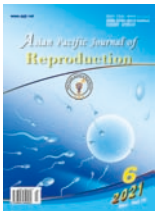

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Association between *eNOS* gene promoter polymorphism (−786T>C) and idiopathic recurrent pregnancy loss in Iranian women

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ABSTRACT

Objective: To investigate the frequency of -786T>C variant in endothelial nitric oxide synthase (*eNOS*) gene promoter in Iranian women with recurrent pregnancy loss.

Methods: Blood samples were obtained from 100 unrelated women affected by recurrent pregnancy loss and 100 unaffected women as the controls. Genomic DNA was extracted and -786T>C polymorphism in *eNOS* gene promoter was investigated by PCR-RFLP method. Statistical analyses and Hardy-Weinberg equilibrium in the groups of patients and controls were performed by *Chi*-square test and SPSS standard software (Version 21).

Results: The frequency of homozygous TT was 40% in cases and 46% in the control group; the frequency of CC was 7% in cases and 5% in the control group; frequency heterozygote TC was 53% in cases and 49% in the control group. Genotype frequencies between the two groups showed no significant differences (P>0.05).

Conclusions: The -786T>C polymorphism is not more frequent in recurrent pregnancy loss in this population.

KEYWORDS: Repeated pregnancy loss; Endothelial nitric oxide synthase; *eNOS*; -786T>C variant; PCR-RFLP; Iranian women

1. Introduction

Recurrent pregnancy loss, one of the most common complications of pregnancy, occurs in 2% to 5% of clinically recognized pregnancies. It defined as two or more consecutive miscarriages before 20 weeks gestation, which is greater than expected by chance (0.34%)[1,2]. Consequently, recurrent pregnancy loss is an extremely stressful condition for couples and physicians and is an important area of research. This is a multifactorial obstetric problem with a polygenic background[3].

The important and evident roles of nitric oxide (NO) within the body and in diseases have been highlighted. NO is produced by the NO synthase (NOS) family of enzymes. There are three separate enzymes each coded by a separate gene, with clear contrasts in location, regulation, catalytic properties, and sensitivity to inhibitors[4]. The three NOS isoforms are named accordingly to their position within the human body; NOS found in the endothelium is known as endothelial NOS (also known as Type Ⅲ, NOS3), NOS found in macrophages is known as inducible NOS (also known as Type Ⅱ, NOS2) and the final type is neuronal NOS (also known as Type Ⅰ, NOS1)[5].

Significance

There is a controversy about the relationship of -786T>C polymorphism in *eNOS* gene promoter and repeated pregnancy loss in different investigations. The results of our study demonstrated that there is no association between the noted variant in *eNOS* gene promoter and repeated pregnancy loss in Iranian women. These results infer that the association of this polymorphism with repeated pregnancy loss among distinct ethnic groups from different countries can be inconsistent.

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The structure of the *eNOS* gene was first determined by Marsden *et al*[6]. It was discovered to contain 26 exons incorporating 21 kb of genomic DNA, encoding for 1 203 amino acids in the form of a 135-kD protein. Also, this gene is located in chromosome 7 (7q35-q36)[7]. The *eNOS* gene has been shown to exhibit different variations. The influence of these variations on *eNOS* function and consequently disease is still disputed. The hypothesis is that the presence of the variation within this gene may individually or collectively reduce *eNOS* function and subsequently NO synthesis.

Numerous studies imply that several gene polymorphisms could be proposed as a risk factor for recurrent pregnancy loss. One of these genes is *eNOS* which produces NOS in the vascular endothelium and causes vasodilation and smooth muscle relaxation[8]. This enzyme catalyzes the biosynthesis of *L*-citrulline and NO from *L*-arginine[9]. The level of NO as a signaling molecule will increase during pregnancy. These result in fetal blood supply without hypertension. Therefore, decreasing serum NO levels at the beginning of pregnancy can interfere with oxygen supply and nutrition for the fetus and lead to abortion[10]. In this study, we aimed to analyze the relationship of -786T>C polymorphism (rs2070744) in *eNOS* gene regulatory region and recurrent pregnancy loss among central and southern Iranian women.

2. Material and methods

2.1. Study participants

One hundred Iranian women with two or more idiopathic spontaneous repeated pregnancy losses which had clinically occurred from the beginning of pregnancy to the end of the 20th week of gestation were recruited in this study. They had attended to the Abortion Research and Clinical Center, Yazd Reproduction Sciences Institute, Shahid Sadoughi University of Medical Sciences. Also, 100 healthy women with no history of abortion with at least two successful pregnancies who were referred to this clinic for routine checkups were selected as the control group. Inclusion criteria included: patients with repeated pregnancy loss based on tests and physician diagnosis and examinations that there was no specific cause for their miscarriage. Moreover, normal people had no history of miscarriage and no disease or factors related to repeated pregnancy loss. Exclusion criteria included: people with one of the types of autoimmune diseases, hormonal disorders, chromosomal defects, hematological diseases, infections, anatomic uterine defects, and other known factors associated with repeated pregnancy loss. All participants were from 20 to 40 years old. In this study, the participants were selected according to the mentioned criteria by the purposive sampling method, and the sample size was calculated using the odds ratio calculation formula.

2.2. Blood collection and DNA extractions

Five mL peripheral blood samples were collected in blood collection tubes containing ethylene diamine tetraacetic acid for anticoagulation. The samples were stored at -20 ℃ until further use for DNA extraction. Genomic DNA extraction was carried out by using a commercially available kit (Qiagen, Germany; Cat. No.51104) according to the manufacturer's protocol.

The quality of DNA was assessed by using 1% agarose gel electrophoresis. Concentration and purity of DNA were estimated in absorbance at 260 nm by spectrophotometer (thermo scientific).

2.3. Polymerase chain reaction (PCR) analyses

The length of the target gene in our study was 180 bp and the genomic DNA was amplified by the following primer sequences. Besides, the melting temperature was considered at 68 ℃.

Forward primer: 5'-TGGAGAGTGCTGGTGTACCCCA- 3'

Reverse primer: 5'-GCCTCCACCCCCACCCTGTC- 3'

Optimized PCR reaction was performed in a total reaction volume of 25 μL containing: 12/5 μL PCR master mix (2×), 1 μL forward primer (10 pmol), 1 μL reverse primer (10 pmol), 3 μL genomic DNA (50 ng), and 7/5 μL distilled water. Finally, the tubes were placed onto a thermocycler (Astec-Japan). Cycling conditions were as follows: initial denaturation at 95 ℃ for 10 min; followed by 35 cycles of amplification; 95 ℃ for 15 s; 68 ℃ for 15 s; 72 ℃ for 30 s. The final extension was done at 72 ℃ for 5 min. Amplification of PCR products was also confirmed by gel electrophoresis (1/5% agarose gel).

2.4. PCR–restriction fragment length polymorphism (RFLP) technique

The final assessment was performed by PCR-RFLP method. In other words, the alleles of -786T>C were detected by digestion of the PCR product with the restriction enzyme NgoMIV. This enzyme cleavage site on the gene is shown in Supplementary Figure 1. The digestion reaction was carried out in a total volume of 25 μL, containing 10 μL PCR product, 0/25 μL R buffer (10×), 0/25 μL NgoMIV enzyme, and 14/5 μL distilled water. The tubes were incubated at 37 ℃ for 3 h. After digestion, the samples were separated by gel electrophoresis (2% agarose gel) so as to visualize the different products.

2.5. Statistical analysis

All data were analyzed by using the SPSS standard software (Version 21.0, IBM, Armonk, NY, USA) and *Chi*-square test. Hardy-Weinberg equilibrium analysis was also done. Additionally, the results were reported as a graphical representation by GraphPad Prism 6 software. $P<0.05$ was considered statistically significant for all data.

2.6. Ethics statement

The study was approved by the Institutional Ethics Committee of the Shahid Sadoughi University of Medical Sciences of Yazd (No. IR.SSU.MEDICINE.REC.1396.221), and all of the controls and subjects who agreed to participate in this study had signed informed consent before the collection of peripheral blood samples.

3. Results

3.1. Study characteristics

PCR was performed and after loading the PCR product on 1/5% agarose gel, the bands were seen at 180 bp (Supplementary Figure 2). Enzymatic digestion by NgoMIV was done to produce two smaller fragments (89 bp, 91 bp) and the sequences containing the T base in that position were not cut (Supplementary Figure 3).

3.2. Allele and genotype frequency distribution

The frequency of TT, TC, CC in cases were 40%, 53%, and 7%, respectively; whereas in the controls, they were matched with 46%, 49%, and 5%, in that order. Table 1 shows that the differences of these genotypes in cases and controls were not significant (P=0.687). Also, Table 2 displays that there were no differences between the frequency of C and T alleles among cases and controls (P=0.091).

Furthermore, calculation of *Chi*-square showed that the case (P=0.058, χ^2=3.592) and control (P=0.075, χ^2=3.169) groups for the distribution of rs2070744 in *eNOS* gene, were in the Hardy-Weinberg equilibrium.

Table 1. Frequency of different *eNOS* genotypes in the control and the case groups of recurrent pregnancy loss.

Genotype	Case (n=100)	Control (n=100)	P-value
TT, n(%)	40 (40)	46 (46)	
TC, n(%)	53 (53)	49 (49)	0.687
CC, n(%)	7 (7)	5 (5)	

Table 2. Distribution of different alleles of *eNOS* gene in the control and the case group of recurrent pregnancy loss.

Alleles	Case	Control	P-value
T, n(%)	134 (67)	140 (70)	0.091
C, n(%)	66 (33)	60 (30)	

Alleles in each group (n=200).

4. Discussion

Recurrent pregnancy loss presents a critical and stressful clinical problem, which affects about 5% of couples around the world[11]. The extensive research had been carrying out about its causes and treatment. Although several reasons had been recognized for it, about 50% of recurrent pregnancy loss cases are still unexplained[12,13].

A normal pregnancy is a complex and dependent process on several essential factors such as cytokines, hormones, growth factors, angiogenesis agents and some signaling pathways. Any dysfunction of these, depending on the stage of pregnancy, can lead to disturbing in adjustments of the pathways involved in fetal development and consequently may cause miscarriage or the other fetus disorders[14].

Some researches disclosed the role of *eNOS* gene in recurrent pregnancy loss. Changes in physiological levels of NO could be related to *eNOS* variants[15]. It had been shown that -786T>C, 27-bp repeat 4b/4a, 894G>T in the *eNOS* gene supported an association with increasing the risk of recurrent pregnancy loss[16], but the role of some of these variants like -786T>C in recurrent pregnancy loss is less studied and there is still a controversy about them.

The present study investigated the prevalence of alleles of the *eNOS* gene promoter -786T>C polymorphism in women mostly from south and center of Iran with recurrent pregnancy loss, compared to normal control of that population. The *Chi*-square test results demonstrated that there were no significant differences between genotype distribution in the controls and women with recurrent pregnancy loss as a whole group. According to -786T>C position in the gene promoter, studies have focused more on this gene expression level. The *in vitro* investigations asserted the substitution T allele by C in -786 position caused a 50% decline in transcription level and maybe this is the result of replication protein A1 binding as a gene suppressor to this region in peoples with mutant allele[17,18]. Also, some researches exposed the lower levels of serum nitrite in patients with endothelial and vascular disease[19,20]. Previous studies had investigated the distribution of -786T>C polymorphism in various groups. No association of this variant and recurrent pregnancy loss in a cohort of Tunisian had been reported[21]. Some other studies among different ethnicities after that had also reported similar findings[22–25]. In contrast, a study on the South Korean population had discovered that -786T-4b-894T haplotype significantly increased in the recurrent pregnancy loss group[23]. Another study on Lebanon women suggested that *eNOS* gene promoter variation (-786T>C) sustained a substantial impact on recurrent pregnancy loss in their women population[26]. An investigation on a group of Egyptian women exposed a significant association between recurrent

pregnancy loss and this variant[27]. A meta-analysis study reported that this variant has a significant correlation with the recurrent pregnancy loss risk[28]. Azani *et al* had analyzed the prevalence of this variant among Iranian women in Tabriz, Iran. They had revealed that there was a significant relationship between this variant and recurrent pregnancy loss[29].

Unlike the studies mentioned so far, one research evaluated the effect of *eNOS* gene polymorphisms on the aborted embryos and it displayed that CC and TC genotypes for -786T>C variant in the *eNOS* gene in fetus had a significant relationship with spontaneous abortion. Indeed, it claimed this gene polymorphisms special -786T>C promoter variant in the fetus is more likely to be related to recurrent pregnancy loss than in women[30].

The limitations of this study were: small sample size and lack of the broadly available data of the participants.

In conclusion, the results of this study suggested that the mentioned polymorphism is not related to recurrent pregnancy loss in the south and center of Iran population. As point out before, the outcomes of various studies are contradictory. These results infer the association of this polymorphism with recurrent pregnancy loss among distinct ethnic groups from different countries can be inconsistent, and it is because of their genetic variations.

Conflict of interest statement

The authors declare no competing interests.

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Authors' contributions

Maryam Sadat Jalili and Samira Asadollahi performed all the main steps of study, collected the samples, carried out the main steps of essay and wrote the manuscript. Hamid Reza Ashrafzadeh helped to perform DNA extraction and PCR. Seyed Morteza Seifati made the analysis of results and conducted statistical tests. Nasrin Ghasemi was the head of team, designed the study and was responsible for monitoring and fixing technical errors during all steps of the study.

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