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Research Article

Angiotensinogen gene (met235thr and thr174met), angiotensin-converting enzyme (intron 16 insertion/deletion) and angiotensin II type 1 receptor (A1166C) gene polymorphisms in patients with pre-eclampsia

Shamaila Habib[1], Maryam Zafar[1], Osheen Sajjad[1], Ali Ammar[2], Saqib Mahmood[1]
1: Department of Medical Laboratory Technology, NUR International University, Raiwind Road, Lahore, Pakistan.
2: Institute of Nursing and Allied Health Sciences, Pakistan Kidney and Liver Institute and Research Centre, Lahore, Pakistan

Abstract
Background: Pre-eclampsia (PE) is a specific disorder of pregnancy characterized by new onset of hypertension (systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg) and proteinuria (>0.3g /day), presenting after 20th weeks of gestation. PE affects 2 to 4% of all pregnancies worldwide.
Methods: Ninety-two pre-eclamptic women and ninety-two controls were genotyped using PCR-RFLP and Allele specific PCR analysis. In the present study, we analyzed allelic and genotypic distribution of *AGT* gene (Met235Thr and Thr174Met), *ACE* I/D and *AT2R1* (A1166C) polymorphisms in patients with PE and healthy controls. Genetic modelling and haplotype analysis was done for these SNPs.
Results: Met235Thr (T704C) polymorphism under dominant and log additive models showed increased risk with pre-eclampsia and reached statistically significant levels for association with the PE but when it was adjusted after Bonferroni correction it became insignificant. While Thr174Met (C521T) polymorphism of *AGT* gene and *AT2R1* A1166C polymorphism did not show any association with PE. *ACE* I/D polymorphism under recessive model was found significantly associated with the development of PE. While dominant and over dominant models showed protective effect which was also statistically significant.
Conclusion: Met235Thr and ACE I/D polymorphisms are significantly associated with the development of pre-eclampsia, while AT2R1 A1166C and Thr174Met polymorphisms are not associated with the disorder in local population.
Key Words: Preeclampsia, Angiotensinogen, Angiotensin Converting Enzyme, Polymorphisms

INTRODUCTION

Pre-eclampsia (PE) is a specific disorder of pregnancy characterized by new onset of hypertension, that is, systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg) and proteinuria (>0.3g/day), presenting after 2nd half of pregnancy in previously normo-tensive non-proteinuric pregnant women [1]. PE occurs in 2 to 4% of all pregnancies and is a leading cause of maternal mortality (46,000 deaths per year), fetal or neonatal death (500,000 deaths per year) [2]. PE is one of the main reasons of maternal mortality, morbidity, perinatal death, premature birth, placental disruption, oligohydramnios and intrauterine growth restriction [3, 4].

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Email: shamaila.habib@niu.edu.pk
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According to the 2021 recommendations of the International Society of the Study of Hypertension in Pregnancy (ISSHP), hypertensive disorders of pregnancy have been classified into four categories including Gestational hypertension, Chronic hypertension, Pre-eclampsia and Pre-eclampsia superimposed of chronic hypertension [5, 6].

PE is a complex disorder that occurs in two stages. First stage is called placental stage which starts in first half of pregnancy. It is an asymptomatic stage in which defect is found in vascular walls of spiral arteries. In normal pregnancy, a process known as pseudo-vasculogenesis starts immediately following implantation. In the placenta of a pre-eclamptic patient, this vascular remodeling is incomplete. Second stage is a systemic stage or a symptomatic stage of PE that arises in 2nd half of gestation. Placental hypoxia releases placental materials into maternal circulation and results in increase maternal

systemic inflammatory response and endothelial dysfunction. This leads to hypertension and proteinuria that mainly affect liver, brain and kidneys. As disease progress patients develop edema especially of face and hands [9, 10]. In chronic stage of disease, patients may develop pulmonary and cerebral edema, eclampsia, renal failure and HELLP (Hemolysis, Elevated Liver Enzymes and Low Platelets) syndrome. In eclampsia there is evidence of central nervous system involvement in addition to hypertension and proteinuria [11, 12].

Different genetic and environmental factors are associated in pathogenesis of PE [7, 8]. A single-center study identified a family history of preeclampsia to have a four-fold increased risk of severe disease. Correspondingly, a Swedish, population-based study also found that the risk of preeclampsia was higher in women who had a family history of preeclampsia (using full sisters or mother-daughter relationships) than that of women with maternal half-sisters or paternal half-sisters [13]. Renin Angiotensin Aldosterone System (RAS) contribute a major part in "physiological remodeling" of uterine spiral arteries throughout pregnancy [14]. RAS is a group of associated hormones that regulate blood pressure and electrolyte balance. RAS system consists of renin, angiotensinogen (AGT), angiotensin converting enzyme (ACE), angiotensin II receptor type I (AT2R1) and angiotensin II receptor type II (AT2R2) [15]. RAS system undergoes major changes during pregnancy. All components of RAS are regulated during normal pregnancy. Vascular sensitivity to Ang II is decreased, so, these women are resistant to the pressor effect of this molecule and remain normo-tensive despite increase in Ang II [16, 17]. RAS components are down regulated in PE including renin, Ang-1 and aldosterone levels but ACE levels remain same. The negative feedback mechanism which results from sustained hypertension ultimately suppresses the RAS system in pre-eclampsia. In pre-eclamptic women vascular sensitivity to Ang II increases due to heterodimerization of the AT1 receptor with bradykinin receptor. Reactive Oxygen Species (ROS) fails to inactivate this heterodimeric form and results in increased sensitivity to Ang II [18]. Genetic involvement in the development of pre-eclampsia is not clear, and no specific contributory gene has been discovered so far. Pattern of inheritance of PE has been reported as single gene, polygenic-multifactorial and mitochondrial inheritance [19]. Renin-angiotensin system (RAS) gene polymorphisms (ACE insertion/deletion (I/D, rs1799752), ACE G2350A (rs4343), AGT Met235Thr (M235T, rs699), AGT Thr174Met (T174M, rs4762), and AT1R A1166C (rs5186) have been shown to mediate changes in tissue and circulating RAS activity [20].

METHODS

Ethics Statement

After approval from ethical committee in the University of Health Sciences, Lahore, study was conducted in the Department of Human Genetics and Molecular Biology, University of Health Sciences, Lahore.

Study Cohort and Sample Collection

In current study 92 pre-eclamptic patients and 92 healthy pregnant females were recruited from Department of Obstetrics & Gynecology, Jinnah Hospital Lahore, Pakistan. After receiving informed consent, 3ml blood was taken in EDTA vacutainers.

DNA Isolation and Genotyping

DNA isolation was done by salting out protocol as mentioned by Fatima and her coworkers [21]. Genotyping for SNPs rs699 (Met235Thr), rs4762 (Thr174Met) and rs5186 (A/C) was done using PCR-RFLP method as mentioned in table 1. SNP rs1799752 was genotyped by Allele specific PCR as mentioned in table 2. PCR products were run on 2-3% agarose gel electrophoresis.

Statistical Analysis

Hardy–Weinberg equilibrium was tested to check the deviance of genotype frequencies with the help of chi-square goodness-of-fit test. Chi-square test assessed statistical differences in genotypic and allelic distribution between the patients with pre-eclampsia and normal pregnant women at 0.05 level of significance. The association of different alleles with disease phenotype was evaluated by calculating odds ratio (OR) and its 95% confidence interval (95% CI). All these analyses were done with OEGE-Online Encyclopedia for Genetic Epidemiology Study and IBM SPSS software (version 20.0, SPSS Inc). P value < 0.05 was taken statistically significant. Genetic modeling was done using Snpstat software (http://bioinfo.iconcologia.net/snpstats/start.htm).

Table 1: Sequence of primers and restriction enzymes used for *AGT* (rs699, rs4762) and *AT2R1* (rs5186) polymorphisms used in this study.

SNP	Sequence	Annealing Tm	Product Size	Enzyme	Product Size
rs699	**F:** 5′-GTTTGTGCAGGGCCTGGCTC -3′	64°C	158bp	Tth111I	**T/T**: 158bp **T/C**: 158, 141, 17 bp **C/C**: 141, 17 bp
	R: 5′-TGCTGTCCACACTGGACCCC -3′				
rs4762	**F**: 5′-GATGCGCACAAGGTCCTGTCT -3′	63.5°C	303bp	NcoI	**C/C**:303bp **C/T**: 303, 211, 92 **T/T**: 211, 92 bps
	R: 5′-CAGGGTGCTGTCCACACTGG -3′				
rs5186	**F:** 5′-GGCTTTGCTTTGTCTTGTTGCA -3′	60°C	856bp	Dde1	**A/A**: 600,256 bp **A/C**: 600,256,146,110bp **C/C**: 600,146,110 bp
	R: 5′-AATGCTTGTAGCCAAAGTCACCT -3′				

Table 2: Sequence of Allele Specific PCR for ACE I/D polymorphism

SNP	Sequence	Annealing Tm	Product Size
rs1799752	**IF**: 5′-TCGGAGACCACTCCCATCCTTTCT- 3′	65°C	**I/I**: 490bp **I/D**: 335bp **D/D**: 190bp
	IR: 5′-GATGTGGCCATCACATTCGTCAGAT -3′		
	OR: 5′-TCGCCAGCCCTCCCATGCCCATAA-3′	73°C	
	OF: 5′-TGGGACCACAGCGCCCGCCACTAC-3′		

RESULTS

Cohort Summary

The ages of the patient range from 19 to 45 years with a mean age of 28±5.12 years. Majority of patients 76.0% (n=68) presented with disease in the age of 25-40 years (Figure 1). Consanguineous marriage was present in 54% (n= 48) of patients and a family history of PE was found in 40% (n= 36) of patients.

Hardy-Weinberg Equilibrium:

For all four SNPs rs699, rs4762, rs5186 and rs1799752 Hardy-Weinberg Equilibrium analysis was applied on population data. Among these four SNPs, genotypic distribution in the population for rs699, rs4762 and rs5186 was consistent with Hardy-Weinberg equilibrium whereas rs1799752 was not. In various studies it has been shown that many polymorphisms may not fall into Hardy-Weinberg equilibrium for non-causal reasons, particularly if a population exhibits ethnic diversity. Probably the wide genetic admixture of Pakistani population could have influenced the Hardy-Weinberg distribution in this study.

Genotypic and Allelic Distribution

The basic information such as chromosome position and minor allele frequency of candidate SNPs analyzed in our study is presented in Table 3. The minor allele of rs699 (T/C) and rs5186 (A/C) showed increased risk to pre-eclampsia but did not reach to statistically significance level of association. The genotypic frequencies of the SNPs under study in the cases and healthy controls are given in Table 4. Data showed that TC and CC genotype of rs699 showed increased risk to develop pre-eclampsia [OR (CI-95%) 1.83 (1.00-3.36) and 2.00 (0.53-7.54) but did not reach a statistically significant level of association. While mutant DD genotype of rs1799752 showed the increases risk to develop pre-eclampsia with OR (CI-95%) 1.08 (0.30-3.92) which was statistically significant (p-value <0.05).

Results of genetic modeling were shown in Table 5. Met235Thr (T704C) polymorphism under dominant and log additive models showed increased risk with pre-eclampsia and reached to statistically significant levels for association with the pre-eclampsia but after Bonferroni correction, the differences were not significant. While Thr174Met (C521T) polymorphism of AGT gene and AT2R1 A1166C polymorphism did not show any association with pre-eclampsia. In case of ACE, I/D polymorphism recessive model showed positive association (p-value <0.05) with the development of pre-eclampsia with the highest ODDs among all the models i.e. 4.14 [1.76-9.73]. Dominant and over dominant model showed the protective nature of these models toward the development of the disease even after Bonferroni correction.

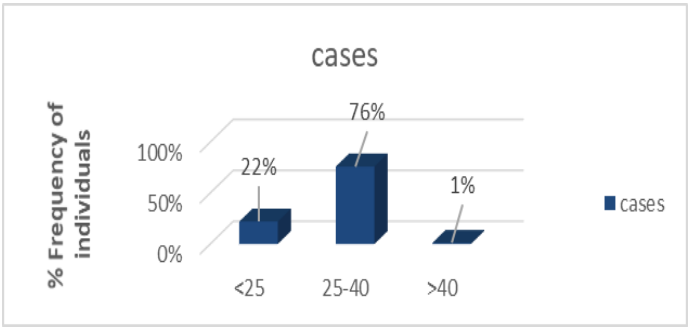

Figure 1: Age distribution of the study population

Table 3: Basic information and minor allele frequency of candidate SNPs in present study

SNP ID	Gene	Position	Base Change	Role	MAF*			OR+ [95% CI] #	*p*- Value
					Cases	Controls	Database		
rs699	AGT	230710048	[T/C]	Exon	0.30	0.22	0.391	1.5750 [0.9839 to 2.5211]	0.0584
rs4762		230710231	[C/T]	Exon	0.15	0.20	0.094	0.7379 [0.4289 to 1.2696]	0.2723
rs1799752	ACE	63488529	[I/D]	Intron	0.44	0.48	0.391	1.1913 [0.7904 to 1.7956]	0.4030
rs5186	AT2R1	148742201	[A/C]	Exon	0.12	0.11	-	0.8537 [0.4513 to 1.6149]	0.6266

*Minor allele frequency, +ODDS ratio, #Confidence Interval, $^{\$}$*p-value* significant <0.05

Table 4: Genotype distribution of the studied SNPs in Cases and healthy Controls.

Genotypes/Allele		Controls n (%)	Cases n (%)	*p*-Value	OR [95% CI]
Genotypes					
rs699	T/T	56 (60.9)	42 (45.6)		1.00
	T/C	32 (34.8)	44 (47.8)	0.12	1.83 (1.00-3.36)
	C/C	4 (4.3)	6 (6.5)		2.00 (0.53-7.54)
rs4762	C/C	59 (64.1)	67(72.8)		1.00
	C/T	30 (32.6)	22 (23.9)	0.42	0.65 (0.34-1.24)
	T/T	3 (3.3)	3 (3.3)		0.88 (0.17-4.53)
rs1799752	I/I	5 (5.4)	15 (16.3)		1
	I/D	79 (85.9)	51 (55.4)	<0.0001*	0.22 (0.07-0.63)
	D/D	8 (8.7)	26 (28.3)		1.08 (0.30-3.92)
rs5186	A/A	72 (78.3)	75 (81.5)		1.00
	A/C	17 (18.5)	14 (15.2)	0.84	0.79 (0.36-1.72)
	C/C	3 (3.3)	3 (3.3)		0.96 (0.19- 4.91)

*p-value significant <0.05

Table 5: ODDs ratios of the genetic models corresponding to the studied SNPs

SNP ID	Dominant Model		Recessive Model		Over-Dominant		Log-Additive	
	OR (95%-CI)	p-value	OR (95%-CI)	p-value	OR (95%-CI)	p-value	OR (95%-CI)	p-value
rs699	1.85 [1.03-3.33]	0.03*	1.53 [0.42-5.63]	0.51	1.72[0.95-3.11]	0.072	1.64 (1.00-2.69)	0.048*
rs4762	0.67 [0.36-1.25]	0.2	1.00 [0.20-5.09]	1	0.65 [0.34-1.24]	0.19	0.74 [0.43-1.27]	0.27
rs1799752	0.30 [0.10-0.85]	0.016*	4.14 [1.76-9.73]	5e-04*	0.20[0.10-0.42]	<0.001*	1.36 [0.79-2.34]	0.27
rs5186	0.82 [0.40-1.68]	0.58	1.00 [0.20-5.09]	NA	0.79 [0.36-1.72]	0.55	0.87 [0.49-1.57]	0.65

*p-value significant <0.05

DISCUSSION

Preeclampsia (PE) is a disorder that develops in pregnant women with a new onset of high blood pressure and proteinuria after 20th weeks of pregnancy. Hypertension (>140/90 mmHg), proteinuria (>0.3g/day) and edema of hands and face are the diagnostic features of pre-eclampsia [22]. Based on these clinical criteria of diagnosis, patients for the present study were selected. In present study, mean systolic (154±35.23 mmHg) and diastolic (99±6.73 mmHg) blood pressure was noted. Proteinuria >0.3g/day (2+) was found in 70% of patients while 30% of patients had >0.5g/day (3+) proteins in their urine. Mean gestational age at which first sign, and symptoms of pre-eclampsia appeared was 26±5.12 weeks. All these parameters are in accordance with the criteria given by the International Society for the Study of Hypertension in Pregnancy (ISSHP) [2].

Familial aggregation of PE has been reported in different studies from different populations of the world [23]. In the current study 40% of patients had family history of pre-eclampsia. This finding consolidates the hypothesis that hereditary and genetics play an essential part in the risk of developing pre-eclampsia. In the present study mean gestational age at delivery was found to be 32 weeks as compared to 34 and 36 weeks in Romanian, Indian and Tanzania studies respectively [24, 25]. It has been suggested that various gene and single nucleotide polymorphisms of the RAS may play an essential role in the modulation of blood pressure and fluid balance in preeclampsia [26]. The present study was performed to find out the association of AGT (Met235Thr & Thr174Met), ACE Intron 16 Insertion/Deletion and Angiotensin II Type 1 Receptor A1166C gene polymorphisms with patients of pre-eclampsia and controls from Lahore Pakistan. Angiotensinogen, encoded by AGT gene has been implicated in the development of pre-eclampsia. The scientific literature has reported many studies that investigated association between susceptibility of pre-eclampsia and polymorphisms in the AGT gene. Two most common polymorphisms, Met235Thr and Thr174Met in the exon 2 of ATG genes have been most studied for this association [27]. In Met235Thr polymorphism (rs 699) of AGT gene 235Thr has been reported to be associated with increased levels of AGT and defective transformation of uterine spiral arteries, which is an early cause of PE [28]. In the present study Met235Thr (T704C) polymorphism under dominant and log additive models showed increased risk with pre-eclampsia and reached statistically significant levels for association with the disease in the studied population but these differences became insignificant after Bonferroni correction. The scientific literature has reported inconsistent results about the association of Met235Thr (T704C) from different populations of the world. Zitouni et al., in 2018 reported a significant association of Thr allele of (T704C) polymorphism with pre-eclampsia in Tunisians [28]. This association was also replicated in Iranian and Pakistani population [27, 29]. However, lack of association of this polymorphism, with the disease, was reported in Chinese and Thai population [30, 31].

In present study Thr174Met polymorphism of AGT gene did not show any association with PE. The findings of the current study are consistent with the meta-analysis report (Lin et al., 2012). On the other hand, positive association between Thr174Met variant and pre-eclampsia was found in Tunisians population [28]. Several studies have showed that women who have D allele of the ACE I/D polymorphism present with greater ACE action and increase resistance of uterine artery, that is an indicator for the development of pre-eclampsia. In the current study positive association was found between DD genotype and development of preeclampsia. These results agree with those presented in Egyptian women [32]. However recent meta-analysis found no statistically significant association between maternal ACE I/D (rs4646994) genotype and preeclampsia risk in most genetic models [33]. In the current study recessive models show the highest ODDs among all other models, i.e. 4.14 (1.76-9.73) and is statistically significant. A previous study in Egypt and Russia reported similar findings [34, 35]. In this study, no association was found between the AT2R1 A1166C polymorphism and pre-eclampsia. These findings revealed concordant results with those presented by another report [36]. However, the study of Wang et al., showed the weak association of AT2R1 A1166C polymorphism and preeclampsia. An updated meta-analysis published in 2023 reported strong association with pre-eclampsia [37, 38].

The results of this research highlight the possible importance of renin angiotensin system gene polymorphisms in the risk evaluation of pre-eclampsia. Such variants as AGT M235T and ACE I/D polymorphism can be utilized as valuable genetic markers to determine women who are at the higher level of risk so that they could be more closely monitored during the ante-natal period and provide preventive measures in time. On the other hand, the absence of correlation with AGT T174M and AT1R A1166C implies that these also have a restricted diagnostic or predictive utility on their own. It is possible that, clinically, the integration of genetic data with conventional risk factors would facilitate more individualized surveillance and early implementation of prophylaxis and customized management of women at high risk of pre-eclampsia. Such learnings can

eventually lead to better maternal and fetal outcomes in the form of precision-based obstetric care.

To conclude, based on the above-mentioned data it is concluded that Met235Thr and ACE I/D polymorphisms are significantly associated with the development of pre-eclampsia, while AT2R1 A1166C and Thr174Met polymorphisms are not associated with the disorder in local population. These findings agree with the studies of different populations of the world. These discrepancies could be ascribed to sample size differences, different clinical presentations of pre-eclampsia disease, difference in ethnic background of the studied populations, and / or Epigenetics and gene-environment interactions. Further research in the future is needed to establish these results and investigate maternal/fetal genetic interactions using larger and more varied populations. Prediction models of pre-eclampsia can be enhanced by combining genetic data, clinical and biochemical markers. Finally, there is need to conduct prospective studies to establish the clinical usefulness of genetic testing in the prevention and personalized care.

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