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Research Article

EVALUATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE GENE G894T POLYMORPHISM IN IRANIAN WOMEN WITH RECURRENT SPONTANEOUS ABORTIONS

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ABSTRACT
Recurrent spontaneous abortion represents the consecutive loss of at least two pregnancies prior to 20th week of gestation. There are many common risk factors such as G894T polymorphism that play key roles in recurrent spontaneous abortions .It has extensively established that nitric oxide (NO) is involved in recurrent spontaneous abortions. Numerous genetic risk factors have been related with recurrent spontaneous abortions, but no study has unraveled a possible association between recurrent spontaneous abortions and G894T polymorphism. Evidence suggests that NOS3 might play a role in recurrent spontaneous abortions, as a result we Studied eNOS common polymorphism (G894T polymorphism) in Iranian women with recurrent spontaneous abortions. In the present case control study, the eNOS polymorphisms (G894T polymorphism) has been investigated in 80 patients with recurrent spontaneous abortions and 100 healthy subjects by using Amplification Refractory Mutation System PCR(ARMS PCR) method. Then, the data were analyzed by pasw statistics 18 (SPSS) software. The present study identified a lack of association between endothelial nitric oxide synthase (eNOS) gene G894T polymorphism in Iranian women with recurrent spontaneous abortions.

Keywords: *Gene Polymorphism, Nitric Oxide, G894T Polymorphism, Recurrent Spontaneous Abortions*

INTRODUCTION
Recurrent spontaneous abortion (RSA) is defined as the spontaneous lack of pregnancy before the 20th gestational week of pregnancy. Pregnancy losses which happen in this time period are said that occurs in about 15 percent of pregnancies. At once, the chance of miscarriage increases consistently to the amount of previous miscarriages experienced. Unfortunately, a certain cause has been difficult to determine (Reznikoff-Etie *et al.*, 1999; Edmonds *et al.*,). A top percentage of early recurrent spontaneous abortion (RSA) before 10 weeks of pregnancy remains unsolved, and specific prevention can't be sufficiently recommended. Recurrent spontaneous abortion has been estimated to take place in almost 1% of all couples (Lee and Silver, 2000). Recurrent spontaneous abortion causes by various genetic and non-genetic factors. Many different factors motivate the occurrence of miscarriage. These generally include genetic, hormonal, anatomic, immunologic and microbiologic variations (Alberman, 1988).
Lifestyle, diet, and, recently maternal genetic characteristics have previously been proposed as determinants of Recurrent spontaneous abortion (RSA). Specifically, maternal and paternal smoking (Chatenoud *et al.*, 1998; Windham *et al.*, 1992). The amount of women with Recurrent spontaneous abortion for non-recurrent reasons will decline exponentially with the amount of previous miscarriages, because their threat of a new miscarriage within their fourth or fifth pregnancy remains only 14 % in contrast to the 35-50% risk in the entire number of Recurrent spontaneous abortion (RSA) patients with an identical quantity of previous miscarriages (Cauchi *et al.*, 1995).
Recurrent Spontaneous Abortions (RSA) supplies a fundamental vision to the processes of embryogenesis and implantation and can also be an irritating and emotionally charged clinical problem. Underneath the best conditions, the reproductive process in humans is fairly inefficient, as approximately 70% of fertilized ova are lost. Spontaneous Abortion is one of many least understood pathological processes regardless of being one of the very most common symptoms. In 50 % or even more of couples with Recurrent spontaneous abortion (RSA) an evaluation testing will undoubtedly be negative. Therefore, a

majority (almost 50 – 75 %) of couples with recurrent spontaneous abortion may have no certain diagnosis. Facts about the frequency, familial distribution and relative to parity and parental age in this dilemma are either unknown or disputable because of the difficulty in obtaining reliable information (Alberman, 1988; Baines and Gendron, 1993; Li *et al.*, 2002). The physical, emotional and financial peal of pregnancy loss is huge. The emotional issues surrounding pregnancy loss become expanded when miscarriage occurs on a repetitive basis (Cougle *et al.*, 2003). Statistical studies declare that, 4-6 % of most women attempting pregnancy will experience at the very least two miscarriages and about 1-2 % may have3 or even more miscarriages. Some studies claim that clinical intrauterine pregnancy losses occur in 12-14 % of all pregnancies (Regan *et al.*, 1989).

NO molecule contributes in cell signaling importantly. In the presence of oxygen, L-arginine, is converted to L- citrulline and release NO. The responsible enzyme for catalyzing this reaction is Nitric oxide synthase (NOS), and it occurs in three isoforms. In the CNS, the neuronal isoform (nNOS) is extensively distributed in neurons, astrocytes and blood vessels. The endothelial isoform (eNOS) is placed in the hippocam-palpyramidal neurons, endothelial cells and some astrocytes (iNOS) expression is typically low but is increased in microglia and astrocytes during neuro- inflammation. Under physiological conditions, it is supposed that NO regulates the release of neurotransmitters and hormones and promotes cell survival and long term potentiation. Even though, high levels of NO are made in inflammatory conditions, which might contribute to synaptic dysfunction, protein and lipid oxidative damage, and neuronal death (Liu *et al.*, 2002; Bishop and Anderson, 2004).

Three different isoforms of NOS derived from various genes generate NO: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). These isoforms are related in function and structure. There is a growing proof that NO is involved in recurrent spontaneous abortions (Marletta, 1994; Akyol *et al.*, 2004).

Many eNOS polymorphisms have been found. Some of these are exposed to be related with recurrent spontaneous abortions. G894T polymorphism is one of the main eNOS polymorphisms, to define a possible role for the NOS3 gene G894T polymorphism in Iranian patients with recurrent spontaneous abortions. As a result we genotyped and analyzed 80 patients with recurrent spontaneous abortions, and 100 healthy controls, from the same population, to define a possible role for G894T polymorphisms in Iranian women with recurrent spontaneous abortions.

MATERIALS AND METHODS

This study is a relationship study with a case-control design and study population consisted of 80 women with recurrent spontaneous abortions, and 100 healthy women matched for age and without previous history of Recurrent Spontaneous Abortions (RSA). Blood samples collection were carried out during the period from July 2012 to September 2013. Blood samples were collected from each subject after fasting for 10-12 hours. DNA was extracted from whole blood samples and then we used Amplification Refractory Mutation System PCR (ARMS PCR) method. Formerly, the data were analyzed by pasw statistics 18 (SPSS) software.

Patients and Controls

DNA was obtained from 80 patients with recurrent spontaneous abortions. A total of 100 healthy controls women without recurrent spontaneous abortions were also analyzed to define a possible role for the NOS3 gene G894T polymorphism in Iranian patients in late pregnancy. Genomic DNA was amplified by polymerase chain reaction (PCR) with proper primers and then, the data were analyzed by (SPSS) software.

DNA Extraction and eNOS G/T894 Polymorphism Genotyping

Genomic DNA from venous blood samples was isolated using Quick Micro Prep Kit (Zymo Research, U.S.A.) according to manufacturer's instructions. The eNOS G/T894 polymorphism genotyping was performed base on ARMS-PCR by eNOS- READY GENE ZG Kit (Zima gene, Iran).The thermal cycling conditions for ARMS-PCR were as follows: 1 cycle at 95°C for 7 min followed by 35 cycles of 94°C for 75 s and 59°C for 55 s and 72ºC for 30 s and final extension step at 72ºC for 5 min. The electrophoresis

was carried out using 2% Gel Red stained agarose gel, at 110V for 45 min. After electrophoresis, the amplified PCR products were visualized under U. V. light (Table).

Table 1: This polymorphism was genotyped by ARMS-PCR, with the primer pairs

Primer Sequence (5'->3')	Length	Tm	GC%
F_C AAGGCAGGAGACAGTGGATG	20	59.38	55.00
R_N TGAAGGAAGAGTTCTGGTGGC	21	59.93	52.38
R_M TGAAGGAAGAGTTCTGGTGGA	21	58.31	47.62

Table 2: Specifications primers used for internal control

Primer Sequence (5'->3')	Length	Tm	GC%
F GTGTACCCCACCTGCATTCT	20	59.67	55.00
R CCCAGCAAGGATGTAGTGAC	20	57.97	55.00

Table 3: PCR program used for ARMS-PCR polymorphism G-T894:

Cycle	Temperature (Celsius)	Time
First	95	7 Minutes
Two to thirty-five	94	1 minute and 15 seconds
	59	55 Seconds
	72	30 seconds
Thirty-six	72	5 Minutes

RESULTS AND DISCUSSION

Results

We genotyped and analyzed 80 patients with recurrent spontaneous abortions, and 100 healthy controls, for the NOS3 and G894T polymorphism.

G894T polymorphism frequencies were in equilibrium in patients and controls. Patients showed an extensively increased frequency of the G894T allele compared with controls. Thus the G894T allele would confer a slightly increased risk of developing recurrent spontaneous abortions in Iranian population. Carriers of the G894T were at a slightly but significantly increased frequency in patients compared with controls. Both groups of healthy controls and women with RAS, had similar gene frequencies, suggesting that this polymorphism is not related with recurrent spontaneous abortions in Iranian population (table).

Genotype Table

Table 4: Genotype Table of G894T polymorphism

Case Processing Summary						
	Valid		Cases Missing		Total	
	N	Percent	N	Percent	N	Percent
Genotype * Group	100	100.0%	0	.0%	100	100.0%

Table 5: Chi-Square Tests for Genotype of G894T polymorphism

Chi-Square Tests			
	Value	**df**	**Asymp. Sig. (2-sided)**
Pearson Chi-Square	4.233[a]	2	0.467
Likelihood Ratio	4.689	2	0.467
Linear-by-Linear Association	1.920	1	0.467
N of Valid Cases	180		

a. 2 cells (33.3%) have expected count less than 5. The minimum expected count is 2.00.

Table 6: Symmetric Measures for Genotype of G894T polymorphism

Symmetric Measures					
		Value	**Asymp. Std. Error[a]**	**Approx. T[b]**	**Approx. Sig.**
Interval by Interval	Pearson's R	.471	.087	5.279	0.467
Ordinal by Ordinal	Spearman Correlation	.491	.088	5.574	0.467
N of Valid Cases		180			

a. Not assuming the null hypothesis.
b. Using the asymptotic standard error assuming the null hypothesis.
c. Based on normal approximation.

Discussion

Our results showed that the genotype and the allele frequencies of G894T polymorphisms were significantly different between patients with recurrent spontaneous abortions and the controls (all P-values were 0.467). On the other hand, neither genotype nor allele frequencies of endothelial nitric oxide synthase gene G894T polymorphism were significantly different between RPL patients and the controls (P-values for genotype and allele frequency: 0.467), so, do not seem to contribute to an increased risk for recurrent spontaneous abortions. As a result Lack of association between recurrent spontaneous abortions and nitric oxide synthase gene G894T polymorphism observed in this study. Contradictory results in associating an allele, genotype and recurrent spontaneous abortions in different populations can be attributed to the variation in the genetic background, in particular linkage disequilibirium to varying genetic elements.

Conclusion

This study was conducted in order to determine the association between G894T polymorphisms of eNOS gene, and recurrent spontaneous abortions. The current study have investigated the relation between G894T polymorphisms of eNOS gene and the development of spontaneous abortions and showed that there is not significantly association between G894T polymorphisms and recurrent spontaneous abortions.

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