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ORIGINAL ARTICLE

Endothelial nitric oxide synthase, angiotensin-converting enzyme and angiotensinogen gene polymorphisms in hypertensive disorders of pregnancy

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We investigated the variations in genes encoding endothelial nitric oxide synthase (*NOS3*), angiotensin-converting enzyme (*ACE*) and angiotensinogen (*AGT*) in hypertensive disorders of pregnancy and the relationship between the polymorphisms and circulating nitric oxide (NO) and ACE levels in pregnant north Indian women. Frequencies of *NOS3* G894T, 4b/a and $T^{-786}\rightarrow C$, *AGT* T704C and *ACE ins/del* polymorphisms were studied in 342 subjects: 120 with preeclampsia (PE), 104 with gestational hypertension and 118 normotensive pregnant women. Variations were evaluated by polymerase chain reaction–restriction fragment length polymorphism. NO and ACE levels were determined using ELISA. There was no difference in the distribution of individual *NOS3* and *ACE* polymorphisms in the study groups. Haplotype analysis showed a global difference in the *NOS3* haplotype distribution between the PE and non-PE subjects ($P=0.03$). The presence of *AGT* 704C allele was associated with a reduced risk of developing PE (odds ratio: 0.33, 95% CI: 0.19–0.59 in recessive mode). Circulating total NO and ACE levels were similar in three groups. No relationship was found between circulating NO levels and any of the *NOS3* polymorphisms, but the circulating ACE levels were higher in those with DD genotype ($P<0.05$). In conclusion, there was no association between individual *NOS3* and the *ACE* gene polymorphisms and hypertensive disorders of pregnancy in north Indian women. The presence of minor alleles at all the three sites in *NOS3* seemed to increase the risk of PE, and *AGT* 704C allele was associated with a reduced PE risk. The complexity of interaction between these genetic abnormalities requires further studies.

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INTRODUCTION

Preeclampsia (PE) is a major complication of pregnancy and increases fetal and maternal morbidity and mortality.[1] PE usually manifests after the 20th week of gestation and regresses postpartum. The clinical manifestations suggest a perturbation of the balance in vasoactive factors in favor of vasoconstriction.

Though the familial component of PE is well documented,[2] the precise function of genetic factors remains unclear. Linkage and epidemiologic analyses suggest a polygenic inheritance pattern with strong nongenetic component.[3]

Renin–angiotensin system and endothelial nitric oxide synthase (*NOS3*) are important regulators of vascular tone and salt and water homeostasis, and contribute to spiral artery remodeling during placentation that reduces uteroplacental resistance typical of normal pregnancy.[4,5] A function for variations in genes encoding endothelial *NOS3*, angiotensinogen (*AGT*) and angiotensin-converting enzyme (*ACE*) in the genesis of PE has been speculated, but studies in different population groups have yielded conflicting results. We evaluated three functionally important polymorphisms in the *NOS3* gene, namely G894T in exon 7, 4b/a in intron 4 and $T^{-786}\rightarrow C$; *AGT* T704C in exon 2 and *ACE* intron 16 *ins/del* (I/D) polymorphism in a cohort of preeclamptic, gestational hypertensive (GH) and normotensive pregnant women. We also measured the circulating total nitric oxide (NO) and ACE levels in the three groups and evaluated their relationship with genotypes.

METHODS

Subjects were recruited from the Antenatal Clinic and Wards of the Department of Obstetrics and Gynecology, Postgraduate Institute of Medical Education and Research, Chandigarh, a large tertiary care hospital in north India.

PE was defined as new onset of elevated blood pressure $>140/90$ mm Hg using a standard mercury sphygmomanometer along with proteinuria $\geqslant 2+$ on dipstick on two occasions at least 6 h apart or >300 mg per day on two occasions after 20 weeks of gestation in earlier normotensive pregnant women

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(American Society of Obstetrics and Gynecology Technical Bulletin # 91, 1986). GH was defined as new onset of elevated blood pressure >140/90 after 20 weeks of gestation on two occasions at least 6 h apart in the absence of any proteinuria in earlier normotensive and nonproteinuric pregnant women. Patients were followed up after delivery to document normalization of blood pressure. The normotensive control group had women with uncomplicated gestation and blood pressure <125/85 mm Hg and no proteinuria. Subjects were excluded if they had one or more of the following: hypertension before 20 weeks of gestation, diabetes, asthma, heart disease, kidney disease, hematological disorder, autoimmune disease, urinary tract infection, current or past history of smoking, twin/molar pregnancy and eclampsia.

Institute Ethics committee approved the study protocol and all subjects provided informed consent.

After establishing the diagnosis through careful history, full clinical examination and urinalysis, venous blood was collected, plasma/serum separated and stored in sterile cryovials at −80 °C. Buffy coat was stored at −20 °C until DNA isolation.

Polymorphism analysis

Genomic DNA was extracted from cells in the buffy coat using QIA*amp* DNA mini kit (Qiagen GmbH, Hinden, Germany) as per manufacturer's instructions. Gene polymorphisms were detected using polymerase chain reaction followed by restriction fragment length polymorphism. Table 1 shows the primer sequences, reaction conditions, restriction enzymes used and fragment sizes for the various reactions. To exclude the possibility of the ACE ID genotypes getting mistyped as DD because of preferential amplification of D allele, all DD were re-amplified using insertion specific primer pairs that gave a 335 bp fragment in the presence of I allele.

Angiotensin-converting enzyme

Serum ACE levels were quantitated in duplicate by a sandwich enzyme immunoassay using Human ACE Immunoassay kit (R&D Systems, Minneapolis, MN, USA). The inter- and intra-assay coefficients of variation were 5.90 and 3.76%, respectively.

Total NO

The plasma total NO levels were quantitated in duplicate using Total NO/Nitrite/Nitrate Assay kit (R&D Systems) as per the instructions of the manufacturer. Briefly, the plasma samples were filtered through 10 kDa cutoff filters to eliminate proteins and the filtrate was used to determine total NO levels after conversion of nitrate into nitrite using nitrate reductase. The inter- and intra-assay coefficients of variation were 3.86 and 1.83%, respectively.

Statistical analysis

Statistical analysis was performed using SPSS 13 (SPSS, Chicago, IL, USA) and *Epi*-info. One-factor *post hoc* ANOVA with Bonferroni corrections was used to compare the means of clinical characteristics. Expected genotype frequencies were determined using the Hardy–Weinberg calculator (http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20%20HW%20calculator.xls) and analyzed using the appropriate χ^2 test. Odds ratios were calculated using *Epi*-Info software. *NOS3* haplotype analysis was performed using SHEsis software.[6] NO and ACE levels were compared using Mann–Whitney *U*-test. A *P*-value of <0.05 was considered as significant.

RESULTS

A total of 342 subjects (120 with PE, 104 with GH and 118 normotensive pregnant women) were included in the study. Table 2 shows the demographic details of the enrolled subjects. Mean age of the pregnant women was similar in the three groups, but gestational age of women with PE was lower compared with the normotensive controls. As expected, the mean systolic and diastolic blood pressures were elevated in PE and GH ($P<0.001$), and proteinuria was present only in PE group. Table 3 shows the distributions of genotype and allele frequencies for all the polymorphisms in the three groups. The genotype and allele frequency distribution for all the studied polymorphisms were in accordance with Hardy–Weinberg equilibrium. No significant variation was observed in the individual genotype and allele distribution of *NOS3* and *ACE*. *AGT* 704 CC genotype and C allele frequencies were significantly reduced in PE ($P=0.0002$ and

Table 1 Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) conditions

Gene (accession no.)	*Polymorphic site*	*Primer sequence (5′–3′)*	*Denaturation, annealing and extension*	*Product size (bp)*	*Restriction enzyme (incubation duration)*	*Genotype and restriction fragment (bp) pattern*
ACE (A28005)	I/D	F:CTGGAGACCACTCCCATCCTTTCT	95 °C for 30 s	490/190		II: 490
		R:GATGTGGCCATCACATTCGTCAGA	57 °C for 30 s			ID: 490 and 190
			72 °C for 45 s			DD: 190
	I allele	F:TGGGACCACAGCGCCCGCCACTAC	95 °C for 30 s	335		II: 335
		R:TCGCCAGCCCTCCCATGCCCATAA	76 °C for 30 s			DD: no product
			72 °C for 45 s			
NOS3 (D26607)	G894T	F:TCCCTGAGGAGGGCATGAGGCT	94 °C for 1.0 min	457	*Ban II* (5U) (37 °C for 5 h)	GG: 320 and 157
		R:TGAGGGTCACACAGGTTCCT	63 °C for 1.0 min			GT: 457, 320 and 157
			72 °C for 1.0 min			TT: 457
	4b/a	F:AGGCCCTATGGTAGTGCCTTT	94 °C for 1.0 min	420/393		4bb: 420
		R:TCTCTTAGTGCTGTGGTCAC	61 °C for 1.0 min			4ba: 420 and 393
			72 °C for 1.0 min			4aa: 393
	$T^{-786}\rightarrow C$	F:GCATGCACTCTGGCCTGAAGTG	95 °C for 45 s	222	*Msp 1* (5U) (37 °C for 5 h)	TT: 161 and 61
		R:CAGGAAGCTGCCTTCCAGTGC	67 °C for 45 s			TC: 161, 116, 61 and 45
			72 °C for 1.0 min			CC: 116, 61 and 45
AGT (AH002594)	T704C	F:CAGGGTGCTGTCCACACTGGACCCC	95 °C for 1.0 min	165	*Tth111 I* (5U) (37 °C for 8 h)	TT: 165
		R: CCGTTTGTGCAGGGCCTGGCTCTCT	67 °C for 45 s			TC: 165, 141 and 24
			72 °C for 1.5 min			CC: 141 and 24

Abbreviations: ACE, angiotensin-converting enzyme; AGT, angiotensinogen; NOS3, nitric oxide synthase.

Table 2 Demographic characteristics of the study subjects

	PE (n=*120*)	*GH* (n=*104*)	*N* (n=*118*)	P-*value* *PE* vs. *N*	P-*value* *GH* vs. *N*
Age (years)	25.7 ± 3.8	26.9 ± 4.0	26.3 ± 4.0	NS	NS
Gestational age at delivery (weeks)	33.2 ± 2.7	35.3 ± 2.4	35.9 ± 2.7	<0.001	NS
Blood pressure (mm Hg)					
Systolic	153.4 ± 10.7	146.5 ± 8.4	117.0 ± 4.6	<0.001	<0.001
Diastolic	101.6 ± 8.4	96.1 ± 7.3	76.2 ± 4.9	<0.001	<0.001
Proteinuria (dipstick)	3.03 ± 0.78	Nil	Nil		

Abbreviations: GH, gestational hypertension; PE, preeclampsia; N, normotensive.

Table 3 Distribution of NOS3, ACE and AGT genotypes and alleles

Polymorphism	*PE*	*GH*	*N*
NOS3 G894T			
GG	64 (53.3)	65 (62.5)	70 (59.3)
GT	46 (38.3)	37 (35.6)	42 (35.6)
TT	10 (8.3)	2 (1.9)	6 (5.1)
Minor allele frequency	0.28	0.20	0.23
NOS3 4b/a			
bb	83 (69.2)	55 (52.9)	74 (62.7)
ba	32 (26.7)	42 (40.4)	39 (33.0)
aa	5 (4.2)	7 (6.7)	5 (4.3)
Minor allele frequency	0.18	0.27	0.21
NOS3 $T^{-786}\rightarrow C$			
TT	80 (66.7)	67 (64.4)	74 (62.7)
TC	35 (29.2)	34 (32.7)	40 (33.9)
CC	5 (4.2)	3 (2.9)	4 (3.4)
Minor allele frequency	0.19	0.19	0.20
ACE I/D			
II	38 (31.7)	41 (39.4)	45 (38.1)
ID	66 (55.0)	45 (43.3)	54 (45.7)
DD	16 (13.3)	18 (17.3)	19 (16.2)
Minor allele frequency	0.41	0.39	0.39
AGT T704C			
TT*	7 (5.8)	2 (1.9)	4 (3.3)
TC	55 (45.8)	28 (26.9)	27 (22.8)
CC	58 (48.3)	74 (71.2)	87 (73.9)
Minor allele frequency**	0.71	0.85	0.85

Abbreviations: ACE, angiotensin-converting enzyme; AGT, angiotensinogen; GH, gestational hypertension; I/D, *ins/del*; PE, preeclampsia; N, normotensive; NOS3, nitric oxide synthase. Numbers in parentheses indicate percentages, *P=0.0002, **P=0.0004 (PE *vs.* N).

0.0004, respectively); and the odds ratio of development of PE in the recessive mode was 0.33 (95% CI: 0.19–0.59, P=0.00005). *NOS3* haplotype analysis showed significant (P=0.003) global differences in the frequency distribution. With reference to normal (G894-4b-T786) haplotype, the 894T-4b-786C was associated with increased PE risk (P=0.03), whereas G894-4b-786C and 894T-4a-T786 haplotypes were protective (P=0.004 and 0.04) (Table 4).

Correlation of ACE and NO levels with genotype

Circulating total NO levels ($\mu mol\,l^{-1}$) were 28.7 ± 11.9 in the normotensive, 29.9 ± 10 in the GH and 30.8 ± 17 in the PE group ($P>0.05$). Similarly, the ACE levels ($ng\,ml^{-1}$) were also not different between the three study groups (141.3 ± 46.3 in normotensive, 130.4 ± 37.9 in GH and 149.7 ± 50.3 in PE). None of the *NOS3* polymorphisms correlated with total NO levels (Table 5). However, compared with II and ID, ACE levels were higher in the subjects with DD genotype (Figure 1).

DISCUSSION

The molecules that we investigated are distinguished by their common property of being important players in influencing endothelial cell function and vasomotor tone. Through their actions, they determine whether blood vessels are in a state of vasoconstriction or vasodilatation, systemic blood pressure levels and fluid distribution between different body compartments. Perturbation of endothelial function impacts coagulation pathway. Angiotensin pathway also determines salt and water homeostasis, both directly and through its influence on aldosterone levels. In combination, these are important determinants of the PE phenotype.

This study is the first one to simultaneously examine the distribution of several functionally important polymorphisms (*NOS3* G894T, 4b/a and $T^{-786}\rightarrow C$, *AGT* T704C and *ACE* I/D), and attempt a correlation between these polymorphisms and circulating total NO and ACE in normotensive and hypertensive pregnancies. In addition, this is the first study to report the NOS3 and AGT polymorphisms in the Indian population. Our population sample was a homogeneous group of north Indian women.

Compared with normotensive pregnancies, we failed to find any difference in the individual frequency of any of the studied *NOS3* and *ACE* polymorphisms in those with PE or GH. Likewise, the circulating total NO as well as ACE levels were similar among the three study groups. However, our data does suggest that subjects that carry the minor alleles at all three loci in the *NOS3* gene have an increased risk of developing PE.

Of the three functionally significant *NOS3* polymorphisms, G894T has been studied most frequently, but the results are inconsistent. Studies in Finnish,[7] North American,[8] Korean[9] and British[10] populations did not show any association between this single-nucleotide polymorphism and occurrence of PE. In contrast, the frequency of T allele was higher in PE in the Japanese.[11] A recent meta-analysis found

Table 4 *NOS3* haplotypes and preeclampsia risk

G894T	*4b/a*	*$T^{-786}\rightarrow C$*	*Frequency in PE*	*Frequency in controls*	*OR (95% CI)*	P-*value*
G	4a	C	0.084	0.059	1.43 (0.67–3.01)	0.34
G	4a	T	0.071	0.101	0.69 (0.33–1.29)	0.22
G	4b	C	0.027	0.087	0.29 (0.11–0.72)	0.004
G	4b	T	0.543	0.523	1.03 (0.70–1.50)	0.89
T	4a	T	0.018	0.053	0.32 (0.11–1.00)	0.04
T	4b	C	0.075	0.028	2.73 (1.04–7.19)	0.03
T	4b	T	0.180	0.122	1.54 (0.89–2.63)	0.11

Abbreviations: CI, confidence interval; NOS3, nitric oxide synthase; OR, odds ratio; PE, preeclampsia.
Global *P*-value=0.03.
Frequency <0.03 in both control and cases has been dropped.

Table 5 *NOS3* G894T, 4b/a and $T^{-786}\rightarrow C$ genotypes and plasma total NO levels ($\mu mol\,l^{-1}$)

Polymorphic site	*Genotype* (n)	*Mean ± s.d.*	*Median*	*Interquartile range*
G894T	TT (17)	25.8 ± 8.32	25.52	18.9–31.1
	GT (119)	30.9 ± 12.8	29.2	19.5–39.3
	GG (180)	29.5 ± 14.4	27.7	20.1–34.5
4b/a	aa (17)	26.9 ± 13.4	24.42	16.1–30.1
	ba (112)	29.9 ± 16.1	27.8	20.5–34.2
	bb (195)	30.1 ± 11.9	28.5	19.9–38.2
$T^{-786}\rightarrow C$	CC (11)	24.1 ± 15.4	17.5	12.8–38.6
	TC (103)	31.8 ± 16.2	29.3	20.8–36.5
	TT (210)	29.2 ± 11.7	27.7	19.9–35.5

Abbreviations: *n*, number of subjects; NO, nitric oxide; NOS3, nitric oxide synthase.

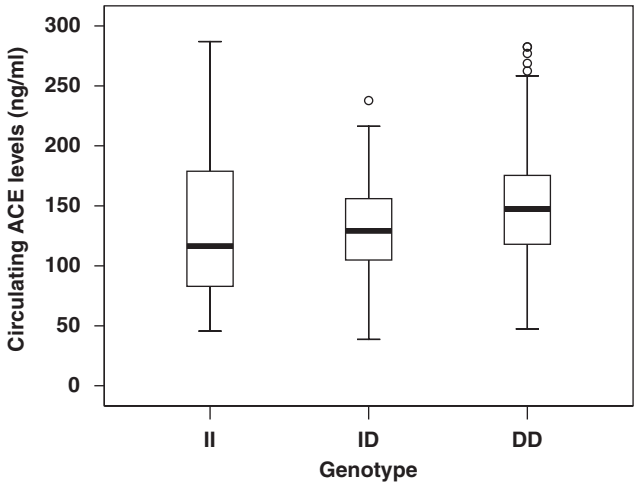

Figure 1 Serum ACE levels in DD, ID and II genotype of ACE gene. The lower and upper bars represent the 10th and 90th percentiles, respectively, and the interquartile range is indicated by the box, the median value being the horizontal line in the box. Significant increase in DD compared with ID and II genotype ($P<0.05$).

the cumulative odds ratio of 1.24 with T allele, but the confidence intervals crossed 1, making it nonsignificant.[12] It can, therefore, be concluded that the association between G894T polymorphism and PE is weak.

Not many studies have examined the association between PE and the other two *NOS3* polymorphisms. We did not find an association between the 4a/b or $T^{-786}\rightarrow C$ polymorphism and PE or GH in either modes of inheritance. Some studies have reported an association between 4b/a polymorphism and PE[13] and higher blood pressure at an earlier gestational age in the presence of 4aa genotype.[14] Seremak-Mrozikiewicz *et al.*[15] reported a weak correlation between *−786C* alleles and PE susceptibility in Polish population. Others, however, have found no association between this polymorphism and PE.[16]

Several studies have measured circulating NO levels in PE and found them to be decreased,[17] increased[5,18] or unchanged.[19] We did not find any significant differences in the total circulating NO levels in any of the groups. However, this endothelium-derived molecule has a short half-life and largely acts in an autocrine or paracrine manner, hence circulating levels may not truly reflect the local NO concentration and/or activity. *NOS3* genotype affects circulating total NO levels,[20] but we did not find significant differences in NO levels between the genotypes of all the three studied polymorphisms that is G894T, 4b/a and $T^{-786}\rightarrow C$ (Table 5). Haplotype analysis suggests a more complex relationship between these variations and PE risk. The presence of minor alleles at all three sites simultaneously predicted an increased risk, a finding that needs to be studied further.

In contrast to several studies, we found a significant underrepresentation of AGT 704C allele in subjects with PE. In our control population, the frequency of this allele was 0.8, compared with 0.4 in European Caucasians,[21] 0.9 in Black Africans,[22] 0.8 in Japanese[21] and 0.7 in Hispanics.[14] Several studies have documented a higher frequency of the 704C variant in women with PE.[12,21,23] Others, however, did not find a difference in the frequency of the two alleles.[24–26] Our findings are similar to those of Hopkins *et al.*[27] who showed a higher frequency of T allele in Hispanic women with PE. A meta-analysis involving 17 studies concluded that C allele homozygotes had a 1.62 times higher risk of developing PE.[28] In our population, the CC genotype and C allele afforded protection against PE. The significance of this finding along with its relation to the circulating angiotensin-II levels needs to be confirmed in larger cohorts.

Whether or not there is an association between *ACE* intron 16 I/D polymorphism and PE is not fully settled. Individuals with DD genotype have ACE activity about twice that of II subjects.[29] It has been suggested that the DD genotype could predispose to the development of PE.[30,31] A recent study reported an association between the I/D polymorphism and mild, but not severe PE.[32] We did confirm the high ACE levels in those with the D allele, but failed to find any difference in the distribution of *ACE* II, DD and ID genotypes between the three groups. A meta-analysis of 11 studies involving 1121 PE and 1361 control subjects calculated the odds ratio of developing PE at

1.51 under the recessive model of inheritance.[12] This was, however, driven largely by two studies, one from Italy[33] and the other from Korea.[31] Single population studies, however, have failed to show association.[12,22,34] Studies that evaluated ACE level in PE and normotensive pregnant women also could not find a difference.[30]

To conclude, we did not find any association between individual polymorphisms in the *NOS3* (Glu298Asp in exon 7, 4b/a in intron 4 and $T^{-786}\rightarrow C$) and the *ACE* (intron 16 I/D) genes and hypertensive disorders of pregnancy. Subjects carrying minor alleles at all the three sites in *NOS3* seem to have an increased risk of developing PE. The presence of *AGT* 704C allele was associated with a reduced PE risk. These findings could be population specific. The complexity of interaction between these genetic abnormalities requires further studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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