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Genetic Variant of Vascular Endothelial Growth Factor (VEGF)-A rs699947 is Associated with Preeclampsia

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Background: Preeclampsia remains as the leading cause of maternal-neonatal mortality and morbidity worldwide. Vascular endothelial growth factor A (VEGF-A) is a proangiogenic factor related to endothelial dysfunction and plays an important role in the preeclampsia pathophysiology. Genetic variants of VEGF-A are associated with VEGF-A expression and preeclampsia risk, however there are still inconsistent results between different populations. The aim of this study was to determine the association of this genetic variant as preeclampsia risk factor.
Materials and methods: A cross-sectional study was performed with 76 pregnant women (29 preeclampsia and 47 normotensive) Jambi-Malay ethnic subjects. Sample DNA was extracted from subject's blood. To determine the genotype, one-step tetra amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) method for VEGF-A rs699947 C/A was used.
Results: We found that pregnant woman with AC genotype (*p*-value=0.045; OR=2.76 ; 95% CI=1.01-7.58) and AA genotype (*p*-value=0.026; OR=12.44; 95% CI=1.23-126.18) had higher risk of preeclampsia than the CC genotype.
Conclusion: Genetic variant VEGF-A rs699947 C/A is associated with preeclampsia. The AC and AA genotype is the risk genotype for preeclampsia in Jambi-Malay ethnics.

Keywords: *preeclampsia, VEGF-A, genetic variant, Jambi-Malay, Indonesia*

Introduction

Preeclampsia, a specific pregnancy syndrome, is signed by hypertension after 20 weeks of pregnancy and followed by clinical signs of endothelial dysfunction in maternal and neonatal.[1] This syndrome is the leading cause of maternal-neonatal morbidity and mortality worldwide, including in Indonesia.[2] Genetic and environmental factors have been known to have contributions for preeclampsia phenotype.[3-5]

Abnormality of trophoblast invasion to myometrium is followed by endothelial disfunction local in placenta, then spreads to maternal circulation. This abnormality involved

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unbalanced proangiogenic and antiangiogenic factors. Vascular endothelial growth factor A (VEGF-A) is one of the proangiogenic factors, which has a role in placenta and maternal vascular remodeling. The role of VEGF-A in trophoblast invasion to myometrium is by promoting trophoblast survival and its normal function.[6-9]

Early genetic risk assessment may enhance better prenatal care and awareness of high-risk pregnancy. VEGF-A gene is highly polymorphic. Genetic variant of VEGF-A rs699947 C/A located in 5' untranslated region (UTR) is associated with vascular related diseases and different VEGF-A expressions. However, the role of this genetic variant in preeclampsia is still inconclusive. Studies in Brazilian and Saudi Arabian pregnant women reported that this genetic variant was associated with the risk of preeclampsia.[10-11] In contrast, a study in Chinese women has shown otherwise.[12-14] Ethnicity and environmental factors may influence this difference.

To the best of our knowledge the association of VEGF-A rs699947 genetic variant with preeclampsia in Jambi-Malay population has never been published before in other Malay ethnicity or other ethnic in Indonesia. Hence, the aim of this study was to determine association of this genetic variant as preeclampsia risk factor.

Materials and methods

Study Design

This was a cross-sectional study with 76 Malay pregnant women who lived in Jambi Province. Twenty-nine women suffered preeclampsia and 47 women were normotensive. Subjects of the maternal period started in March to December 2020, and all subjects gave birth in Jambi Province General Hospital, Jambi, Indonesia. We determined racial background of subjects with anamneses, subjects were grouped as Jambi-Malay if they had grandparents from both parents with Jambi-Malay ethnicity.

Preeclampsia was defined based on American College of Obstetricians and Gynecologist (ACOG) 2013 criteria. Subjects were diagnosed as preeclamptic when they had hypertension (Systolic blood pressure ≥140 mmHg and or diastolic blood pressure ≥100 mmHg) after 20 weeks of pregnancy with one or more of the following conditions (proteinuria >+1; acute kidney failure sign by creatinine >1.1 gr/dL; serum glutamate oxaloacetate transferase (SGOT) and or serum glutamate pyruvate transferase (SGPT) >40 Iu/dL; or thrombocytes <100,000 cell/mm^3). Meanwhile, the control subjects were the normotensive pregnant women. Our exclusion criteria were multiple pregnancy, mother with sign clinical active infection, pregnancy with assisted technology for fertilization, history of kidney and hepatic failure, intrauterine growth retardation. All of the subjects were followed until the delivery of the baby.

Blood pressure was the highest recorded blood pressure during antenatal care after 20 weeks of pregnancy until baby delivery. It was taken twice by sphygmomanometer and the highest blood pressure was recorded.

Proteinuria was determined after the random urine sample, as much 10 mL was taken. The Combur-ux TestR strip was dipped into the urine sample. Then quantified using Urisys 1100R analyzer (Roche Diagnostic, Basel, Switzerland) and semiquantitative result was taken.

Fasting blood vein sample was taken as much 5 mL then centrifuge with 3000 RPM was performed to yield clear serum. Serum was used for creatinine, SGOT and SGPT measurement. Creatinine serum measurement was based on colorimetric enzymatic hydrolase assay using Creatinine PAP FSR (Proline, Cikarang, Indonesia). The SGOT and SGPT measurements were based on International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) colometric enzymatic assay with GOT and GPT FS kit (Proline). Urea, creatinine, SGPT and SGOT were quantified by automated chemical analyzer TRX 7010 (TokyoBoeki, Tokyo, Japan). Thrombocytes were measured with automated hematoanalyzer Sysmex XNLR, based on principle of flowcytometer.

All the subjects had signed informed consent forms after receiving detailed information concerning the purpose of the study. The protocol was arranged based on Helsinki Declaration and had approval from the Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Dr. Sardjito Public Hospital, Yogyakarta, Indonesia (Ethical approval number: KE/FK/1189/EC/2020).

DNA Extraction and Genotyping

Deoxyribonucleic acid (DNA) was extracted from venous blood buffy-coat, solid-phase with FavorPrep proteinase K DNA extraction kit (Favorgene, Ping-Tung, Taiwan) was used. DNA quality was measured by agarose gel electrophoresis which showed a continuous band above 1000 bp.

The genotyping process used one-step tetra amplification refractory mutation system (ARMS)

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polymerase chain reaction (PCR) which showed general product and allele specific product. The primer design was adapted from previous study[15], and the primer sequences and its product shown in Table 1. General product was 353 bp, A allele was shown by 149 bp PCR product and C allele was shown by 243 PCR product.

The PCR conditions were 95°C for 7 minutes as initial denaturation and 1 minute as denaturation; 60°C for 1 minute as annealing; 72°C as extension for 1 minutes and 7 minutes as final extension. The PCR product was then visualized with 2.5% agarose gel for 45 minutes with 100 mV.

Data Analysis

Normality distribution for continuous data scale was tested with Saphiro-Wilk, while data that was not normally distributed was transformed with log 10. Data that was not normally distributed was presented as median (min-max) and tested with Mann-Whitney test. Normally distributed data was presented as mean±SD and was tested with independent t-test. Association between genetic variants with preeclampsia and neonatal outcome were analyzed by bivariate Pearson chi-square and Fisher tests were used for bivariate analysis. The SPSS 25 (IBM Corporation, Armonk, NY, USA) was used as a statistical analysis tool.

Results

Baseline Subjects Characteristic

The baseline characteristics of study subjects were shown in Table 2. Maternal characteristic and neonatal outcome was described. Maternal and neonatal characteristics showed that the mean of age between preeclampsia and normotensive did not differ significantly. The parity and gravid also did not differ significantly between the two groups. Meanwhile systolic and diastolic blood pressure were higher in preeclampsia than normotensive as one of criteria for diagnosing preeclampsia. The gestational age did not differ between preeclampsia and normotensive women. But, the preeclampsia group had a higher proportion of baby birth weight less than 2,500 gram and asphyxia (Table 2).

Table 1. Primer and product of tetra ARMS PCR VEGF-A rs699947 C/A.

Primer Sequences	Product
FO : 5'-CCTTTTCCTCATAAGGGCCTTAG-3'	353 bp
RO : 5'-AGGAAGCAGCTTGGAAAAATTC-3'	
FI : 5'-TAGGCCAGACCCTGGCAA-3'	149 bp
RI : 5'-GTCTGATTATCCACCCAGATCG-3'	243 bp

Genotyping and Genotype Distribution

We performed one-step tetra ARMS PCR for genotyping of VEGF-A rs699947 C/A. PCR products were then visualized with electrophoresis. Specific allele was shown by different base pair. Our general product was 353 bp. Specific A allele PCR product was 149 bp and specific C allele PCR product was 243 bp (Figure 1). Heterozygote AC was shown with 3 different PCR products in electrophoresis 353 bp, 243 bp and 149 bp. Heterozygote AA and CC were shown with 2 different PCR products: general product and allele specific product. Genotype distribution of VEGF-A rs699947 C/A shown in Table 3. Minor allele of our population was A allele with frequency as much as 29%.

Hardy-Weinberg equation for all populations was calculated. The genotype distribution in our population was not deviated from Hardy-Weinberg equilibrium (p-value > 0.05) (Table 3).

Association of Genotype with Preeclampsia

The association phenotype and genotype for preeclampsia and severe preeclampsia were shown in Table 4 and Table 5. Pregnant women with AC and AA genotype had higher risk for suffering preeclampsia than CC genotype (Table 5).

The risk for suffering preeclampsia was higher 2.76 to 3.27 in additive and recessive models in all preeclampsia group. The risk was higher up to 12.44 in codominant model (Table 4). In addition, we performed analysis for severe preeclampsia group as shown in table 5. Pregnant women with AC and AA genotype have higher risk for suffering preeclampsia than CC genotype in additive, codominant and recessive models.

The highest risk for suffering preeclampsia was reached by codominant model, the risk up to 17.14 but has large confidence interval. The risk in additive and recessive model for preeclampsia was 2.65-3.31 (Table 5). The risk of this genetic variant as a risk factor for severe preeclampsia was slightly lower than all preeclampsia group.

Discussion

Baseline subject characteristic of our population showed that age between preeclampsia and normotensive does not

Table 2. Baseline sample characteristics.

Characteristic	Preeclampsia (n=29)	Normotensive (n=47)	*p* -value
Maternal characteristic			
Age, years old	29 (19-41)	30 (19-41)	0.728[a]
Pregnancy			
First pregnancy (n,%)	12	17	0.650[b]
Later pregnancy (n,%)	17	30	
Parous			
Nulliparous (n,%)	20	33	0.908[b]
Multiparous (n,%)	9	20	
Blood Pressure			
Systolic blood pressure, mmHg	170 (140-210)	110 (100-130)	<0.001[a]
Diastolic blood pressure, mmHg	100 (80-150)	80 (60-80)	<0.001[a]
Neonatal outcome			
Gestational age			
<37 weeks (n, %)	12 (41.38%)	17 (36.17%)	0.650[b]
≥37 weeks (n, %)	17 (58.62%)	30 (63.83%)	
Birth weight			
<2500 gr (n, %)	7 (24.14%)	3 (6.38%)	0.032[c]
≥2500 gr (n, %)	22 (75.86%)	44 (93.62%)	
APGAR score			
Asphyxia (<7) (n, %)	5 (17.24 %)	0 (0%)	0.006[c]
Normal (≥7) (n, %)	24 (82.76%)	47 (100%)	

[a]Nonparametric test due to not normally distributed data; [b]Chi-square test; [c]Fisher exact test.

differ significantly. Previous studies reported increased preeclampsia in pregnant women 20 years old or older than 35 years old.[6,16] Other environmental factors such as nulliparous and primigravida increase risk for preeclampsia. This condition is associated with higher expression of antiangiogenic factor and lower expression of proangiogenic

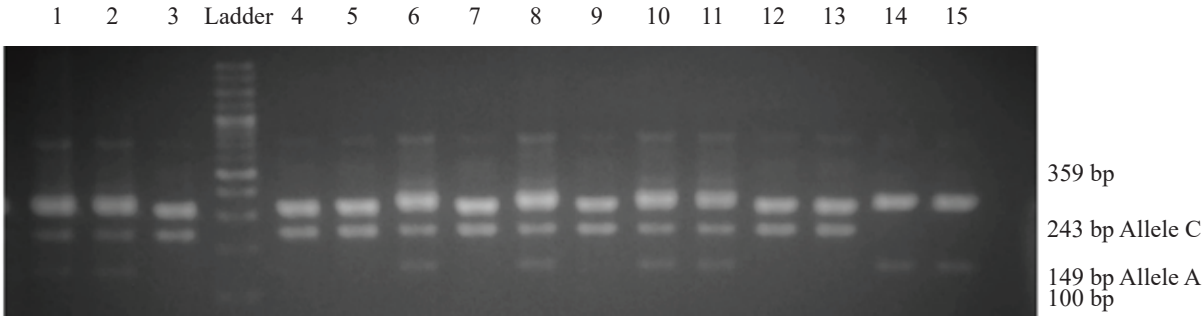

Figure 1. Electrophoresis of tetra ARMS PCR VEGF-A rs699947 C/A. A allele shown by PCR product 149 bp, C allele shown by PCR product 243 and general product shown by PCR product 359 bp. Lane 1,2,7,10,12,13 shown heterozygote AC. Lane 3, 5, 9 15,16 shown homozygote CC. Lane 17 and 18 shown homozygote AA.

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Table 3. Genotype distribution in Jambi-Malay population.

Genotype	Observed Value	Expected Value	X^2 (DF)	*p*- value	MAF
AA	5	6			
AC	34	32	0.58	0.44	0.29
CC	37	38			

Chi-square value with degree of freedom (DF)=1; MAF: minor allele frequency.

factor in nulliparous and first pregnancy than multiparous women.[17] This similar trend was not found in our population.

This study reported higher percentage of prematurity, low birth weight and asphyxia in preeclampsia group than normotensive group. According to previous research, there is an increase in cases of premature birth in preeclampsia patients. It is important to identify factor(s) that might increase the possibility of miscarriage in woman with preeclampsia to save mother and fetus's condition.[18] Preeclampsia is caused by a spasm in blood vessels, which, if prolonged, can cause an increase in uterine muscle tone and an increase in the sensitivity of the uterus to stimulation, resulting in premature labor.[19,20]

Preeclampsia is associated with neonatal outcomes such as low birth weight and asphyxia, in addition to premature birth. Preeclampsia is caused by a decrease in uteroplacental blood flow, which results in spasm. Continued spasm reduces uteroplacental blood flow. This can result in the inhibition of intrauterine fetal growth and development.[21,22] Fetal development has a significant impact on the baby's condition at birth. There is a perfusion disorder between the mother and the fetus in preeclampsia conditions. Inhibition of oxygen transport to the fetus resulting in hypoxia during the intrauterine period, which causes additional symptoms emerging in the form of asphyxia.[23,24]

Genotype distribution in our population reported A allele as minor allele. The frequency minor allele (MAF) of this genotype variant was 30%. This frequency is near MAF in Asian but lower than European population based on NCBI database. Our genotype was not deviated from Hardy-Weinberg equilibrium despite a small number of participated subjects. This may reflect our findings that the genotype and phenotype association were not different from the findings of other populations (Table 2).

One-step tetra-primer ARMS-PCR method allows fast, reliable and low cost for SNP genotyping than PCR-RFLP, two step ARM-PCR or HRM-PCR. This method uses outer forward and reverse forward primers to gain general product. Mismatch inner primer was designed to show specific allele in single nucleotide polymorphism.[25,26] Then, *in silico* analysis was performed to measure the PCR product. PCR condition optimization was based on our laboratory resources.

Genetic variant of VEGF-A rs699947 C/A is genetic variant in 5'UTR. Previous study reports this genetic variant modulates VEGF-A expression.[10,27] Different levels of VEGF-A may contribute to preeclampsia pathophysiology. Decreased serum VEGF-A was found in preeclampsia women compared to normotensive women. VEGF-A plays a key role in placental angiogenesis and secreted by trophoblast cells. The VEGF-A is also essential protein for integrity of the maternal endothelial cell. VEGF-A modulates vascular integrity by suppressing endothelial apoptosis, inhibiting inflammation and platelet aggregation.[7, 28-30]

This study reported AC and AA genotype as risk factors for preeclampsia and severe preeclampsia in Jambi-Malay population. Study in Brazilian and Saudi Arabian women reported that this genetic variant is associated with

Table 4. Association of genotype with preeclampsia.

Genotype	Preeclampsia (n=29)	Control (n=47)	*p* -value	OR (95% CI)
CC	9	28	ref	
AC	16	18	0.045[a]	2.76 (1.01-7.58)
AA	4	1	0.026[b]	12.44 (1.23-126.18)
AA+AC	20	19	0.016[a]	3.27 (1.23-8.72)

[a]Chi-square test; [b]Fisher exact test.

Table 5. Association of genotype with severe preeclampsia.

Genotype	Severe Preeclampsia (n=24)	Non Severe Preeclampsia (n=52)	*p* -value	OR (95% CI)
CC	7	30	ref	
AC	13	21	0.071[a]	2.65 (0.91-7.77)
AA	4	1	0.013[b]	17.14 (1.65-178.08)
AA+AC	17	22	0.021[a]	3.31 (1.17-9.35)

[a]Chi-square test; [b]Fisher exact test.

preeclampsia. Meanwhile, CC genotype and C allele carrier serve as protective factors for preeclampsia.[10,11] It may be related to higher expression of VEGF-A in women with C allele carrier, which appears to be a protective angiogenic factor for endothelial health.[10] In contrast, studies in China, Korea and Greece report that this genetic variant is not associated with preeclampsia.[12,14,31] This might be caused by the differences in ethnicity, subject recruitment criteria, interaction with other genetic variants and environmental factors.

To confirm genetic risk factor(s) of preeclampsia in this population, further research with larger sample size and more single nucleotide polymorphism in VEGF-A gene or other gene is needed.

Conclusion

Genetic variant VEGF-A rs699947 C/A is associated with preeclampsia. Subjects with AC and AA genotypes have higher risk for suffering preeclampsia than CC genotype.

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