Original Article

Role of Endothelial Nitric Oxide Synthase Gene Polymorphisms (Glu298Asp) in Egyptian Patients with Recurrent Spontaneous Abortion

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ABSTRACT

Background: Previous studies indicated an association between endothelial nitric oxide synthase (eNOS) activity and maintenance of pregnancy, but it is rather controversial whether polymorphisms of the gene encoding for eNOS are associated with recurrent spontaneous abortions (RSAs). **Aim:** The aim was to investigate whether the presence of maternal polymorphism Glu298Asp in exon seven of the eNOS gene increase the risk of RSA in Egyptian women. **Subjects and Methods:** Hundreds women were randomly selected as the case group. They had at least three RSA before 20th weeks of gestation, same partner and at least one live birth and compared with 100 women, same age range, with no history of abortions or complicated pregnancy as control group. All were investigated for the polymorphism using the polymerase chain reaction-restriction fragment length polymorphisms method. Data were expressed descriptively as percentages for qualitative values and mean ± standard deviation for quantitative parametric data and comparison of qualitative data was done. **Results:** Frequency of GG genotype 50/100% in cases and 67/100% in control group. Heterozygous GT frequency was 46/100% in cases and 26/100% in control ($P \leq 0.01$, odds ratio [OR] =2.37, and 95% confidence interval [CI] =1.30–4.34). Homozygous TT was 4/100% in the cases and at 7/100% in control ($P = 0.68$, OR = 0.77, and 95% CI = 0.21–2.76). GG genotype versus GT and GG ($P = 0.01$, OR = 2.03, and 95% CI = 1.15–3.60). Cases with TT genotype were more susceptible to abortion at an older age with a mean of 29 (4.76) ($P = 0.02$). **Conclusion:** In conclusion, (eNOS) Glu298Asp polymorphism was found to be associated with increased risk of RSA in this sample of Egyptian women.

KEY WORDS: Endothelial nitric oxide, polymorphism, recurrent spontaneous abortions, synthase

INTRODUCTION

Recurrent spontaneous abortion (RSA) affects only 5% of all couples; it is a most frustrating experience for the patient as well as for the clinician. Recently, the spectrum of etiologies for RSA has been changing. Some causes such as luteal insufficiency and infectious diseases have lost much of their previous importance, but some new exciting findings have enriched our understanding of possible mechanisms of RSA. The sharing of parental antigens has become a hot topic again with the finding of specific human leukocyte antigen alleles that seem to be associated with RSA. Although genetic abnormalities have been implicated in recurrent loss of pregnancy for some time, skewed X-inactivation was recently found to be an additional genetic factor.

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During pregnancy, nitric oxide (NO) production in endothelial cells increases, probably playing an important role in implantation, decidualization, and regulation of blood flow in the placenta by means of vasodilatation and myometrial relaxation. NO is synthesized by the trophoblast cells, NO prevents the contractions of the uterine myometrium directly or by an interaction with the cyclooxygenase. NO is also apparently crucial for maintenance of the maternal systemic vasodilatation and reduced vascular reactivity seen during normal pregnancy. It was shown that in the early stage of pregnancy, trophoblasts express high amounts of NO synthase (NOS) activity, the isoform being endothelial NOS (eNOS). Furthermore, placental production of gonadotropic hormone is modulated by eNOS expression and the subsequent NO release by cytotrophoblast and syncytiotrophoblast cells.[1]

In humans, abnormal NO levels have been shown to play a role in hypertension, vasospasm, infraction and preeclampsia. Several recent studies were performed to

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investigate whether polymorphisms of the eNOS gene could be risk factors for RSA. An impaired oxygen and nutrient supply might affect the ability of the embryo to resist maternal rejection in early pregnancy.[2] Among the various eNOS gene polymorphisms is Glu298Asp polymorphism in exon 7. It is investigated for its potential association with RSA in some population. Glu298Asp polymorphism alters the stability of the enzyme[3] and is associated with various diseases such as myocardial infarction,[4] placental abruption, and preeclampsia.[5]

The association between eNOS gene polymorphism and RSA has been studied among various ethnic groups, including Caucasian, South Indian, North Indian, Chinese, Greek, and Taiwanese.[2,6-9] The results of the various studies conflicted with each other. Some studies showed positive associations between certain genotypes and RSA,[2,6-9] while others did not.[2] It has to be stated that the aim of our present study was to investigate whether presence of maternal polymorphism of the eNOS gene increase the risk of RSA in Egyptian women.Thus, we made an effort to reduce an error in the interpretation of our result by only considering Egyptian women in the study and control groups. Some other studies investigating RSA have taken women with no history of live births. This strategy does not rule out that RSA might be of an anatomical factor, to avoid this possible bias all candidates in our study had at least one history of live birth. We also took in consideration that our candidates have the same partner during their marital life in order to exclude paternal chromosomal abnormality that might be a cause of RSA. The aim of our study was to investigate whether the presence of maternal polymorphism Glu298Asp in exon seven of the eNOS gene increase the risk of RSA in Egyptian women.

SUBJECTS AND METHODS

Our study population included 100 women, selected by simple random sampling way, with three or more RSA before 20 weeks of gestation, with their ages ranging (20–35) years as a case group against 100 women with no history of spontaneous abortions or complicated pregnancy as a control group. All candidates were referred from the outpatient clinic of the Prenatal Diagnosis and Fetal Medicine Department, National Research Centre during the period from June 2010 to May 2011. The study was approved by the Scientific and Ethical Committees of the National Research Center, Cairo University. An informed consent has been obtained from all patients according to the National Research Centre Ethical Committee and underwent a standard diagnostic workup including: Pedigree analysis with detailed medical, obstetric, past and family history, serum progesterone, follicle-stimulating hormone and luteinizing hormone, TORCH, anticardiolipin (IgM and IgG), paternal and maternal karyotyping, and abdominal ultrasound.

Collection of samples and isolation of genomic DNA

A volume of 5 ml venous blood samples were withdrawn from all candidates under complete aseptic conditions and collected in a polypropylene tube containing 0.5 M ethylenediaminetetraacetic acid (pH 8.0) to prevent clotting and nuclease activity and genomic DNA was extracted using salting-out method.

Genotype analysis

Polymerase chain reaction (PCR) amplification of DNA samples was performed using exon seven of the eNOS gene-specific primers (forward [5'-TCCCTGAGGGCATGAGGCT-3¢] and the reverse [5'-TGAGGGTCACACAGGTTCCT-3¢.]). PCR products were then confirmed by electrophoresis on 2% agarose gel. PCR product variants were then digested by specific restriction enzymes (Sau3AI enzyme) and analyzed on 2% agarose gel stained with Ethidium bromide (7, 25, 26).

Interpretation

The gene coding for eNOS is located on chromosome 7q35–36 with 26 exons spanning 21 kb. The E/E genotype contained 277, 150, and 30 bp SAU3AI fragments reflecting. The D/D genotype also contained the 30, 132, 150, and 145 bp fragments. Fragment150 and 145 were too close so detected as one band). Each of the samples from heterozygotic combinations contained both sets of fragments from each eNOS allele. E/D 277, 150, and 30 as well as 132 bp fragments from cleavage at the SAU3AI site [Figures 1 and 2].

Statistical methods

Data were expressed descriptively as percentages for qualitative values and mean ± standard deviation for quantitative parametric data. The compiled data were computerized and analyzed by SPSS software package, version 2, Echosoft Corporation, (Chicago, USA). The

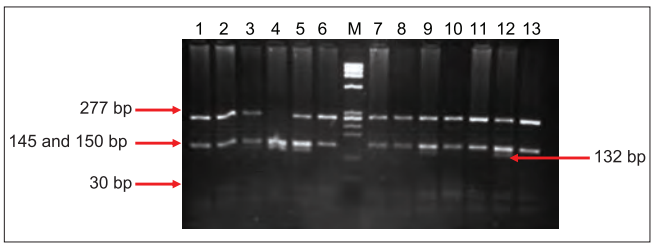

Figure 1: A 2% agarose gel showing digestion of polymerase chain reaction products of exon seven of the endothelial nitric oxide synthase gene with SAU3AI for detection of the different genotypes in 13 cases. Lanes 1, 2, 6, 7, 8, 10, 11, 13: Eight cases with G/G genotype (30, 277, 150 bp). Lane 4: One case subjects with T/T genotype (30, 132, 145, 150 bp). Lanes 3, 5, 9, 12: Four cases with G/T genotype (30,132,145,150,277bp).M:Molecularweightmarker(PhiX174DNA/HaeIIIdigest) 1 2 3 4 5 6 M 7 8 9 10 11 12 13

following tests of significance were used: Analysis of variance test between more than two means, *t*-test between means was used to analyze mean difference. Comparison of qualitative data was done using Chi-square test, crosstabs, and least significant difference. A level of significance with $P \leq 0.05$ was considered significant, $P \leq 0.01$ was considered of high significance and $P > 0.05$ insignificant.

RESULTS

Cases population

All patients included in the cases group had three or more RSA before 20th weeks of gestation with a range of three to 10 abortions and the most frequent cases were patients with three RSA showing a mode of three, 37/100, (37%) of the cases. Their ages ranged from 20 to 35 years, mean 27.32 (4.5).

Control population

All control candidates included in the study group had normal live births with no history of spontaneous abortions or complicated pregnancy. Their ages ranged from 20 to 35 years, mean 25.43 (3.78).

Genotypes distribution and allele frequencies within groups

The frequency of GG genotype coding for the glu298 isoform of eNOS was found to be 50/100 (50%) in cases as compared to 67/100 (67%) in control group. The heterozygous genotype GT frequencies were 46/100 (46%) in cases and 26/100 (26%) in control, ($P \leq 0.01$, odds ratio [OR] =2.37, and 95% confidence interval [CI] =1.30–4.34). Homozygous TT was present at a frequency of 4/100 (4%) in the cases and at 7/100 (7%) in control, ($P = 0.68$, OR = 0.77, and 95% CI = 0.21–2.76). GG genotype versus GT and TT genotypes with ($P = 0.01$, OR = 2.03, and 95% CI = 1.15–3.60) and the frequency of G allele was 0.65% for the cases while 0.74% for control group, while T allele was 0.35% for the cases and 0.26% for control group. With ($P = 0.15$, OR = 1.47, and 95% CI = 0.87–2.48) [Table 1].

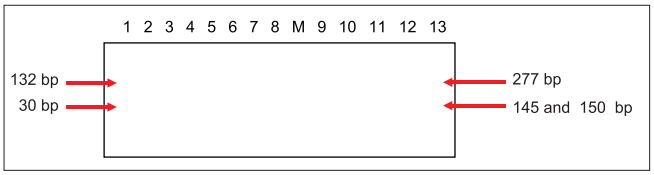

Figure 2: A 2% agarose gel showing digestion of polymerase chain reaction products of exon seven of the endothelial nitric oxide synthase gene with SAU3AI for detection of the different genotypes in 12 normal controls. Lanes 1, 5, 6, 8, 9, 10: Five cases with G/T genotype (30, 132, 145, 150, 277 bp). Lanes 2, 3, 4, 11, 12: Eight cases with G/G genotype (30, 277, 150 bp). Lane 7: One case subjects with T/T genotype (30,132,145,150bp).M:Molecularweightmarker(PhiX174DNA/HaeIIIdigest) 1 2 3 4 5 6 7 8 M 9 10 11 12

Table 1: Distribution and allele frequencies of eNOS polymorphism Glu298Asp in exon 7 among case and control groups

Genotype Glu298Asp in exon 7	Cases (*n*=100) (%)	Controls (*n*=100) (%)	OR (95% CI)	*P**
GG	50 (50.0)	67 (67.0)	Reference**	
GT	46 (46.0)	26 (26.0)	2.37 (1.30-4.34)	<0.01 (S)
TT	4 (4.0)	7 (7.0)	0.77 (0.21-2.76)	0.68 (NS)
GG versus GT + TT	50 versus 46+4	67 versus 26+7	2.03 (1.15-3.60)	0.01 (S)
G	0.66	0.74	Reference***	
T	0.34	0.26	1.47 (0.87-2.48)	0.15 (NS)

*P value is significant at the ≤0.05 level; **The genotype GG was used as a reference in calculating the OR and *P* value of genotypes GG and GT; ***The G allele was used as a reference to calculate the OR and *P* value for the T allele. eNOS=Endothelial nitric oxide synthase, OR=Odds ratio, CI=Confidence interval, NS=Nonsignificant, S=Significant

Correlation of the genotypes with age of the patient at time of the abortion in the cases group (*n* = 100)

Cases with TT genotype were more susceptible to abortion at an older age with the respect to GT and GG genotypes. RSA in TT genotype cases had a mean of 29 (4.76) years, with a significant ($P = 0.02$) when compared to GT and GG cases with ($P = 0.86$).

DISCUSSION

The outcome of pregnancy depends to a great extent on the success rate of early events, which includes implantation, establishment of fetomaternal circulation, and the maintenance of increased blood flow to the implantation site. A reduction in NO production can lead to impaired placental perfusion and a compromised oxygen and nutrient supply to the fetus. In humans, abnormal NO levels as well as the polymorphic variants have been shown to play an important role in preeclampsia.[6]

The association between eNOS gene polymorphism and recurrent spontaneous miscarriage (RSM) has been studied among various ethnic groups including Caucasians, South Indians, North Indians, Chinese, Greeks, South Koreans, and Taiwanese, and the results of the various studies conflicted with each other. Some studies showed positive associations between certain genotypes and RSM while others did not.

It has to be stated that the aim of our present study was to investigate whether presence of maternal polymorphism of the eNOS gene increase the risk of RSA in Egyptian women. Thus, we made an effort to reduce an error in the interpretation of our result by only considering Egyptian women in the study and control groups. Some other studies investigating RSA have taken women with no history of live births. This strategy does not rule out that RSA might be of an anatomical factor, to avoid this possible bias all candidates in our study had at least one history of live birth. We also took into consideration that our candidates have the same partner during their marital life in order to exclude paternal chromosomal abnormality that might be a cause of RSA.

Our study had shown that the GT genotype coding for the Glu298 isoform of eNOS was found to be more associated

with the increased incidence of RSA as it had a significant *P* value when comparing to other genotypes. RSA cases holding TT genotype were more susceptible to abortion at an older age with the respect to GT and GG genotypes.

The results of our study are supported with the published results of Tempfer *et al*., 2001, Suryanarayana *et al*., 2006, Shin *et al*. and Parveen *et al*., 2011,[6-8,10] and it is opposite to Karvela *et al.*, 2008[2] and Pereza *et al*. 2015.[11]

In another published study, Su *et al*., 2011 stated that angiogenesis and an adequate blood supply are critical for several steps in human early pregnancy. Angiogenesis- and vasoconstriction-related genes are associated with RSA and eNOS is one of these genes.[9]

Our data indicate that the investigated eNOS polymorphism confers a small but significantly increased risk of developing RSA. However, this polymorphism is not sufficient alone to explain the development of RSA, as in some of the candidates in the control group had the polymorphism and did not complain of RSA. Mechanisms that might explain the relationship between eNOS polymorphism and RSA include linkage disequilibrium with a mutation within coding region or promoter region in eNOS gene, the possibility of neighboring gene and altered splicing or processing of the primary transcript.

It cannot specify at what level an increase or decrease in protein expression regulated by the investigated gene could affect the risk of developing RSA. With the respect to the eNOS gene, impaired NO production could happen at gonadal level, placental level or at the level of the placental vasculature. Furthermore, it could be argued that evaluating polymorphism only in mother does not reflect the pathophysiology process leading to RSA. Ethnic variation and genetic admixture need to be considered in an evaluation of genetic background of RSA.[12]

CONCLUSION

We were able to find a correlation between the increased risk for the occurrence of RSA and polymorphism Glu298Asp in eNOS gene exon seven. GT genotype was more significant in cases associated with RSA, increasing the risk for RSA by 2.3 folds. While GG and TT genotypes were insignificant in cases of RSA. Cases holding TT genotype are more susceptible to abortion at an older age with the respect to GT and GG genotype. These results cannot exclude that the TT genotypes have not role in the RSA, but additional genetic association and functional studies in different populations with larger numbers of participants and a uniformly defined idiopathic RSA (IRSA) are needed to clarify the contribution of NOS3 + 894 G/T gene variation to IRSA.

Molecular genetics diagnosis of the male partner of patients diagnosed with RSM in order to define the possible new correlation between the incidence of abortion and the paternal genotype.

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