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Lack of Association of the Asp298 Variant of the Endothelial Nitric Oxide Synthase Gene with Pre-Eclampsia in a Malay Population

Julia FV Ho[1#]
Sherry SY Ho[2#]
Wei-Rong Chua[2]
Chew Kiat Heng[1]
Annamalai Loganath[2]
Caroline G Lee[3]
Siew-Yee Ng[2]
Arijit Biswas[2]
Mahesh A Choolani[2]
Samuel S Chong[1*]
Yap-Seng Chong[2]

ABSTRACT

Pre-eclampsia is one of the most common serious complications of pregnancy. Yet its etiology and pathogenesis remain unknown. Polymorphisms in the eNOS gene, in particular the Glu298Asp variant, have been reported to be associated with hypertension and preeclampsia. Using PCR and sequencing strategies, we compared the incidence of the wild type G allele and the variant T allele at codon 298 of the eNOS gene in 126 normal and 66 preeclamptic Malay women and their babies. Allele frequencies in both the control and preeclamptic groups were in Hardy-Weinberg equilibrium, with no significant difference in the frequency of the variant T allele between the control and pre-eclamptic groups. We conclude that there is no association between the Asp298 variant of the eNOS gene and pre-eclampsia in the Malays.

Departments of [1]Pediatrics,
[2]0bstetrics & Gynecology,
and [3]Biochemistry,
National University of Singapore

[#]Both authors contributed equally to this work, and are listed in alphabetical order.

Correspondence:
Samuel S Chong, PhD, FACMG
Department of Pediatrics
National University of Singapore
5 Lower Kent Ridge Road
Singapore 119074

INTRODUCTION

Pre-eclampsia (PE) is a leading cause of maternal and fetal mortality and morbidity. This condition affects pregnant women after 20 weeks of gestation and resolves soon after delivery of the placenta. Pre-eclamptic mothers suffer from pregnancy-induced hypertension and proteinuria, which may develop into potentially fatal eclampsia. Although PE has been widely studied, its etiology and pathogenesis remain uncertain, occurring in about 5% of healthy nulliparous women and 18% of previously pre-eclamptic women (Hnat, Sibai et al. 2002).

Pre-eclampsia can be said to be due to a combination of maternal and fetal factors. It has been generally accepted that PE is a consequence of placental ischemia that leads to a maternal systemic reaction in the form of generalized endothelial cell dysfunction, since it is promptly resolved after the delivery of the placenta. Moreover, studies have shown that the risk for developing PE is higher in asthmatics and people residing in higher altitudes (Moore, Hershey et al. 1982; Lehrer, Stone et al. 1993), suggesting a possible link between hypoxia and PE. Shallow trophoblastic invasion of the spiral arteries at the maternal-fetal interface is generally thought to contribute to its pathogenesis (Gerretsen, Huisjes et al. 1981), as this abnormal process of placentation may lead to fetal hypoxia following reduced blood supply to the fetus. It has also been suggested that maternal immune maladaptation to fetal antigens is involved in its pathogenesis, as the incidence of PE is higher in nulliparous women (Campbell, MacGillivray et al. 1985), and in women who are less exposed to their partners' antigens (Robillard, Hulsey et al. 1994; Trupin, Simon et al. 1996; Smith, Walker et al. 1997; Lie, Rasmussen et al. 1998; Saftlas, Levine et al. 2003). A genetic basis for PE has also been shown; a family history of PE increases the risk for developing the condition (Chesley, Annitto et al. 1968; Trupin, Simon et al. 1996).

Many studies have shown that it is likely that the nitric oxide system plays an important role in nommal pregnancy. It has been documented that there is increased nitric oxide (NO) production, and increased responsiveness of the vasculature to NO in nommal vascular adaptation during pregnancy (Izumi, Garfield et al. 1994; Weiner, Knowles et al. 1994) (Nathan, Cuevas et al. 1995; Nelson, Steinsland et al. 1995; Zhou, Li et al. 1999), implying that NO may help to reduce peripheral vascular resistance. Indeed, it has been shown that NO is important in the regulation of vascular tone and exhibits vasoprotective effects (Lefer 1997; Dorup, Skajaa et al. 1999). Therefore, alterations like mutations in the eNOS gene may affect the expression of NO during pregnancy, resulting in matemal vascular maladaptation and endothelial injury during this penod of increased maternal cardiac output. These may present as hypertension and proteinuria if the kidneys are affected as well.

The association between the eNOS gene and pre-eclampsia has been studied widely, but the results have been rather contradictory. Placental tissues studied by immunohistochemistry show localization of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) (Zhou, Li et al. 1999). Moreover, a longitudinal study that follows the changes in endothelial vasomotor function also supports the idea that NO may contribute to the decrease in peripheral resistance in the normal course of pregnancy (Dorup, Skajaa et al. 1999). Additionally, it has been found that the amount of eNOS mRNA and expression are significantly less than normal in pre-eclamptic placental villi (Tong, Li et al. 1998; Steinert, Wyatt et al. 2002), which could possibly be due to genetic alterations in the eNOS gene. Indeed, some studies have observed an association of the T allele in the Glu298Asp polymorphism in exon 7, and the A allele in intron 4, of the eNOS gene with PE and pregnancy induced hypertension (Yoshimura, Yoshimura et al. 2000; Kobashi, Yamada et al. 2001; Tempfer, Dorman et al. 2001) and hypertension (Hingorani 2003)(Arngrimsson, Hayward et al. 1997). We wished to determine if a similar association exists in the Malay population, to explore the potential of using the eNOS gene as a genetic marker to predict risk for PE.

MATERIALS AND METHODS

Recruitment and phenotyping of patients

A total of 66 pre-eclamptic and 126 healthy normotensive Malay mother-baby pairs were recruited for this study. Diagnosis of pre-eclampsia was made if there was gestational hypertension of at least 140mmHg (systolic) or 90mmHg (diastolic), measured on two occasions at least 6 hours apart, and proteinuria of at least 300mg per day (Sibai 2003). All patients were examined for the presence of other causes of hypertension such as autoimmune diseases and cardiac or renal complications. Questions on other potential confounding factors like demographic details and obstetric history, and risk factors like a family history of hypertension and PE, were also asked. For both case and control groups, venous blood was collected from the mother either in the delivery suite or in the antenatal ward. Fetal blood was collected from the umbilical cord after the delivery of the baby. DNA was extracted from the collected bloods according to standard protocols. This study was approved by the Institutional Review Board of the National University Hospital.

Polymerase chain reaction,
DNA sequencing and genotyping

To genotype the single nucleotide polymorphism (SNP) at Glu298Asp, part of exon 7 of the eNOS gene was amplified using previously described primer sequences (Yoshimura, Yoshimura et al. 2000) and polymerase chain reaction (PCR) conditions (Hakli, Romppanen et al. 2003). A 5 ul aliquot of each PCR product was analyzed by electrophoresis through a 2% agarose gel in 0.5 x Tris-Borate-EDTA buffer at 15 volts/cm for 90 min to confirm successful DNA amplification. The remaining PCR product was purified using the

QIAquick™ kit (Qiagen) and sequenced using the same primers described for the PCR. The cycle sequencing conditions were as follows: an initial l min denaturation at 96 °C, followed by 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 4 min. Sequencing products were precipitated with ethanol and resuspended in Hi-Di™ formamide (Applied Biosystems), and analyzed by automated capillary electrophoresis on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

Statistical analysis

Deviations of allele frequencies from Hardy-Weinberg expectation were evaluated using the chi-square goodness of fit method. Comparisons were made between control and preeclamptic mothers, as well as between the babies of the control and pre-eclamptic mothers. The test for significance of the results was based on chi-square statistics.

Results

All samples were genotyped by PCR amplification and sequencing. Typical sequencing results from two samples are shown in Figure 1.

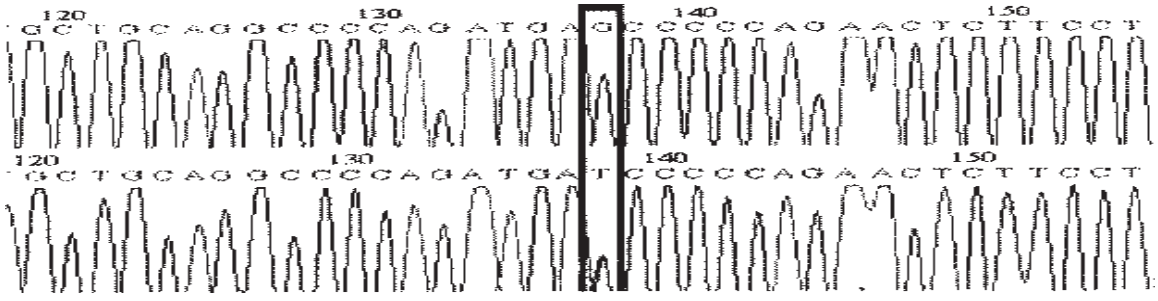

Figure 1. Sequence chromatograms showing homozygosity for the G-allele (top tracing) and heterozygous G/T (bottom tracing) in the boxed nucleotide position. The T-allele alters the amino at codon 298 from Glu to Asp.

The codon 298 genotype frequencies (G/G, G/T or T/T) were determined for each sample, and the total number of individuals with each genotype are listed in Table 1. Genotype frequencies within the control and case groups were in Hardy Weinberg equilibrium. There was no significant difference in the frequency of the variant T allele between control and pre-eclamptic mothers (0.143 vs 0.144), or between the babies of the control mothers and babies of pre-eclamptic mothers (0.155 vs 0.152).

Table 1. Individuals with each genotype at the eNOS gene codon 298 G/T polymorphism.

Samples	Control				Pre-eclamptic			
	GG	GT	TT	Total	GG	GT	TT	Total
Mother	94	28	4	126	50	13	3	66
Baby	88	37	1	126	47	18	1	66
Total	182	65	5	252	97	31	4	132

DISCUSSION

There are marked differences in the distribution of the Asp298 variant (the T-allele) of the eNOSgene in different ethnic groups: 34.5% in Caucasians, 15.5% in African Americans, and 8.6% in Asians (Tanus-Santos, Desai et al. 2001). However, the difference in the incidence of PE between these ethnic groups is comparatively similar: 1.04% in Caucasians, 2.52% in Africans Americans, and 2.19% in Asians (Knuist, Bonsel et al. 1998). Although the eNOS gene 298Asp variant has been reported to be linked to severe PE (28.8% in preeclamptic and 14.1% in normal women, $p<0.01$), no association was found with women with mild PE (Yoshimura, Yoshimura et al. 2000). Several other studies in Japanese, Caucasian and Hispanic women have also found no association between this variant and PE (Hakli, Romppanen et al. 2003) (Lade, Moses et al. 1999; Yoshimura, Chowdhury et al. 2003; Landau, Xie et al. 2004). Our results are consistent with the findings of the majority of previous studies.

Another study reported a positive association between a polymorphism in intron 4 of the eNOSgene and PE (Tempfer, Dorman et al. 2001), suggesting that the underlying genetic predisposition to PE may be in linkage disequilibrium with different SNPs of the eNOS gene in different ethnic population groups. Therefore, the lack of association of PE with the exon 7 SNP in some populations does not necessarily indicate the absence of involvement of the eNOS gene in PE.

It has been proposed that nitric oxide contributes to the normal adaptive decrease in peripheral resistance in pregnancy (Dorup, Skajaa et al. 1999). Expression studies using immunoblots for endothelial nitric oxide synthase also found that there is reduced eNOS . expression in preterm and PE cells (Steinert, Wyatt et al. 2002). However, this could be a secondary response to endothelial cell dysfunction in PE instead of the precipitating factor (Lade, Moses et al. 1999). More extensive longitudinal studies tracing the levels of NO and expression of the eNOS gene throughout the period of pregnancy in normal and pre-eclamptic women would be necessary to determine its true role in PE.

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