Investigation of the association between *eNOS* gene promoter polymorphism (-786 T>C) and idiopathic recurrent pregnancy loss in Iranian women

Maryam Jalili
Shahid Sadoughi University of Medical Sciences and Health Services Yazd Research and Clinical Centre for Infertility

Samira Asadollahi
Shahid Sadoughi University of Medical Sciences and Health Services Yazd Research and Clinical Centre for Infertility

Seyed Morteza Seifati
Shahid Sadoughi University of Medical Sciences and Health Services Yazd Research and Clinical Centre for Infertility

Hamid Reza Ashrafzadeh
Shahid Sadoughi University of Medical Sciences and Health Services Yazd Research and Clinical Centre for Infertility

Nasrin Ghasemi (nghasemi479@gmail.com)
Shahid Sadoughi University of Medical Sciences and Health Services Yazd Research and Clinical Centre for Infertility https://orcid.org/0000-0003-3392-7999

Research article

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Abstract

Background: Repeated Pregnancy Loss (RPL) is defined as 2 or more consecutive spontaneous losses of pregnancy before 20 weeks. Some genetic polymorphisms such as endothelial nitric oxide synthase (*eNOS*) gene, which lead to the synthesis of nitric oxide, could be the reasons for RPL. This case-control study was investigated the frequency of -786 T>C variant in *eNOS* gene promoter in Iranian women with RPL.

Methods : Blood samples were obtained from 100 unrelated women affected by recurrent pregnancy loss and 100 unaffected women as controls. Genomic DNA was extracted and -786 T>C polymorphism in *eNOS* gene promoter investigated by PCR-RFLP method in all of the samples. Statistical analysis in the group patients and controls were performed by chi-square test and P-values of <0.05 were considered significant.

Results: Frequency of homozygous TT was 40% in cases and 46% in control group and frequency of CC was 6% in cases and 5% in the control group and frequency heterozygote TC was 54% in cases and 46% in control group. Genotype frequencies between the two groups showed no significant differences (P>0/05).

Conclusion: The result of this study showed that this polymorphism is not more frequent in recurrent pregnancy loss in this population.

Background

Recurrent pregnancy loss (RPL), is one of the most common complications of pregnancy, occurs in 2 to 5% of clinically recognized pregnancies. It defined as 2 or more consecutive miscarriages before 20 weeks gestation, which is greater than expected by chance (0.34%) [1, 2]. Consequently, RPL 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 nitric oxide synthase (eNOS) (also known as Type III, NOS3), NOS found in macrophages is known as inducible nitric oxide synthase (iNOS) (also known as Type II, NOS2) and the final type is neuronal nitric oxide synthase (nNOS) (also known as Type I, NOS1)[5].

The structure of the *eNOS* gene was first determined by Marsden et al. (1993) [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 influences 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 RPL. One of these genes is *eNOS* which produces nitric oxide synthase in the vascular endothelium and causes vasodilation and smooth muscle relaxation [8]. This enzyme catalyzes the biosynthesis of L-citrulline and Nitric oxide (NO) from L-arginine [9]. The level of NO as a signaling molecule will increase during pregnancy. This results in fetal blood supply without hypertension. Therefore, decreasing serum nitric oxide levels at the beginning of pregnancy can interfere with oxygen supply and nutrition for the fetus and leads to abortion [10]. In this study, we aimed to analyze the relationship of -786T > C polymorphism (rs2070744) in *eNOS* gene regulatory region and RPL among central and southern Iranian women.

Methods

Study participants

The present study was approved by the Human Ethics Research Committee of the Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences.

All of the controls and subjects who agreed to participate in this study have signed informed consent before the collection of peripheral blood samples.

One hundred women with idiopathic spontaneous repeated pregnancy loss who were attending the recurrent miscarriage clinic and 100 women without any miscarriage who had at least one successful pregnancy were recruited in this study. Control blood samples were collected in routine checkup test.

Blood collection and DNA extractions

Five ml peripheral blood samples were collected in blood collection tubes containing EDTA for anticoagulation. They stored at -20°c until further used for DNA extraction. Genomic DNA extraction was carried out using a commercially available kit (Qiagen, Germany. cat.No.51104) according to the manufacturer's protocol.

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

Polymerase chain reaction analyses (PCR)

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 Tm temperature was considered at 68°C.

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 (2X), 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 °C for 10 min; followed by 35 cycles of amplification; 95 °C for 15 s; 68 °C for 15 s; 72 °C for 30 s. The final extension was done at 72 °C for 5 min. Amplification of PCR products were also confirmed by gel electrophoresis (1/5% agarose gel).

PCR-RFLP technique

Final assessing was performed by PCR-RFLP method. In other words, digestion of the PCR product with the restriction enzyme NgoMIV allowed detection of the alleles of -786T > C. this enzyme cleavage site on the gene is shown in Fig. 1. The digestion reaction was carried out in a total volume of 25 µl, containing 10 µl PCR product, 0/25 µl R buffer (10X), 0/25 µl NgoMIV enzyme, and 14/5 µl distilled water. The tubes were incubated at 37°C for 3 hours. After digestion, the samples were separated by gel electrophoresis (2% agarose gel) so as to visualize the different products.

Statistical analysis

All data were analyzed using the SPSS standard software (Version 21.0, IBM, Armonk, NY, USA) and chi-square test. Additionally, the results were reported as a graphical representation by GraphPad Prism 6 software. P-values of < 0.05 were considered statistically significant for all data.

Results

Study characteristic

Present study was performed on 100 Iranian women who had spontaneous recurrent pregnancy loss and 100 women without history of abortion with one normal pregnancy as control for detection of the frequency of -786C > T variant in RPL.

Extracted DNA evaluated on 1% agarose gel (Fig. 2). PCR reaction was performed and after loading the PCR product on 1/5% agarose gel, the bands were seen at 180 bp (Fig. 3). 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 (Fig. 4).

Allele and genotype frequency distribution

The frequency of TT, TC, CC in cases were 40%, 54%, and 6%, respectively; whereas in control were matched with 46%, 49%, and 5%, in that order. Figure 5 shows that the differences of these genotypes in cases and control were not significant (P-value = 0/687). Figure 6 shows that there were no differences between the frequency of C and T alleles among case and control (P-value = 0/091) (Table 1).

Table 1
Distribution of different alleles of *eNOS* gene in control and the case group of RPL.

Alleles	Case	Control	P-value
	NO = 200	NO = 200	
T	134 (67%)	140 (70%)	0/091
C	66 (33%)	60 (30%)	
Notes: NO, number			

Discussion

Recurrent pregnancy loss (RPL), present a critical clinical and stressful 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 RPL 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 RPL. 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 RPL[16], but the role of some of these variants like − 786T > C, in RPL, 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 RPL in compared this with normal control of that population. The $\chi 2$ test results demonstrated that there were no significant differences between genotype distribution in controls and women with RPL 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 fifty-percent decline in transcription level and maybe this is the result of RPA1 (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 the 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 RPL 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, in the study on the South Korean population had been discovered that − 786T-4b-894T haplotype significantly increased in the RPL group [23]. Another study on Lebanon women suggested that *eNOS* gene promoter variation (-786T > C)

sustained a substantial impact on RPL in their women population. [26]. An investigation on a group of Egyptian women exposed a significantly association between RPL and this variant [27]. A meta-analysis study reported that this variant has a significantly correlation with the RPL risk [28]. Azani et al had analyzed the prevalence of this variant among Iranian women in Tabriz. They had revealed that there was a significant relationship between this variant and RPL [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 RPL than women [30].

Conclusion

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

Abbreviations

NOS: Nitric Oxide Synthase

eNOS: endothelial Nitric Oxide Synthase

iNOS: inducible Nitric Oxide Synthase

nNOS: neuronal Nitric Oxide Synthase

NO: Nitric Oxide

RPL: Recurrent Pregnancy Loss

EDTA: Ethylenediaminetetraacetic acid

RPA1: Replication protein A1

Declarations

Ethics approval and consent to participate

The Human Ethics Research Committee of the Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences of Yazd approved the study design (IR.SSU.MEDICINE.REC.1394.230) and written informed consent was obtained from each participant before the collection of blood samples.

Consent for publication

Not Applicable.

Availability of data and materials

Not Applicable.

Competing interests

The author reports no conflicts of interest in this work.

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No funding has been received for this experiment.

Authors' contributions

All authors have read and approved the manuscript;

M.J and S.A: performing the main steps of essay and writing the manuscript.

M.J and H.A: Collecting the samples and helping to perform DNA extraction and PCR.

S.M.S: Analysis of results and doing statistical tests.

N.G: Head of team and monitoring and fixing technical errors during all steps of the study.

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Figures

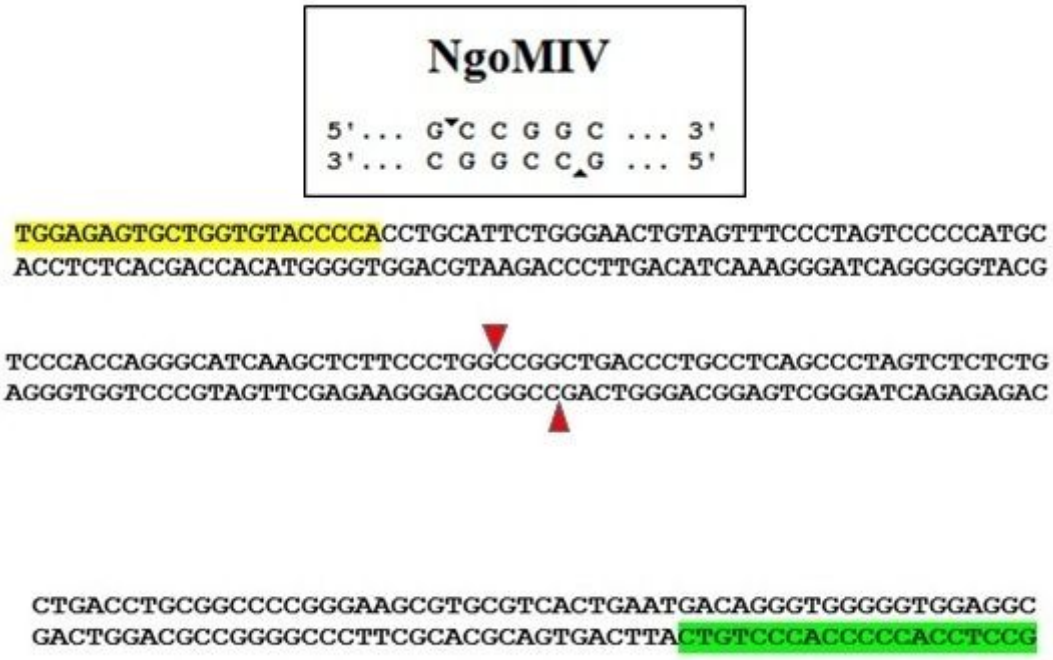

Figure 1

Recognition and cleavage site of NgoMIV enzyme on 180bp PCR-product (NEB Cutter site version 2).

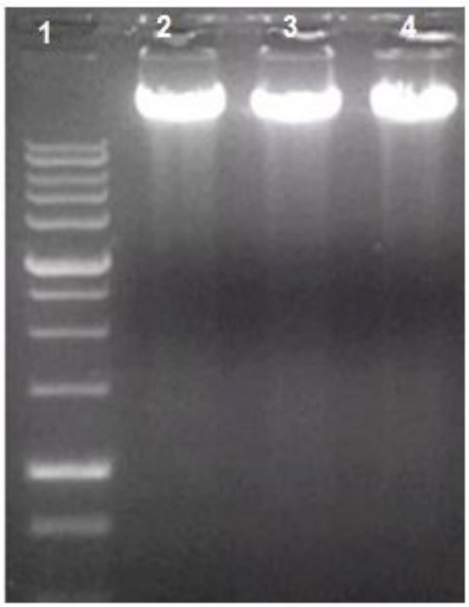

Figure 2

Results of DNA extraction. Case and control DNA samples were separated on 1%agarose gel. Lane No.1 is DNA marker and No.2-4 is three DNA extracted samples.

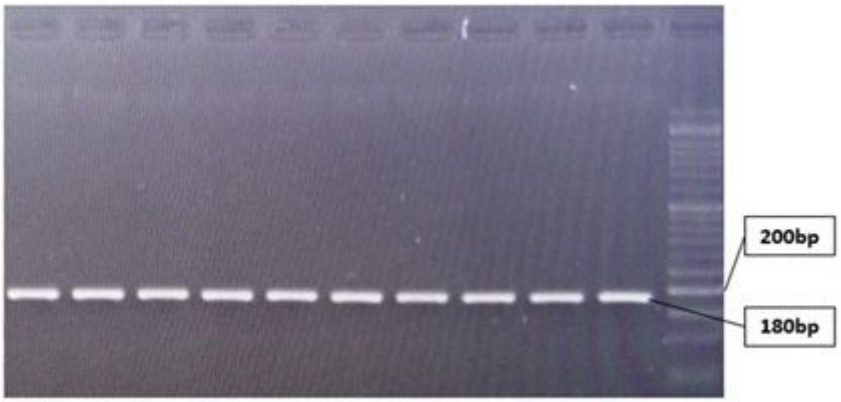

Figure 3

Results of electrophoresis gel from 180bp PCR product of eNOS gene. Right lane is DNA marker and the others being ten samples of PCR products.

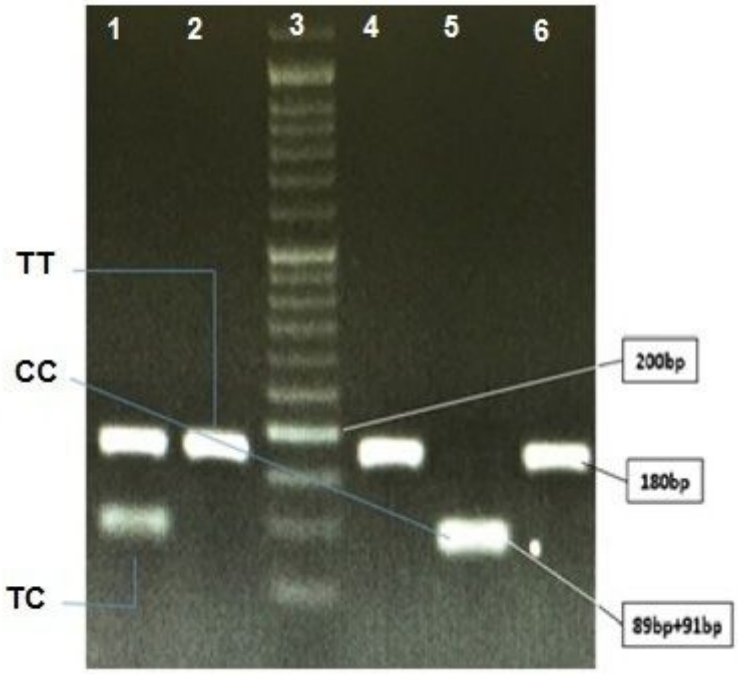

Figure 4

A 2% agarose gel showing the PCR product digested by NgoMIV for -786T>C polymorphism of eNOS gene. Lane 3 is DNA marker and lanes No.2, 4, 6 are TT genotype, lane 5 is CC genotype and lane 1 is TC genotype.

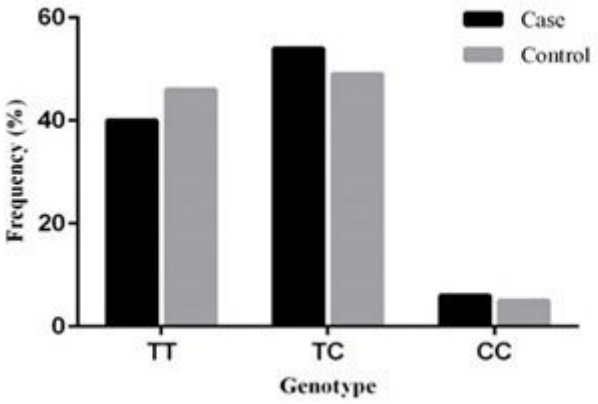

Figure 5

Frequency of different eNOS genotypes in control and the case group of RPL (P=0/687).

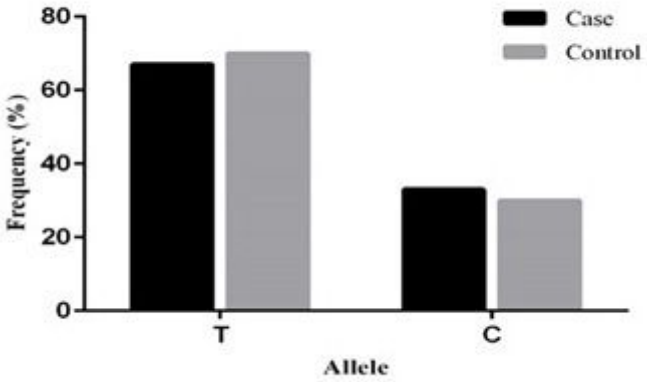

Figure 6

T and C alleles frequency in control and the case group of RPL (P=0/091).

Supplementary Files

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