African Journal of Biotechnology Vol. 10(52), pp. 10712-10717, 12 September, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.895
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Endothelial nitric oxide synthase gene Glu298Asp polymorphism and risk of preeclampsia in South East of Iran

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Accepted 22 July, 2011

Preeclampsia (PE) is the most serious complication of pregnancy that causes maternal and fetal morbidity and mortality. Although the exact pathophysiology of PE is unknown, a large number of studies have shown that abnormalities in nitric oxide (NO) synthesis may contribute to the development of this disorder. There are some evidences that polymorphisms of the endothelial nitric oxide synthase (eNOS) gene affect NO production and have been associated with hypertension and PE in some populations. Therefore the aim of this study was to assess the relation of the Glu298Asp eNOS polymorphism and PE in an Iranian population. We compared the frequency of the Glu298Asp polymorphism in 147 women with PE and 137 healthy pregnant control subjects by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) method. The frequencies of Glu298Asp genotypes were significantly different between PE women and controls ($p < 0.001$). The frequency of Asp allele was 0.32 in PE patients and 0.20 in controls and was significantly different ($p < 0.001$). The risk of PE was 2.4 fold in pregnant women with Asp allele. In conclusion, the Asp allele could be a risk factor for PE in South East of Iran.

Key words: Nitric oxide synthase, polymorphism, preeclampsia, pregnancy.

INTRODUCTION

Preeclampsia (PE) is one of the most severe problems of pregnancy and has a familial predisposition. Although the exact pathophysiology of preeclampsia is not clear, several maternal genetic variations, in conjunction with environmental factors, may predispose to the development of the disease (Salonen et al., 2000). Genetic factors and mutilation of nitric oxide (NO)–mediated vasodilation appear to have important roles in progress of PE (Salonen et al., 2000; Broughton et al., 2001). The role of endothelial nitric oxide synthase (eNOS) gene as a candidate gene for the development of PE has been investigated by many studies (Arngrimsson et al., 1997; Lade et al., 1999; Yoshimura et al., 2000; Tempfer et al., 2001). Furthermore, some evidences demonstrated familial pregnancy-induced hypertension associated with a locus in the region of chromosome 7q36, which also encodes the eNOS gene (Lade et al., 1999; Lewis et al., 1999). The eNOS is expressed in the endothelium, encoded by a 26 exon gene with a total size of 21 kb and encodes a mRNA of 4052 nucleotides (Marsden et al., 1993). The eNOS gene has a common polymorphism at position 298 (Glu298Asp) which has been associated with both altered NO production (Wang et al., 1997) and with vascular disorders including hypertension (Benjafield and Morris, 1999), myocardial infarction (Hibi et al.,

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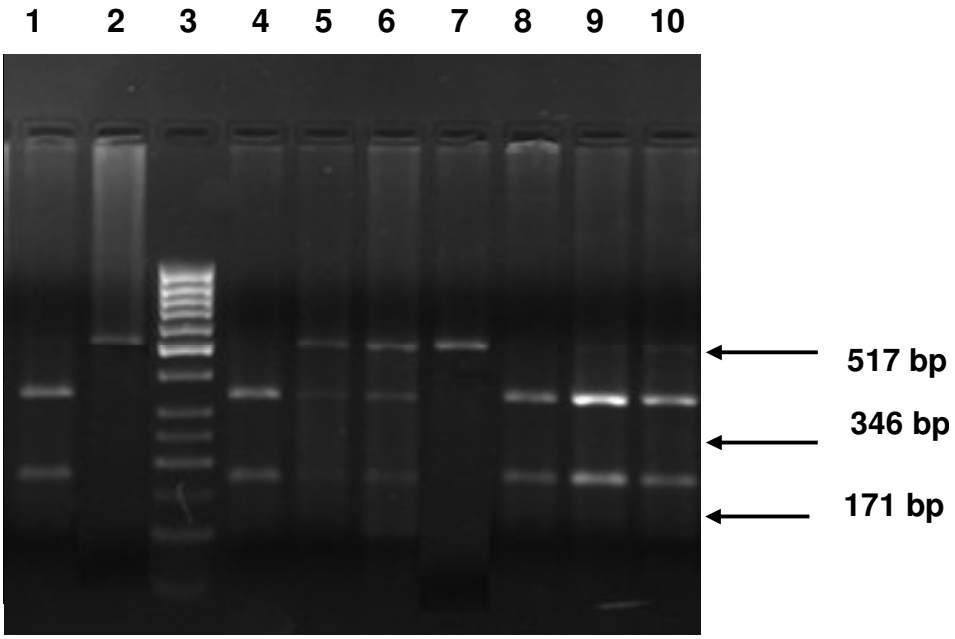

Figure 1. The Glu298Asp polymorphism of eNOS gene was shown by electrophoresis on 2% agarose gel. Lane 3: 50-bp DNA ladder; lane 2 and 7: Glu/Glu genotype; lane 5 and 6: Glu/Asp genotype; lane 1, 4, 8 to 10: Asp/Asp genotype.

1998), coronary artery spasm (Yoshimura et al., 2000), stroke (Elbaz et al., 2000), and renal disease (Noiri et al., 2002). Yoshimura et al. (2000) reported the association of the Asp allele with severe PE in Japanese but they did not observe any association between this polymorphism and PE in Bangladesh (Yoshimura et al., 2003). There are conflicting results about the correlation between Glu298 Asp polymorphism and PE in different ethnic groups. In Eastern Finland and USA, it was revealed that there was no evidence for the association between this polymorphism and PE (Hakli et al., 2003; Landau et al., 2004) while Tempfer et al. (2004) showed a significant differences between them in USA.

Since there is not any report in Iranian population, the aim of this study was to investigate the association between Glu298 Asp polymorphism of the eNOS gene and PE in South East of Iran.

MATERIALS AND METHODS

Subjects

With Institutional Review Board approval and written informed consent from all subjects, we obtained blood samples for genotyping from women delivering at Ali-ebn-abitaleb hospital in Zahedan. Women were considered to have PE if they met the 1996/2000 American College of Obstetricians and Gynecologists criteria for the definition of PE: Systolic blood pressure > 140 mm Hg or diastolic blood pressure > 90 mm Hg, occurring on at least two occasions 6 h apart, with proteinuria > 0.3 g/L in a 24 h specimen, or a proteinuria dipstick reading of 2+ on a random urine collection, with no pre-pregnancy history of essential hypertension or hypertension before 20 weeks gestation. Blood pressures were determined using the automated blood pressure module of a Hewlett-Packard M1176A model 66 (Hewlett-Packard, Andover, MA) with the patient in the supine position. A total of 147 women with PE were genotyped and compared with 137 healthy normotensive women delivering at term.

DNA genotyping

Blood samples were obtained and genomic DNA was isolated from peripheral blood by DNA extraction Kit (Roche, Germany). The eNOS polymorphism of interest, Glu298Asp, was detected using a polymerase chain reaction (PCR)–based restriction enzyme analysis in Zahedan Cellular and Molecular Research Center.

In brief, this exonic fragment was amplified by PCR with a forward primer 5´- GAC CCT GGA GAT GAA GGC AGG AGA-3´ and reverse primer 5´- ACC TCC AGG ATG TTG TAG CGG TGA-3´ (Hibi et al., 1998). The reaction performed according to the protocol as previously described, except for the annealing step which was at 60ºC (Salimi et al., 2010). The 517 bp PCR product was digested overnight at 37°C with 10 units of the *BanII* restriction enzyme (Fermentas, Lithuania). The digested PCR products were run on 2% agarose gel and visualized by ethidium bromide staining. The G wild type allele was not digested and produced a 517 bp fragment, whereas the variant T allele was digested into 346 and 171 bp fragments (Figure 1).

Statistical analysis

All statistical analyses were performed with SPSS V-11.5. The differences between groups were examined by χ^2 tests or an independent student t-test for quantitative parameters. Allele frequencies were calculated by the gene counting method. The frequencies of the alleles and genotypes were analyzed between

Table 1. Demographic characteristics of preeclamptic patients and controls.

Demographic characteristic	Preeclampsia, N = 147	Controls, N = 137	p Value
Age (year)	28.1±7.7	26.3±6.1	NS
Gestational age (weeks)	36.6 ± 3.6	38.2 ± 2.8	0.002
Birth weight (g)	2789 ± 829	2993 ± 666	NS
Diastolic blood pressure (mm Hg)	96 ± 8.7	69.7 ± 9.1	0.0001
Systolic blood pressure (mm Hg)	152.2 ± 14.5	111.9 ± 11.9	0.0001
Primiparity (%)	0.42	0.29	0.015
Family history of preeclampsia (%)	0.31	0.37	NS
Race			
Persian (%)	25	39	
Baloch (%)	44	43	0.011
Afghan (%)	31	18	

NS, Not significant; gestational age at onset of preeclampsia.

Table 2. Genotype and allele frequencies of Glu298Asp polymorphism of the eNOS gene in preeclamptic patients and controls.

4b/a polymorphism	Pre-eclampsia, N = 147	Controls, N = 137	χ^2	P Value	OR (95%)
Glu/Glu, n (%)	61 (41.5)	86(63)			
Glu/Asp, n (%)	78(53)	48(35)	13.3	0.001	
Asp/Asp, n (%)	8(5.5)	3(2)			
Glu/Asp + Asp/Asp (%)	86(58.5)	51(37)	12.8	0.0001	2.4(1.5 - 3.8)
Glu (%)	68	80	11.1	0.001	
Asp (%)	32	20			

patients and control groups by the χ^2 test. The odds ratio (OR) and 95% confidence intervals (CI) were also estimated. The χ^2 test was used for deviation of genotype distribution from Hardy-Weinberg equilibrium. Logistic regression analysis was employed to determine the relations of gene polymorphisms and other risk factors with PE. A p value of < 0.05 was considered as statistically significant.

RESULTS

The clinical and biochemical parameters of the controls and PE women are shown in Table 1.

Maternal age and family history of PE did not differ significantly between two groups. As expected, systolic and diastolic blood pressure and primiparity were significantly higher and gestational age was significantly lower in PE women. Although birth weight was lower in PE women, the difference was not significant.

The frequencies of ethnic groups (Persian, Balooch and Afghan) were significantly different between PE women and controls (P = 0.03) and the risk of PE was two fold in Afghan women in contrast to other groups (OR, 2 [95% CI, 1.1 to 3.8]; P = 0.01).

Allele frequencies of Glu298Asp polymorphism were in Hardy Weinberg equilibrium. The distribution of genotype and allele frequencies of Glu298Asp polymorphism of eNOS gene was compared between PE patients and controls (Table 2).

The frequencies of Glu/Glu, Glu/Asp and Asp/Asp genotypes were 46, 47 and 10% in PE patients and 63, 35 and 2 in healthy pregnant women, respectively and were significantly different ($p < 0.001$). The frequency of Asp allele was 0.32 in PE patients and 0.20 in controls and was significantly different ($p < 0.001$). The risk of PE was 2.4 fold in pregnant women with Asp allele (Glu/Asp + Asp/Asp) in contrast to control women without Asp allele (OR, 2.4 [95% CI, 1.5 to 3.8]; P = 0.0001).

There was no correlation between Glu298Asp polymerphism of eNOS gene and the onset and severity of PE. Moreover, there was no variation in Glu298Asp polymorphism in different races. Multiple regression analysis revealed that Afghan race, gravity and presence of Asp allele were independent risk factors of PE (Table 3).

DISCUSSION

PE is the most serious complication of pregnancy influencing both fetal and maternal morbidity and

Table 3. Multiple logistic regression analysis with forward stepwise selection (Wald).

Risk factor	B	S. E.	Wald	df	Sig.	Exp (B)	95% CI	
							Lower	Upper
Afghan race	0.8	0.3	7.1	1	0.008	1.5	2.5	4
Primigravida	0.72	0. 27	7.4	1	0.007	2.1	1.2	3.5
Glu/Asp + Asp/Asp genotype	0.95	0.25	13.9	1	0.0001	2.6	1.6	4.2
Constant	0.89	0.3	8.6	1	0.003	2.4		

mortality. The exact pathophysiology of PE remains unknown, however genetic and environmental factors are reported to be important (Broughton, 2001). There is several maternal genetic variations in conjunction with environmental factors that may predispose to the development of the disease (Salonen et al., 2000; Broughton, 2001). In the first half of the pregnancy, systemic arteriolar vasodilation increase blood volume and cardiac output and decrease blood pressure. These changes are probably dependent on endothelial NO production and deficiency in NO production could be a risk factor for PE (Buhimschi et al., 1998; Landau et al., 2004). NO is synthesized by eNOS from L-Arginine and molecular oxygen in the endothelium. Therefore eNOS gene that encodes endothelial NO synthase is a candidate gene for PE (Lade et al., 1998; Buhimschi et al.; 1998; Kobashi et al., 2001). Arngrfmsson et al. (1997) reported the localization of a familial pregnancy-induced hypertension-susceptibility locus in the region of chromosome 7q36 encoding the eNOS gene.

In this study, we assessed the relation between the common G894T polymorphism of eNOS gene, which encodes an amino acid substitution (Glu298Asp) between preeclamptic patients and controls. The mechanisms by which eNOS Asp298 polymorphism might reduce NO bioavailability have been studied. The most probable mechanism have shown that eNOS Asp298 is exposed to selective proteolytic cleavage in endothelial cells and vascular tissues, and this could explain reduced vascular NO generation in homozygote subjects for this variant (Tesauro et al., 2000). Furthermore, some evidences suggest that preeclampsia is associated with decreased levels of NO and increased levels of circulating inflammatory cytokines due to single nucleotide polymorphisms (Sharma et al., 2011).

In the present study, we found that the Glu298Asp polymorphism of eNOS gene genotype ($p < 0.001$) and allele ($p < 0.001$) frequencies were significantly different between the two groups and Asp allele being more frequent in PE women. In women with Asp allele (Glu/Aspl + Asp/Asp), the risk of PE was 2.4 fold (OR, 2.4 [95% CI, 1.5 to 3.8]; P = 0.0001).

There are several studies about the association of Glu298Asp polymorphism of eNOS gene and PE in different countries. However, most of the studies failed to document an association between rare allele (298Asp) frequency and PE; in other studies, the investigators found an association between Glu298Asp polymorphism and PE.

Yoshimura et al. (2000) reported this polymorphism as a risk factor for developing severe PE in Japanese population. Their study revealed that the frequency of the Glu298Asp variant was 28.8% in severe PE group and 14.1% in control group. Moreover, in another study, they demonstrated an association between placental abruption and Asp298 allele in Japanese women (Yoshimura et al., 2001). Later, they replicated their investigation in a developing country (Bangladesh) and they did not find any association between PE and rare allele frequency (Yoshimura et al., 2003). In Eastern Finland, it was demonstrated that there was no evidence for the association between this polymorphism and PE (Hakli et al., 2003).

Moreover in other studies in USA (Landau et al., 2004), Korea (Kim et al., 2006), United Kingdom (Yu et al., 2006), India (Singh et al., 2009) and Germany (Hocher et al., 2008) did not find any relation between PE and this polymorphism.

In a meta analysis study, Medica et al. (2007) carried out a systematic research on published case-control studies on Glu298Asp polymorphism of eNOS gene (9 analyses involving 1055 patients and 1788 controls) and reported that single polymorphism did not have a major effect on PE.

Chen et al. (2007) in Taiwan concluded that the polymorphisms in the eNOS gene may be protective against PE in a Chinese population, in contrast to the results in the Japanese population.

In another study, a significant difference in the allele frequency and genotype distribution of this polymorphism and PE have been shown in USA (Tempfer et al., 2001). Furthermore, other investigators evaluated the relation of three common polymorphisms of eNOS gene (4a/b, Glu298Asp and T-786C) and assessed synergic effect of these SNPs. Serrano et al. (2004) in Colombia concluded that the eNOS Glu298Asp polymorphism and the Asp298-786C-4b haplotype are risk factors for PE; in consistent with our results, they found in women homozygous for the Asp298 allele, the adjusted OR for PE was 4.60 (95% confidence interval [CI], 1.73 to 12.22) compared with carriers of the Glu298 allele. Sandrim et al. (2008) in Brazil showed that the haplotype 'C Glu a' was more common in women with gestational hypertension and PE than in healthy controls. Recent study in

India showed that there was no relation between individual eNOS gene polymorphisms and hypertensive disorders of pregnancy in North Indian women. But the presence of rare alleles at all the three sites in eNOS seemed to increase the risk of PE (Aggrawal et al., 2010). What we have seen in South East of Iran was in agreement with studies in Colombia, USA and Japan and was in contrast to with studies in Finland, Korea, United Kingdom and India.

This discrepancy is common in association studies and is due to the unlike genetic and environmental back-grounds, inclusion and exclusion criteria for PE women and controls and sample size volume; however, with attention to meta analysis study, it is more probable that the small study volume is the most important reason of this discrepancy.

In conclusion, this study demonstrated that maternal eNOS Glu298Asp polymorphism is associated with PE and the frequency of Asp allele was significantly higher in PE patients than healthy controls. The risk of PE was 2.4 fold in pregnant women with Asp allele contrast to control women without Asp allele in the South East of Iran.

ACKNOWLEDGMENTS

This research was supported by a grant from the Zahedan University of Medical Sciences.

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