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Endothelial nitric oxide synthase gene polymorphisms (−786T>C, 4a4b, 894G>T) and haplotypes in Korean patients with recurrent spontaneous abortion

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ABSTRACT

Objective: To investigate the association of three common polymorphisms (−786T>C, 4a4b, 894G>T) of the endothelial nitric oxide synthase (eNOS) gene with idiopathic recurrent spontaneous abortion (RSA).
Study design: In a prospective case–control study, 340 patients with unexplained recurrent spontaneous abortion and 115 controls with at least one live birth and no history of pregnancy loss were enrolled. Polymerase chain reaction and restriction fragment length polymorphism analysis were performed to identify the genotypes.
Results: The recurrent spontaneous abortion patients exhibited a significantly higher frequency of the eNOS 894GT + TT genotype (Odds ratio (OR), 2.39; 95% confidence interval (CI), 1.25–4.58; $p = 0.008$) compared to the control group; no significant differences in the −786T>C and 4a4b genotype frequencies were observed. The eNOS 894GT genotype (OR, 1.94; 95% CI, 1.00–3.75; $p = 0.056$) was marginally different between recurrent spontaneous abortion and control groups. The frequency of the −786T-4b-894T haplotype ($p = 0.001$) was significantly higher in the idiopathic RSA group than in the control group.
Conclusion: The eNOS 894GT + TT genotype and the −786T-4b-894T haplotype are significantly associated with idiopathic recurrent spontaneous abortion in Korean women.

1. Introduction

Clinically recognized pregnancies end in miscarriage in 15–20% of cases. One to 5% of pregnant women experience recurrent spontaneous abortions (RSA), of which 40–55% are induced by unknown causes [1,2]. Known etiologic factors of RSA are parental chromosome abnormalities, uterine abnormalities, hereditary thrombophilias, endocrinologic disorders, immunologic factors, infections, and nutritional and environmental factors [3–6].

Endothelial nitric oxide synthase (eNOS) is the main enzyme required for vascular nitric oxide (NO) production. NO is released during the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) enzymes. NOS enzymes exist as three isoforms: endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) [7]. NO inhibits platelet aggregation and leukocyte adhesion to the vascular endothelium and limits the oxidation of atherogenic LDL [8–10]. NO-generating vasodilators also inhibit vascular smooth muscle cell migration and proliferation [11]. Lack of endothelium-derived nitric oxide is associated with the development of endothelial damage, hypertension, coronary spasm, myocardial infarction, coronary artery disease and ischemic stroke [12–16].

Endothelial nitric oxide synthase (eNOS) is expressed in terminal villous vessels and in the syncytiotrophoblast of pregnant women [17]. In mice, lipopolysaccharide (LPS)-induced abortion is mediated by placental NO production [18], and pharmacological inhibition of NO release by aminoguanidine successfully rescues LPS-induced abortion [19]. Accordingly, polymorphisms of the eNOS gene have been observed in various populations. A single nucleotide polymorphism (SNP), −786T>C, was identified in the 5′-flanking region of the eNOS gene involving a substitution of thymine (T) to cytosine (C) at a locus 786 bp upstream of eNOS [12]. Another common variant of eNOS has a G to T transversion at

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nucleotide position 894 (894G>T), leading to a change of amino acid at position 298 (Glu298Asp) [14]. A polymorphism in intron 4 (4a4b) of the gene encoding eNOS has been shown to segregate with lower plasma NO metabolites in nonpregnant Japanese females [20]. Also, this polymorphism was associated with recurrent miscarriage in Caucasians [21]. Tempter et al. [21] reported that the distribution of genotype frequencies was significantly different between the study and control groups for 4b4a heterozygotes. Recent reports indicate a role for these polymorphisms in human reproductive diseases including intrauterine fetal death, placental abruption, pre-eclampsia, as well as RSA [22–31].

Previous studies have evaluated the eNOS polymorphisms in patients with RSA, but the results are often conflicting. Despite the association studies, the effect of eNOS polymorphisms on the risk of Korean patients with RSA has not been reported yet. Therefore, in the present study, we examined these three polymorphisms of the eNOS gene and evaluated the relationship between the polymorphisms and the development of RSA in Korean women.

2. Materials and methods

2.1. Patients

The study group consisted of 340 women (age range, 22–45 years; mean age ± SD, 32.45 ± 4.26 years) who were diagnosed with at least three consecutive spontaneous abortions; these patients were enrolled in the study at the Infertility Medical Center of CHA Bundang Medical Center from March 1999 to February 2008. The age and ethnicity matched control group consisted of 115 women (age range, 23–43 years; mean age ± SD, 31.22 ± 4.30 years), each of whom had had at least one live birth and no history of pregnancy loss. All of the patients and controls were Korean and the institutional review board of CHA Bundang Medical Center approved the study in 1999, and all subjects gave written informed consent.

2.2. Genetic analysis of eNOS polymorphisms

Genomic DNA was extracted from anticoagulated peripheral blood using the G-DEX blood extraction kit (Intron, Seongnam, South Korea). The nucleotide changes were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis using the isolated genomic DNA as a template.

The PCR of the eNOS −786T>C polymorphism was performed using primers to generate a 236-bp (bp) product: forward 5′-ATG CTC CCA CCA GGG CAT CA-3′ and reverse 5′-GTC CTT GAA TCT GAC ATT AGG G-3′. For the eNOS 4a4b polymorphism, the following primers were used to generate a 420- or 393-bp product: forward 5′-AGG CCC TAT GGT AGT GCC TTT-3′ and reverse 5′-TCT CTT TAG TGC TGT GGT CAC-3′. For the eNOS 894G>T polymorphism, the following primers amplified a 206-bp fragment: forward 5′-CAT GAG GCT CAG CCC CAG AAC-3′ and reverse 5′-AGT CAA TCC CTT TGG TGC TCA C-3′.

The eNOS −786T>C polymorphism was analyzed by digesting the PCR product with the restriction endonuclease *Ngo*MIV (New England Biolabs, Beverly, MA) at 37 °C for 16 h. The −786T allele remains uncut (236-bp), whereas the −786C allele is cut into two fragments of 203- and 33-bp. The eNOS 894G>T polymorphism was analyzed by digesting the PCR product with the restriction endonuclease *Mbo*I (New England Biolabs, Beverly, MA) at 37 °C for 16 h. The 894G allele remains uncut (206-bp), whereas the 894T allele is cut into two fragments of 119- and 87-bp.

The amplified DNA fragments of the eNOS 4a4b polymorphism were analyzed without digesting the PCR product with any restriction enzymes. The wild-type allele (allele 4b) generates a 420-bp band (five copies of a 27-bp repeat). The polymorphic allele (allele 4a) generates a 393-bp band (four copies of the same repeat).

2.3. Statistical analysis

Differences in the frequencies of the eNOS −786T>C, 4a4b, and 894G>T alleles and haplotypes in the study and control groups were analyzed by the X^2 test. The odds ratio (OR) was used as a measure of the strength of the association between allele frequencies and RSA. All *p*-values were two-tailed and 95% confidence intervals (CI) were calculated. $p \leq 0.05$ was considered statistically significant. We used the StatsDirect statistical software version 2.4.4 (StatsDirect Ltd., Altrincham, UK) to perform the calculations. Haplotype analysis was performed using SNPAlyze Ver.5.1 Standard/Pro (DYNACOM Co., Ltd., Yokohama, Japan).

3. Results

The eNOS −786T>C, 4a4b and 894G>T polymorphisms were investigated, and their genotype distributions and allele frequencies in RSA patients and controls are shown in Table 1. The eNOS genotype and allele frequencies in patients and controls were

Table 1
Genotype and haplotype frequencies of eNOS −786T>C, 4a4b and 894G>T polymorphisms in Korean patients with idiopathic recurrent spontaneous abortion (RSA) and in controls.

Genotype	Controls (%)	Cases (%)	OR (95%CI)	*p* value
eNOS −786T>C				
TT	93 (80.9)	278 (81.8)	1.0	
TC	21 (18.3)	61 (17.9)	0.97 (0.56–1.68)	0.889
TC + CC	22 (19.1)	62 (18.2)	0.94 (0.55–1.62)	0.890
C allele	0.100	0.093		
eNOS 4a4b				
4b4b	90 (78.3)	275 (80.9)	1.0	
4b4a	24 (20.9)	63 (18.5)	0.86 (0.51–1.46)	0.584
4b4a + 4a4a	25 (21.7)	65 (19.1)	0.85 (0.51–1.43)	0.588
Allele frequency (4a)	0.113	0.099		
eNOS 894G>T				
GG	103 (89.6)	266 (78.2)	1.0	
GT	12 (10.4)	60 (17.6)	1.94 (1.00–3.75)	0.056
GT + TT	12 (10.4)	74 (21.8)	2.39 (1.25–4.58)	0.008
T allele	0.052	0.129		
Total	115 (100)	340 (100)		