affinity. The ERE sequence is GGTCAnnnTGACC in which "n" can stand for any nucleotide (Nelson et al. 1999, Dickson and Stancel 2000). The main objective of present study was to study of polymorphisms in promoter region of C3 gene in patients with asymptomatic miscarriage. This study is the first challenge on the C3 gene promoter as a candidate region involved in abortion.

Material and Methods

Peripheral blood and tissue Samples

Peripheral blood samples were collected from40 women (mean age 31 years, ranging between 25-37 years) registered to Infertility Center, Shahid Beheshti Hospital in Isfahan, Iran with positive historyof more than two asymptomatic miscarriage. In addition, 15 control subjects were healthy women with two successful pregnancies those involved in this study. All the individuals provided informed consent for their participation in this study.

Blood samples were collected in EDTA-containing tubes and stored in a -20°C.

DNA extraction and Polymerase chain reaction

Total genomic DNA was extracted from peripheral blood using standard Phenol-Chloroform protocol (sambrook J and Rusell DW, 2001). The promotersequence of C3 was obtained from NCBIgenome database. The relevant fragments including the ERE of C3 promoter were amplified using two pair of primers designed by Genrunner software version 4.0.9.67. The sequence of primers was Forward ERE1C3 5'-CAAAGTGGGTCCAACAGAG-3' Reverse ERE1C3 5'- CTCAATACCATT TCCTAAGC-3; and forward ERE2C3 5'-AGCTTAGGAAATGGTATTGAG-3' reverse ERE2C3 5'- TGGGTTAGTAGCAGGAGC AG –3' respectively. Each reaction mixture(25 µl)contained 30 ng of genomic DNA,1X PCR buffer, 0.2 mM of dNTP, 1.5 mM ofMgCl2 0.4pM of each primer(forward and reverse) and 1.5U of Taq DNA polymerase. Following an initial denaturation step(5 min at 95°C), samples were subjected to 35 rounds of PCR at 94°Cfor 30s, 51°C (ERE1) or 53°C (ERE2)for 40s, and72°C for 30 s with a final extension time of 5 min at 72°C followed by a 4°C hold cycle. The specificity of the amplification was confirmed by acrylamid gel electrophoresis prior to further analyses.

SSCP analysis

The PCR products were diluted with ddH2O and formamide loading buffer according to the strength of the DNA bands. Prior to loading, the samples were denatured at 95C for 3min, then immediately snap-cooled on ice and then analyzed on a non-denaturing 10% poly-acrylamide gel and run overnight at 350V. This analysis was carried out on various buffer condition and acrylamide gel concentration. DNA bands on the SSCP gel were visualized by silver staining method (Barnini et al 2004).

Results

EREC3 polymorphisms were analyzed in 40 women with recurrent miscarriage and 15 ethically matched healthy control subjects. The integrity of isolated DNA was evaluated in agarose gel 1% (Fig.1). The PCR condition was optimized. For assurance of any non-specific amplification product that disturb SSCP procedure, PCR products were visualized using silver staining method after 7% acrylamide gel electrophoresis (Fig. 2).The PCR products were analyzed by SSCP method. Several sets of conditions tried empirically to optimize mutation detection. Finally there was not any significant difference in promoter region of c3 gene in patient compared with control(Figure 3). To evaluate preservation of this region, multi-alignment was done for ERE of C3 promoter gene in Musmusculus,

BosTaurusandHomo sapiens (Fig. 4a). This analysis showed low variability in the amplicon.

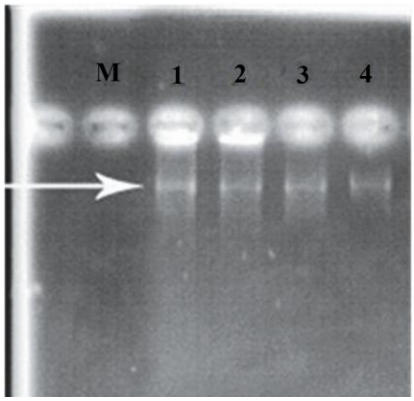

Figure 1. The electrophoresis of DNA extracted from blood samples. Separation was done on 1% agarose gel. Lane M shows DNA marker (SMO373). Lanes 1-4 DNA samples extracted from patients. Arrow shows the DNA extracted.

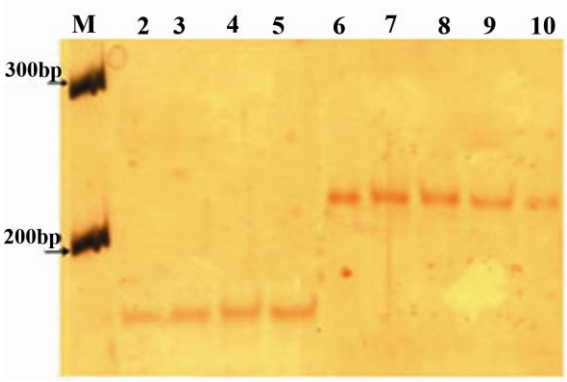

Figure 2. The PCR products of the patient's samples on acrylamide gel. Lane M is DNA marker (SM0373). Lanes 2-5 the ERE1 gene (170bp). Lanes 6-10 ERE2 gene (236bp).

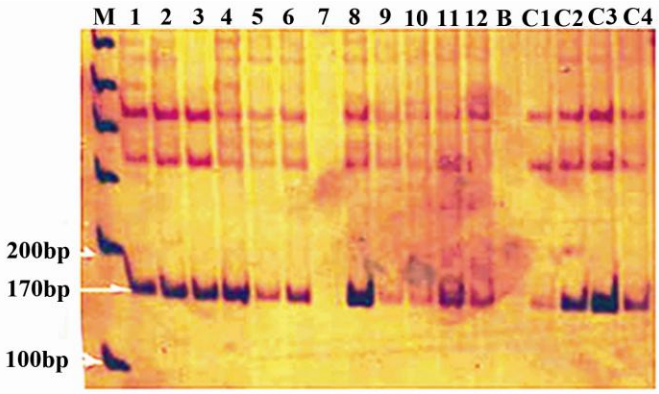

Figure 3. SSCP results of the first fragment of PCR products. SSCP results for the first fragment of PCR product (170bp). Lane M is DNA marker (SM0373). Lanes 1-4 and 5-12 selected samples and lanes C1-C4 are controls.

Discussion

This study is focused on the polymorphisms in the promoter region of C3 gene as an embryo-trophic factor. Loss of such embryo-trophic factor or low concentration of these factors may be an important cause of conception failure. At least 50 present of all recurrent miscarriage cases are unexplained. However several types of polymorphism have recently been reported to be associated with recurrent miscarriages (Sugiura-Ogasawara et al. 2006). Fertilization and early development of the embryo occur in the oviduct. Oviduct epithelium composed of ciliated and secretary cells. The luminal fluid of oviduct is a suitable microenvironments for Oocyte maturation, capacity of sperm, fertilization and transport of gametes and embryos (Leese et al. 2001).

As mentioned above the ETF-3 is a complex of C3 and its derivatives (Lee et al. 2004). Human oviduct epithelial cells synthesize and secrete C3. C3 gene is transcribed actively in the oviduct around ovulation. Entry of early embryos leads to rapid release of C3. Also embryos stimulate further synthesis of oviductal C3 for supporting the development of early embryos. The level of C3 transcript on day 1 and day 2 of pregnancy was higher than on the corresponding days of pseudo-pregnancy (Lee et al. 2009). It has been reported that the levels of CRP, C4d C3a, SC5b9, C3, C9 and H antigen factor were significantly higher in healthy pregnant than non-pregnant women (Derzsy et al. 2010). In this connection, it was assumed that the polymorphisms in promoter region of these genes is associated with abnormal expression levels of the related genes. Steroids are regulator of the synthesis and secretion of C3 gene in the mouse endometrium (Li, et al., 2002). Estrogens mediate this function through binding to the estrogen receptor (ER). Ligand receptor combination (E2-ER) interacts directly with specific estrogen response elements. This complex interacts with co-activator proteins and components of the RNA polymerase II. This interaction enhances the transcription (Klinge, 2000). In the mouse oviduct, E2 treatment significantly induced C3 mRNA expression(Lee et al. 2009). Estrogen response element in C3 promoter and the importance of this protein in cell proliferation in the linings of the uterus, certainly, underlines a prominent role of this promoter in the stability of the wall of the uterus. Hence any polymorphisms in response elements can disrupt efficiency of the promoter.

The idea presented here is justifiable and may open a new field of study in infertility. Such studies confirm the role of promoter but the supporting data for this hypothesis are insufficient and require further studies with larger clinical samples.

Based on SSCP analysis there was no

significant association between the ERE polymorphism and infertility symptoms in this small study population, however there are evidences which show that early abortion is associated with components of the immune system.

Based on previous studies and bioinformatics, the C3 gene polymorphism in promoter regions can be considered as a factor associated with early miscarriage. The data in figure 4 indicate a low variability rate in promoter region of C3 gene suggesting a low toleration for accurate of functional mutations in these elements.

In conclusion based on these data there was no significant correlation between the ERE polymorphism and infertility symptoms in control and cases. Further studies using a larger sample size warrant the identification of relationship between infertility symptoms and polymorphism in ERE-related genes.

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