Research Article

Polymorphisms in renin-angiotensin-aldosterone system and vascular endothelial growth factor may cross talk in preeclampsia: a pilot study of maternal and fetal dyads in Indian population

Sangeeta Sharma[1], Sruthi Bhaskaran[2], Shashi Bhushan Tripathi[3], Neelam Vaid[2], Binuja Varma[4]*

[1]TERI University, New Delhi, India
[2]Department of Obstetrics and Gynecology, UCMS and GTB Hospital, New Delhi, India
[3]The Energy and Resources Institute, New Delhi, India
[4]CSIR-TRISUTRA, CSIR-Institute of Genomics and Integrative Biology, New Delhi, India

*Correspondence:
Dr. Binuja Varma,
E-mail: binuja.varma@igib.in

ABSTRACT

Background: Preeclampsia (PE) is a multi-system disorder complicating 5-7% pregnancies and one of the leading causes of fetal/maternal morbidity and mortality worldwide.
Methods: We have analyzed the association of common genetic polymorphisms of renin-angiotensin-aldosterone system (RAAS) and angiogenesis pathway in preeclamptic/eclamptic mother-fetal dyad samples, as we have hypothesized that there is a cross-talk between the maternal and fetal genotypes. Maternal venous and fetal umbilical vein blood of 50 primigravidae with preeclampsia /eclampsia and 100 matched normotensive controls of north Indian origin were collected.
Results: A significant association was observed in the prevalence of VEGFA (vascular endothelial growth factor) polymorphism (rs25648 T>C) with PE. Moreover, difference in the prevalence of the AGT (rs7079 A>C) polymorphism was observed between mild vs. severe PE. Fetal genotypes showed a strong association with respect to RENIN (rs11240688 A>G) and AGT (rs11122576 G>A) to preeclampsia.
Conclusions: Preeclamptic mothers and their fetuses have shown association with genes that are interacting partners in the regulation of angiogenesis and it would be interesting to expand the study to include more genes and connected pathways for the better understanding of the interplay of the biological process that goes dysregulated in this multi-systemic disorder.

Keywords: Preeclampsia, Eclampsia, RAAS, Angiogenesis pathway, VEGFA

INTRODUCTION

Preeclampsia is a pregnancy-specific disease characterized by hypertension (blood pressure >140/90 mmHg) and proteinuria (>0.3 g/day). It affects 5-8% of all pregnancies and forms one of the deadly triad, along with hemorrhage and infection, which contributes greatly to maternal and fetal morbidity and mortality.[1] The disease usually develops at any time after 20 weeks of pregnancy, and depending on the time of the onset of symptoms (earlier or later than 34 weeks), it can be categorized as early onset preeclampsia (EOPE) or late onset preeclampsia (LOPE). The genetic predisposition of the disease has been established by epidemiological and family based studies in different populations and has been elucidated that preeclampsia is multifactorial with

familial tendency and influenced by race, ethnicity and environment.[2]

Increasing evidence, that inadequate placentation is the primary pathogenic factor in preeclampsia, makes the study of genes involved in renin angiotensin (RAS) and angiogenesis pathway very critical. Data in support of this include 1) lower levels of all circulating components of the RAS, and activation of cellular and tissue renin-angiotensin components at the same time and 2) pregnancy-associated blunting of angiotensin II (AT II) pressor responsiveness owing to excessive up regulation of AT II receptors in a variety of tissues.[3] Another important component, Vascular endothelial growth factor (VEGF) is hypoxia induced growth factor produced by the cytotrophoblasts plays an important role in the regulation of placentation through its effect on vascular remodelling and invasion of cytotrophoblasts . Zhou et al. found that cyotrophoblastic differentiation and invasion during pregnancy is regulated through VEGF receptor-2 and its expression is deregulated in severe preeclampsia.[2] The VEGF gene is highly polymorphic. Banyasz et al. found carriers of allele VEGF G405C occurred less frequently in pre-eclampsia than in controls and that the progression of preeclampsia may be modified by the presence of VEGF 2578A allele.[4] Shim et al (2007) evaluated the distribution of +936 C/T polymorphism in VEGF gene in Korean women and found that its carriage may be associated with increased susceptibility to the development of PE and may be an independent risk factor.[5]

Preeclampsia presents a peculiar challenge, in that both the maternal and fetal genotype may contribute to the clinical phenotype. Very few feto-maternal genetic studies have been done so far and moreover, the feto-maternal genetic interaction is essential as the role of paternity in preeclampsia (paternal genes being expressed through the fetus)[6,7] is very important. Towards understanding the contribution of common genetic polymorphisms in maternal and fetal genes, we have explored the genetic analysis of renin angiotensin system (renin, angiotensinogen, angiotensin converting enzyme, angiotensin-II type-I and type 2 receptor genes, SCNN1B) and angiogenesis (vascular endothelial growth factor) in preeclampsia.

METHODS

Study subjects

The case control study was conducted in the Department of Obstetrics & Gynaecology at University College of Medical Sciences (UCMS) & Guru TegBahadur (GTB) Hospital and The Center for Genomic Applications (TCGA) (IMM-IGIB collaboration), Delhi. This study was done in primigravida women of North India (Indo European origin). A total of 150 mother/fetal dyads were recruited. Inclusion criteria for cases: blood pressure ≥140/90 mmHg and urine albumin ≥1+ by Dipstick,

repeated after 6 hours, diagnosed after 20 weeks gestation with or without seizures, admitted for induction of labour. Criteria for severe preeclampsia were as follows: BP of >160/110 mm of Hg and persistent proteinuria of 2+ or more with at least one of the additional symptoms: headache, epigastric pain, dyspnea, oligouria, and visual disturbance and HELLP syndrome. Controls were healthy age and ethnicity matched normotensive women admitted for labour induction at same hospital. Patients with chronic hypertension, chronic renal disease, >35 years age, BMI>35 Kg/m2, smoking, diabetes mellitus, UTI, fetal congenital anomaly and intra uterine death were excluded from the study.

The medical/family history and necessary blood investigations were carried out. Labour was induced as per the hospital protocol and maternal-fetal outcome were recorded. Written informed consent was obtained from each subject to collect venous and cord blood samples for diagnostic and genetic experiments and the ethic committee of UCMS approved the study.

Table 1: Genes and SNPs selected in the study.

Gene name	Chr. location	No. of SNPs
AGT (Angiotensinogen)	1q42-q43	10
REN (Renin)	1q32	5
ACE (Angiotensin converting enzyme)	17q23.3	5
AGTR1 (Angiotensin II type I receptor)	3q21-q25	4
AGTR2 (Angiotensin II type 2 receptor)	xq22-q23	2
SCNN1B (Sodium channel, nonvoltage-gated 1, beta)	16p12.2-12.1	7
PRCP (Prolylcarboxypeptidase (angiotensinase C))	11q14	1
NOS3 (Endothelial nitric oxide synthase)	7q36	5
EDNI (Endothelin 1)	6p24.1	1
GNB3 (Guanine nucleotide binding protein (G protein), beta polypeptide 3)	12p13	1
VEGF A (Vascular endothelial growth factor A)	6p12	10
ESR2 (Estrogen receptor 2 (ER beta)	14q23.2	2
SYNE2 (Spectrin repeat containing, nuclear envelope 2)	14q23.2	1
ADRB2 (Adrenoceptor beta 2, surface)	5q31-q32	1
ADD1 (Adducin 1 (alpha))	4p16.3	2

Sample collection

10 ml of maternal venous blood at the time of induction & fetal umbilical venous blood from placental site at the time of delivery were collected in ACD vacutainers. Genomic DNA was extracted from the samples using Flexigene DNA extraction kit as per the recommended protocol (Qiagen).

SNP selection and primer design

Single nucleotide polymorphisms (SNPs) of REN, AGT, ACE, AGRT1, AGRT2, SCNN1B, VEGFA, ADD1, ADRB2, EDN1, ESR2, GNB3, MTHFR, NOS3, PRCP and SYNE2 were selected. We have included of tag SNPs and other informative SNPs which have been reported in the literature for the development of assays. Tag SNPs were selected using Tagger Software8 (Table 1). A total of 74 SNPs were selected for the study and their flanking sequences were downloaded from NCBI dbSNP9. Primers were designed using Mass Array design software (Sequenom Assay Design v3.1)

Genotyping

SNP genotyping assays were optimized using iPLEX chemistry of Mass Array Sequenom platform (Sequenom Inc., San Diego, CA, USA), which utilizes MALDI-TOF MS technology. SNP genotyping experiments were performed as per the manufacturer recommended protocols. In brief, multiplex PCR was performed in 5 µl volumes and unincorporated dNTPs were deactivated using shrimp alkaline phosphatase. This was followed by primer extension with normalized (200-500 nM) primers. Extended products were treated with resin and the purified products were spotted on to a SpectroCHIP. The data was visualized, extracted and analyzed using SpectroTYPER-RT software.

Statistical analysis

Clinical history including socio-demographic characteristics and laboratory parameters of maternal subjects were analyzed using chi-square and student t-student.

Hardy-Weinberg equilibrium was checked for genotype distribution in the control group. Chi-square test of proportion was used to compare each genotype frequency between preeclampsia cases and normotensive controls. Odd ratios (OR) and their 95% confidence intervals were used to measure the strength of association between polymorphisms and risk of preeclampsia.

All the clinical analysis was conducted using SPSS 20.0 software and genetic analysis using PLINK software.[10] All the tests were two sided and P values <0.05 was considered statistically significant.

RESULTS

The preeclampsia group comprised of 30 mild PE, 18 severe PE and 2 eclampsia patients. For analysis, both the cases of eclampsia were included in severe preeclampsia category. No significant difference was observed with respect to socio-demographic characteristics-age, residence, religion, occupation & socio economic status of in both cases and control subjects.

Analysis of clinical and laboratory parameters

There was significant difference (p<0.001) in the mean gestational age at presentation in preeclampsia group (38.52 ± 1.4 weeks) as compared to controls (39.74 ± 1.29 weeks), with 7 patients having preterm delivery. The mean BMI was similar in both PE and control group (20.98±1.45 vs 20.49±1.44 Kg/ m2) and was well within the normal range. Among symptoms, headache was the commonest and significantly associated with severe preeclampsia (55%). Mean SBP and DBP in severe preeclampsia category was 154.80 mmHg and 108.80 mmHg, respectively. The difference in blood parameters including platelet count, serum creatinine and liver enzymes(AST,ALT) between preeclampsia and normotensive control groups was statistically significant (Table:2). On comparing the same in mild and severe preeclampsia category, liver enzymes (AST and ALT) were higher in the latter group. The mean AST was 98.15±21.87 IU/l in severe PE category as compared to 54.57±9.53 IU/l in mild category (Table 2).

Women in the PE group had more cesareans as compared to normotensive controls (32% vs 10%, p<0.001). Moreover, 50% percent of women with severe PE underwent cesarean sections as compared to 20% in the mild PE group (p=0.026). Number of low birth weight babies (birth weight <2.5 kg) in PE group was also significantly more (58%, 29/50) (14%). Sixteen percent of the babies born to women with preeclampsia required NICU admission as compared to 2% in the non preeclamptic women (p=0.031) (Table 2).

Genotype distribution and their association with preeclampsia

Case-control analysis of preeclampsia

Mothers: Genotype analysis of 16 genes, showed a significant difference in the prevalence of the VEGFA (rs25648 T>C) polymorphism among women with PE compared to control women (Chi-square= 5.661, p=0.017) with OR=0.345 (CI is 0.139 to 0.856) with C as risk allele for PE. . Manhattan plot shows the overview of p-value of all the SNPs analyzed in different sets. (Figure 1A).

Fetuses: Genotype analysis of fetus (n=129) observed a significant difference in the prevalence of the RENIN polymorphism (rs11240688, A>G) among fetus of PE women (Chi-square= 9.673, p=0.002) with OR=3.042 (CI is 1.476 to 6.267) with the implication that A allele is risk allele for PE. One intronic SNP of AGT gene (rs11122576, G>A) was also significant with G allele being associated with PE (Chi-square= 4.071, p=0.044) (Figure 1C).

Table 2: Comparison of laboratory parameters, mode of delivery and fetal outcome.

Laboratory parameter	PE (n=50)	Control (n=99)	p-value	Mild PE (n=30)	Severe PE (n=20)	p-value
Platelet count (/mm^3)	1.55±0.51	2.34±0.57	<0.001	1.59±0.39	1.50±1.65	0.543
S. creatinine (mg/dl)	0.88±0.29	0.7±0.11	<0.001	0.897±0.34	0.864±0.20	0.697
AST (IU/l)	72.00±26.54	24.92±7.65	<0.001	54.57±9.53	98.15±21.87	<0.001
ALT (IU/l)	58.61±27.01	25.37±8.92	<0.001	44.42±20.33	79.90±21.34	<0.001
S. Bilirubin (mg/dl)	0.78±0.14	0.76±0.11	0.135	0.78±0.11	0.78±0.18	0.965
Mode of delivery						
LSCS	16 (32%)	10 (10.1%)	0.001	06 (20%)	10 (50%)	0.026
Vaginal	34 (68%)	89 (89.9%)		24 (80%)	10 (50%)	
Birth weight (kgs)						
<1.5	01 (02%)	0 (0)	<0.001	0 (0)	1 (2%)	0.637
1.5-2.5	28 (56%)	18 (18.2%)		17 (34%)	11 (22%)	
>2.5	21 (42%)	81 (81.8%)		13 (26%)	8 (16%)	
NICU admission	08 (16%)	02 (2%)	0.018	2 (4%)	6 (12%)	0.047

LSCS=Lower Segment Caesarean Section, NICU=Neonatal Intensive Care Unit, AST=Aspartate Transaminase, ALT= Alanine transaminase, Data expressed as mean±SD

Table 3: Allelic association in the study group.

Group	CHR	SNP ID	Gene ID	A1	A2	CHISQ	P	OR (95% CI)
Case vs Control (Mother)	6	rs25648	VEGFA	T	C	5.661	0.017	0.3451 (0.1391-0.8562)
Mild vs Severe (Mother)	1	rs7079	AGT	A	C	5.771	0.016	0.2381 (0.07027-0.8067)
Case vs Control (Fetus)	1	rs11122576	AGT	G	A	4.071	0.044	2.292 (1.007-5.216)
	1	rs11240688	REN	A	G	9.673	0.002	3.042 (1.476-6.267)
Mild vs Severe (Fetus)	1	rs11571081	REN	G	A	4.396	0.036	NA
	12	rs5443	GNB3	T	C	4.731	0.03	0.3214 (0.1127-0.9164)
	14	rs928554	ESR2	A	G	4.404	0.036	2.605 (1.055-6.43)
	16	rs7204560	SCNN1B	T	C	3.98	0.046	4.013 (0.9513-16.93)
	17	rs4354	ACE	T	C	6.176	0.013	NA
	17	rs8066276	ACE	C	T	7.666	0.006	3.8 (1.443-10)

A1=Minor allele, A2=Major allele, OR=Odds ratio, CI=Confidence interval.

Table 4: Haplotypes frequencies and their association with PE (Mother).

Haplotype Block*	Haplotype Freq	Chi Square	p Value
AGCAA	0.312	0.792	0.3736
CCCGG	0.256	0.151	0.6975
CGCGA	0.226	0.485	0.486
AGTAA	0.141	4.434	0.0352
CGCAA	0.029	6.407	0.0114
AGCGA	0.013	0.106	0.7447
AGTAG	0.012	1.446	0.2291

*The SNP order which defined the VEGF haplotype structure was rs699947, rs2010963, rs25648, rs1413711 and rs833069, respectively.
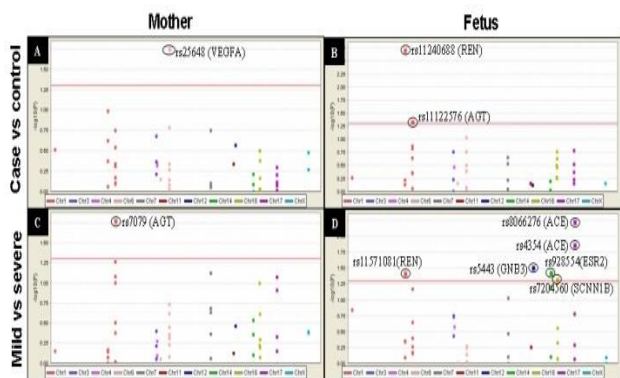
Figure 1: Manhattan plot of the p values obtained from analysis of different groups. The plot shows -log10 p values for each snp against chromosomal location. Values for each chromosome (chr) are shown in different colors for visual effect.
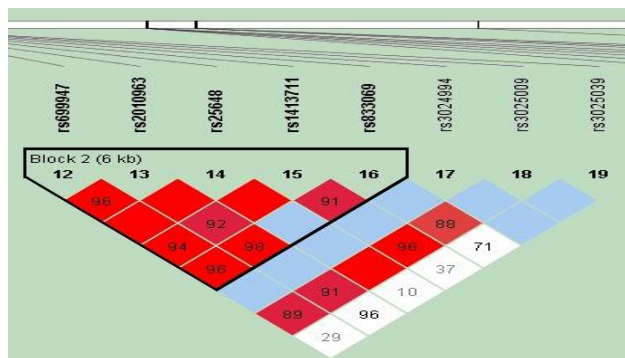

Figure 2: Pairwise linkage disequilibrium (LD) among five SNPs of VEGFA. The LD plots were generated by Haploview 4.2. The value within each diamond represents the pairwise correlation (R^2) between SNPs defined by the upper left and upper right sides of the diamond. The diamonds without value mean the $R^2=1.0$, showed complete linkage disequilibrium.

II. Analysis of mild vs. severe types of Preeclampsia

Mothers: When we compared severe preeclampsia (n=16) with mild preeclampsia (n=26) there was significant difference in the prevalence of the AGT (rs7079, A>C) polymorphism (Chi-square= 5.771, p=0.016) with OR=0.238 (CI is 0.070 to 0.807) which implies that the C allele being more present in severe PE (Figure 1B).

Fetuses: Furthermore, when we compared genotype calls of fetus of severe mothers (n=17) with that of mild preeclampsia mothers (n=25). It was interesting to note that that a number of associations were observed for SNPs of REN, GNB3, ESR2, SCNN1B, ACE which differed between the two groups (Figure 1D).

The details of significant SNPs are given in Table 3.

III. Haplotype analysis

Haplotype analysis of maternal genotypes for all the gene variants showed a LD association between the rs699947, rs2010963, rs25648, rs1413711 and rs833069 of VEGF. A comparison between haplotypes frequencies of VEGF SNPs and their association with PE is summarized in Table 4, Figure 2. The p values of two haplotype score tests indicate a significant difference in VEGF haplotype frequency profiles between cases and controls (p<0.05).

DISCUSSION

In this study, we have examined the inter-individual variability of genes involved in hypertension and angiogenesis in susceptibility to preeclampsia. The case and control groups were matched with respect to ethnicity, age, demographic and socio economic conditions. Based on the existing literature, genes of the renin-angiotensin system (important in regulation of blood pressure) and angiogenesis pathway (VEGF) (regulators of placentation through effects on vascular remodeling), are important candidates for investigations of the genetic basis of preeclampsia.[11-17]

The analysis of clinical parameters showed marked difference between preeclamptic and normotensive pregnancies that was correlating with the observed physiology. Besides, a statistically significant elevation in the liver enzymes (AST-54.57 vs. 98.15; ALT-44.42 vs. 79.90 in mild vs. severe preeclampsia respectively), 5 cases of partial HELLP (platelet count <100,000 and increased AST ≥72 IU/l) in severe preeclampsia were also observed. These observations were similar to some of the other studies (Wong et al, 2004), where preeclampsia and partial HELLP syndrome were found to be the commonest cause of raised LFT in the third trimester.[18] In our study low weight babies were associated with PE, the observation very similar to earlier reports where it was found that PE and severe PE increase the risk of IUGR and low birth weight, whereas, gestational hypertension has no such association.[19]

Maternal genotype alone is insufficient to account for Preeclampsia as the disease presents a peculiar challenge with both maternal and fetal genotype contributing to the clinical phenotype. Hence including the fetal genotypes for analysis is important as the role of paternity in preeclampsia (paternal genes being expressed through the fetuses) is an emerging hypothesis.[6,7]

Our analysis of gene variations have found VEGFA polymorphism (rs25648, synonymous codon, upstream variant 2KB, 5' UTR variant) to have a risk allele (C allele) which is prevalent in Preeclampsia mothers. Frequency of rs25648, 'A' allele in Indian Genome variation database for healthy north Indian population range from 0.15 to 0.26.[20] This is in concordance with allele frequency in study control samples (0.19). A central role of VEGF in fetal and placental angiogenesis was suggested from murine gene knockout studies, where it was shown that mice lacking VEGF expression died in

utero due to inadequate vascularization.[12] In human early pregnancy, VEGF is important for proper trophoblast proliferation, and the establishment of enough maternal and fetal circulation.[15] Several polymorphisms have been reported in the VEGFA gene, which include rs699947 and 1570360 (promoter region), rs2010963 and rs25648 (5' untranslated region) which are associated with altered VEGF secretion.[11] Banyasz I et al (2006) proposed that bearers of the VEGF (rs2010963) allele in a Hungarian population have less tendency to PE and while nulliparous pregnant women with VEGF (rs699947) allele could have increase tendency for the development of disease in severe PE.[4]

Analysis of genetic variations of fetuses showed two SNPs with significant difference 1) RENIN (rs11240688) (Chi-square= 9.673, p=0.002) with OR=3.042 (CI is 1.476 to 6.267).[2] AGT (rs11122576) (Chi-square= 4.071, p=0.044) with OR=2.292 (CI is 1.007 to 5.216). OR >1 in both the cases implies that the A and G allele are the disease causing allele for PE. Renin catalyzes the initial step of angiotensinogen pathway, which is a cascade that results in release of aldosterone, vasoconstriction, and increase in blood pressure. In humans, during to pregnancy RAS undergoes major changes. Extra renal release by the ovaries and maternal decidua causes an early increase in rennin.[13]

It is interesting to observe the associations observed here, implicate that fetal REN and AGT genotypes may contribute to the dysregulation of maternal RAAS system and disruption of angiogenesis, thereby evoking the maternal features of PE. It was also interesting to observe that variation in AGT (rs7079) has significant difference in the prevalence of severe preeclampsia as compared to mild group where C allele is risk allele for severe PE. Previously AGT gene polymorphisms (Met235Thr (rs699) and Thr174Met (rs4762)) have been reported to be associated with severe preeclampsia.[21,22] Although the same polymorphisms have not been associated here, AGT polymorphisms play a significant role in the severity of the disease. Allele frequency of each SNP was also analyzed in fetuses of mild and severe PE and a number of variations of ACE, GNB3, ESR2 and SCNN1Bshowed significant differences between the two groups. The study of these genes in more fetal samples of different subtypes of preeclampsia might provide more insights into the contribution of fetal genotypes to the severity of maternal phenotype.

CONCLUSION

The result of the present study showed interesting cross talk between fetal and maternal genotypes especially when a plausible defect in maternal angiogenesis is present. The distribution of allele frequencies of VEGFA and Renin was statistically different in PE mothers and fetuses respectively, as compared to normotensive mothers and their fetuses. The interplay of RAAS and angiogenic pathway along with other interacting partners have to be explored to provide interesting leads to this multi-systemic disease which has a burden on maternal and fetal mortality and morbidity. This is a pilot study which provided some leads in maternal and fetal interplay in preeclampsia. Further studies involving large sample set and multilayered modelling involving different genetic, epigenetic and metabolic profiles are needed to elucidate the maternal-fetal contribution to the disease. It is important to understand the disease pathophysiology and develop markers for early diagnosis and management, more so with the occurrence of shared etiology of preeclampsia and cardiovascular and cerebrovascular events and its impact on public health well beyond the affected pregnancies.

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