Polymorphisms in the endothelial nitric oxide synthase gene associated with recurrent miscarriage

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ABSTRACT. Endothelial nitric oxide synthase (eNOS) is an enzyme that influences placental human chorionic gonadotropin production during gestation. Previous studies have indicated an association between eNOS activity, implantation, and maintenance of pregnancy, but proposed associations between polymorphisms of the eNOS gene and recurrent miscarriage (RM) are controversial. To identify markers contributing to the genetic susceptibility to RM, we examined the potential association between RM and 8 single nucleotide polymorphisms (SNPs; rs1799983, rs2070744, rs11771443, rs3918188, rs2853796, rs7830, rs1541861, and rs2853792) of the eNOS gene using the MassARRAY system (Sequenom, USA). The enrolled participants included 192 RM patients and 201 women with normal fertility as controls. The results showed that rs1799983 at exon 7 of the eNOS gene was significantly associated with RM (genotype: chi-square = 15.071, P = 0.001; allele: chi-square = 6.250, P = 0.016). Another significant association was observed for rs11771443 in the

promoter (genotype: chi-square = 6.259, P = 0.044; allele: chi-square = 7.076, P = 0.008). Furthermore, strong linkage disequilibrium was observed in 3 blocks (D' > 0.9), and significantly fewer T-T-G haplotypes (chi-square = 5.981, P = 0.015) residing in block 1 were found in RM patients. These findings point to a role for eNOS gene polymorphisms in RM in the Chinese Han population and may be informative for future genetic or neurobiological studies of RM.

Key words: Endothelial nitric oxide synthase; Recurrent miscarriage; Single-nucleotide polymorphisms

INTRODUCTION

Approximately 15 to 20% of clinically recognized pregnancies end in miscarriage, and approximately 1 to 5% of pregnant women experience recurrent miscarriage (RM), of which 40 to 55% are induced by unknown causes (Yamada et al., 2001). Although many etiological factors causing RM are known, such as parental chromosome abnormalities, uterine abnormalities, hereditary thrombophilias, endocrinologic disorders, immunologic factors, infections, and nutritional and environmental factors (Li et al., 2002), most RM cases remain idiopathic.

Endothelial nitric oxide synthase (eNOS), which oxidizes its substrate L-arginine to L-citrulline, is the main enzyme responsible for the production of vascular nitric oxide (NO) (Moncada and Higgs, 1993). During the first trimester, eNOS is expressed in the terminal chorionic villous vessels and in the cyto- and syncytiotrophoblast layers. The level of eNOS increases as gestation progresses and is significantly enhanced in certain pathological conditions (Ayuk et al., 2002). Furthermore, the placental production of human chorionic gonadotropin, an important angiogenic factor for neoangiogenic interaction and trophoblast migration (Zygmunt et al., 2002), is modulated by eNOS during gestation (Sanyal et al., 2000). Abnormal levels of NO have been reported in the placentae from preeclamptic pregnancies as well as from term pregnancies with fetal growth retardation (Myatt et al., 1997). In mice, placental NO production mediates abortion induced by lipopolysaccharide (Haddad et al., 1995), whereas pharmacological inhibition of NO release with aminoguanidine successfully rescues lipopolysaccharide-induced abortion (Athanassakis et al., 1999). Thus, eNOS and NO may contribute to pregnancy maintenance by controlling both endocrine function and vascular tone.

Two single-nucleotide polymorphisms (SNPs) associated with eNOS protein expression, including rs1799983 (894G>T, Glu298Asp) in exon 7 and rs2070744 (-786T>C) in the promoter region have been well described in the eNOS gene (Nakayama et al., 1999; Hefler et al., 2002). The rs1799983 SNP is associated with various vascular diseases related to pregnancy (Yoshimura et al., 2001; Hefler et al., 2002; Hillermann et al., 2005; Huber et al., 2005; Suryanarayana et al., 2006). A systematic review has indicated that rs1799983 may be correlated with recurrent spontaneous abortion (Su et al., 2011). The rs2070744 SNP has been observed in patients with vascular disease (Khurana et al., 2003; Rossi et al., 2003; Guang-Da et al., 2005); it inhibits the promoter activity of eNOS. However, whether rs2070744 is associated with RM remains controversial (Serrano et al., 2004; Shin et al., 2010). In addition to reports of these variants, a limited number of studies have investigated a possible association between RM and other polymorphisms of the eNOS gene. Thus, a larger number of patients must be assessed for conclusive results.

In the present study, we investigated the crucial and controversial role of the eNOS polymorphisms in RM. We systematically identified 8 genetic polymorphisms in eNOS and

conducted a case-control association analysis of the variants and their haplotypes to determine the susceptibility of variants and haplotypes to RM.

MATERIAL AND METHODS

Subjects

The study group consisted of 340 women (age range, 22-43 years; mean age ± SD, 30.56 ± 4.58 years) who were diagnosed with at least 3 consecutive spontaneous abortions; these patients were enrolled in the study at the Fourth Hospital of Xi'an (Xi'an, China). All RM patients underwent careful clinical examinations, including hysteroscopy or hysterosalpingography, transvaginal ultrasound, serial endometrial biopsies, and analyses of tissue antibodies and autoantibodies. The results of the clinical examinations showed that they had no identifiable causes for their miscarriages, including chromosomal abnormalities or anatomic, endocrine, cytogenetic, or autoimmune causes, and antibody screens for toxoplasmosis, cytomegalovirus, rubella, human immunodeficiency virus, group B streptococci, chlamydia trachomatis, and hepatitis B and C were negative. Age- and ethnicity-matched subjects included 115 women (age range, 21-44 years; mean age ± SD, 31.02 ± 4.36 years), each of whom had a history of at least 1 live birth and no pregnancy loss, were recruited as healthy controls at the Fourth Hospital of Xi'an. The following exclusion criteria were used: taking prescribed medications that could affect the central nervous system; a history of seizures, hematological disease, or severe liver or kidney damage; smoking; hypertension; and previous use of oral contraceptives. This study was approved by the ethics committee of the Fourth Hospital of Xi'an, and written informed consent was obtained from all participants.

SNP selection

The genomic length of eNOS is 21 kb. SNPs in the promoter region, untranslated region, exons, and introns of the eNOS gene were systematically screened. Marker selection was based on the following criteria. First, we examined tagSNPs in Haploview version 4.2 (Daly Lab Inc., Cambridge, MA, USA) using the Han Chinese in Beijing population and a minor allele frequency cut-off of below 5% (HapMap Data Release 27). Second, the linkage disequilibrium (LD) pattern of the gene was determined in the Chinese population using the preliminary data from HapMap. Third, 8 SNPs were selected for genotyping. Previous studies have shown significant associations between SNPs and RM. The rs1799983 SNP is located in exon 7, rs2070744 and rs11771443 in the promoter, rs1541861 in intron 8, rs3918188 and rs2853792 in intron 12, rs2853796 in intron 13, and rs7830 in intron 22. These SNPs were further analyzed in an association study.

Genotyping

Genomic DNA was extracted from blood leukocytes using an EZNATM Blood DNA Midi Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer protocol. Genotyping for 8 SNPs was performed using the MassARRAY platform (Sequenom, San Diego, CA, USA) by means of a matrix-assisted laser desorption ionization-time of flight mass spectrometry method according to manufacturer instructions. Primers were designed using Sequenom software (Table 1).

Table 1. Primer sequences used for genotyping eNOS gene SNPs with the MALDI-TOF Sequenom platform.

SNPs	Forward primers	Reverse primers	Extension primers
rs1799983	ACGTTGGATGAAACGGTCGCTTCGACGTG	ACGTTGGATGACCTCAAGGACCAGCTCGG	TGCAGGCCCCAGATGAG
rs2070744	ACGTTGGATGACTGTAGTTTCCCTAGTCCC	ACGTTGGATGAGGTCAGCAGAGAGACTAGG	CAAGCTCTTCCCTGGCC
rs11771443	ACGTTGGATGCGTCTGTGGGCGTAACATC	ACGTTGGATGGAAGGATCAGGCCCACAATG	CCGCTGGGCTGATGTA
rs3918188	ACGTTGGATGAAAAGTGGGAGCAAGGCACA	ACGTTGGATGACTTCACTGAGACTGAAGGG	AGGCACACGTACAAGGGC
rs2853796	ACGTTGGATGAAGACAAGGTTGTCACAGGG	ACGTTGGATGAGAGGCAGAGACCCTGAAG	ACAGGCTAGGCCCAGC
rs7830	ACGTTGGATGCTGTCCCTAGATTGTGTGAC	ACGTTGGATGCGGCTGCATGACATTGAGAG	CCTTCAGGCAGTCCTTTAGTCC
rs1541861	ACGTTGGATGATCAGATTCTCAAAGGGGAC	ACGTTGGATGACCTCAGAGCAGAGAGGGA	ATTCTCAAAGGGGACCTTGACTCGGC
rs2853792	ACGTTGGATGCAAATAAAATAAAATGCTGG	ACGTTGGATGGGTTTGTAAAAATCCACAGAGC	AATGCTGGAATAGTGTTGC

Statistical analysis

Each SNP was tested for deviation from Hardy-Weinberg equilibrium using Pearson's chi-square test or Fisher's exact test. The association between genetic polymorphisms was examined using the chi-square test and Fisher's exact test. The odds ratios (ORs) and 95% confidence intervals (95%CIs) were calculated to estimate the strength of association through unconditional logistic regression analysis. The common genotype/allele served as a reference for calculating OR. All statistical tests were conducted in SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences were considered significant if the P value was smaller than 0.05. For haplotype analyses, the P value was based on a comparison of the frequency distribution of all possible combinations of haplotypes that contained a certain SNP that was significantly associated with RM. Pairwise LD statistics (D' and r^2) and haplotype frequencies were computed using Haploview 4.0 to construct haplotype blocks. Haplotypes with a frequency of less than 5% were combined with the rare haplotype group. The frequency of all haplotypes was compared between case and control participants using Pearson's chi-square test.

RESULTS

The distribution frequencies of the 8 genotyped SNPs were in agreement with Hardy-Weinberg equilibrium. LD analyses of RM patients and healthy controls revealed that 3 SNPs (rs11771443, rs2070744, rs1799983), 2 SNPs (rs1541861, rs2853792), 2 SNPs (rs3918188, rs2853796) were located in haplotype blocks 1, 2, and 3, respectively (D' > 0.9; Figure 1).
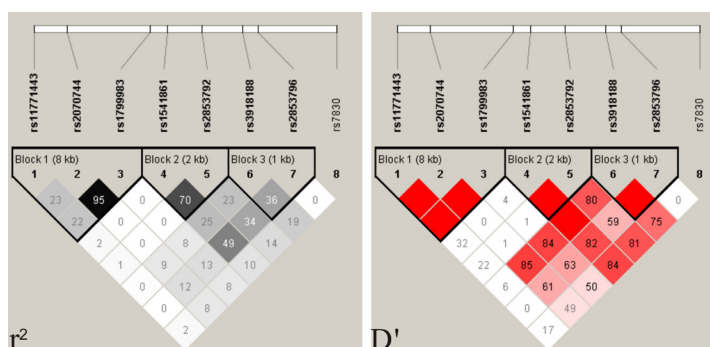

Figure 1. Linkage disequilibrium (LD) plot of 8 single nucleotide polymorphisms (SNPs) in the endothelial nitric oxide synthase gene. Values in squares are the pairwise calculation of r^2 (left) or D' (right). Black squares indicate r^2 = 1 (i.e., perfect LD between a pair of SNPs). Empty squares indicate D' = 1 (i.e., complete LD between a pair of SNPs).

The genotype distribution, allelic frequencies, and haplotypes in RM patients and healthy controls along with the results of statistical analysis are shown in Tables 2 and 3.

Table 2. Genotype and allele frequencies of eNOS gene polymorphisms in cases (N = 192) and controls (N = 201) and the results of their associations with risk of recurrent spontaneous abortion.

Identity	Genotype/allele	Controls [N (%)]	Case [N (%)]	OR, 95%CI
rs1799983	G/G	154 (76.617)[#]	118 (61.458)	1.0 (reference)
	G/T	41 (20.398)	72 (37.500)	2.292, 1.458-3.603
	T/T	6 (2.985)	2 (1.042)	0.435, 0.086-2.194
	χ^2, P	15.071, 0.001*		
	G	349 (86.816)	308 (80.208)	1.625, 1.108-2.383
	T	53 (13.184)	76 (19.792)	
	χ^2, P	6.250, 0.016*		
rs2070744	T/T	153 (76.119)	128 (66.667)[#]	1.0 (reference)
	T/C	41 (20.398)	55 (28.646)	1.603, 1.005-2.559
	C/C	7 (3.483)	9 (4.688)	1.537, 0.557-4.242
	χ^2, P	4.312, 0.116		
	T	347 (86.318)	311 (80.990)	1.481, 1.011-2.170
	C	55 (13.682)	73 (19.010)	
	χ^2, P	4.091, 0.053		
rs11771443	T/T	78 (38.806)	57 (29.688)[#]	1.0 (reference)
	T/C	84 (41.791)	79 (41.146)	1.287, 0.813-2.037
	C/C	39 (19.403)	56 (29.167)	1.965, 1.153-3.347
	χ^2, P	6.259, 0.044*		
	T	240 (59.701)	193 (50.260)	1.497, 1.128-1.986
	C	162 (40.299)	191 (49.740)	
	χ^2, P	7.076, 0.008*		
rs3918188	C/C	69 (34.328)	62 (32.292)[#]	1.0 (reference)
	C/A	89 (44.279)	97 (50.521)	1.213, 0.775-1.898
	A/A	43 (21.393)	33 (17.188)	0.854, 0.484-1.508
	χ^2, P	1.829, 0.401		
	C	227 (56.468)	221 (57.552)	0.957, 0.721-1.269
	A	175 (43.532)	163 (42.448)	
	χ^2, P	0.094, 0.773		
rs2853796	T/T	101 (50.249)	91 (47.396)[#]	1.0 (reference)
	T/G	72 (35.821)	74 (38.542)	1.141, 0.742-1.755
	G/G	28 (13.930)	27 (14.063)	1.070, 0.588-1.950
	χ^2, P	0.360, 0.835		
	T	274 (68.159)	256 (66.667)	1.070, 0.794-1.442
	G	128 (31.841)	128 (33.333)	
	χ^2, P	0.199, 0.704		
rs7830	C/C	95 (47.264)	83 (43.229)	1.0 (reference)
	C/A	88 (43.781)	89 (46.354)	1.158, 0.763-1.756
	A/A	18 (8.955)	20 (10.417)	1.272, 0.631-2.565
	χ^2, P	0.714, 0.700		
	C	278 (69.154)	255 (66.406)	1.134, 0.841-1.530
	A	124 (30.846)	129 (33.594)	
	χ^2, P	0.679, 0.445		
rs1541861	A/A	119 (59.204)	120 (62.500)	1.0 (reference)
	A/C	63 (31.343)	55 (28.646)	0.866, 0.557-1.347
	C/C	19 (9.453)	17 (8.854)	0.887, 0.440-1.790
	χ^2, P	0.452, 0.798		
	A	301 (74.876)	295 (76.823)	0.899, 0.648-1.247
	C	101 (25,124)	89 (23.177)	
	χ^2, P	0.406, 0.560		
rs2853792	A/A	93 (46.269)	93 (48.438)[#]	1.0 (reference)
	A/G	86 (42.786)	80 (41.667)	0.930, 0.612-1.414
	G/G	22 (10.945)	19 (9.896)	0.864, 0.438-1.701
	χ^2, P	0.230, 0.891		
	A	272 (67.662)	266 (69.271)	0.928, 0.687-1.254
	G	130 (32.338)	118 (30.729)	
	χ^2, P	0.235, 0.646		

*Significant results (P < 0.05). [#]Rounded off to three decimal.

Table 3. eNOS haplotype in block frequencies and their associations with risk of recurrent miscarriage.

Block	Haplotype[a]	Controls (N, %)	Cases (N, %)	Statistics			
				χ^2	P[b]	OR	95%CI
1	T-T-G	120 (59.701)	91 (47.396)	5.981	0.015	0.608	0.408-0.907
	C-T-G	53 (26.368)	58 (30.208)	0.715	0.433	1.209	0.779-1.876
	C-C-T	27 (13.433)	31 (16.146)	0.575	0.479	1.241	0.710-2.169
2	A-A	136 (67.662)	133 (69.271)	0.118	0.746	1.077	0.704-1.649
	C-G	50 (24.876)	45 (23.438)	0.111	0.814	0.924	0.582-1.468
	A-G	14 (6.965)	15 (7.813)	0.103	0.848	1.132	0.531-2.413
3	A-T	87 (43.284)	81 (42.188)	0.048	0.839	0.956	0.641-1.426
	C-G	64 (31.841)	64 (33.333)	0.100	0.830	1.070	0.702-1.632
	C-T	49 (24.378)	46 (23.958)	0.009	1.000	0.977	0.616-1.551

[a]Haplotypes with a frequency lower than 0.05 were excluded. [b]P value is adjusted by Bonferroni's correction and statistically significant. Results (P < 0.0017 in block 1; P < 0.025 in blocks 2 and 3).

The results revealed a significant association between the rs1799983 genotype distribution and RM patients (chi-square = 15.071, P = 0.001). RM patients had a significantly lower frequency of the G allele (chi-square = 6.250, P = 0.016, OR = 1.625, 95%CI = 1.108-2.383). A significant between-group difference was found in the genotype distribution of rs11771443 (chi-square = 6.259, P = 0.044). RM patients had a significantly higher frequency of the C allele of rs11771443 (chi-square = 7. 076, P = 0.008, OR = 1.497, 95%CI = 1.128-1.986).

Tests of the 3-marker haplotype (block 1) association using rs11771443, rs2070744, and rs179998 provided evidence for significant associations between these SNPs and RM (P < 0.017). Owing to higher frequencies in healthy controls, a protective effect may be implicated with T-T-G haplotypes (P = 0.015, after Bonferroni correction).

DISCUSSION

Recently, eNOS has been reported to be overexpressed in the glandular and luminal epithelium of the endometrium in women with RM and unexplained infertility, suggesting a detrimental effect of excess NO on endometrial receptivity and implantation (Najafi et al., 2012). In humans, abnormal levels of NO as well as polymorphic variants of eNOS have been shown to play important roles in pregnancy-related vascular disease (Hefler et al., 2002; Hillermann et al., 2005; Huber et al., 2005; Suryanarayana et al., 2006; Yoshimura et al., 2001).

The rs1799983 SNP alters the stability of eNOS (Tesauro et al., 2000) and is associated with reduced NO production (Veldman et al., 2002). This reduction is reportedly associated with various vascular diseases such as myocardial infarction (Shimasaki et al., 1998), placental abruption (Yoshimura et al., 2001), and pre-eclampsia (Yoshimura et al., 2000; Savvidou et al., 2001). A meta-analysis has revealed a close association between rs1799983 and idiopathic RM (Su et al., 2011). Our results demonstrated that RM patients had a significantly lower frequency of the G allele of rs1799983, which is consistent with previous studies in Koreans (Shin et al., 2010), whites (Hefler et al., 2002), and Indians (Parveen et al., 2011). By contrast, some studies have not confirmed this association (Daher et al., 2012).

Population stratification and sample size are important concerns in human genetic surveys (Thomas and Witte, 2002). In the present study, the experimental and control groups were ethnically matched by drawing from a homogeneous population. Our study had sufficient statistical power to argue an epidemiologically relevant impact of hereditary variations among

the studied genes. The statistical difference remained significant when the data were subjected to Bonferroni correction. Furthermore, T-T-G (rs11771443-rs2070744-rs1799983) haplotypes may be involved in a protective effect. Against our expectation, the G allele of rs1799983 made no other haplotype in RM patients or controls. Thus, we could safely conclude that the G allele of rs1799983 had the greatest effect on rs11771443 and rs2070744 alleles in Chinese women.

The rs2070744 SNP in the promoter region of eNOS has been observed in patients with vascular disease (Khurana et al., 2003; Rossi et al., 2003; Guang-Da et al., 2005), and this polymorphism inhibits the promoter activity of eNOS, reducing NO production in blood vessels and causing endothelial dysfunction (Brown et al., 2003; Kimura et al., 2003; Senthil et al., 2005). Previous studies have demonstrated a lack of consistent association between the rs2070744 polymorphism and recurrent pregnancy loss in Tunisians (Zammiti et al., 2008) and Koreans (Shin et al., 2010). The current study suggests that this polymorphism was unassociated with the development of RSA in a Chinese population sample. The most intriguing finding was the significant association between rs11771443 in the promoter of the eNOS gene and RM. To our knowledge, this study is the first to associate rs11771443 with RM. The result suggests that this polymorphism might play a critical role in the development of RM through regulation of eNOS transcription. The functional importance of this polymorphism, however, requires further studies.

In conclusion, in a homogeneous population sample with a relatively large sample size, we found that 2 eNOS polymorphisms (rs1799983 and rs11771443) were associated with RM. These findings encourage the search for functional polymorphisms within and close to the eNOS gene using a systemic approach in a larger population sample.

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