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Research Paper

Endothelial Nitric Oxide Synthase "eNOS" Gene Polymorphisms in Preeclampsia in South Indian Population.

Human Genetics

KEYWORDS :

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ABSTRACT

Introduction: Pregnancy is a hypercoagulable state with increased tendency for thrombus formation, a condition that is increased when combined with acquired or inherited risk factors that lead to thrombophilia. The association between endothelial nitric oxide synthase (eNOS) polymorphism, their haplotypes, serum nitric oxide (NO) levels and RPL, were studied in different ethnic populations. The results, however, were contradictory. Objective: This study was conducted in order to determine the association between promoter -786 T>C, exon 7 Glu298Asp (894 G>T) polymorphisms of eNOS gene in idiopathic Preeclampsia in women of Coastal Andhra Pradesh, Visakhapatnam, India. Materials and Methods: Our study included 45 (30 non-pregnant and 15 pregnant) women who suffered from unexplained preeclampsia, and 45 (30 non-pregnant and 15 pregnant) healthy women matched for age and without previous history of preeclampsia. DNA was extracted from whole blood samples. The PCR products of exon 7 Glu298Asp (894 G>T) and promoter -786T>C polymorphisms by PCR-RFLP, where they were digested using specific restriction enzymes and then separated electrophoretically using 2% agarose gel. Results: The C allele carrier which represented by (CC + CT) genotypes and the C allele of the promoter -786T>C polymorphism are significantly associated with increased risk of preeclampsia, where they presented with a higher frequency in this women. Neither exon 7 Glu298Asp(894G>T) was significantly associated with preeclampsia risk in the study population. Conclusion: The (CC + CT) genotypes (C allele carrier)and the C allele of the promoter -786T>C polymorphism are possible risk factors for preeclampsia. The study showed that the (C allele carrier) which represented by (CC + CT) genotypes is associated with preeclampsia.

Introduction

Preeclampsia is one of the leading cause of maternal and perinatal mortality and morbidity in developing and developed countries. [1, 2] It is thought to be multifactorial in origin with environmental, social and genetic factors acting in conjunction to cause disease. In normal pregnancy successful implantation depends on the receptivity of maternal endometrium which is influenced by the synergistic actions of progesterone and nitric oxide (NO) [1].

NO is a short-lived free-radical gas synthesized by a family of nitric oxide synthase (NOS) [2], with an extremely short half life of approximately 4 seconds [3]. The level of NO has been shown to be influenced by various polymorphisms in the eNOS gene [4,5]. The initial demonstration of the role of NO in penile erection led to investigation of its role in various reproductive processes. NO has been identified throughout the reproductive tract and is involved in ovarian folliculogenesis, ovulation, gonadotropin releasing hormone secretion, sperm motility, fertilization and embryo development. The identification of NO in the uterus and cyclic change in the endometrial levels of NOS suggest a role for this molecule in the events of implantation [6].

Nitric Oxide (NO) is well-known to mediate vascular smooth muscle relaxation and contributes to maternal systemic vasodilation during pregnancy, regulates uterine and fetoplacental blood flow, and is involved in uterine quiescence before parturition[7]. Lack of endothelial-derived NO is associated with vasospasm, and vascular infarction[8]. Lack of NO has also been associated with the development of endothelial damage, hypertension, coronary spasm, myocardial infarction, coronary artery disease and ischemic stroke[2].

Exogenous NO promotes uterine relaxation and has prompted interest in the use of NO donors as tocolytic agents. Thus, endogenous production of NO may be involved in the regulation of myometrial tone in pregnancy, and a decline in NO production at term could play an important role in the initiation of, or preparation for, parturition[9].

Endothelial nitric oxide synthase (eNOS) is expressed in terminal villous vessels and in the syncytiotrophoblast of pregnant women. In mice, lipopolysaccharide (LPS)-induced abortion is mediated by placental NO production, and pharmacological inhibition of NO release by aminoguanidine successfully rescued LPS-induced abortion[2,6,7] .Alteration in NO metabolism may be a contributing factor in the pathogenesis of hypertension. Thus, abnormalities in the activity of the eNOS enzyme that synthesizes NO in endothelial cells may lead to NO deficiency with severe consequences[10].

The etiology of preeclampsia is often multi-factorial[7], regulated by multiple genetic pathways[10,11,12], and different genes encoding for proteins involved in various biological pathways have been reported to be associated with preeclampsia [9,10,13].The most intensively studied eNOS gene polymorphisms are -786T>C in the promoter region of the gene[2], the Glu298Asp missense mutation in exon 7 of the gene[13].

There is paucity of data in the correlation of *eNOS* with vascular related pregnancy complications. There are few studies available in International literature about the association of polymorphism of the *eNOS* gene on the risk of placental vasculopathies particularly preeclampsia . There is scarce existing data from Indian population in this context. The main objective of this study was to determine the association between promoter -786 T>C, exon 7 Glu298Asp (894 G>T) and preeclampsia in East Coastal Andhra Pradesh, India.

Materials & Methods:
Our study was preliminary with Sample size of 45 patients diagnosed with preeclampsia and eclampsia. An equal number of controls with low risk singleton pregnancy and who were neither siblings nor relatives with the index cases were recruited. Patients with singleton pregnancy and patients diagnosed with preeclampsia or eclampsia were included in the study. **Preeclampsia** - Presence of Blood pressure higher than 140/90 mm Hg on at least two occasions more than 6 hours apart, and a proteinuria higher than 300 mg/24 h, after the 20th week of pregnancy. **Eclampsia** - Onset of convulsions in a woman with preeclampsia that cannot be attributed to other causes is termed as eclampsia. The study was approved by the institutional ethical Committee and the work has been performed at Department of Human Genetics, Andhra University.

For *exon 7 Glu298Asp (894G>T),* the wild-type allele *(894G allele)* remained uncut upon *BanII* digestion and was detected as a 317-bp band, whereas the polymorphic allele *(894T allele)* was cut into two fragments detected as a 223- and a 139-bp bands. Therefore, wild-type homozygous individuals should generate a single 317-bp product, heterozygous individuals should generate three fragments 317-, a 223- and a 139-bp bands, while mutant homozygous individuals should generate a two; 223- and a 139-bp fragments.

For *promoter -786 T>C polymorphism,* The wild-type allele *(-786T allele)* is uncut upon *Msp I* digestion and was detected as a 178-bp band, whereas the polymorphic allele *(-786C allele)* was cut into two fragments detected as a 137- and a 41-bp bands. Therefore, wild-type homozygous individuals should generate a single 178-bp product, heterozygous individuals should generate three fragments 178-, 137-and a 41-bp, while mutant homozygous individuals should generate a two; 137- and a 41-bp fragments.

Statistical analysis
Genetic power calculation has been determined to estimate the representative sample size for each of the two polymorphisms included in the current study, they summarized as shown in table

Statistical analysis were carried out using Chi *(X2)* square test and independent samples t-test test of the *Statistical Package for Social Sciences (SPSS) version 13* for Windows. Chi *(X2)* square test was used to assess the frequencies of genotypes and alleles. While independent samples t-test was used to compare the difference in the mean levels of NO. Odds Ratio (OR) and odds ratio (9 % CI) were analyzed by Fisher's exact test using the *Stats Direct software Version 2.7.2* to measure the strength of association between *eNOS* genotypes, NO and preeclampsia. The Hardy-Weinberg equilibrium (HWE) was used to calculate estimated genotype frequency and experienced genotype frequency. *P-value* less than 0.05 was considered statistically significant.

Results
In our study results showed that the genotype and the allele frequencies of *promoter -786 T>C* were significantly different between preeclampsia patients and the controls (all P-values were < 0.001). On the other hand, neither genotype nor allele frequencies of *exon 7 Glu298Asp (894 G>T* were significantly different between preeclampsia patients and the controls (P-values for genotype and allele frequency of *exon 7 Glu298Asp* were 0.398 and 0.402, respectively). It can be inferred that the mutant *C* allele of the *promoter -786 T>C* variant of the *eNOS* gene is related to an increased risk for preeclampsia. The mutant *T* allele of the *exon 7 Glu298Asp* missense variant, however, does not seem to contribute to an increased risk for preeclampsia.

Frequency of the *promoter -786T>C* polymorphism among Preeclampsia patients and Control subjects

The frequency of the polymorphic *C* allele carrier which represented by *(CC + CT)* genotypes was 46.7% in Preeclampsia patients and 24.5% in controls, while the frequency of wild-type *TT* genotype was 53.3% in Preeclampsia patients and 75.5% in controls. The statistical analysis of frequency of the *promoter -786T>C* polymorphism among the Preeclampsia patients and controls by Chi *(X2)* square test showed that a statistical significance was evident between the two groups (P-value < 0.02). Fisher's exact test was used to assess the odds ratio (95% CI) and indicated a significant difference between the frequency of *(CC + CT)* genotypes and the frequency of the wild-type *TT* genotype (P-value <0.02), Odds Ratio (95% CI) for *(CC + CT)* genotypes= 0.63 (0.42- 0.94). (Table: 1)

Allele frequencies of the *eNOS* gene *promoter -786T>C* polymorphism among preeclampsia patients and control subjects
The frequency of the polymorphic *C* allele was 26.7% in Preeclampsia patients and 6.7% in controls, while the frequency of the wild-type *T* allele was 73.3% in Preeclampsia patients and 93.3% in controls. The statistical analysis of allele frequencies of the *promoter -786T>C* polymorphism among the Preeclampsia patients and controls by Chi *(X2)* square test showed that a statistical significance was evident between the two groups (P-value < 0.001). Fisher's exact test indicated a significant difference between the frequency of polymorphic *C* allele and the frequency of the wild-type *T* allele (P-value < 0.001), Odds Ratio (95% CI) for *C* allele= 0.55 (0.43- 0.71). (Table:2)

Frequency of the *exon 7 (894 G>T)* polymorphism among Preeclampsia patients and control subjects
The frequency of polymorphic T allele carrier which represented by *(TT + GT)* genotypes was 42.2% in Preeclampsia patients and 51.1% in controls, while the frequency of wild-type *GG* genotype was 57.8% in Preeclampsia patients and 48.9% in controls. The statistical analysis of frequency of the *exon 7 (894 G>T)* polymorphism among the Preeclampsia patients and controls by Chi *(X2)*square test showed that there is no statistically significant difference between the two groups (P-value= 0.398). Fisher's exact test was used to assess the odds ratio (9 % CI) and indicated that there is no statistically significant difference between the frequency of *(TT + GT)* genotypes and the frequency of the of wild-type *GG* genotype (P-value= 0.), Odds Ratio (95% CI) for *(TT + GT)* genotypes= 1.20 (0.78 - 1.83). (Table 3)

Allele frequencies of the *eNOS* gene *exon 7 (894 G>T)* polymorphism among Preeclampsia patients and control subjects
The frequency of the polymorphic *T* allele was 24.4% in Preeclampsia patients and 30.0% in controls. While the frequency of the wild-type *G* allele was 75.6% in Preeclampsia patients and 70.0% in controls. (Table 4)

The statistical analysis of allele frequencies of the *exon 7 (894 G>T)* polymorphism among the Preeclampsia patients and controls by Chi *(X2)* square test showed that there is no statistically significant difference between the two groups (P-value= 0.402). Fisher's exact test indicated that there is no statistically significant difference between the frequency of polymorphic *T* allele and the frequency of the wild-type *G* allele (P-value= 0.402), Odds Ratio (95% CI) for *T* allele= 1.16 (0.81- 1.64).

Discussion
Preeclampsia is an important clinical and stressful problem that has been studied tremendously but the causes and treatment have not been fully resolved [12]. Preeclampsia affects about 1-5% of women who conceive [14] and accounts for about 20% of clinically recognized pregnancy losses[1]. Despite extensive researches to explain the causative effects of Preeclampsia , about 50%-60% of Preeclampsia are still idiopathic. Endothelial

damage, impaired placental vascularization and resultant oxidative stress have been proposed to play a role in the pathophysiology of Preeclampsia .

In case of normal pregnancy, the NO pathway is activated and leads to increased NO availability and level which is further responsible for maternal vasodilation required to accommodate the increase in circulating volume during pregnancy without a rise in blood pressure[15,16,17]. *eNOS* has been regarded as the source of endothelial NO, which has a critical role in vascular physiology and impaired placental vascularization [18].

In recent years much attention was paid to determine the association between *eNOS* gene *[promoter -786 T>C and exon 7 Glu298Asp (894 G>T)* polymorphisms and preeclampsia . However, the results of these studies have been controversial among different ethnic groups [2]. Lack of association between preeclampsia and *exon 7 Glu298Asp* polymorphism observed in this study was in agreement with the findings recorded for women from Palestain[1], Austrian[11], Greek[12], Chinese[19] and Tunisian[20] populations. In the contrary, results for women from Korea[2] and North India[21] indicated that *exon 7 Glu298Asp* polymorphisms is significantly associated with preeclampsia .

Few studies have investigated the relation between *eNOS promoter -786T>C* polymorphism and the development of preeclampsia and other reproductive complications in women from various populations[2]. Our results for the *promoter -786 T>C* polymorphism are in agreement with those published by *Shim, et al.(2010)* who showed that *the promoter -786 T>C* polymorphism is associated with the risk of spontaneously aborted fetuses[22]. This result is also consistent with those recorded for Indian women origin where a significant association between the *promoter -786 T>C* and preeclamptic pregnancy complication was observed.

In contrast, our results do not support the previously published results for women from Korean[2] and Tunisian[20] populations which indicated that *promoter -786 T>C* polymorphisms is not significantly associated with Preeclampsia .

Results of the present study showed that the *-786C*-allele of the *promoter -786T>C* polymorphism is associated with lowering NO level. Reporter gene studies have shown that *promoter -786T>C* substitution markedly blunts the transcription rate of the *eNOS* gene, and hence NO production, likely because the *C* allele creates a binding site for a replication protein A1 (RPA1) that acts as a suppressor of *eNOS* transcription. Furthermore, it has been shown that RPA1 protein is present not only in endothelial cells but also in placenta, which is rich in vasculature, and that the level of *eNOS* mRNA in placentas with *promoter -786T>C* substitution mutation is significantly lower than in placentas without the mutation [23,24]. These findings confirm our results that Preeclampsia women are associated with a high frequency of *promoter-786T>C* polymorphism *C* allele and might explain why this polymorphism is associated with a low serum NO levels.

Our study group was small and has a high frequency of consanguineous marriage. Thus, we expect that our group gene pool is homogenous; we also expect that the alleles frequency are low. Therefore, the small sample size recruited in the current study reflects the genotype and allele frequency.

The conflicting outcomes of preeclampsia genetic association studies may be attributed to differences in genetic background and gene environment interactions among various populations. Therefore, the present results cannot be considered contradictory to some of the previous studies as there is considerable ethnic variability in each of the studied polymorphic loci. The present data add to the importance of ethnic as well as intra-regional variability in such studies concerning multifactorial disorders including preeclampsia . Our findings regarding the two investigated *eNOS* polymorphisms and their associations with preeclampsia clearly showed that the *promoter -786T>C* polymorphism of the *eNOS* gene, namely *"allele -786C"* is associated with preeclampsia in Coastal Andhra Pradesh women residing in South India.

Our study showed that the *C* allele carriers which represented by *(CC + CT)* genotypes and the *C* allele of the promoter *-786T>C* polymorphism are a possible risk factor for Preeclampsia . Where they presented with a high frequency in Preeclampsia women and were associated with decreased serum NO levels in this group. The present study confirmed that *exon7 Glu298Asp (894G>T)* polymorphism is not associated with the risk of preeclampsia in Indian women.

We recommend for testing the promoter *-786T>C* polymorphism of *eNOS* gene in all Indian women experiencing preeclampsia or unexplained hypertension during pregnancy. Since NO pathway plays an important role in the pathophysiology of preeclampsia , thus, any factors balancing NO metabolism could be useful in the treatment of preeclampsia , consequently, reducing the substantial morbidity and associated maternal and fetal mortality.

TABLE 1.GENETIC POWER CALCULATION OF ENOS GENE POLYMYMORPHISM.

Polymorphism	**Promoter -786 T>C**	**Exon 7 (894 G>T)**
Frequency of risk allele	0.44	0.20
Odds ratio (OD)	1.36	1.39
N cases for 80% power	398	588

Table:2
Frequency of the *eNOS* gene *promoter -786T>C* polymorphism among Preeclampsia patients and control subjects.

Polymorphism	**Frequency**		**X2 Test**	**Fisher's exact test**
	Preeclampsia	**Control**	**p-value**	**Odds Ratio (95% CI)**
TT	24|(53.3%)	34 (75.5%)	0.02	0.37(0.14-0.99)
CC + CT	24|(46.7%)	11 (24.5%)		0.63(0.42-0.94)
Total	45	45		

Table: 3 Allele frequencies of the *eNOS* gene *promoter -786T>C* polymorphism among Preeclampsia patients and control subjects.

Polymorphism	**Frequency**		**X2 Test**	**Fisher's exact test**
	Preeclampsia	**Control**	**p-value**	**Odds Ratio (95% CI)**
T allele	66|(73.3%)	84 (93.3%)	<0.0003	0.20 (0.07-0.54)
C allele	24|(26.7%)	6 (6.7%)		0.55 (0.43-0.71)
Total	90	90		

Table:4
Allele frequencies of the *eNOS* gene *promoter -786T>C* polymorphism among Preeclampsia patients and control subjects.

Polymorphism	Frequency		X2 Test	Fisher's exact test
	Preeclampsia	Control	p-value	Odds Ratio (95% CI)
GG	26|(57.8%)	22 (48.9%)	0.398	1.00|(wild-type)
TT + GT	19|(42.2%)	23 (51.1%)		1.20(0.78-1.83)
Total	45	45		

Table:5
Allele frequencies of the *eNOS* gene *exon 7 (894 G>T)* polymorphism among Preeclampsia patient and control subjects.

Polymorphism	Frequency		X2 Test	Fisher's exact test
	Preeclampsia	Control	p-value	Odds Ratio (95% CI)
G allele	68 (75.6%)	63 (70.0%)	0.402	1.00| (wild-type)
T allele	22 (24.4%)	27 (30.0%)		1.16 (0.81 – 1.64)
Total	90	90		

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