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Research paper

Novel association of SNP rs479200 in *EGLN1* gene with predisposition to preeclampsia

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ABSTRACT

Objectives: Placental hypoxia is a hallmark of preeclampsia. SNP rs479200 in the *EGLN1* gene is associated with reduced responsiveness to hypoxia. Whether this translates into an association between SNP rs479200 and preeclampsia is not known. We evaluated the association of SNP rs479200 (T > C) with the risk of preeclampsia.
Methods: This case-control study involved 600 pregnant women of whom 300 were preeclamptic and 300 were normotensive. SNP rs479200 was genotyped by PCR-RFLP method.
Result: Minor allele frequency was 44% in preeclamptic women and 53% in normotensive pregnant women ($P = 1.8 \times 10^{-3}$; odds ratio = 1.43). The odds ratio was heterogeneous when compared after categorization of the preeclamptic group into clinical sub-groups. The association was significant with both mild ($P = 6.2 \times 10^{-5}$) and severe (3.8×10^{-3}) preeclampsia. However, the odds ratio was 0.52 for mild preeclampsia and 1.43 for severe preeclampsia.
Conclusion: The minor allele of SNP rs479200 is associated with the predisposition to preeclampsia. This association underlines the importance of oxygen sensing in the pathogenesis of preeclampsia.

1. Introduction

Preeclampsia (PE) is a pregnancy-related multisystem complication involving new-onset hypertension in a woman without any history of hypertension. PE is a major cause for maternal and perinatal mortality and morbidity. The worldwide prevalence of PE ranges from 2 to 8% with an average of about 5% (Abalos et al., 2013).

PE is a multifactorial disorder involving risk factors like obesity, ethnicity, maternal age, primigravida, environmental factors and genetic variations. Insufficient placental vascularization and shallow trophoblast invasion are the hallmarks of PE. These events arise due to elevated levels of circulating anti-angiogenic molecules soluble like Fms-like tyrosine kinase-1 (sFLT-1) and reduced levels of pro-angiogenic molecules like placental growth factor (PIGF). Placental abnormalities lead to hypoxia and the eventual maternal complications (Roberts and Escudero, 2012).

Hypoxia-inducible factor-1 (HIF-1) is a key transcription factor that regulates the cellular response to hypoxia. HIF-1 is responsible for the regulation of the genes that are involved in the crucial aspects of placental health like angiogenesis, energy metabolism and vasomotor function (Tekin et al., 2010). HIF-1 is a heterodimeric protein comprising of α and β subunits. The β subunit is constitutively expressed while the expression of α subunit is regulated by hypoxia. Under normoxic conditions, HIF-1 α subunit undergoes hydroxylation and rapid degradation through von Hippel-Lindau mediated ubiquitin-proteasome pathway. Hypoxia results in stabilization and rapid accumulation of HIF-1 α subunit (Tekin et al., 2010). HIF-1α is overexpressed in the placenta of preeclamptic women (Tal, 2012). Also, the expression of HIF-1 α subunit shows an inverse correlation with the expression of PIGF in the preeclamptic placenta (Rath et al., 2016).

The hydroxylation of HIF-1 α subunit is catalyzed by the enzyme prolyl hydroxylase domain-containing protein 2 (PHD2). This enzyme thus plays a key role in maintaining the steady-state levels of HIF-1 α subunit by promoting its down-regulation. Changes in PHD2 can, therefore, affect the ability of the cell to respond to hypoxia. PHD2 enzyme is coded by the *EGLN1* gene. Recent studies have linked single nucleotide polymorphism (SNP) rs479200 in the *EGLN1* gene with hypoxia (Aggarwal et al., 2010). The minor allele of SNP rs479200 is linked to the significantly higher expression of PHD2 (Aggarwal et al., 2010). Higher expression of PHD2 is inversely correlated to HIF-1

Abbreviations: bp, base pair; HIF-1, hypoxia-inducible factor-1; PE, preeclampsia; PCR, Polymerase Chain Reaction; RFLP, restriction fragment length polymorphism; PHD2, prolyl hydroxylase domain-containing protein 2; SNP, single nucleotide polymorphism; UV, ultra violet

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activity. Therefore, individuals with genetic variations in the *EGLN1* gene that are linked to the higher expression of PHD2 are expected to perform poorly under hypoxic conditions (Aggarwal et al., 2010).

The preeclamptic placenta is under immense hypoxia stress and therefore any factor that affects the activation of counteractive response would be detrimental to placental health. We hypothesized that SNP rs479200 may be associated with PE since it disrupts responsiveness to hypoxia. However, there is a paucity of literature on the status of this SNP in PE. Therefore this study was undertaken with the aim of evaluating the association between SNP rs479200 (C > T) and PE.

2. Materials and methods

2.1. Study design and participants

We carried out a case-control study by enrolling a total of 300 preeclamptic and 300 normotensive pregnant women. The study subjects were enrolled from the Department of Obstetrics and Gynaecology, R. L. Jalappa Hospital and Research Centre, Kolar, Karnataka, India. The study was approved by the Institutional Ethics Committee of Sri Devaraj Urs Medical College, Kolar, India. Informed consent was obtained from each participant before enrolling into the study. Diagnosis and severity assessment of PE was based on the criteria of The American College of Obstetricians and Gynaecologists (Roberts et al., 2013). Normotensive pregnant women who had no complication till delivery and no history of hypertension were included as the control. SNP rs479200 was genotyped in both the groups and subjected to statistical analysis to measure its association with PE.

2.2. Patient selection

Pregnant women were diagnosed with PE on the basis of the criteria listed in Supplementary Table 1 (Li et al., 2018). PE was classified as severe on the presence of one of the following features: systolic pressure ≥ 160 mm Hg or diastolic pressure ≥ 110 mm Hg on two occasions at least 4 h apart, thrombocytopenia, elevated liver enzyme level, pulmonary edema, renal insufficiency, new-onset cerebral or visual disturbance. In the absence of these feature, PE was classified as mild.

2.3. Genotyping of SNP rs479200

Genomic DNA was prepared from peripheral blood samples by using the salting out method (Mishra et al., 2013). Purity and concentration of the genomic DNA preparation were determined by UV spectrophotometry (Perkin Elmer model Lambda 35, Waltham, USA). PCR was set-up with the primers: 5′ CTC CCA GCA CAT CTG TGA AT 3′ and 5′ CAT GCT GAC CTG GGC TAT T 3′. 20 µl reaction mixture included 1 × assay buffer, 100 ng genomic DNA, 0.2 mM dNTP, 10 pmol of each primer, 1.5 mM $MgCl_2$ and 1 unit *Taq* DNA polymerase (Bangalore Genei, Bengaluru, India). The program comprised of an initial denaturation at 95 °C for 3 min followed by 28 cycles at 95 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min; final extension involved 7 min at 72 °C. The PCR product was analyzed on 1% Agarose gel. The 501 bp amplicon was subjected to restriction digestion with 5 units of *Bsr*GI (New England BioLabs, Ipswich, USA) at 37 °C for 8 h and analyzed on 2% agarose gel with ethidium bromide staining (Supplementary Fig. 1). T allele was visible as an uncut 501 bp fragment while the C allele is cleaved to produce a 270 bp and 231 bp fragments. A Sanger sequenced sample with CC genotype was used as positive control.

2.4. Statistical analysis

Statistical analysis was carried out by using the OpenEpi web-tool (Dean et al., n.d.). Differences in allele and genotype distribution between the groups were compared by calculating P-value from Fisher's exact test. A P-value < 0.05 was considered as significant. The study population was tested for conformity to Hardy-Weinberg Equilibrium using the web-tool 'Simple Hardy-Weinberg Calculator' (Rodriguez et al., 2009).

Table 1
Clinical profile of the study participants.

Parameter	Preeclamptic pregnant women (n = 300)	Normotensive pregnant women (n = 300)
Age (years)	24.7 ± 3.6	24.5 ± 3.3
Gravida		
Primigravida	53.3%	39%
Multigravida	46.7%	61%
Gestation (weeks)	35.5 ± 4.1	37.8 ± 2.9
Severity		
Mild	118 (39.3%)	NA
Severe	182 (60.6%)	
Blood pressure (SBP/DBP; mm Hg)		
Mild PE	153.9 ± 22/105.0 ± 53	120.5 ± 10/78.8 ± 8
Severe PE	174.0 ± 14/113.0 ± 8	
Proteinuria		
Mild PE	+1 (73), +2 (45)	NA
Severe PE	+3 (134), +4 (48)	
Comorbidity		
Eclampsia	59 (20%)	NA
IUGR	29 (9.6%)	
Still birth	25 (8.3%)	
HELLP syndrome	8 (2.6%)	

SBP: systolic blood pressure; DBP: diastolic blood pressure; IUGR: intrauterine growth restriction; HELLP: hemolysis, elevated liver enzymes and low platelet count.

3. Results

The clinical and demographic details of the study participants are given in Table 1. The distribution of the alleles and the genotypes of SNP rs479200 C > T among preeclamptic and normotensive pregnant women is shown in Table 2. The genotype frequencies in the control group were in conformity with the Hardy-Weinberg equilibrium ($\chi^2 = 2.43$). The homozygous major allele genotype (CC) was 2.1 times higher in the normotensive pregnant. The heterozygous genotype (CT) and the homozygous minor allele genotypes (TT) were 1.3 and 1.1 times higher in the preeclamptic women. The genotype frequencies of the preeclamptic and normotensive pregnant groups were significantly different ($P = 9.8 \times 10^{-6}$).

The frequency of the minor allele (T) was higher in preeclamptic women (56%) than in the normotensive women (47%). The difference in the distribution of the alleles between the two groups was statistically significant ($P = 1.8 \times 10^{-3}$; odds ratio 1.43). The distribution of the alleles was also analyzed after stratification of the preeclamptic women in terms of severity and the results are summarized in Table 3.

Table 2
Profile of SNP rs479200 in the study groups.

Genotype/ allele	Preeclamptic pregnant women (n = 300)	Normotensive pregnant women[a] (n = 300)	P-value[b]	OR (0.95 CI)[c]
CC	43 (14.3%)	91 (30.3%)	9.8×10^{-6}*	NA
CT	178 (59.3%)	136 (45.3%)		
TT	79 (26.3%)	73 (24.3%)		
C	264 (44.0%)	318 (53.0%)	1.8×10^{-3}*	1.43 (1.14–1.80)
T	336 (56.0%)	282 (47.0%)		

[a] Hardy Weinberg Equilibrium: $\chi^2 = 2.43$.
[b] Chi-square test (two-tailed).
[c] OR: odds ratio; CI: confidence interval; NA: not applicable.
* Significant (P-value < 0.05).

Table 3
Effect of PE severity on the association with SNP rs479200.

Comparative groups	P-value[c]	Odd's ratio
No. PE[a] vs. PE_{mild}[b]	6.2×10^{-5}	0.52 (0.38–0.72)
No. PE vs. PE_{severe}	3.8×10^{-3}	1.47 (1.13–1.90)
No. PE vs. $PE_{overall}$	1.8×10^{-3}	1.43 (1.14–1.80)

[a] Normotensive pregnant women.
[b] Preeclamptic pregnant women.
[c] Two-tailed.

Statistically significant difference was found with both mild and severe subgroups. The odds ratio showed considerable variation between the two subgroups. The odds ratio was < 1 in the case of mild PE and > 1 in the case of severe PE.

4. Discussion

The purpose of this study was to evaluate the association of SNP rs479200 with the predisposition to of PE. The main findings of this study are: (i) the frequency of the minor allele T was significantly higher in PE women indicating that SNP rs479200 is associated with the predisposition to PE and (ii) PE severity affected the association in a heterogeneous manner.

The minor allele T is linked to the higher expression of PHD2, therefore, lower HIF-1 activity and risk of hypoxia. As a consequence, the frequency of the minor allele T would be expected to be higher in the preeclamptic women. In contrast, the frequency of the major allele would be expected to be higher in the normotensive women (Fig. 1). In agreement with this, the minor allele T was found to be a risk factor for the development of PE.

The heterogeneity in the direction of association with respect to the severity of PE is a noteworthy aspect of this study. The discrepancy between the subgroups was perplexing. However, we found several examples of heterogeneity caused by clinical sub-phenotypes. Gender affects association between *RNF212* variant and the genome-wide recombination rate, age of onset affects association between polygenic risk score and Crohn's disease, organ of disease manifestation affects association between 11q, 5q, and 2q33 markers and gluten sensitivity (Kong et al., 2008; Li et al., 2018; Holopainen et al., 2001). This indicates that multifactorial diseases commonly show wide phenotypic variation; and, that phenotypically defined subgroups represent unique genetic architectures with disease-associated variants having different effect sizes and direction in the subgroups (Wen and Stephens, 2014; Liley et al., 2017).

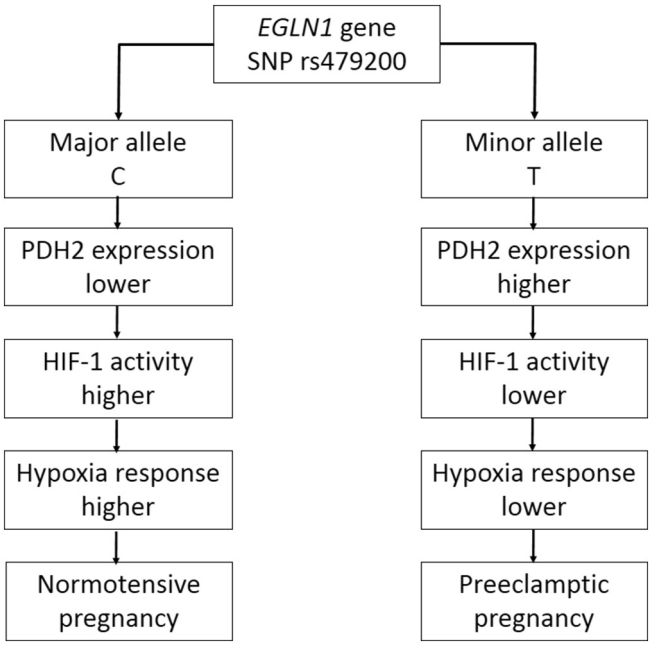

Fig. 1. Mechanism of action of SNP rs479200.

The heterogeneity in the nature of association observed in this study agrees with the heterogeneous nature of PE. Bioinformatics analysis indicates that PE represents a collection of several distinct phenotypes, with both discrete and overlapping genetic contributions (Triche et al., 2014). Genetic variants associated with PE have been noticed to segregate with the clinical variables like severity, superposition by eclampsia and stillbirth. For example, heterogeneity in the genetic association of PE has been noticed in the case of the 4q25 variant near *PITX2* gene (SNP rs2200733). The association was higher when PE was accompanied by stillbirth and lower when superposed by eclampsia (Rani et al., 2018). The heterogeneity may represent a fundamental difference in the causal determinants of mild and severe PE. The positive association between the variant linked to inadequate responsiveness to hypoxia and severe PE indicates a causal role for reduced oxygen availability at the feto-placental interface. In contrast, hypoxia may not be a causal determinant in mild PE. Hypoxia response involves upregulation of angiogenic factors for neovascularization and also increased endothelial permeability for increased blood flow to the intervillous space (Eskild et al., 2016). The latter process is also supported by hypertension (Eskild et al., 2016). If hypoxia is not the primary causal determinant in mild PE, then, promoting maternal endothelial injury without collateral benefit to the fetus would be detrimental to maternal health. Thus, factors linked to reduced responsiveness to hypoxia would assume a protective role in mild PE. If our argument is correct, then hypoxia is an important cause of PE but only in the severe group. Furthermore, the opposing association at the subgroup level is a result of maternal-fetal tradeoff.

SNP rs479200 has been linked to the lack of responsiveness to hypoxia. It is associated with high altitude adaptation in the Indian population (Aggarwal et al., 2010). The major allele C was significantly higher in the Tibeto-Burman sub-population residing at an altitude 3500 m above sea level than in other members of the same genetic cluster but residing at lower altitudes. In addition, the minor allele T is associated with an increased risk of high altitude pulmonary edema when the lowland residents undertake highland sojourn (Aggarwal et al., 2010). Also, SNP rs479200 correlates with the expression levels of PHD2 and decreased arterial oxygen saturation (Aggarwal et al., 2010; Mishra et al., 2013). This study adds to the spectrum of phenotypic manifestation of SNP rs479200.

The enormous public health burden of PE warrant detailed understanding of its pathophysiology. However, very little is known in this direction and PE continues to be labeled as a disease of theories. The transient occurrence during pregnancy and also ethical concerns of an uncontrollable complication prevents undertaking physiological and interventional studies. In this light, genetic association studies provide a means to probe potential pathways and critical modulators by examining risk variants. Both candidate gene studies and GWAS have been undertaken with this intention. Candidate gene studies have linked PE with coagulation and hypertension pathways (Staines-Urias et al., 2012). GWAS, which is not biased by the selection of candidate genes on the basis of pre-existing knowledge has linked PE with 2q14 locus, *PSG11* and *FLT1* genes (Johnson et al., 2012; Zhao et al., 2012; McGinnis et al., 2017; Gray et al., 2018). The existing candidate gene studies are limited by the inadequate emphasis for the role of genetic determinants of angiogenic factors that have emerged as potential causal factors in PE (Staines-Urias et al., 2012). This study assumes importance in this context since hypoxia is an important inducer of angiogenesis (Eskild et al., 2016).

There are some limitations to this study. Firstly, all the patients were recruited from a single center. Furthermore, there were more patients with severe than mild PE though the incidence of the former is

relatively lower. The site of patient recruited was a tertiary care hospital and involved both antenatal registrants and late referrals from smaller centers upon the manifestation of the complications. Therefore, the study group may not be a complete sample of the PE population. Replication studies preferably in other nationalities are necessary to validate the association at the global level. Secondly, we chose only one SNP for the study. Since several SNPs contribute incrementally to a multifactorial disease like PE, complete sequencing of *EGLN1* gene along with other genes in the hypoxia response pathway may be necessary to obtain a complete picture of hypoxia genetics in PE.

5. Conclusions

The results presented herein show that SNP rs479200 is associated with the predisposition to PE and that the association is modified by the severity. This genetic association underlines the importance of oxygen sensing in the hypoxia response pathway in the pathogenesis of PE.

CRediT authorship contribution statement

Praveen Kumar K. S.: Validation, Formal analysis, Investigation, Data curation, Visualization. **Madhuri Arcot:** Formal analysis, Investigation, Writing - original draft, Visualization. **Munikrishna Munisamaiah:** Resources. **Sharath Balakrishna:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Disclosure statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2019.04.049.

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