

No Association of the Genetic Polymorphisms of Endothelial Nitric Oxide Synthase, Dimethylarginine Dimethylaminohydrolase, and Vascular Endothelial Growth Factor With Preeclampsia in Korean Populations

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The purpose of this study was to investigate whether there is any association between preeclampsia and eNOS, DDAH, and VEGF gene polymorphisms, and also to search for a possible association between haplotypes in eNOS, DDHA, and VEGF genes and the risk for preeclampsia. DNA was extracted from whole blood of 223 preeclampsia patients and 237 healthy pregnant women. The genotypes were analyzed by a single base primer extension assay using a SNaPSHOT assay kit. Results were analyzed with the Student's t-test, Chi-square test, and Logistic regression analysis. Haplotype analyses were performed using Haploview 3.2 version. There were no significant differences in genotype or allele frequencies of eNOS, DDAH, and VEGF gene polymorphisms between preeclampsia patients and controls. No increase in the risk of preeclampsia for those genes was observed under any model of inheritance and there were no statistically significant associations between any haplotypes and preeclampsia risk. Polymorphisms in eNOS, DDAH, and VEGF gene do not seem to be risk factors for preeclampsia.

Preeclampsia is a serious complication of the second half of human pregnancy that occurs at frequencies of 5% to 7% throughout the world. Although it is a leading cause of maternal and perinatal morbidity and mortality, the pathogenesis of preeclampsia remains unclear.

The search for genetic factors predisposing women to preeclampsia has been intense, and is

believed to be related to one or more genes. Candidate genes for preeclampsia susceptibility include those involved in systemic arteriolar vasodilatation associated with endothelial nitric oxide (NO) and the regulation of vascular remodeling (Banyasz et al., 2006; Serrano et al., 2004).

NO synthesized by endothelial NO synthase (eNOS) is apparently important for maintenance of the maternal systemic vasodilatation and reduced vascular reactivity during normal pregnancy (Magness et al., 1996; Sladek et al., 1997). Chronic NO synthase inhibition in pregnant rats is known to produce a hypertensive state associated with vasoconstriction, proteinuria, and intrauterine growth retardation, a pattern that resembles preeclampsia (Sladek et al., 1997). Arngrimsson et al. (1997) showed that there was localization of a familial pregnancy-induced hypertension-susceptibility locus in the region of chromosome 7q36 encoding the eNOS gene. Recently, the Glu298Asp and -786 T/C gene polymorphisms in the eNOS gene have been associated with the risk of preeclampsia (Serrano et al., 2004).

In humans, NO synthesis is inhibited by endogenous asymmetric methylarginines (Buhimschi et al., 1995). Asymmetric dimethylarginine (ADMA) inhibits NO synthesis and the primary route of

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elimination of ADMA is carried out by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), to citrulline and di- or mono-methylamine (Akbar et al., 2005). When DDAH is inhibited, NO synthesis is decreased because of increased concentration of ADMA, and elevated levels of circulating ADMA may be of immense pathophysiological importance in preeclamptic pregnancies (Murray-Rust et al., 2001). The enzyme is encoded by a gene which has two isoforms, DDAH1 and DDAH2, located on chromosome 1p22 and 6p21.3, respectively, and the haplotypic association of DDAH1 with preeclampsia has been reported by Akbar et al. (2005).

Vascular endothelial growth factor (VEGF) is an important regulator of vasculogenesis and angiogenesis. It is also a key component in the regulation of vascular remodeling and the survival of cytotrophoblasts in the placenta (Banyasz et al., 2006). The human VEGF gene is located on chromosome 6 at location 6p21.3, and consists of eight exons (Vincenti et al., 1996). Many polymorphisms of the VEGF gene have been identified. There are some reports about the association between VEGF gene polymorphism and preeclampsia (Banyasz et al., 2006; Papazoglou et al., 2004). However, there are no reports about the association between preeclampsia and eNOS, DDHA, and VEGF gene polymorphisms in Korean pregnant women. Therefore, in the present study we investigated whether there is any association between preeclampsia and eNOS, DDAH, and VEGF gene polymorphisms, and also searched for a possible association between haplotypes in eNOS, DDHA, and VEGF genes and the risk for preeclampsia in pregnant Korean women.

Methods

Subjects

Participants were recruited when admitted for delivery at Ewha Womans University, MokDong Hospital, and Cheil General Hospital in Seoul, Korea, between 2003 and 2004.

This is a retrospective, hospital based, case-control study, and was approved by the Institutional Review Board at Ewha Womans University and Cheil General Hospital. Informed consent was obtained from all subjects. The study included 223 patients with preeclampsia and 237 controls without pregnancy complications.

Preeclampsia was defined according to the Working Group (2000) criteria as high blood pressure ($\geq 140/90$ mmHg after 20 weeks gestation) and proteinuria (≥ 300 mm/24 hours). The controls were 237 pregnant women monitored from the first trimester at the Department of Obstetrics and Gynecology of Ewha Womans University, MokDong Hospital, and Cheil General Hospital, and who completed their pregnancy without complications. Exclusion criteria were as follows: altered renal function, diabetes or chronic disease, twin pregnancy, recurrent miscar-

riage, fetal growth retardation, abruptio placenta, and thrombophilia. Women with a history of essential hypertension were also excluded from the study. Gestational age was defined as the interval between the first day of the mother's last menstrual period and the date of delivery. Maternal and infant medical records were reviewed to collect detailed information concerning antepartum, labor, and delivery characteristics, and conditions of the newborn, including birth weight and gestational age at delivery.

DNA Extraction and Genotyping

At the time of delivery, blood was collected in sterile ethylenediaminetetraacetic acid vacutainer tubes and stored immediately after collection at -80 °C until required for analysis. Genomic DNA was extracted from whole blood using the PUREGENE® DNA Purification Kit (Gentra Systems, USA) according to the manufacturer's instructions. The genotyping was analyzed by single base primer extension assay using the SNaPshot assay kit according to manufacturer's recommendations (ABI, USA). Briefly, the genomic DNA region containing both of the SNPs was amplified by PCR reaction. Each PCR reaction contained: 10.0 ng of DNA, 1X PCR Buffer, 0.125 units of *AmpliTaq Gold* DNA polymerase (ABI, USA), 3.0 mM MgCl₂, 0.25 mM of each dNTP, and 0.5 pmol of each primer in a 10 ul reaction volume. Reactions were incubated at 95 °C for 10 minutes, then cycled 30 times at 95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, followed by 72 °C for 5 min. The primer sequences are shown in Table 1. For selection of SNPs in this study, we used the KSNP database system (www.ngri.go.kr/SNP).

After amplification, the PCR products were treated with 1 unit each of shrimp alkaline phosphatase (SAP; Roche, Germany) and exonuclease I (USB Corporation, USA) at 37 °C for 60 minutes, and 72 °C for 15 minutes, to purify the amplified products. One microliter of the purified amplification products was added to a SNaPshot Multiplex Ready reaction mixture containing 0.15 pmols of genotyping primer. The primer extension reaction was carried out for 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 30 seconds. To remove excess fluorescent dye terminators, the reaction products were treated with 1 unit of SAP for 37 °C, 1 hour, followed by 72 °C, 15 minutes. One microliter of the final reaction samples containing the extension products were added to 9 microliters of Hi-Di formamide (ABI, USA). The mixture was incubated at 95 °C for 5 minutes, followed by 5 minutes on ice, and then analyzed by electrophoresis in an ABI Prism 3730 DNA analyzer. Results were analyzed using Gene Mapper software (ABI, USA).

Statistical Analysis

Case-control comparisons of clinical characteristics were performed using the Student's *t*-test and Chi-square test. All polymorphisms were tested for

Table 1

Nucleotide Sequences of the SNaPshot Primers Used for Amplification and Studied Gene Sequence Variations

Gene	SNP location	RS No	Sequence variant	Snap Shot primers
eNOS1	5' flanking		-786T > C	GACTAGGGCTGAGGCAGGGTCAGCC
eNOS2	Exon 7	1799983	Glu298Asp (894G/T)	CCCTGCTGCTGCAGGCCCCAGATGA
DDAH1	IVS2	2076699	IVS2 - 33C/T	AGATGTCATGGAACATAGTGAGCAG
DDAH2	IVS5	1498375	IVS5 - 71A/T	ACCATCCTCAGAAGTGGAGAATCAA
DDAH3	3' UTR	3087894	3UTR + 16C/G	TGCCTGTGCGGTCTTGCCGGCWACC
VEGF1	5' UTR	2010963	-634G/C	GYAGGTCACTCACTTTGCCCTGTC
VEGF2	3' flanking	3025039	936C/T	GGCGAATCCAATTCCAAGAGGGACC

Note: SNP: Single nucleotide polymorphism

eNOS: Endothelial nitric oxide

DDAH: Dimethylarginine dimethylaminohydrolase

VEGF: Vascular endothelial growth factor.

conformation with the Hardy-Weinberg Equilibrium test by Chi-square test analysis. Linkage disequilibrium (LD) plots were generated with the program Haploview 3.2 version, and block structure was defined by the confidence interval algorithm (Gabriel et al., 2002). Standardized LD coefficients for DDAH gene pairs were plotted. We calculated the odds ratio (OR) of each genotype, allele, haplotype, and alternative models (recessive, dominant, additive models) using logistic regression models.

For recessive inheritance, genotypes were coded as 1 (homozygote for the minor allele) or 0 (heterozygote or homozygote for the major allele). For dominant inheritance, genotypes were coded as 1 (heterozygote or homozygote for the minor allele) or 0 (homozygote for the major allele). For additive inheritance, genotypes were coded as 1 (homozygote for the minor allele) or 0 (homozygote for the major allele). All statistical analysis was performed using the SAS 9.1 version. $P < .05$ was considered statistically significant.

Results

The clinical characteristics of controls and preeclampsia patients are described in Table 2. Delivery weeks, birth weight, body mass index (BMI), systolic blood pressure, and diastolic blood pressure were significantly different between preeclampsia patients and controls ($p = .0001$). However, age, gravida, and para were not significantly different between preeclampsia patients and controls ($p > .05$).

The results regarding the gene frequencies and allele frequencies in preeclampsia patients and controls are presented in Table 3 and Table 4, respectively. All control and study samples included where genotype frequency was available were in Hardy-Weinberg equilibrium ($p > .05$). There were no significant differences in genotype or allele frequencies of the eNOS gene, DDAH gene, and VEGF gene polymorphisms between preeclampsia patients and controls ($p > .05$; Table 3, 4).

However, haplotypes were constructed on the basis of maximum likelihood. Among SNPs of the eNOS, DDAH, and VEGF genes, only the DDAH gene SNPs (DDAH 2 and DDAH 3) were in a strong linkage disequilibrium with each other (Lod score > 2.0).

Table 5 shows the odds ratios and 95% confidence intervals from multiple logistic regression analyses for the association between genotypes and haplotypes and preeclampsia risk according to dominant, recessive, and additive models, respectively. We adjusted for age, Body Mass Index (BMI), gravida, and para. There were no significant differences in the eNOS, DDAH, and VEGF genes and preeclampsia risk under dominant, recessive, and additive models ($p > .05$; Table 5).

In haplotype analysis, those who had the -/- genotype of DDAH AC haplotype were more likely to have preeclampsia risk under all models, although there were no significant differences in haplotypes and preeclampsia risk ($p > .05$; Table 5). Those who had the -/- genotype of DDAH-TG haplotype were more likely to have preeclampsia risk under the recessive model. However, those who had the -/- genotype of

Table 2

Clinical Characteristics of Controls and Preeclampsia Patients

	Control (n = 237)	Patients (n = 223)	p value
Age (year)	31.1 ± 4.2	31.0 ± 4.5	.68
Delivery weeks (wks)	39.1 ± 1.6	35.7 ± 4.4	.0001
Birth weight (g)	3196 ± 490	2459 ± 912	.0001
BMI (kg/m ²)	26.3 ± 3.4	28.0 ± 4.2	.0001
Systolic BP (mmHg)	116.9 ± 13.3	158.0 ± 18.4	.0001
Diastolic BP (mmHg)	76.1 ± 9.8	99.4 ± 12.5	.0001

Note: BP: Blood pressure

BMI: Body mass index

Values are given as mean ± SD

We used student *t* tests.

Table 3
Gene Frequencies in Preeclampsia Patients and Controls

Gene	Genotypes	Patients <i>N</i> (%)	Control <i>N</i> (%)	<i>p</i> value	Unadjusted * OR (95% CIs)
eNOS1	TT	176 (80.7)	185 (78.4)	.74	0.89 (0.56-6.43)
	CT	39 (17.9)	46 (19.5)		
	CC	3 (1.4)	5 (2.1)		
eNOS2	GG	192 (85.0)	188 (83.6)	.78	0.87 (0.51-1.46)
	GT	31 (13.7)	35 (15.5)		
	TT	9 (1.3)	2 (0.9)		
DDAH1	CC	184 (82.5)	189 (81.5)	.95	0.93 (0.57-1.58)
	CT	38 (17.0)	42 (18.1)		
	TT	1 (0.5)	1 (0.4)		
DDAH2	AA	128 (57.4)	142 (59.9)	.27	1.00 (0.68-1.49)
	AT	74 (33.2)	82 (34.6)		
	TT	21 (9.4)	13 (5.5)		
DDAH3	CC	129 (57.8)	142 (60.2)	.80	1.07 (0.73-1.56)
	CG	80 (35.9)	82 (34.7)		
	GG	14 (6.3)	12 (5.1)		
VEGF1	GG	72 (31.8)	83 (37.9)	.22	1.08 (0.64-1.81)
	CG	110 (48.7)	89 (40.6)		
	CC	44 (19.5)	47 (21.5)		
VEGF2	CC	166 (73.8)	153 (66.0)	.18	0.69 (0.45-1.08)
	CT	51 (22.7)	68 (29.3)		
	TT	8 (3.5)	11 (4.7)		

Note: SNP: Single nucleotide polymorphism

OR: Odds ratios

CIs: Confidence intervals.

Table 4
Allele Frequencies in Preeclampsia Patients and Controls

Gene	Genotypes	Patients	Control	<i>p</i> value	Unadjusted * OR (95% CIs)
eNOS1	T	391 (89.7)	416 (88.1)	.46	1.0
	C	45 (10.3)	56 (11.9)		
eNOS2	G	415 (89.4)	411 (91.3)	.33	1.0
	T	49 (10.6)	39 (8.7)		
DDAH1	C	406 (91.0)	420 (90.5)	.78	1.0
	T	40 (9.0)	44 (9.5)		
DDAH2	A	330 (74.0)	366 (77.2)	.27	1.0
	T	116 (26.0)	108 (22.8)		
DDAH3	C	338 (75.8)	366 (77.5)	.39	1.0
	G	108 (24.2)	106 (22.5)		
VEGF1	G	254 (56.2)	255 (58.2)	.54	1.0
	C	198 (43.8)	183 (41.8)		
VEGF2	C	383 (85.1)	374 (80.6)	.07	1.0
	T	67 (14.9)	90 (19.4)		

Note: SNP: Single nucleotide polymorphism

OR: Odds ratios

CIs: Confidence intervals

Table 5

Genotype and Haplotype Analysis According to Different Models

SNP	Recessive	Dominant	Additive
	Unadjusted* OR (95% CIs)	Unadjusted* OR (95% CIs)	Unadjusted* OR (95% CIs)
Genotype			
eNOS1	0.65 (0.15–2.73)	0.87 (0.55–1.37)	0.74 (0.33–1.67)
eNOS2	1.50 (0.25–9.06)	0.90 (0.54–1.49)	0.89 (0.36–2.22)
DDAH1	1.04 (0.07–10.74)	0.93 (0.58–1.50)	0.88 (0.35–2.21)
DDAH2	1.79 (0.87–3.67)	1.11 (0.77–1.61)	1.38 (0.77–2.47)
DDAH3	1.25 (0.57–2.77)	1.10 (0.76–1.60)	1.21 (0.66–2.23)
VEGF1	0.89 (0.56–1.40)	1.31 (0.88–1.93)	1.16 (0.70–1.94)
VEGF2	0.74 (0.29–1.88)	0.69 (0.46–1.03)	0.55 (0.28–1.08)
Haplotype			
DDAH-AC	1.66 (0.78–3.54)	1.11 (0.76–1.61)	1.34 (0.74–2.43)
DDAH-TG	0.96 (0.66–1.39)	0.79 (0.36–1.74)	0.88 (0.48–1.62)

Note: SNP: Single nucleotide polymorphism

OR: Odds ratios

CIs: Confidence intervals

DDAH-TG haplotype were less likely to have preeclampsia risk under the dominant model (Table 5).

Discussion

These results indicate that polymorphisms in eNOS, DDAH, and VEGF gene do not seem to increase the risk of preeclampsia. We also found that no increase in the risk of preeclampsia for those genes was observed under any model of inheritance, and there were no statistically significant associations between any haplotypes and preeclampsia risk.

Family studies have shown that genetic factors play a role in preeclampsia, although the exact inheritance pattern is unknown. There are many candidate factors for the explanation of genetic factors in the risk of preeclampsia. Previous linkage studies in affected sibling pairs have implicated the eNOS gene locus on chromosome 7q35 to 36 (Guo et al., 1999). Recently, a common Glu298Asp polymorphism of eNOS has been associated with the development of preeclampsia, in which endothelium-dependent vasodilation and NO bioactivity are impaired (Savvidou et al., 2001). The Asp298 variant has been shown to be susceptible to enhanced proteolytic cleavage, and this might contribute to abnormally low NO generation and diminished NO bioactivity (Savvidou et al., 2001; Tesaro et al., 2000). In case of the -786 T/C mutation, Miyamoto et al. found that the -786 T/C mutation induced a modest (~40%), but significant decline in eNOS gene promoter activity in cultured human endothelial cells, and was related to reduced placental eNOS mRNA and serum nitrite/nitrate levels (Miyamoto et al., 2000). Therefore, we hypothesized that women carrying eNOS Asp298 mutation or -786 T/C promoter mutation would be a risk factor for preeclampsia.

However, the results from this study showed that there were no significant differences in genotype or allele frequencies of eNOS gene polymorphisms between preeclampsia patients and controls. These results for the eNOS polymorphism are consistent with previous findings in British (Yu et al., 2006), Finnish (Hakli et al., 2003), and Bangladeshi populations (Yoshimura et al., 2003). However, they are inconsistent with the American and Japanese populations (Kobashi et al., 2001). Recently, Yu et al. tried to include 12 genetic associations of the Glu298Asp polymorphism in preeclampsia involving 1334 cases and 2984 controls by meta-analysis (Yu et al., 2006). They found that the eNOS Glu298Asp polymorphism is not associated with a significant increased risk of preeclampsia (Yu et al., 2006). In their report, the frequency in control samples of women homozygous for the Asp298 allele was 3.2% overall, and this frequency was different among ethnic groups as follows: Caucasians (9.5%), Asians (1.41%), Hispanics (2.55%), and Afro-Caribbean (1.11%; Yu et al., 2006). This frequency for Asians for the Asp298 allele is similar to our findings.

Although there are some reports about an association with coronary disease and myocardial infarction and the -876 T/C mutation, (Miyamoto et al., 2000; Nakayama et al., 1999; Nakayama et al., 2000), this is the first report in the world concerning the association between the -786T/C mutation and preeclampsia. From our results, we did not find any association between the -786T/C mutation and preeclampsia. Miyamoto et al. demonstrated that the -786T/C mutation of the eNOS gene is positively associated with coronary spastic angina and myocardial infarction (Miyamoto et al., 2000). They also showed that the replication protein A1 functionally represses transcription of eNOS carrying the -786T/C mutation, that replication protein A1 is present not only in endothelial cells but also in placenta, and that the level of eNOS mRNA in placenta with the -786T/C mutation is significantly lower than in placentas without the mutation (Miyamoto et al., 2000). The frequency of C/C, C/T, and T/T genotypes in the -786T/C eNOS polymorphism was 2%, 20%, and 78%, respectively, in our results here, and this frequency is very similar to that found in the Japanese population (Nakayama et al., 2000).

The inhibition of DDAH activity should induce decreased NO synthesis, because of increased ADMA concentration, and these findings may be a pathophysiology of preeclampsia (Murray-Rust et al., 2001). Therefore, the DDAH gene can be supposed as a candidate gene for risk of preeclampsia. Previously, Akbar et al. (2005) reported on a haplotypic association of DDAH1 with susceptibility to preeclampsia in a Finnish sample. They found that the DDAH1 haplotype was associated with preeclampsia, although genotype distributions of the SNPs did not reveal statistically significant single-point association of the

DDAH gene with preeclampsia (Akbar et al., 2005). However, we could not find any association of DDAH genotypes or alleles with preeclampsia in our study. We also tried to perform haplotype analysis among these DDAH genes, and although a strong linkage association disequilibrium was observed between the DDAH IVS 5-71 A/T and DDAH 3'UTR + 16C/G variants, there was no significant difference between any haplotypes and preeclampsia risk.

The frequency of C and T alleles in DDAH IVS2-33 C/T polymorphisms was 90% and 10%, respectively, in our study, while the frequency was very different within the Finnish sample (79% for C allele, 21% for T allele; Akbar et al., 2005). Similarly, the frequency of A and T alleles in DDAH IVS5-71 A/T polymorphisms was 77% for the A allele and 23% for the T allele in our study, while the corresponding frequencies were 66%, and 34% within the Finnish study. The frequency of C and G alleles in the DDAH 3'UTR + 16C/G polymorphism was 70% and 23%, respectively, in our study, while the Finnish frequencies were 66% for C allele, and 34% for G allele. Because there was a strong linkage association disequilibrium between the DDAH IVS 5-71 A/T and DDAH 3'UTR + 16C/G variants, the allele frequencies for the DDAH IVS 5-71 A/T and DDAH 3'UTR + 16C/G variants were similar. This discrepancy of the genotype or allele frequencies for the DDAH gene polymorphism is most likely due to different genetic backgrounds in the two samples, and environmental factors.

VEGF has a central role in many processes that are involved in the development and progression of preeclampsia (Banyasz et al., 2006). Because VEGF is known to play a role in the regulation of cytotrophoblast invasion and placentation, and there is evidence of abnormal placentation in preeclamptic placenta, we can suppose that the genes related to VEGF activity would be a risk factor for preeclampsia (Zhou et al., 2002). Our results suggest that there is no significant difference for the 936 C/T polymorphism between preeclampsia patients and controls. This result is discordant with previous findings in the Australian and Chinese populations where women with the T allele of the 936 C/T polymorphism of VEGF had an increased risk for developing severe preeclampsia (Guo et al., 1999). These researchers investigated -2578A/C, -634G/C, and 936C/T polymorphisms, and found there was no significant association between these SNPs and preeclampsia (Guo et al., 1999). The frequencies of the T allele of the 936 C/T polymorphism of VEGF in our results were similar to a previous Greek study (Guo et al., 1999). Banyasz et al. suggested that the presence of the VEGF 405G allele in nulliparous pregnant women is associated with a decreased risk of severe preeclampsia (Banyasz et al., 2006). Recently, there was a report that VEGF 405 G/C polymorphisms determined the production of VEGF, and highest VEGF production was associated with the VEGF 405

GG genotype (Watson et al., 2000). The discrepancy between our findings and the data of other groups for eNOS, DDAH, and VEGF gene polymorphisms may be explained by ethnic variation and gene-environmental interaction.

This study has limited power — in order for the differences revealed to have statistical significance with over 80% power, more than 6000 samples would be needed, a number which could not be collected here.

In summary, this study suggests that polymorphisms in eNOS, DDAH, and VEGF genes do not seem to be risk factors for preeclampsia. However, this finding will need to be confirmed in a large cohort of preeclampsia patients.

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