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Polymorphism of the *Klotho* G-395a Gene Promoter with the Incidence of Preeclampsia

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ABSTRACT

Background. Preeclampsia is one of the leading causes of maternal and perinatal morbidity and mortality and is still a disease of theory. *Klotho* is a new gene, in several biological processes in the pathophysiology of preeclampsia that play a role in regulating endothelial nitric oxide production, angiogenesis, production of antioxidant enzymes and protection against endothelial dysfunction. The *Klotho* G-395A genotype AA promoter polymorphism is the cause of hypertension. This study aims to determine the relationship of the *Klotho* G-395A promoter polymorphism to the incidence of preeclampsia. **Methods.** This study is an analytical study with a case-control design. The research was conducted at Pembina community health centre of Palembang and the public hospital of Prabumulih in February - July 2020 and involving 50 case group and 50 control group. To determine the genotype and allotype of the *Klotho* G-395A gene promoter polymorphism, using polymerase chain reaction examination. **Result.** The results showed that the risk factors for maternal age and maternal gestational age had a significant relationship with the incidence of preeclampsia (p-value 0.015; p-value 0.000). There was a significant relationship between the *Klotho* G-395A genotype GA + AA promoter polymorphism and the incidence of preeclampsia (p-value 0.024; OR = 2.571; 95% CI = 1.122-5.895), while allotypes in the study sample also had a significant relationship with the incidence of preeclampsia. preeclampsia (p-value 0.025; OR = 1.978; 95% CI = 1.087-3.599). **Conclusion:** There is a significant relationship between the *Klotho* G-395A gene promoter polymorphism and the incidence of preeclampsia.

1. Introduction

Maternal mortality rate (MMR) and infant mortality rate (IMR) are indicators of the success of a country's development in the health sector. According to the *World Health Organization* (WHO), the global maternal mortality ratio has decreased by 38 per cent, from 451.000 women who died in 2000 to 295.000 women who died in 2017 or around 810 women worldwide die every day due to complications in pregnancy and childbirth.¹ In Indonesia, based on data obtained from the Indonesian Health Profile in 2017, there was a decrease in MMR from 359 in 2012 to 305 in 2015.

While the expected target based on the Sustainable Development Goals (SDGs) in 2030 is 70 / 100.000 live births.²

Hypertension in pregnancy, preeclampsia and eclampsia rank third (14%) of causes of maternal death after haemorrhage (27%) and indirect causes of pre-existing medical conditions exacerbated by pregnancy (28%).³ In South Sumatra Province, the number of maternal deaths by December 2017 reached 107 cases. Hypertension in pregnancy is the second cause of maternal death in 35 cases.²

Preeclampsia is still a disease of theory. Various studies have not been able to explain the exact cause of preeclampsia clearly. There are several hypotheses regarding the aetiology of preeclampsia, including placental ischemia, vascular disease, immunological maladaptation and genetic factors. The placenta plays a vital role in the pathogenesis of preeclampsia. Placental dysfunction often presents as a placental endocrine deficiency, silting placental invasion, and abnormal vascularization leading to systemic changes in preeclampsia.⁴⁻⁵

The imbalance between the production of free radicals and the antioxidant defence system due to placental ischemia causes oxidative stress. The lipid peroxidation process is considered to have an essential role in it. Ideally, during normal pregnancy, the balance of increased free radical production is always maintained through adequate production of antioxidants, however, in preeclampsia there is an increase in excessive free radical production and a decrease in antioxidant levels, causing oxidative stress.⁶

In a normal pregnancy, vascular homeostasis is maintained by physiological concentrations of *vascular endothelial growth factor* (VEGF) and signalling by transforming growth factor- β 1 (TGF- β 1). In preeclamptic conditions, excessive secretion of soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) (two endogenous anti-angiogenic proteins) inhibits VEGF and TGF- β 1 signalling. As a result, there will be endothelial cell dysfunction, decreased prostacyclin, decreased nitric oxide (NO), and pro-coagulant protein release.^{5,7}

Nitric oxide (NO) production and oxidative stress-regulated by α -Klotho (α -KL) can be significant components in the development of preeclampsia (PE). The function of α -Klotho in several biological processes in the pathophysiology of PE, including endothelial nitric oxide production, angiogenesis, production of antioxidant enzymes and protection against endothelial dysfunction.⁸ Klotho is a gene that was discovered by chance in 1997 by a Japanese group exploring the mechanisms of ageing.⁹ A known polymorphism of the Klotho gene, SNPs G-395A is widespread in Asian populations. SNPs in the G-395A

promoter region of the Klotho gene are frequently reported in Korean populations. G-395A (rs1207568) in the Klotho promoter region is associated with various disorders including hypertension, metabolic syndrome and reduced cognitive impairment.¹⁰ The purpose of this study was to determine the association of the Klotho G-395A promoter polymorphism with the incidence of preeclampsia.

2. Methods

This research is an analytical study with a case-control design. This research was conducted in February-July 2020 at Pembina community health centre of Palembang City and the Regional General Hospital of Prabumulih City, South Sumatra. This research has been through the study of the ethics of RSUP Mohammad Hoesin Palembang and Faculty of Medicine, Sriwijaya University Palembang with No. 019/kepkrmsfhkunsri/2020.

The population in this study were normotensive pregnant women as controls and preeclampsia as cases, with exclusion criteria, namely pregnant women with chronic hypertension, heart disease, kidney disease, gestational diabetes mellitus, thyroid function disorders, placental abruption, uterine malformations, multiple pregnancies, treatment. In vitro fertilization, infection, cancer, or other systemic diseases, including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Samples were taken using consecutive sampling technique, and age matching was carried out in the two study groups. A total of 100 samples were obtained, with 50 subjects of normotensive pregnant women in the control group and 50 subjects of pregnant women with preeclampsia in the case group.

The risk factors that were also studied were the age of the mother, the number of pregnancies (gravida), and the gestational age of the mother. Polymorphism examination consists of several stages, namely DNA isolation, *polymerase chain reaction* (PCR), and *Restriction Fragment Length Polymorphism* (RFLP). DNA isolation was performed using the DNA extraction method Wizard® Genomic DNA Purification Kit (Promega, USA), which used 300 μ l whole blood with EDTA.

The PCR kit used was the Promega Go Taq Green Master Mix. The primers used for the Klotho G-395A gene promoter polymorphisms are: Forward primer 5'-CGT GGA CGC TCA GGT TCA TTC-3' and Reverse primer 5'-TCC CTC TAG GAT TTC GGC CAG T-3'. Amplification was performed using a Biorad T100™ thermal cycler PCR machine (Bio-Rad, California, USA). The PCR conditions at the time of the study were initial denaturation of 94°C for 3 minutes, 35 cycles, denaturation at 94°C for 30 seconds, annealing at 61.7°C for 45 seconds and extension at 72°C for 45 seconds, additional extension at 72°C for 10 minutes after all cycles.

Detection of the Klotho G-395A gene polymorphism can be identified through the RFLP technique using the Hpy188III enzyme. The resulting PCR products were detected by electrophoresis in 2% agarose gel with ethidium bromide staining. Processing data using the SPSS 20.0 program. Bivariate analysis with *Chi-Square* test and multivariate analysis with Multiple Logistic Regression.

3. Results

Table 1 shows the comparison of demographic characteristics data between cases and controls. The results showed that maternal age and maternal gestational age had a significant relationship with the incidence of preeclampsia (p-value 0.015; p-value 0.000), while for gravida risk factors there was no significant relationship with the incidence of preeclampsia (p-value 0.817).

The results of the Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) as a method used to determine polymorphisms in the Klotho G-395A gene promoter are seen at the 247 bp position. In **figure 1**, there are three variations of the genotype resulting from the cutting of the Hpy188III inhibition enzyme on PCR (amplicon) products, the GG (wild type GG) genotype shows one band at 247bp, the

GA (Heterozygote mutant) genotype shows three bands, at 247bp, 175bp and 121bp, Genotype AA (Homozygote mutant) shows a two-band image, at 247bp and 121bp.

Of all study subjects who had AA genotype 7 subjects (7%), GA genotype 53 subjects (53%) and GG genotype 40 subjects (40%).

From the results of the chi-square test in **Table 2** above, it was found that there was a relationship between the AA, GA and GG genotypes of the Klotho G-395A gene and the incidence of preeclampsia. The group with the GA + AA genotype was found to have an increased risk factor of 2.571 times for the occurrence of preeclampsia compared to the GG genotype group (p-value = 0.024; 95% CI = 1.122-5.895). The presence of the A allele in a person is also known to increase the risk of preeclampsia by 1.978 times compared to individuals with the G allele (p-value = 0.025; 95% CI = 1.087-3.599).

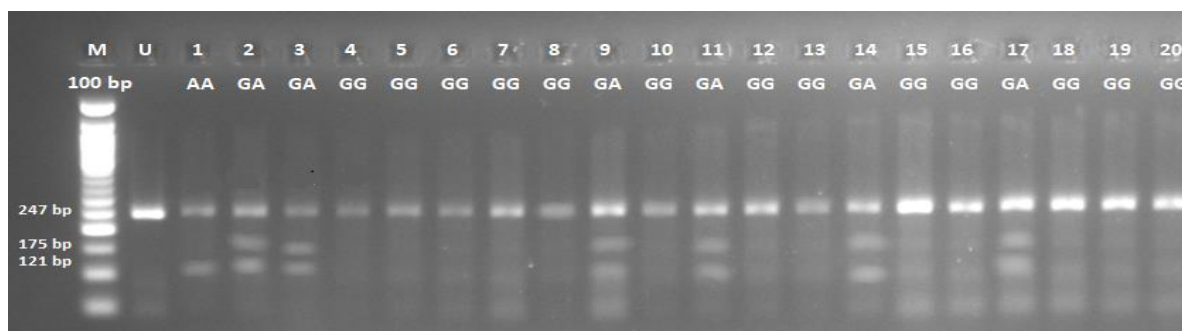
After conducting a bivariate analysis to determine the relationship between risk factors and the incidence of preeclampsia, it was found that the promoter polymorphisms of the Klotho G-395A genotype GA + AA, the age of pregnant women and the gestational age of the mothers were related to the incidence of preeclampsia with p values of 0.024 each; 0.015 and 0.000. Thus, these three risk factors, which have a p-value of <0.25, will be included in the multivariate analysis.

The multivariate analysis in **table 3** was carried out using logistic regression tests for the dominant risk factors affecting the incidence of preeclampsia. So it was found that gestational age is the dominant risk that affects the incidence of preeclampsia.

Based on these statistical tests, a formulation can be made to predict the occurrence of preeclampsia, as follows: probability of preeclampsia = $-5.842 + (1.110 \text{ polymorphisms of the Klotho G-395A genotype GA+AA}) + (1.112 \text{ maternal age}) + (1.799 \text{ gestational age})$.

Table 1. Analysis of the relationship between demographic characteristics and the incidence of preeclampsia

Characteristics	PE	Control	p-value	OR	95% CI
Maternal age (years)					
• < 20 years or > 35 years	20 (40%)	9 (18%)	0.015	3.037	1.214-7.597
• 20-35 years	30 (60%)	41 (82%)			
Gravidity					
• 1 times or ≥ 5 times	13 (26%)	12 (24%)	0.817	1.113	0.450-2.753
• 2-4 times	37 (74%)	38 (76%)			
Gestational age					
• ≥ 37 weeks	42 (84%)	23 (46%)	0.000	6.163	2.411-15.755
• 20-37 weeks	8 (16%)	27 (54%)			

**Figure 1.** Visualization of the Klotho G-395A gene promoter RFLP after administration of the Hpy188II restriction enzyme**Table 2.** Klotho G-395A Gene Promoter Polymorphisms With The Incidence Of Preeclampsia

Genotype/Alotype <i>Klotho</i> Gene G-395A	Preeclampsia	Normotensive	p	OR	95%CI
• AA	6 (12%)	1 (2%)	0.038	-	-
• GA	29 (58%)	24 (48%)			
• GG	15 (30%)	25 (50%)			
• GA+AA	36 (72%)	25 (50%)	0.024	2.571	1.122-5.895
• GG	14 (28%)	25 (50%)			
• Alele A	45 (40.9%)	30 (27.3%)	0.025	1.978	1.087-3.599
• Alele G	65 (59.1%)	80 (72.7%)			

Table 3. Risk factor associated with preeclampsia

Risk factor	B	Wald	p*	OR	CI 95%
Polymorphisms of the <i>Klotho</i> G-395A genotype GA+AA	1.110	5.318	0.021	3.036	1.181 - 7.801
Maternal age	1.112	4.514	0.034	3.040	1.090 - 8.479
Gestational age	1.799	12.651	0.000	6.042	2.242 - 16.281
Constanta	-5.842				

4. Discussion

Preeclampsia (PE) is a multisystem disorder in pregnancy that affects both the mother and the fetus. Maternal changes include hypertension and proteinuria with an onset after the 20th week of pregnancy. This also affects the condition of the fetus so that the fetus is at high risk of developing intrauterine growth disorders or even death in the womb.¹¹ Preeclampsia has been described as a pregnancy-specific syndrome of decreased organ perfusion that occurs due to vasospasm and endothelial activation. If not appropriately treated, preeclampsia can develop into eclampsia which is characterized by seizures. Preeclampsia is often unpredictable, can occur in mid-pregnancy, in labour or the early puerperium.⁴

Some of the risk factors for preeclampsia in pregnancy are a history of pregnancy; the maternal age is too young (less than 20 years) or too old (more than 35 years), family history, a history of maternal disease and obesity. The frequency of preeclampsia in primigravida is higher than in multigravida, especially primigravida at a young age.¹²⁻¹⁴

The results of the bivariate analysis in this study showed that maternal age had a significant relationship with the incidence of preeclampsia. A good and safe reproductive age for pregnancy and childbirth is 20-35 years at this age, the risk of complications during pregnancy is low. Preeclampsia is expected in the early and late reproductive years. Age <20 years, the reproductive organs are not ready for pregnancy. The size of the uterus has not reached the average size for pregnancy and is not ready and able to protect pregnancy and is not mentally ready and mature psychologically so that the possibility of disorders in pregnancy such as preeclampsia is more significant. At the same time, the age of the mother > 35 years of age occurs a degenerative process that occurs. resulting in structural and functional changes that occur in peripheral blood vessels that are responsible for changes in blood pressure, making it more susceptible to preeclampsia.¹⁵

Based on statistical analysis using the chi-square test, it was found that there was no significant relationship between gravida and the incidence of

preeclampsia. The results of this study are not by the theory which states that first pregnant women (primigravidas) often experience stress in dealing with childbirth, emotional stress that occurs in nulligravida, primigravida, multigravida causes an increase in the release of a corticotropic-releasing hormone (CRH) by the hypothalamus, which then causes increased cortisol. The effect of cortisol is to prepare the body to respond to all stressors by increasing sympathetic responses, including responses aimed at increasing cardiac output and maintaining blood pressure. Also, primigravidas have a very high chance of blocking antibodies from the mother's body with placental antigens, which can lead to hypertension to preeclampsia/eclampsia.

In pregnant women with ≥five times (multigravida) pregnancies, the endometrial environment around the implantation site is less than perfect and not ready to accept the conception, so the provision of nutrition and oxygenation to the results of conception is less than perfect and results in impaired growth of the results of the conception so that it can increase the risk of preeclampsia.¹⁵ Differences in the results of this study may occur because economic factors support them. Mothers who have high economic status in primigravida or grand multigravida can easily access health services during pregnancy when compared to mothers with no risk parity but have low economic status.

The risk factors for gestational age in this study have a significant relationship with the incidence of preeclampsia. The results of this study are under the theory of placental implantation ischemia, namely that the incidence of preeclampsia increases in late gestational age because at old gestational age fibrinogen levels increase and further increase in mothers with preeclampsia.¹⁶

Preeclampsia is still a disease of theory. Various studies have not been able to explain the exact cause of preeclampsia clearly. There are several hypotheses regarding the aetiology of preeclampsia, including placental ischemia, vascular disease, immunological maladaptation and genetic factors.⁵ Klotho is a gene that was discovered by chance in 1997 by a Japanese group exploring the mechanisms of ageing.⁹ The Klotho

gene (KL) was initially defined as an anti-ageing gene in mice. This gene encodes a single transmembrane protein type I. The human Klotho gene is located on chromosome 13q12 and five exons.¹⁷

In humans, α -klotho is mostly expressed in the kidneys, parathyroid glands, adipose tissue and choroid plexus. It is also distributed in the small intestine, placenta, and cord blood.¹⁸ In the female reproductive system, α -klotho is expressed in the prostate web of the breast, ovary, salpinx and uterus. During pregnancy, α -klotho is expressed in the placenta and is located at the border of the syncytiotrophoblast brush, while much less in the cytotrophoblast.¹⁹⁻²⁰

The functions of α -Klotho in several biological processes in the pathophysiology of PE include regulating endothelial nitric oxide production, angiogenesis, production of antioxidant enzymes and protection against endothelial dysfunction.⁸ α -KL regulates nitric oxide (NO) production or exhibits anti-inflammatory action in protecting endothelium and oxidative stress which can be significant components in the development of PE. Ischemic spasm and vascular lesions are the main pathological changes in hypertensive pregnancy, and NO plays a vital role in this process. α -KL can affect NO production. Animal studies have revealed that angiogenesis, which is dependent on endothelium-derived NO, is impaired in α -KL deficient mice. With the presence of Klotho protein, it can increase the availability of NO and protect against the incidence of endothelial dysfunction.²¹⁻²²

In humans, there are more than 10 SNP polymorphisms in the Klotho gene and have been linked to arteriosclerotic diseases, including hypertension. G-395A is an SNP in the promoter area that is associated with cardiovascular disease in women.²³⁻²⁵ This study shows that there is a relationship between the AA, GA and GG genotypes of the Klotho G-395A gene and the incidence of preeclampsia. Allele A in pregnant women is also known to increase the risk of preeclampsia by 1.978 times compared to pregnant women with the G allele (p-value = 0.025; 95% CI = 1.087-3.599).

The presence of the A allele in the Klotho G-395A

gene promoter polymorphism resulted in reduced transcription factor binding and reduced expression of the Klotho gene in human blood vessels in subjects with the A allele compared to subjects with the G allele. low in blood vessels, which will promote the development of endothelial dysfunction.²⁶ There are several different opinions regarding the impact of G/A substitution on expression -395 in the promoter part of the Klotho gene; the A allele has been reported to formless DNA protein complexes compared to the G allele indicating that protein bonds on the promoter part can be weakened by G/A substitution, which may be due to a decrease in the production of protein klotho.²⁷

From some of the above theories, the researchers concluded that the occurrence of preeclampsia is due to the G allele which forms a lot of DNA-protein complexes, transcription factor binding and Klotho gene expression, G/A substitution occurs at -395 expression resulting in decreased function of the Klotho gene due to decreased production of Klotho protein and reduced expression of the Klotho gene in blood vessels which promotes the development of endothelial dysfunction in mothers with preeclampsia.

5. Conclusion

From the results of the discussion, it can be concluded that there is a significant relationship between the Klotho G-395A gene promoter polymorphism and the incidence of preeclampsia. It is necessary to carry out further research using larger sample size and using more varied characteristics, such as research related to different polymorphisms so that genetic variants can be found in preeclampsia

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