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Original Article

Genetics Section

Maternal Factor V Leiden Mutation in Pre-eclampsia: A Case-Control South Eastern Indian Tertiary Care Hospital Based Study

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ABSTRACT

Introduction: Pre-Eclampsia (PE) is a pregnancy-specific disorder which further complicates and leads to eclampsia. The Factor V Leiden (FVL) is an autosomal dominant genetic abnormality with incomplete penetrance predisposes to thrombosis. It codes for Factor V, as it is a missense mutation where arginine is replaced by glutamine. The FVL is a heterozygous condition which has risk of complicated pregnancy outcomes.

Aim: To find out association between the Factor V Leiden Mutation (FVLM) and PE.

Materials and Methods: The study was designed as case-control, where 150 PE gravid women were cases and 150 healthy normotensive gravid women were controls enrolled from the Department of Obstetrics and Gynaecology in RL Jalappa Hospital and Research Centre, Tamaka, Kolar, Karnataka, India. The methodology for maternal FVLM adopted was, isolation of the Deoxyribo Nucleic Acid (DNA) by using salting-out method followed by Polymerase Chain Reaction and Restricted Fragment Length Polymorphism (PCR-RFLP) with MNL1 enzyme. On digestion FVL allele was visible as an uncut 268 base pair fragment with PCR while the Leiden was cleaved to produce 163 and 67 base pair fragments (wild type). The 37 base pair fragment was not visible on the gel due to its small size. Homozygous Leiden mutation produces two bands corresponding to 200 base pair and 67 base pair (homozygous Leiden) for heterozygous Leiden Mutation four bands corresponding to 200 base pair, 163 base pair, 67 base pair, 37 base pair (heterozygous/Wild Type/mutant Leiden). Statistical analysis was done by using the SPSS software 13. The difference in frequency between two groups was not statistically significant.

Results: Frequency of the leiden variant was 5.3% among cases and 6.7% in the control groups. Leiden variant of factor V in homozygous condition was not found in either of the study groups.

Conclusion: FVLM is not a significant marker for PE in the Kolar population.

Keywords: Blood coagulation, Missense, Mutation, Pregnancy

INTRODUCTION

PE is a pregnancy complicated hypertensive disorder which usually occurs before eclampsia [1,2]. PE is a syndrome characterised by an increase in Blood Pressure (BP) 140/90 mmHg, oedema, proteinuria 300 mg/L for 24 hours of the urinary collection which occurs after the 20th week of gestation and often complicated by renal failure and coagulopathy [2]. The global prevalence of PE is 2-8% [3]. In India, its incidence is about 28.7%, whereas in the southern India particularly in Karnataka and Andhra Pradesh, it is 19.8%, 21.0%, respectively [4]. It is a foremost obstetric confront as it majorly contributes to maternal and perinatal mortality and morbidity by complicating the pregnancy with seizures, increase in BP further complicates to eclampsia and death [1]. However, it affects even the growth of foetus by causing intra uterine growth retardation, foetal insufficiency, intra uterine death. At present, the only available therapeutic option is the removal of placenta [5].

PE is a complex genetic disorder, follows the autosomal dominant pattern of Mendelian inheritance. PE occurs as a result of numerous common variants at different loci which individually have small effects collectively add to an individual's susceptibility. By previous studies, it is evident that no single cause or genetic variant will account for all the cases of PE [6]. For the development of PE apart from genetic risk factors like genotypes of mother and foetus individually and combined, a heterozygous condition in mother or family, family history of hypertension and PE. Other factors like endothelial dysfunction, inflammation, and coagulopathy will have combined effect [7]. However, the different variants and candidate gene approaches are associated with various subsets of disease conditions [6]. Overall, 70 biological candidate, genes were examined. The list of genes is as follows:

Vasoactive proteins: Angiotensinogen, Angiotensin-converting enzyme.

Thrombophilia and hypofibrinolysis: FVL, Methylene Tetra Hydro Folate Reductase, Prothrombin, and Plasminogen Activator Inhibitor-I.

Oxidative stress and lipid metabolism: Apolipoprotein E, Microsomal epoxide hydrolase, glutathione S-transferase.

Endothelial injury: Vascular Endothelial Growth Factor Receptor-1, Vascular Endothelial Growth Factor.

Immunogenetics: Tumour necrosis factor α, Interleukin 10 [6].

FVLM of Thrombophilia is an autosomal dominant genetic condition that exhibits incomplete variable penetrance [8]. FVLM is a gene for clotting Factor V. In coagulation cascade Factor V is converted to Factor Va in presence of active protein C. Due to FVLM there will be resistance in acquired protein C. This leads to thrombophilic condition in veins and spiral arteries. As pregnancy itself is a stressful condition, FVLM increases the risk of clotting [8]. As in PE placental ischemia and endothelial damage occur and reduces the uteroplacental perfusion. This alters the uteroplacental vasculature like an incomplete trophoblastic invasion of spiral arteries. This leads to an imbalance in haemostasis system [7]. For FVLM with thrombophilia is an added risk for PE. The changes like endothelial damage, uteroplacental insufficiency were initiated during placentation or due to any pre-existing factors are still imprecise. As some changes are evident in PE after the 20th week of gestation, but some pre-existing factors like thrombophilia, abnormality in blood clotting proteins these factors are there before pregnancy or it is expressing during PE pregnancy is left over as indistinct [9]. FVLM in thrombophilia was observed 1 in 1000 of the homozygous condition [10] and heterozygous condition is 1 in 500 [11]. There was a paucity of data regarding the study

of FVLM and PE due to inconsistency in results, especially in the southern part of India. This is the second study in the south eastern part of India and the first study in Karnataka, India. So this makes us find out the fact of whether there was an association between FVLM and PE in Kolar population. The study aim was to find out the association between FVLM in PE (Kolar population).

MATERIALS AND METHODS

Study Design and Participants

In this prospective case-control study 300 subjects, (150 in each group) were enrolled from the Department of Obstetrics and Gynaecology from August 2014 to July 2015 in RL Jalappa Hospital attached to Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka, India. The sample size was calculated by using Open Epiweb tool www.OpenEpi.com with 95% confidence interval and 80% power, (updated and accessed April 04, 2013, May 23, 2016). The diagnostic criteria of PE by Department of Obstetrics and Gynaecology in RL Jalappa Hospital and Research Centre was based on American College of Obstetrics and Gynaecologist is as follows [12]:

1. Systolic blood pressure of ≥140 and160 mmHg; and
2. Diastolic blood pressure of ≥90 and110 mm Hg on 2 occasions with 2/hours, 2 weeks of gap after 20th week of gestation;
3. Proteinuria with ≥300 mg per 24 hours of urine collection or protein/creatinine ratio of ≥0.3 on dip stick reading is +1;
4. In absence of proteinuria thrombocytopenia with a platelet count of ≤100000/ microliter;
5. Renal insufficiency (Serum creatinine ≥1.1 mg/dL/doubling concentration without any renal disorders);
6. Impaired liver functions (double the values of the normal range of liver transaminases);
7. Pulmonary oedema, cerebral or visual disturbances.

Inclusion Criteria

Both primigravida and multigravida in the age group of 18-45 years with clinically diagnosed as PE.

Exclusion Criteria

Pregnant women with eclampsia, chronic hypertension, and a pregnant woman less than 20 weeks of gestation.

Control Group

Normotensive pregnant women who had no complication till delivery, no history of hypertension, PE or Eclampsia.

Ethics: After approval from the institutional research ethical committee clearance with number: No.DMC/KLR/IEC-CEC/167/2015-2016 the study was initiated. Informed consent was obtained from each participant before enrolment.

Sample collection and DNA isolation: About 3 mL of peripheral venous blood was collected in EDTA vacutainer and stored at 4°C till further analysis. DNA was isolated from the peripheral blood lymphocytes using salting-out method [13]. The quality of sample was determined by UV spectrophotometry (Perkin Elmer model Lambda 35, USA).

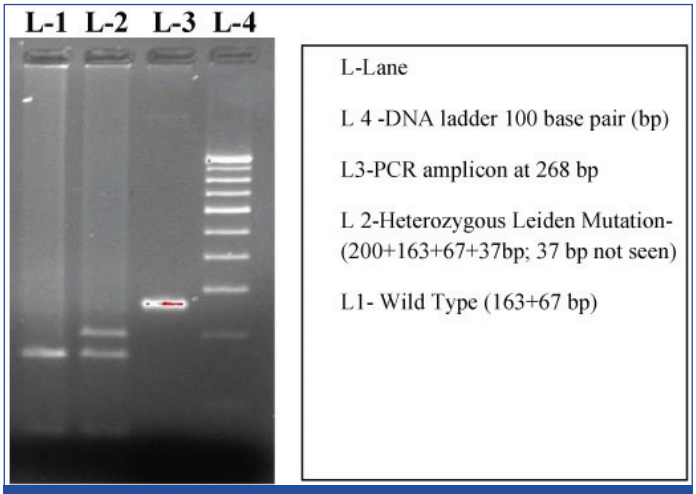
Genotyping of FVL: FVLM gene polymorphism with forward primers 5'TGCCCAGTGCTTA ACAAGACCA3" and reverse primers are 5' TGTTAT CACACTG GTGCTAA 3" (Bangalore Genei, India) explained in [Table/Fig-1]. Genomic DNA was amplified by PCR [14] on Bio-Rad C1000 Touch Thermal Cycler. The polymorphic region was amplified with primer pairs explained in [Table/Fig-1]. The 20 µL reaction mixture included 1x assay buffer, 100 ng genomic DNA, 0.2 mMdNTP, 1 picomole of each primer, 2.5 mM $MgCl_2$ and 1 unit Taq DNA polymerase (Bangalore Genei, India). The program comprised of an initial denaturation at 95°C for 3 minutes followed by 35 cycles at 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds; final extension involved 10 minutes at 72°C. The 268 bp amplicon was subjected to restriction digestion with 5 units of Mnl I (New England Biolabs, USA) at 37°C for 3 hours and analysed on 3% agarose gel with ethidium bromide staining. On digestion of the FVL allele, PCR products with heterozygous, homozygous Leiden mutation, wild type was explained in [Table/Fig-2]. The diagrammatic representation of PCR products and RFLP with heterozygous Leiden mutation, wild type was explained in [Table/Fig-3].

Gene	Chromosome location	Nucleotide	Aminoacid substitution	SNP/rs no	Forward primers	Reverse primers	RFLP enzyme
Factor V	1q24.2	1691	Arginine to glutamine at 506	Exon 10/rs 6020	5'TGCCCAGTGCTTAACAAGACCA3"	5'TGTTATCACACTGGTGCTAA3"	MNL1 37°C 3 hours

[Table/Fig-1]: Representing Factor V Gene polymorphism, location on chromosome, nucleotide, amino acid substitution with PCR and RFLP.

PCR amplicon	Homozygous	Heterozygous	Wild type
268 bp	200 bp, 67 bp	200 bp,163 bp, 67 bp, 37 bp	163 bp, 67 bp

[Table/Fig-2]: FVLM PCR products.



[Table/Fig-3]: Representing agarose gel image with PCR product and RFLP for FVLM.

STATISTICAL ANALYSIS

Statistical analysis was done using the Statistical Packages for Social Sciences software (SPSS, Windows version release 13, SPSS Inc., Chicago, Illinois, USA). Differences in allele frequencies and genotype distribution between cases and controls were compared using relevant contingency tables by Fisher's-exact test. Quantitative variables were compared with student's t-test.

RESULTS

A total of 300 participants were included in the study. Of these, 150 samples were from PE women as cases and the remaining 150 were controls as normotensive pregnant women. The maternal age, gestational week, blood pressure readings were taken in both cases and in controls. The mean of maternal age did not show much significance in cases to controls as in cases the mean was 24.2±4.1 and controls were 25.2±3.7 so maternal age is not a major factor in PE. Gestational weeks in cases were 35.4±2.0 in controls were 38.0±1.4 with significant p-value so, it means with PE more chance for early delivery. Around 99% of the cases had oedema. The summary of the clinical profile of the patients was given in [Table/Fig-4]. Due to PE, the complications were not only

in mother but even foetus had adverse effects [Table/Fig-5]. Total of 88 cases has shown complications due to PE like foetal distress, Intra Uterine Fetal Death/Demise (IUFD), Intra Uterine Death (IUD), Feto-Placental Insufficiency (FPI), Intra Uterine Growth Restriction (IUGR) has explained with type of PE and type of gravida in [Table/Fig-5]. (Remaining 62 cases of foetus did not showed above complications due to PE). The profile of FVL genotype in the two study groups was shown in [Table/Fig-6]. Frequency of the Leiden variant was 5.3% among cases and 6.7% in the control groups. The Leiden variant of factor V was not found in homozygous condition in either of the study groups. As the heterozygous condition is more in controls the FVLM was not significant in PE of South eastern part of India (Kolar). The distribution of Factor V genotypes in the study population explained in [Table/Fig-6,7] shows the Gravida and type of PE affecting the Wild type mutation and mutant Leiden in cases [Table/Fig-8], explains about the Gravida of PE effecting the Wild type mutation and mutant Leiden (heterozygous type) in controls. The statistical evaluation of the FVL genotypes with PE was explained in [Table/Fig-9].

Clinical parameters	Cases	Controls	p-value
Maternal age	24.2±4.1	25.2±3.7	0.025
Gestational age at delivery (weeks)	35.4±2.0	38.0±1.4	0.001*
Systole blood pressure (mm/hg)	149.93±19.1	111.7±6.89	0.001*
Diastole blood pressure (mm/hg)	101.77±13.3	75.33±4.39	0.001*
Blood pressure (mm/hg) Systolic/Diastolic			
Mild PE	141.3±11.6/94.7±5.8		
Severe PE	170.0±18.5/112.4±5.6		

[Table/Fig-4]: Clinical profile of PE in the FVL study.
p-value calculated by Fischer's-exact test; *p=0.001 (significant)

Clinical complications	No. of subjects (88)	Primi gravida +Mild PE	Primi gravida +Severe PE	Multi gravida +Mild PE	Multi gravida +Severe PE
Fetal distress	26	12	07	03	04
IUGR	13	04	05	02	02
Twin pregnancy	02	-	01	-	01
First pregnancy PE, repeated in second pregnancy	04	-	-	02	02
Anaemia with PE	09	03	02	04	-
FPI	08	02	02	01	03
PE pregnancy complicated to eclampsia	06	-	-	03	03
Preterm Delivery due to PE	02	-	02	-	-
IUFD	09	02	01	03	04
Hypothyroidism with PE	09	02	02	03	02

[Table/Fig-5]: Maternal and Neonatal complications due to PE.
IUGR: Intra uterine growth restriction; FPI: Foeto-placental insufficiency; IUFD: Intra uterine fetal death/demise

Factor V genotype	Cases (n=150)	Controls (n=150)	p-value
WT/WT	142 (94.7%)	140 (93.3%)	1.0
WT/Mut Leiden	8 (5.3%)	10 (6.7%)	
Mut Leiden/Mut Leiden	0	0	

[Table/Fig-6]: Distribution of factor V genotypes in the study population.
p-value calculated by Fischer's-exact test

Gravida and Type of PE	WT/MutLeiden	WT/WT
Multi gravida-mild	01	34
Severe	02	25
Total	03	59
Primi gravida-mild	04	52
Severe	01	31
Total	05	83

[Table/Fig-7]: Gravida and type of PE effecting the wild type mutation and mutant leiden in cases.

Gravida	WT/Mut Leiden	WT/WT
Multi gravida	05	100
Primigravida	05	40

[Table/Fig-8]: Gravida of PE effecting the wild type mutation and mutant Leiden (heterozygous type) in controls.

Genetic model	Dominant WT/WT+WT/MutLeidenvs. MutLeiden/MutLeiden	Recessive WT/WT vs. WT/Mut Leiden+MutLeiden/MutLeiden
Cases	150 vs. 0	142 vs. 8
Control	150 vs. 0	140 vs.10
p-value	-	0.4

[Table/Fig-9]: Statistical evaluation of FVL genotypes with PE.
p-value calculated by Fischer's-exact test

DISCUSSION

The risk factors differ with ethnicity, maternal age and dietary factors. So each subject is not same even of same age group and same gravida. Few studies have been piloted on FVLM and PE. Hardly studies were evaluated on FVLM and thrombophilia in relation to PE. But none of the studies proved that FVLM can be a genetic marker for PE. As PE itself is a multifactorial disorder, genetically complex to understand and often associated with ethnicity may be difficult to find out specific marker [15]. So the present study focused on association of FVLM and PE. Aggarwal S et al., in his study mentioned that there was association between PE and FVL SNP. The study analysis proved the positive association between PE and FVLM with Odds Ratio (OR) of 2.08. Separate analysis of mild and severe PE showed higher association with the mild PE (OR=2.15) than the severe PE (OR=1.9) [16]. The frequency in the south Indian population fails to indicate a statistically significant association between PE and FVLM [17]. A study from south Indian population from Hyderabad with 105 PE subjects and 100 normotensive pregnant women author found no statistical significance with FVLM in PE [17]. Present study matches with these results. Previous studies from outside India have found both positive and negative association between PE and FVLM. In a meta-analysis conducted by Kosmas IP et al., included 19 studies revealed a positive association between FVLM and PE with 2.5 fold increase risk [18]. Karimi S et al., in their study, mentioned about German and Indonesian women in which German women had association and Indonesian women were not associated. So FVL carrier frequency has been shown to vary between ethnicities [15]. The polymorphism of FVLM in thrombophilia has not been observed in Japan, Southeast Asia and Africa. The frequency of FVLM is as high as 15% was reported in the European populations [19]. In another meta-analysis done by Wang X et al., with 37 studies with 5048 PE patients and 6796 controls in Portuguese population the OR for the association between FVL and all PE patients was 1.60 (95%CI 1.28-2.00) and 2.45 (95%CI 1.63-3.69) for the cases of severe PE [20]. A massive meta-analysis of 31 studies involving 7522 patients revealed a positive association between FVLM and PE. The pooled OR association of FVL was 1.81 but the value of OR increased to 2.24 when the cases of severe PE tested separately [21]. The studies with association and disassociation with FVLM in PE were explained in [Table/Fig-10] [15-17,18,20-34]. However in India, FVLM has been examined for association with PE in North Indian population- Lucknow by Aggarwal S et al., with 200 PE and 200 normotensive pregnant women were compared for the frequency of Leiden mutation and found to be 4%. Further mutation increased the risk by two fold (OR: 2.08, p-value 0.03) [16]. Present

study findings were same as study done in south Indian population by Kamineni V et al., in south-eastern Indian population. Both studies showed FVLM was not statistically significant. In this light, the data obtained in this study suggest that FVL may not be a relevant marker for PE in the south Indian population [17]. Present data suggest that carriers of the FVL mutation are at increased risk for severe PE.

Sl. No.	Author-Year	Country	Associated/Not
1.	Wang X et al., 2014 (M) [20]	Portugal	Associated with severe PE
2.	Karimi S et al., 2012 [15]	Iran	Associated
3.	Hiltunen LM et al., 2010 [22]	Finland	Associated
4.	Kahn SR et al., 2009 (MC) [23]	Montreal-Cannada	Not associated
5.	Dudding T et al., 2008 (M) [24]	Australia	Associated
6.	Mutze S et al., 2008 (R) [25]	Germany	Not associated
7.	Zahed LF et al., 2006 [26]	Lebanon	Not associated
8.	Nurk E et al., 2006 [27]	Hordaland-Bergan	Associated
9.	Mello G et al., 2005 [28]	Italy	Associated with severe PE
10.	Davalos IP et al., 2005 [29]	Mexico	Not associated
11.	Lin J et al., 2005 [21] (M)	USA	Associated with severe PE
12.	Prasmusinto D et al., 2004 [30]	Germany	Associated
13.	Prasmusinto et al., 2004 [30]	Indonesia	Not associated
14.	Salomon O et al., 2004 [31]	Israel	Not associated
15.	Kosmos IP et al., 2003 (M) [18]	Loannia-Greece	Associated
16.	Rigó J Jr et al., 2000 [32]	Hungary	Associated
17.	Kupferminc MJ et al., 2000 [33]	Israel	Associated
18.	Dizon-Townson DS et al., 1996 [34]	USA	Associated with severe PE
19.	Aggarwal S et al., 2011 [16]	Lucknow-North India	Associated
20.	Kamineni V et al., 2015 [17]	Hyderabad-South India	Not associated
21.	Present study	Kolar-South India	Not associated

[Table/Fig-10]: Studies in relation with FVL mutation in PE [15-17,18,20-34].
MC: Multi-centric cohor; M-Meta-analysis; R-Review

Limitation(s) and Future Research Aspects

Genetic screening on FVLM in both mother and foetus and also in couples (mother and father) was to be performed. Women who carry FVLM along with thrombophilia are at increased risk for PE so FVLM with thrombophilia in PE subjects has to be screened. The Carriers of thrombophilic mutations has to be screened.

CONCLUSION(S)

In this case-control study the FVL gene polymorphism lack significant association in the PE women (Kolar population) so it may not be a relevant genetic marker for South Indian population.

Declaration: Author has presented the article in 2016 at Natcon Conference.

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- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

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