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Research Article

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# Influence of thrombophilic genes; *MTHFR* (C677T), *FVL* (G1691A) and *ACE* (I28005D) in pregnant women with pre-eclampsia

## Abstract

**Objective:** Pre-eclampsia (PE) is a pregnancy complication and vascular disorder recognized by new onset gestational hypertension and proteinuria. It may lack of immunological mother and fetus signalization. The exact pathogenesis of PE remains uncertain. The aim of this study was to identify the selected polymorphisms in the genesis of PE.

**Methods:** PE was identified in the second half of pregnancy, often in the latter part of the second or final trimesters, although it may occur earlier. This is a case-control study carried out in 105 cases and 100 ages matched normal pregnant women (controls). We have selected three different genes: MTHFR, FVL, ACE to evaluate mutation/ polymorphisms in 677C>T, 1691G>A and I>D28005 genotypes by PCR followed by Restriction fragment length polymorphism and gel electrophoresis.

**Results:** Statistical analysis was performed by Openepi software. Both the allele and genotype frequencies between PE cases and controls were contradictory and odds ratio with 95% confidence interval did not reveal statistical significant in either alleles or genotypes of any of the three snips. MDR analysis also failed to show the disease marker association in the PE women. It has found an interaction between MTHFR and FVL. These findings suggest the existence of population based differences in the association of candidate gene variants with PE emphasizes the importance of studying specific polymorphisms, which can be used as biomarkers uniquely for an ethnic group.

**Conclusions:** In conclusion, the present case-control study in pregnant women appears as lack of association with the selected polymorphisms in the study population.

**Keywords:** preeclampsia, pregnancy induced hypertension, methylene tetrahydro folate reductase, factor V leiden, angiotensin converting enzyme

Volume 2 Issue 1 - 2015

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**Received:** November 3, 2014 | **Published:** February 03, 2015

**Abbreviations:** PE, pre eclampsia; PIH, pregnancy induced hypertension; E, eclampsia; FVL, factor V leiden; APC, activated protein C; I/D, insertion-deletion; PCR, polymerase chain reaction; Ors, odds ratio; HWE, hardy-weinberg equilibrium; ACE, angiotensin converting enzyme; *MTHFR*, methylene tetrahydro folate reductase; MDR, multifactor dimensionality reduction

## Introduction

Preeclampsia (PE) is a hypertensive disorder associated with elevated blood pressure and proteinuria frequently develops after 20 weeks of gestation.[1] Pregnancy Induced Hypertension (PIH) was defined as persistently raised blood pressure (≥140/90mmHg) starting after the 20th week of gestation in an otherwise normotensive woman with proteinuria (≥300mg/24h).[2] The incidence of PIH in India is 7-10% of all antenatal admissions.[3] It can progress to PE or eclampsia (E) which are life threatening conditions leading to both maternal and perinatal morbidity and mortality. The pathophysiology of PE is still unclear; however, studies have demonstrated an association with a cluster of metabolic abnormalities such as dyslipidemia and hyper-insulinaemia.[4] Toxemia in pregnancy which includes PE and E contributes to 12% of maternal deaths in India. The general risk factors associated with PE are advanced maternal age, history of PE, obesity, multiple pregnancies and women with diabetes/ gestational diabetes.

Genetic polymorphisms are markers of biologic diversity and genotypic variations, which correlate with specific phenotypes are sometimes associated with the development of human disease in different ethnic groups.[5] Polymorphisms of various genes encoding thrombophilic factors like Methylenetetrahydrofolate reductase (*MTHFR;* OMIM: 607093), Factor V Leiden (*FVL;* OMIM) and Angiotensin converting enzyme (*ACE* -OMIM) have been evaluated in numerous studies to assess their association with several complex disorders. *MTHFR* is a critical folate-metabolizing enzyme which requires riboflavin as its co-factor.[6] The 5, 10-MTHFR enzyme catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the primary circulatory form of folate, and a cosubstrate for homocysteine remethylation to methionine.[7] The thermolabile variant occurs at 677th position substitutes from C-T and this variant involves an exchange of a valine for an alanine residue at amino acid position 226 of the *MTHFR* enzyme.[8] G1691 is a common mutation in the *FVL* gene involving a substitution from guanine to adenine at amino acid position 506, which results in a defective

*FVL* protein unable to react with activated protein C (APC), has been previously associated with increased coagulation activity and susceptibility to thromboembolism.[9]

*ACE* is a zinc metallopeptidase widely distributed on the surface of endothelial and epithelial cells, which plays an important role in renin-angiotensin system cascade by converting angiotensin I to angiotensin II.[10] One of these polymorphisms is an insertion-deletion (I/D) of the 287-bp Alurepetitive element in intron 16 of the gene located on chromosome 17, has been identified and this polymorphism does not affect the protein structure but influences expression of this gene.[11] The D-allele is associated with higher concentrations of ACE compared with the I-allele. The DD genotype was found to be a genetic marker associated with human hypertension.[12] The aim of this present study was to investigate the distribution of allele and genotype frequencies of the C677T, G1691A and I28005D mutation/polymorphisms with PE in south Indian population.

## Methods

### Ethics

The study was approved by Ethics committee of the Kamineni Hospitals, Hyderabad, India. Written informed consent was obtained from all the pregnant women who have participated in this study.

### Selection of pregnant women

In order to evaluate the allele and genotype frequencies of selected polymorphisms in the south Indian population we carried out a hospital based case-control study during the period (January 2006- December 2009). The sample consists of 205 pregnant women; 105 of them were diagnosed with PE and acted as a "study group" while the other 100 pregnant women were normal and acted as a "control group". All the subjects belonged to the same geographic region. PE was diagnosed based on two consecutive measurements of systolic and diastolic blood pressure taken, after the 20th week of pregnancy, at least 6 hours apart. Increase in diastolic blood pressure to >110mmHg or a rise of 15-30mmHg above the normal pre-pregnancy values indicates PE (Figure 1). This could be accompanied with 300 mg protein in the 24 hour urine specimen or urine dipstick >1+.[4,13] The women with normal systolic and diastolic blood pressure and absence of proteinuria were considered as controls. Edema was established with an increase of 500g in weight during a week or clinically defined by facial, hand or generalized swelling. Eclampsia was defined as preeclampsia associated with convulsive syndrome or coma. The exclusion criteria were pregnant women with chronic hypertension.

### Sampling

Two milliliter of peripheral blood was collected in the EDTA vacutainer from all the pregnant women. Instantly after collection, whole blood was sent to the Department of Genetics and Molecular medicine for isolation of DNA.

### DNA extraction and genotyping

Salting out technique was used to extract the DNA from peripheral blood leucocytes as routinely used in our lab.[14] *ACE* I28005D (rs 4646994) polymorphism was detected by Polymerase chain reaction (PCR) (Applied Biosystems), amplification and gel electrophoresis, while C677T (rs 1801133) and *FVL* (G1691A) variants were studied by PCR amplification followed by Restriction Fragment Length Polymorphism (RFLP) and agarose gel electrophoresis.[10,15] The details of primers, annealing temperatures, fragment sizes and restriction enzymes used in this study are tabulated in Table 1 & Table 2. PCR products for C677T and G1691A variants are digested at 37˚C, after electrophoresis, the digested products were visualized on 2% agarose gel with ethidium bromide staining.

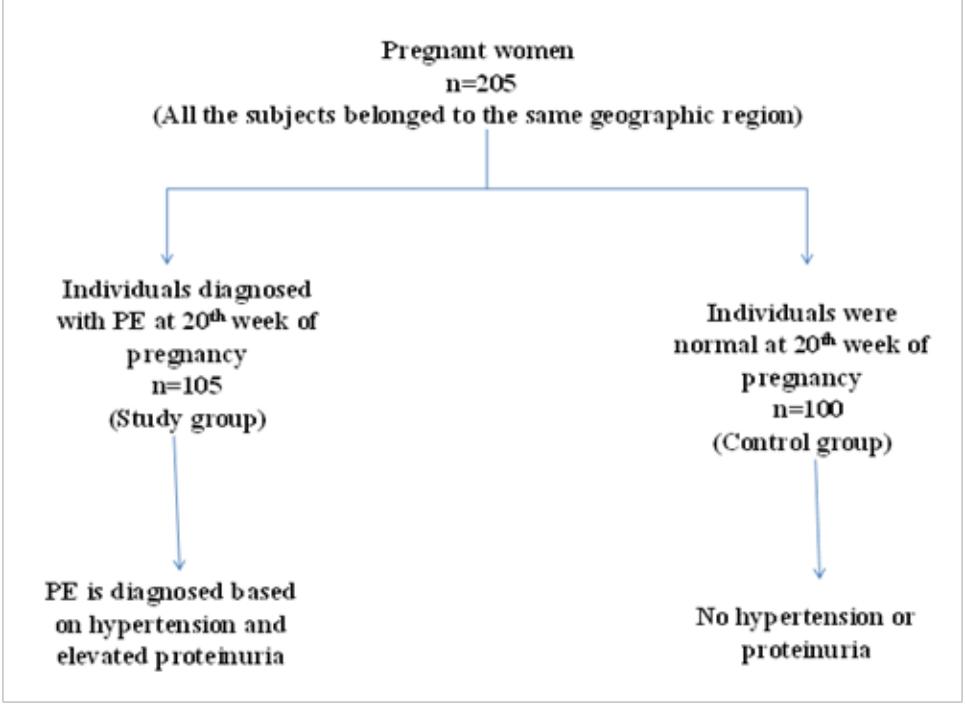


**Figure 1** Patient selection criteria.

**Table 1** Selection of genes (SNPs) and primers involved in this study

| Gene | SNP | rs no | Nucleotide change | Amino acid change | Forward primer | Reverse primer | Fragment |
|---|---|---|---|---|---|---|---|
| MTHFR | EXON 5 | rs1801133 | C>T1 | Ala-Val | TGAAGGAGA AGGTGTCTGCGGGA | GGACGGTGCG GTGAGAGTG | 198bp |
| FACTOR V | EXON 10 | rs6020 | G>A1 | Gua-Ade | TCAGGCAGGAA CAACACCAT | GGTTACTTCAAGGAC AAAATACCTGTAAAGCT | 241bp |
| ACE | INTRON 16 | rs 4646994 | I>D1 | - | CTGGAGACCACT CCCATCCTTTCT | GATGTGGCCATCAC ATTCGTCACGAT | 490bp |

[1]Risk allele

**Table 2** Reaction conditions for PCR-RFLP of C677T and G1691A polymorphisms

| Gene | Band | Amino acid variation | Enzyme | Digestion | Temperature | Sizes (bp) |
|---|---|---|---|---|---|---|
| MTHFR | 1p36.22 | C677T | HinfI | 2h | 37°C | C: 198bp<br>T:175/23bp |
| FACTOR V | 1q24.2 | G1691A | HIndIII | 16h | 37°C | G: 241bp<br>A: 209/32bp |
| ACE | 17q23 | - | - | - | - | I: 490bp<br>D: 190bp |

## Statistical analysis

We used Openepi software system (Openepi, version 2.3.1, Atlanta, GA, USA) to perform all the statistical analysis. Allele and genotype frequency differences between the cases and controls were tested for each SNP using a chi-square test. Odds ratio (ORs) and 95% confidence intervals are calculated to estimate the strength of the association between polymorphism genotype alleles in pregnancy women by binomial logistic regression. Independent sample *t*-test was used to test the cases and controls. Yates correction was also performed. Clinical data are expressed as mean±standard deviation (M±SD). A *p* value less than 0.05 ($p<0.05$) was used as the criterion of significance. Pearson's Chi-square ($\chi^2$) or Fischer's exact two test (two-sided) was used to test the deviations of genotype distribution from Hardy-Weinberg equilibrium (HWE) and to determine the allele and genotype frequencies. Gene-gene interaction was performed by multifactor dimensionality reduction (MDR) analysis software (Linux version).

# Results

Clinical data of the pregnant women with and without PE are listed in Table 3. The mean age in the study group (28.9±4.5 years) and mean period of gestation (24.9±5.0) at the time of recruitment in the study was comparable with the control group (26.9±4.3 years) and 24.1±4.9 weeks, respectively. The BMI of the pregnant women as well as SBP and DBP were significantly different between the two groups ($p<0.05$).

## Amino acids substitution analysis

Amino acid changes at position 677 and 1691 in the exon 5 and 10 regions of *MTHFR* and *FVL*, respectively, were investigated using PCR-RFLP. Distribution of genotype frequencies of the C677T *MTHFR* polymorphisms in controls satisfies the HWE. The genotype frequencies of *MTHFR*in cases are 78.1% CC, 19% CT and 2.9% TT respectively, whereas in the control group the homozygous CC, variants TT and heterozygous CT genotypes are found at frequencies of 82%, 17% and 1%, respectively. The allele frequencies observed in the cases are 0.88 (C) and 0.12 (T) and in controls 0.90 (C) and 0.10 (T). The genotypes and allele frequencies of C677T were not statistically different between the cases and controls nor were they associated with PE (OR-1.27, 95%CI=0.64-2.54; $p=0.48$). Similarly, the frequency of Factor V Leiden mutation G1691A was seen in 2.9% of the PE cases and none of the controls (Table 4) & (Table 5).

## Insertion/deletion polymorphism analysis

The frequencies of *ACE* I and D alleles were 0.56 and 0.43 in the cases and 0.49 and 0.50 in the controls. The genotype and allele distribution of I28005D polymorphisms are summarized in Table 4 & Table 5. Distribution of genotypes and allele frequencies of I28005D polymorphism in cases and controls are satisfied by the HWE. The genotype frequencies for I28005D polymorphisms were not significantly different between cases and controls. The results from this study show a high prevalence of DD genotype in the controls (31%) when compared to the PE women (24.8%).

## MDR analysis

Gene-gene interaction was performed by MDR analysis to explore the pathogenesis of PE women. MDR analysis was carried out between three genes and the interaction appears to be between *MTHFR* and Factor V Leiden (Figure 2).

**Table 3** Clinical Data for PE cases and controls involved in this study

| | PE (n=105) | Controls (n=100) | p value |
|---|---|---|---|
| Age (Years) | 28.9±4.5 | 26.9±4.3 | p=0.64 |
| BMI (Kg/m$^2$) | 27.1±3.9 | 26.2±3.7 | p=0.59 |
| Gestational age | 24.1±4.9 | 24.9±5.0 | p=0.84 |
| SBP (mmHg) | 158 | 119 | p=0.0001 |
| DBP (mmHg) | 101 | 79 | p=0.0001 |

**Table 4** Genotype distribution of 677C>T, 1691G>A, and 28005 I>D polymorphisms in the pregnancy women enrolled in the study

| Genotypes | PE (n=105) | Controls (n=100) | Odds ratio (95% CI) | $\chi^2$ | p value |
|---|---|---|---|---|---|
| **677C>T** | | | | | |
| CC | 82 (78.1) | 82 (82) | Reference | | |
| CT | 20 (19.0) | 17 (17) | 1.17 (0.57, 2.4) | 0.6567 | 0.6559 |
| TT | 03 (2.9) | 01 (1) | 3 (0.30, 29.44) | 0.3246 | 0.3231 |
| **1691G>A** | | | | | |
| GG | 102 (97.1%) | 100 (100%) | Reference | | |
| GA | 3 (2.9%) | 0 (0%) | 6.8 (0.3, 135.2) | 0.1423 | 0.1414* |
| AA | 0 (0%) | 0 (0%) | 0.03 (0.0005, 1.9) | 0.015 | 0.014* |
| **ACE I>D** | | | | | |
| II | 39 (37.1) | 30 (30) | Reference | | |
| ID | 40 (38.1) | 39 (39) | 0.78 (0.4, 1.5) | 0.4752 | 0.4737 |
| DD | 26 (24.8) | 31(31) | 0.64 (0.3, 1.3) | 0.225 | 0.2232 |

*indicates Yates correction

**Table 5** Allele distribution of 677C>T, 1691G>A, and 28005 I>D polymorphisms in the pregnancy women enrolled in the study

| Alleles | PE (n=105) | Controls (n=100) | Odds ratio (95% CI) | $\chi^2$ | p value |
|---|---|---|---|---|---|
| **677C>T** | | | | | |
| C | 184(0.88) | 181 (0.90) | Reference | | |
| T | 26 (0.12) | 19 (0.10) | 1.34 (0.71, 2.51) | 0.3515 | 0.3509 |
| **1691G>A** | | | | | |
| G | 207 (0.99) | 200 (100) | Reference | | |
| A | 3 (0.1) | 0 (0) | 6.76 (0.34, 131.8) | 0.1452 | 0.1446* |
| **ACE I>D** | | | | | |
| I | 118 (0.56) | 99 (0.495) | Reference | | |
| D | 92 (0.44) | 101 (0.505) | 0.76 (0.51,1.12) | 0.1756 | 0.1751 |

*indicates Yates correction

**Figure 2** Dendogram representation of interaction of *MTHFR*, *FVL* and *ACE* genes by MDR analysis.

## Discussion

In this prospective case-control study, we scrutinized the association of three common and widely studied mutation/polymorphisms of *MTHFR* (677 C>T), Factor V Leiden (1691G>A) and *ACE* (28005 I>D) genes with PE cases and control subjects in south Indian population. PE has been associated with insufficient trophoblast invasion of maternal spiral arteries, impaired placental perfusion, and widespread endothelial cell dysfunction.[16] The causes leading to these pathological alterations remain unclear. PE is believed to be a multifactorial disorder with a strong genetic component.[17] The incidence of PE is three times higher in women with a family history of the disease,[18] nearly two and a half times more common in daughters of women with PE than in their daughters-in-law;[19] twice as frequent in pregnancies fathered by men who are themselves the product of pregnancies complicated by PE compared with pregnancies fathered by men who were born of normotensive pregnancies.[18]

PE is a significant obstetric problem in India, however, the contribution of genetic polymorphisms to PE have not been well studied. Some papers have looked at the role of eNOS (Glu298Asp), FOXp3 (-3279C>A) and thrombophilic (*MTHFR+FVL+ACE*) gene polymorphisms[13,20,21] in PE. The prevalence of two thrombophilic gene polymorphisms in our study are similar to previously reported figures for *MTHFR* and *FVL*in the North Indian population.[22] The Factor V Leiden mutation was seen in 3 cases in this population. *MTHFR* 677 T allele was found to be heterozygous in 21% and homozygous TT in 3% of cases despite the fact that these percentages are higher than the controls the values are not statistically significant. This may be because the women with MTHFR CT or TT genotype in the control group may be those who have adequate folic acid in their diet or have received folic acid supplementation and hence do not show symptoms of PE. The result indicates that *MTHFR*, *FVL* and *ACE* variants were not significantly associated. Our results are not similar to those of Agarrwal et al.[13] which suggested that ACE polymorphism has a protective effect. A recent meta-analysis has shown that *MTHFR* is significantly increases the risk in PE in Caucasian and East Asian population.[23]

In our study *ACE* I28005D polymorphism has no role in PE either in allele or genotype frequencies and this was supported by earlier studies.[24–27] On the other hand, few studies have addressed the positive association with higher incidence of DD genotype and/or D allele in PE and/or PIH.[28–32] One of the possible reasons for the inconsistency among these reports may be a genetic basis that causes different susceptibilities among different populations.[33] Table 6 represents the earlier studies with their association. This study has not found any association in the *ACE* I28005D polymorphism with PE in the population studied, although D allele of *ACE* is found to be higher in controls (50.5%). A previous study from Indian population had evaluated the association of *ACE*I28005D.[32] The other possibility may be that DD genotype plays a permissive role in the development of PE in those individuals who are at the development of PE/PIH due to other factors. We have excluded the women who are predisposing to the development of PIH, chronic hypertension, collagen disorders, diabetes and multiple pregnancies.

**Table 6** Earlier studies involved in the ACE gene with PE/control subjects

| Prior studies | Country | PE | Controls | Association |
|---|---|---|---|---|
| Agarrwal et al.[13] | India | 120 | 118 | No |
| Agarrwal et al.[22] | India | 200 | 200 | No |
| Morgan et al.[24] | Europe | 72 | 83 | Yes |
| Galao et al.[25] | Brazil | 51 | 71 | No |
| Kobashi et al.[26] | Japan | 122 | 291 | No |
| Mando et al.[28] | Italy | 197 | 410 | No |
| Gurdol et al.[29] | Turkey | 95 | 89 | No |
| Mello et al.[30] | Italy | 17 | 58 | Yes |
| Choi et al.[31] | Korea | 90 | 98 | Yes |
| Kaur et al.[32] | India | 50 | 50 | No |
| Bereketoglu et al.[33] | Turkey | 120 | 116 | Yes |
| Heiskanen et al.[37] | Finland | 133 | 115 | Yes |
| Bouba et al.[38] | Greece | 41 | 102 | Yes |
| Roberts et al.[39] | South Africa | 271 | 338 | No |
| Kim et al.[40] | Korean | 94 | 105 | No |
| El Shafie et al.[41] | Egypt | 117 | 102 | No |
| Velloso et al.[42] | Brazil | 20 | 20 | Yes |
| Miskovic et al.[43] | Croatia | 60 | 50 | Yes |
| Procopciuc et al.[44] | Romania | 36 | 71 | No |
| Salimi et al.[45] | Iran | 125 | 132 | Yes |
| Serrano et al.[46] | Columbia | 665 | 1046 | No |
| Pfab et al.[47] | Germany | 1068 | - | - |
| Benedetto et al.[48] | Italy | 120 | 108 | Yes |
| Present study | India | 105 | 100 | No |

MDR analysis is used for detecting and characterizing gene-gene and gene-environment interaction effects on risk of common complex multifactorial diseases in case-control and discordant-sib-pair studies with relatively small samples.[34] MDR is nonparametric in both statistical and genetic sense, balances accurately as the evaluation to measure the potential risks.[35] We performed the MDR analysis using the three genes in combination to analyze the risk of the disease. It revealed only an interaction between *MTHFR* and *FVL* genotypes have showed an association in combination of genes, but turns to be protective for the PE. In conclusion, the results of the present study suggest no relation between PE susceptibility and *MTHFR* (C677T), *FVL*(G1691A), *ACE*(I28005D)gene polymorphisms in the examined south Indian population.

## Acknowledgments

This study was supported by Kamineni hospitals, Hyderabad, India. Gratitude is expressed to all the volunteers who have participated in this study.

## Authors contribution

KV is a gynecological clinician, selected and recruited the cases and controls. KIA has prepared the manuscript, helped in the interpretation of the data and executed the statistics. VKK and PS has performed the genotyping and analyzed the data. HQ was head of the project, planned the experiment, and revised the draft. All the author's approved the final version of manuscript.

## Conflicts of interest

The authors declare there is no conflict of interests.

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