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RESEARCH ARTICLE

A STUDY ON EVALUATION OF PHOSPHOLIPASE A_2 GENE POLYMORPHISM AND THE EXPRESSION IN TERMS OF PHOSPHOLIPASE A_2 ENZYME IN PREECLAMPSIA

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ABSTRACT

Aim: Our purpose was to know phospholipase A_2 activity and its gene $PLA_2G_4\alpha$ polymorphism was an inflammatory marker in preeclampsia compared with normotensive pregnancies. Efforts were made to determine the association of PLA_2 and its gene.
Study Design: Longitudinal observational study
Methods: 4ml of blood was obtained from preeclampsia (n=51) and normotensive pregnants (n=51). They were maternal age, gestational age, parity matched. Phospholipase A_2 activity was determined by colorimetric method and $PLA_2G_4\alpha$ polymorphism was evaluated by PCR-RFLP techniques.
Results: When HW equation is applied to this observation of controls population (p=0.66) and in preeclampsia cases (p=0.76) it states that allele and genotype frequencies of $PLA_2G_4\alpha$ gene in a population will remain constant. Phospholipase A_2 enzyme activity in control population was 1.80units/mg with SD ±0.225, in preeclampsia was 2.47 unit/mg with SD ±0.0112 with significance of P<0.001.
Conclusion: Results indicated that there was no polymorphism in the $PLA_2G_4\alpha$ gene structure, but the difference in gene expression in comparison of two groups was marked in terms of measuring the enzyme activity with p value (p<0.001). PLA_2 can be used to understand the intensity of inflammation in preeclampsia. However, further studies are necessary to evaluate PLA_2 activity in each trimester of preeclampsia, eclampsia and placental contribution to any PLA activity.

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INTRODUCTION

Preeclampsia is a pregnancy syndrome characterized by hypertension, proteinuria, and oedema after 20 weeks of pregnancy (Ghulmiyyah *et al.,* 2011). The symptoms of Preeclampsia can range from mild to severe due to slow or rapid progress of disease condition. They include persistent headache, blurred vision, Vomiting and abdominal pain (Roberts *et al.,* 1993). The complications of preeclampsia lead to foetal uterine growth restriction, preterm delivery, Inflammation maternal and foetal morbidity and mortality (Sibai, 2008; Cindrova *et al.,* 2009). It is a multisystem disorder, clearly shows the involvement of utero-placental blood flow, vascular resistance, endothelial integrity, endothelial damage, and coagulation system in preeclampsia. Potential causes and mechanisms behind preeclampsia remain unknown, but the involvement of maternal, immune, genetic factors and placenta have been implicated (Price, 2007) Preeclampsia as leading cause of hypertension results in complication up to 10% pregnancies. Preeclampsia and eclampsia account for 24% of all maternal deaths in India (Henderson *et al.,* 2004). In the developing and developed countries, approximately 800 women die from pregnancy and child birth related complications around the world every day (World Health Organization, 2012). Early detection and management helps in reducing the complications of preeclampsia. Despite its prevalence, pathophysiology is poorly understood and etiology has to be elucidated. Both hypertension and preeclampsia (PE) are considered as inflammatory diseases, the role of phospholipase A_2 (PLA_2) is evident as mediator in inflammatory process by virtue of releasing cell signaling molecule arachidonic acid. During pregnancy, prostanoids production increases in both maternal and fetal placental tissues. Prostacyclin is produced by the vascular endothelium as well as in the renal cortex. It is a potent vasodilator and inhibitor of platelet aggregation. Thromboxane A_2 (TXA_2) is produced by the platelets and trophoblasts. It is a potent vasoconstrictor and platelet

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aggregator. These eicosanoids are the products of Arachidonic acid metabolism hence; these eicosanoids mainly prostacyclin and thromboxane have opposite effects and play a major role in regulating vascular tone and vascular blood flow as shown in Figure 10. An imbalance in prostanoids production or catabolism has been suggested as being responsible for the pathophysiological changes in preeclampsia. However, the precise role by which prostaglandins is involved in the etiology of preeclampsia remains unclear (Sibai *et al.,* 2005).

MATERIALS AND METHODS

The present study design is an observational longitudinal study comprised of preeclampsia women as a case group and normal pregnant woman as a control group those are visiting to R.L. Jalappa Hospital at Dept. of OBG, Kolar. Inclusion criteria of the present study were Primigravida, Multiple pregnancies. Age-18-45, preeclampsia subjects and the exclusion criteria were Eclampsia, Diabetes Mellitus, Hydatidiform mole, Chronic hypertension, Pregnant woman less than 20^{th} week in the period of 6months (April to September 2015). Four ml of blood was collected using EDTA vacutainer from the preeclampsia patients and normal healthy pregnant women after obtaining the patient information sheet and consent form. Samples were transferred in to clean dry sterile centrifuge tubes, centrifuged at 3000 rpm for 10 minutes to obtain the clear plasma. The plasma was separated and stored at -80^0C until analysis.

Molecular analysis

Extraction of Genomic DNA: Blood samples were collected from preeclampsia subjects and the control subjects into sterile EDTA vacutainer and were stored at 4°C until processing. Genomic DNA was isolated from the peripheral blood as per the standard procedure (Jivraj *et al.,* 2006). That consists of erythrocyte lysis by Erythrocyte Lysis Buffer (ELB) till a white pellet free of heme was obtained. Leukocytes /lymphocytes lysis carried out by suspending white pellet in ELB and on mixing with 270µl of 20% SDS and 30µl of proteinase K (10mg/ml) were added and mixed. Samples were incubated at 37^0C, in water bath for overnight. After 16 h of incubation, DNA was precipitated by isopropyl alcohol. The total precipitated DNA appeared as a white thread like structure was completely transferred to a sterile micro centrifuge tube containing 500µl of 80% alcohol and incubated at room temperature for 15 min. It was centrifuged at 12000 rpm for 5 min. The supernatant obtained was discarded and this step was repeated for three times to obtain purified form of DNA. The DNA was then air dried and dissolved in 500µl of Tris-EDTA buffer and then incubated at 65^0C for 30 min. The dissolved fraction was refrigerated at 4^0C for one day and stored at -20^0C until use.

DNA Quantification by Agarose Gel Electrophoresis: The quantity of the DNA samples was checked in a 1% agarose gel. The solution was allowed to become lukewarm and 0.1mg/ml ethidium bromide was added. The gel was then poured on a gel-casting tray and allowed to solidify. The gel was placed in an electrophoresis tank with 1X TAE buffer. The DNA samples were mixed with 6X DNA loading dye and loaded on the gel. The gel was electrophoresed at 2 volts/cm and images were captured in a gel documentation system (Bio Rad Gel Doc) (Rousseau *et al.,* 1994).

DNA purity - Spectrophotometric method: The quality and quantity of the DNA samples were assessed by spectrophotometric method by using Perkin Elmer Lambda 35 model. 50µl of TE buffer was pipette into quartz cuvette and subjected for auto zero correction. 48µl of TE buffer and 2 µl of DNA sample were added in quartz cuvette, the absorbance was measured at 260 and 280nm. The absorbance at 260nm gives DNA concentration and the ratio between 260/280 gives the purity of DNA. DNA sample with 260/280 absorbance ratio between 1.7-1.9 were considered for PCR procedure. However, DNA samples with absorbance ratio less than 1.7 were subjected for precipitation until the desired absorbance was obtained. Then after, DNA sample of expected purity was used for PCR procedure.

Genotype Screening

PCR-RFLP –Based Screening: PCR amplification of the PLA_2G_4A gene was amplified by polymerase chain reaction using primers as shown below.

- Forward: 5'- AAGGGATATTTGTAGAGGACT-3'
- Reverse: 5'- TAGATGATTCGATTTTATGACT-3'

Sequence of primers, PCR product size, restriction enzymes and size of digested fragments that are used for screening by PCR-RFLP

PCR was carried out on 50µl volume, in an Eppendorf thermal cycler and subjected to standardized PCR. Initial denaturation at 95^0C for 5 min followed by 35 cycles of denaturation at 95^0C for 1 min; annealing at 61^0C (PLA_2G_4A) and extension at 72^0C for one minute and final extension at 72^0C for seven minutes were performed. PCR Amplification was confirmed by 2% agarose gel electrophoresis. 100bp DNA molecular weight marker was used to confirm the amplicon size. Electrophoresis was carried out at 80V for one hour and the gel was visualized in the gel documentation system. Finally, the amplified products were digested with 10U Ban I-SNP restriction enzyme and will be performed by using agarose gel electrophoresis and visualized in the Gel Doc System (Peet, *et al.,* 1998)

Biochemical analysis: PLA_2 Levels will be measured by colorimetric method described by Price JA 2007. Practical colorimetric assay for PLA2 was done where the acids generated created the measured absorbance change. The substrate (phosphatidyl choline) allows maximal detection of many PLA2. Yet, by using a buffer–dye system at pH 8.2. The absorbance of yellow color produced by acid-bromothymol blue complex measured at 620nm in spectrophotometer.

Statistical Analysis: Statistical analysis will be performed by using Mean, percentage, student- t test and its level of significance presented by p-value. $P \geq 0.05$ is considered to be statistically significant.

RESULTS

In the present case-control study, to measure PLA_2 levels in plasma and genotypic pattern of $PLA_2G_4\alpha$ the following criteria have been taken into consideration during the collection of blood samples. The criteria are Mean age (23.8±3.6years vs. 23.9±3.7years), Parity (0 vs. 0) and Gestational age (36.8±3.5weeks vs. 40.8±11.7 weeks). Mean

systolic (149.8±18.1 vs. 116.2±9.9mm Hg) and diastolic blood pressure (101.76±11.08 vs. 86.47±11.10 mm Hg), as shown in Table 3. PLA_2 genotypic pattern of $PLA_2G_4\alpha$ and Plasma phospholipase A_2 enzyme activity were determined between normotensive Pregnants and in preeclampsia. The PCR amplified product on agarose gel electrophoresis clearly represents the molecular size of 788bp in control and cases. Whereas the RFLP band patterns of PCR amplified product of $PLA_2G_4\alpha$ gene indicated homozygous A1/A1 allele uncut, A1/A2 heterozygous allele with three cuts and A2/A2 homozygous allele with two cuts. The RFLP band patterns were compared between control and cases was shown in Figure 1. Out of 51 normotensive pregnant women, homozygous genotype allele A1/A1 was observed as 47.1% and A2/A2 was observed as 13.7% whereas heterozygous genotype allele A1/A2 was 39.2% are depicted in Table 1. Similarly, out of 51 preeclamptic cases, homozygous genotype allele was A1/A1 was observed as 60.8%, heterozygous genotype A1/A2 was 31.4%, homozygous genotype allele was A2/A2 was 7.8% as presented in Table 2. When Hardy Weinberg equation is applied to this observations in controls (p=0.66) and in preeclampsia cases (p=0.76) it states that allele and genotype frequencies of $PLA_2G_4\alpha$ gene in a population will remain constant. These Genotype distributions in control group expressed the phospholipase A_2 enzyme activity was 1.80units/mg with SD ±0.225. The similar genotypic distribution without showing any polymorphism and the expression of phospholipase A_2 activity was 2.47 unit/mg with SD ±0.0112 with significance of P<0.001 as shown in Table 4.

Table 3. Distribution of Age group, Gestational age weeks and Blood pressure of Normal and Preeclampsia women

Age group				
	Cases		Controls	
Mean	23.86		23.96	
Standard Deviation	3.61		3.79	
Gestational age week				
	Cases		Controls	
Mean	36.80		40.87	
Standard Deviation	3.59		11.77	
Blood group				
	Controls		Cases	
	Systolic	Diastolic	Systolic	Diastolic
Mean	116.27	86.47	149.80	101.77
Standard Deviation	9.10	11.10	18.16	11.08

Table 4. Group Statistics of Phospholipase A_2 activity in Normotensive Pregnant Women

PLA_2 Assay				
Group	No. of subjects	Mean (Units/mg)	Standard Deviation	P-value
Cases	51	2.467	0.01125	<0.001*
Controls	51	1.80	0.22518	

* Statistically significant

DISCUSSION

Etiology of preeclampsia is complex that involves multiple genes in addition to other exogenous environmental factors. There are limited reports on the relation between phospholipase A_2 and its gene polymorphism in preeclampsia.

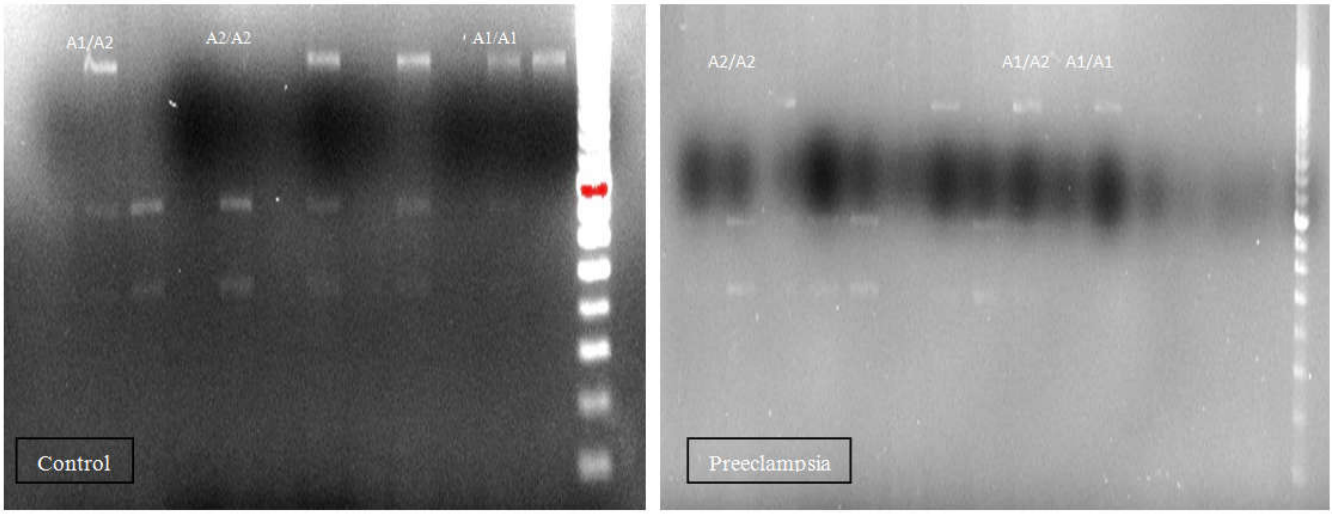

Figure 1. Representing pattern of RFLP product on agarose gel in control and preeclampsia cases. Control showing Uncut (A1/A1), 2 cut (A2/A2), 3cut (A1/A2) for $PLA_2G_4\alpha$ gene alleles. Preeclampsia RFLP pattern resembles same as control

Table 1. Phospholipase A_2 Allele Frequencies in Normotensive Pregnant Women

Allele	Frequency	Percentage%
A1/A1	24	47.1%
A1/A2	20	39.2%
A2/A2	7	13.7%
Total	51	100.0%

Table 2. Phospholipase A2 Allele Frequencies in Preeclamptic Women

Allele	Frequency	Percentage
A1/A1	31	60.8
A1/A2	16	31.4
A2/A2	4	7.8
Total	51	100.0

Therefore the primary objective of this study was to observe the plasma PLA_2 enzyme levels and PLA_2 gene alteration. We aimed at observing polymorphism of $PLA_2G_4\alpha$ gene levels in preeclampsia and normal pregnant women. Preeclampsia is associated with change in prostacyclin, thromboxane ratio, and Phospholipase A_2. In the present study we observed significantly higher activity of phospholipase A_2 in preeclampsia when compared to normotensive pregnant. But Kee- Hak Lim et al. reported there was no significant difference in PLA_2 activity in preeclampsia when compared to normotensive pregnant (Lim et al., 1995). There is an association between an increased plasma free fatty acid and impairment in vasodilation by altering phospholipid turn over (Hoegh *et al.,* 2006). Even though the activity of

phospholipase A_2 has been measured in many disease conditions, the information available is limited about plasma phospholipase A_2 activity and its relation with the Gene polymorphism in normotensive and preeclampsia groups. In current study, we reported that there is no association with gene polymorphism but the significant elevation of Phospholipase A_2 enzyme is observed in preeclampsia explained the possible under lying causes of plasma phospholipase A_2 as an inflammatory marker in preeclampsia.

Conclusion

The study objectives were tested with experimental evidence, and Results of this study conclude that the $PLA_2G_4\alpha$ gene polymorphism is not observed in preeclampsia compared to normotensive group. The assay of phospholipase A_2 enzyme activity (a product of $PLA_2G_4\alpha$ gene) is hypothesized to be expressed as an inflammatory marker in preeclampsia. Further studies are needed to study the factors that influence the transcription of $PLA_2G_4\alpha$ gene in preeclampsia.

Summary

Preeclampsia is a pregnancy related complication characterized by hypertension, proteinuria, and edema after 20 weeks of gestation. In India, 8-10% of the pregnancies are reported to be affected by preeclampsia. Phospholipase A_2 activity and its gene $PLA_2G_4\alpha$ polymorphism present if any, (as an inflammatory marker) needs to be elucidated in preeclampsia, therefore this concept became the main objective of the current study. Efforts were made to determine the association of PLA_2 and its gene. The study was conducted in preeclampsia (n=51) and normotensive pregnant women (n=51). $PLA_2G_4\alpha$ polymorphism was evaluated by advanced molecular biology technique PCR-RFLP using appropriate primer. However, PLA_2 activity in plasma was determined by colorimetric method. This study results indicate that there was no polymorphism in the $PLA_2G_4\alpha$ gene structure, but the difference in expression of the gene in preeclampsia compared to non-preeclampsia is evident in terms of the measurement of the enzyme activity with p value ($p<0.001$) between control and cases groups. PLA_2 can be used to understand the intensity of inflammation in preeclampsia. However further studies are necessary to evaluate PLA_2 activity in each trimester of preeclampsia, eclampsia and placental contribution to any PLA_2 activity.

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