

The Relationship between Vascular Endothelial Growth Factor A (VEGFA) Gene Polymorphism and Pre-Eclampsia in Egyptian Pregnant Women

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Abstract

Pre-eclampsia (PE) is a complicated heterogeneous disorder associated with an increased risk of maternal death. There is a major variation in the literature about the genetics of pre-eclampsia (PE). This study aims to estimate the association between Vascular Endothelial Growth Factor A (VEGFA) gene single nucleotide polymorphism (rs1570360) and the development of PE in the Egyptian population. Genotyping was performed using the tetra-primer amplification-refractory mutation system (ARMS-PCR) and the results were confirmed by the PCR-RFLP method in 100 patients with PE and 100 PE-free patients. Results revealed that GG (wild type) genotype of rs1570360 was identified in 100 patients, (42%) of them had pre-eclampsia. SNP genotype AA was found in 84 patients and (42%) of them had pre-eclampsia. The frequency of AG genotypes was significantly low (16 %). However, the odds ratio was 40.37 for homozygosity and 9.5 for heterozygous alleles. Our study concluded that the AA and AG genotypes were not associated with PE. There is a significant relationship between the AA allele polymorphism and the severity of PE.

Keywords: Pre-eclampsia, Vascular Endothelial Growth Factor A (VEGFA), Single nucleotide polymorphisms.

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INTRODUCTION

Pre-eclampsia is a medical disorder that occur in the second half of pregnancy including the development of new-onset hypertension, new-onset proteinuria and linked with many other symptoms, including headaches, visual disturbances, epigastric pain and the rapid oedema development. Diagnostic criteria include the development of hypertension, defined as persistent systolic blood pressure (BP) of 140 mm Hg or higher, or a diastolic BP of 90 mm Hg or higher after 20 weeks of gestation in women with previously normal blood pressure [1, 2]. The occurrence in non-hypertensive pregnant women is four to five times [3]. Pre-eclampsia may worsen all other hypertensive disorders. Although the evidence from renal biopsy studies suggest that the diagnosis of superimposed pre-eclampsia may be often erroneous [4]. The probability of pre-eclampsia is double to four times greater if a patient has a first-degree relative with a history of the disorder and is sevenfold greater if a

previous pregnancy was pre-eclampsia-complicated [5, 6]. Severe pre-eclampsia can induce acute as well as long-term complications for both the woman and her child. The maternal complications may be stroke, pulmonary oedema, myocardial infarction, acute respiratory distress syndrome, coagulopathy, severe renal failure and retinal injury. Such conditions are more likely to occur in the presence of pre-existing medical conditions and with severe pre-eclampsia-related maternal organ dysfunction. Fetal and new-born complications of severe pre-eclampsia result from exposure to uteroplacental insufficiency or from preterm birth, or both [7-9]. In recent years, significant steps have been taken for the prediction of many diseases and to their response to the specific medications throughout the incidence of certain gene polymorphisms [10, 11].

PATIENTS AND METHODS

The study population consisted of 200 patients who were divided equally into two groups. The first

consisted of patients with pre-eclampsia, and the second was the pre-eclampsia-free patients. All participants were genotyped for the VEGFA gene, rs1570360. The study plan was reviewed and approved by the Ethical Committee at the Faculty of Medicine, Menoufia University (No: 5/2020OBSGN3). Patients were enrolled from the Department of Obstetrics and Gynecology, Menoufia University Hospital, Egypt.

Samples Collection and clinical investigations

The venous peripheral blood samples were obtained using sterile tubes containing K2-EDTA (KEMICO vacutainer, Egypt). All samples were labeled by numbers (N) that matched the number of other investigations. Clinical evaluation of the patients, including complete history taking (age, parity and gravidity), associated medical disorders and PE in previous pregnancies. Moreover, the measurement of blood pressure (BP), body mass index (BMI) and presence of oedema were done. Biochemical laboratory assessments such as complete blood count (CBC), Alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, prothrombin time and creatinine were investigated by using other coagulated sample serum aliquots. Albumin level by urine dipstick also was done and the results were expressed in (+) mark.

Isolation of Peripheral Blood Leucocytes

About 1.5 mL of venous blood samples were incubated with five volumes of erythrocyte lysing buffer (0.015 M NH_4Cl , 1mM NaHCO_3 , 0.1 mM EDTA). Samples were centrifuged for 5 min at 1200 rpm using cooling centrifuge (Sigma 3K30, Germany). These steps were repeated until the appearance of a white leucocytes pellet [12].

Total genomic DNA isolation

Genomic DNA was isolated from the peripheral blood leukocytes according to the salting-out extraction method [13]. Polymerase chain reaction (PCR) products were directly stained with ethidium bromide and then loaded into the 1.5% agarose gel [14].

Genotyping

The tetra primers amplification-refractory mutation system (ARMS-PCR) and confirmation by PCR-restriction fragment length polymorphism (RFLP-PCR) method were carried out to investigate the SNP (rs1570360) polymorphisms in the VEGFA gene. All amplifications were performed in a thermocycler Master cycler gradient (Eppendorf, Germany). DNA samples were processed for initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C, annealing at 55°C and extension at 72°C for 1 min each. For ARMS-PCR, the oligonucleotide primers were 5' CAGGGGTCACTCCAGGATTCCAA -3' (Outer forward), 5'-CAAGTGGGGAATGGCAAGCAAA -3' (Outer reverse), 5' GCCCGAGCCGCGTGTGTAA 3' (Inner

forward) and 5 GACAGGCGAGCCTCAGCACC 3' (Inner reverse).

The amplification reactions were carried out using the four primers in one reaction tube at the same time and the amplicons were 684, 379 (A allele) and 344 (G allele) base pairs.

For RFLP-PCR, the oligonucleotide primers 5' CAGGGGTCACTCCAGGATTCCAA 3' (Outer forward), 5' CAAGTGGGGAATGGCAAGCAAA 3' (Outer reverse), were used. The procedure of the restriction enzyme digestion consisted of 10 μL of PCR reaction mixture, 18 μL of nuclease-free water, 2 μL 10 \times Buffer R and 2 μL of MnlI restriction endonuclease (Thermo Scientific, US) at 37 °C for 2 h. MnlI digestion of allele GG yields fragments of 426 and 258 base pairs, whereas DNA containing the allele AA yields fragments of 684 base pairs. The fragments of heterozygous alleles (GA) appeared at 684, 426, 258 and base pairs. The Restriction digestion products were separated on 2.5% agarose gels (Sigma-Aldrich, Germany) and stained with ethidium bromide for visualization on a UV transilluminator.

Statistical Analysis

Data were collected, tabulated and statistically analyzed by an IBM compatible personal computer with SPSS statistical package version 23 (SPSS Inc. Released 2015. IBM SPSS statistics for windows, version 23.0, Armonk, NY: IBM Corp). Two types of statistical analysis were done; Descriptive statistics, the quantitative data was represented in the form of mean (\bar{X}), standard deviation (SD), range and qualitative data was represented in the form numbers (N) and percentages (%). While the analytic statistics, including *Chi* square test (χ^2), was used to study the association between two qualitative variables. *P* value of ≤ 0.05 was considered statistically significant.

RESULTS

Clinical evaluation of the pre-eclampsia cases (n=100)

- The number of cases diagnosed with PE was 100 participants, most of them were 28.2 ± 5.83 years old. The Systolic blood pressure (BP) was 157 ± 19.5 while the Diastolic BP was 100.6 ± 11.96 .
- Most of cases according to their Gravidity were gravid a 3(G3) about 32% then gravid a 1(G1) by 24%, G2 (22%), G4 (12%), primi gravid a (PG) 8% and the last one was G5 2%.

Clinical evaluation of control group (n=100)

- The number of control cases was 100 women with mean age 27 years (27.5 ± 5.82), Systolic BP was within normal range (115 ± 5.03) and Diastolic BP was less than 95 mm Hg (95 ± 5.03).
- According to gravidity of the participants: PG

was 8 women (8%), G1 was 20 women (20%), G2 was 30 women (30%), G3 was 32 women (32%), G4 was 8 women (8%) and the last one was G5 was 2 women (2%).

- Normal cases have no protein detected in urine and normal range of laboratory kidney and liver functions were recorded.
- They had no history of medical disorders or previous history of PE in previous pregnancy.

Evaluation of liver functions, kidney functions and proteinuria in cases with pre-eclampsia

- The protein in urine was measured with urine dipstick and most cases showed protein in urine (+) in (36%), participant; (+ + +) in 26 % of the cases; (+ + + +) in 16 %; (+ +) in 14% and Traces in 8 cases (8%).
- Liver enzymes were normal in 90 cases (90%) and Higher than normal range in 10%.
- Creatinine level was normal in 88 women (88%), low in 10% and high in 2%.

Severity of complications and previous history in the pre-eclampsia cases (n=100)

- According to the BP, degree of protein in urine and the laboratory investigations, the 100 PE

women were diagnosed as mild PE (54%) and 46% were severe.

- Complications: There were No Complications in 72 cases (72%), Fits (Eclampsia) were in 8 women (8%), Preterm labor was in 8 (8%), IUFD was in 4 cases (4%), eclampsia and IUFD were 2 cases (2%), Renal affection & dialysis (HELP) were in 4 cases (4%) and Oligohydraminos was in 2 cases (2%).
- The history of PE in previous pregnancy was not recorded in 90 women (90%) but the previous history was present in 6 cases (6%), SLE in 2 cases which was diagnosed as medical disorder associated with pregnancy (2%), and CHTN in 2 cases (2%) as they had hypertension disease before pregnancy.

Genotyping of rs1570360

Table-1 shows that there was no association of rs1570360 with the PE. The GG (wild type) genotype of rs1570360 was identified in 90 patients, 42 of them had pre-eclampsia. SNP genotype AA was found in 84 patients and 42 of them had pre-eclampsia. There is no statistical significance between the occurrence of pre-eclampsia and the mutant homo/heterozygous allelic forms of the rs1570360 SNP.

Table-1: Genotyping of control and pre-eclampsia cases

Genotyping	Cases (PE) (n=100) N (%)	Control (n= 100) N (%)	Total (n= 200) N (%)	Test of significance (χ^2)	P value
GG	42 (42)	48 (48)	90 (45)	1.785	0.41
AA	42 (42)	42 (42)	84 (42)		
AG	16 (16)	10 (10)	26 (13)		

χ^2 : Chi square test

P value >0.05: Non-significant

Table-2 shows that there is a significant ($P \leq 0.001$) association between the mutant allele (homo/heterozygous) and the severity of pre-eclampsia. Results revealed a strong relationship between AA and severe cases, whereas, AA was found in 73.9%, GG (8.7%) and AG (17.4%). However, the odds ratio was

40.37 for homozygosity (AA) and 9.5 for the AG heterozygous allele (Table-3). The polymorphism homozygosity (AA) was significantly ($P \leq 0.001$) associated with the most serious complications in PE cases such as HELP and IUFD (Table-4).

Table-2: The relationship between rs1570360 genotyping and the severity of pre-eclampsia

Genotyping	Pre-eclampsia in cases		Total (n= 100) N (%)	Test of significance (χ^2)	P value
	Mild (n=54) N (%)	Severe (n= 46) N (%)			
GG (wild type)	38 (70.4)	4 (8.7)	42 (42)	43.256	<0.001**
AA	8 (14.8)	34 (73.9)	42 (42)		
AG	8 (14.8)	8 (17.4)	16 (16)		

**P value ≤ 0.001 : Highly significant

Table-3: Binary logistic regression of rs1570360 genotyping and the severity of pre-eclampsia

Genotyping	Pre-eclampsia		Odds ratio	95% CI (lower-upper)	P value
	Mild (n=54) N (%)	Severe (n= 46) N (%)			
AA	8 (14.8)	34 (73.9)	40.375	11.155-146.135	<0.001**
AG	8 (14.8)	8 (17.4)	9.5	2.292-39.378	0.002*
GG (wild type)	38 (70.4)	4 (8.7)	-		

**: highly significant

*P value ≤0.05: Significant

Table-4: Relation between the polymorphism homozygosity and the occurrence of most serious complications in PE cases

Complications	AA		Total N (%)	Test of significance (χ^2)	P value
	Present N (%)	Absent N (%)			
Eclampsia	8 (22.2)	-	8 (8.9)	33.75	<0.001**
IUFD	4 (11.1)	-	4 (4.4)		
Eclampsia+IUFD	2 (5.6)	-	2 (2.2)		
HELP	4 (11.1)	-	4 (4.4)		
No complications	18 (50)	54 (100)	72 (80)		

**: highly significant

DISCUSSION

Pre-eclampsia (PE), a human-pregnancy-related disease described as the appearance of hypertension and significant proteinuria in a previously healthy pregnant woman after the 20th week of gestation. It occurs in about 2-8% of pregnancies [15]. Despite its prevalence and severity, there is no definite theory explaining PE's pathophysiology. Different theories includes a combination of immunological, inflammatory, dietary and genetic factors leading to the failure of normal trophoblastic invasion and uterine spiral artery remodeling [16].

The cause of PE remains unclear but there is increasing evidence that an imbalance between the VEGF family and potential anti-angiogenic factors is closely linked to PE pathogenesis. A highly coordinated system ensures that the placenta and fetus receive adequate blood supply needed for proper oxygen and nutrient delivery during a regular and healthy pregnancy. Developing evidence indicates that VEGF is elevated in PE and associates with disease severity and complications [17]. Unlike some reports have found decreased VEGF rates in PE-women's sera [18].

As a member of the VEGF family, VEGF A is a significant angiogenic factor and potent regulator of the proliferation of endothelial cells [19]. There are many popular single nucleotide polymorphisms (SNPs) in the VEGF gene, including positions + 936 (rs3025039), -634 (rs2010963), -1154 (rs1570360), and -2578 (rs699947), which may alter the gene expression and affect the risk of developing diseases defined by deranged angiogenesis [20]. Hence, abnormal angiogenesis and related fetoplacental vascular dysfunction are considered essential steps in

the development of PE [21]. Thus, in recent years, one field of intensive work has concentrated on the VEGF signaling pathway in the progression of PE, which is essential to maintain proper endothelial cell function and health. Case-control studies have recently shown associations between polymorphisms of the VEGF genes and PE [22].

In the present study, the associations between the polymorphisms in VEGF genes (rs1570360) and PE were investigated which might play a role in this disease. To the best of our knowledge, this is the first analysis to evaluate the relationship between SNPs in the VEGF gene and the risk of PE in Egyptians.

The VEGF is a multifunctional cytokine that plays a key role in the *in vivo* angiogenesis and placental development [23]. The VEGF gene has many polymorphisms, and several polymorphisms are associated with the development of proteins [24]. In addition, there was a lack of association of the genotypes of VEGFA SNPs with PE, regardless of the genetic model used. However, among seven-locus studied, results suggest that VEGFA haplotypes, more so than individual SNPs, play a role in PE pathogenesis in Tunisian women [25].

The VEGFA gene rs1570360 (-1154G/A), in the promoter region, was reported to modulate VEGF [26]. Herein, the rs1570360 (-1154G/A) polymorphisms had no association with PE risk in all examined patients. However, there was a relation between genotyping and the severity of pre-eclampsia. A strong association was found between AA and severe cases even with the development of complications in these cases.

CONCLUSION

Our results suggested that VEGFA gene -rs1570360 or (-1154G/A) polymorphism had no association with PE risk in all examined patients. In order to warrant and confirm the associations between VEGFA gene polymorphisms and PE risk, further studies are therefore required. However, AA genotype was found in each severe case associated with serious complications of PE disorder.

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