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RESEARCH ARTICLE

The Polymorphism of rs2549782 gene Endoplasmic Reticulum aminopeptidase-2, high Concentration of Free Fetal DNA, and Cystatin C as Risk Factors of Preeclampsia

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Abstract

Background: Recently, the mechanism of the preeclampsia (PE) occurrence has not gained widespread agreement. This is related to the variations in treatment, maternal factor, and fetal morbidities as well as mortality. However, the studies of the placenta associated with PE mechanism have derived serious attention. This study aims to determine the role of rs2549782 ERAP 2 gene polymorphism, extracellular fetal DNA content, and high-level C cystatin in maternal serum as a risk factor for PE Methods: A matched case-control study among 62 maternal over 20 weeks of gestation was conducted in the Department of Obstetrics and Gynecology, Faculty of Medicine, Sanglah General Hospital, Biomolecular Laboratory of Medical Faculty, Universitas Udayana, Bali, Indonesia and Prodia Laboratory Denpasar. A 12 months-period of the study was enrolled from August 1st 2016-2017. The polymorphism examination of rs2549782 ERAP 2 gene, extracellular fetal DNA content and C cystatin respectively were evaluated among 31 maternal with PE as case and 31 maternal as control (Normal). Data were analyzed using SPSS version 16 for Windows to determine the association of rs2549782 ERAP2 gene polymorphism, extracellular fetal DNA level, and maternal serum cystatin C level using Chi-square Test and Odds Ratio (OR). Discriminant tests with logistic regression were displayed in table and narrative form. Results: The ERAP 2 gene polymorphism in the case was found 7.5 times (OR = 7.50 IK95%: 3.53-15.92; p = 0.002) higher significantly compared to the control. The cell-free fetal (cff) DNA level in the case was 6.67 times (OR, 6.67 IK; 95% 2.67-26.33, p; 0.001) higher significantly than the control. In addition, the level of cystatin C in the case was also 6.5 times (OR, 6.5 IK; 95% 3.04-13, 89, p; 0.007) higher significantly when compared to the control. However, there was no significant difference in the polymorphism of rs2549782 ERAP2 gene, extracellular fetal DNA, and cystatin C between groups (P>0.05).Conclusion: In can be concluded that the polymorphism of rs2549782 ERAP2 gene, extracellular fetal DNA, and high maternal serum C cystatin increase the risk of PE.

Keywords: *Preeclampsia, Rs2549782 polymorphism, ERAP2, DNA, cystatin C.*

Introduction

Until now, preeclampsia (PE) is still a maternal health problem associated with a relatively persistent prevalence with high maternal and fetal morbidity and mortality [1].The aetiology of PE itself is pregnancy, but the exact mechanism of the occurrence of PE has not yet obtained broad agreement so that PE is still the disease of theories. Many theories have evolved but all these theories have not been able to answer the exact aetiology of PE, such as hormonal theory, free radical theory, oxidative theory, immunological theory, and the theory of placental ischemia [1, 3].

However, the development of molecular biology affects many genetic theories, as well as the role of high levels of extracellular fetal DNA in the blood of pregnant women, are not determined yet. Worldwide, the prevalence of PE ranges from 2-10% of all pregnancies, whereas in developing countries, it is estimated to be seven times higher than in developed countries [3].

In Indonesia, the prevalence of PE is 3-7% and is the third-largest contributor to maternal mortality (AKI) after bleeding and infection [4].Recently, the cause of AKI appears to shift from obstetric to non-obstetric [4].In the last decade, PE studies have focused on the hypoperfusion of placenta with multifactorial triggering factors. Those factors are genetic, vascular, inflammation, immune and the environment [5].In PE, invasion of trophoblast cells is impaired so that the spiral artery remodelling is incomplete.

This causes the high resistance of uteroplacental circulation and results in placental hipoperfusion [6].Placental hypoperfusion triggers placental oxidative stress and syncytiotrophoblast cell apoptosis in which the cell fragment enters the maternal blood circulation [6].Furthermore, it activates endothelial cells and leukocytes release free radicals and cytokines [7].

Free radicals and cytokines in maternal blood plasma reactivate endothelial producing free radicals [8].This is a continuous causal cycle of PE. Based on the prevalence of PE is relatively persistent and is still a disease of theory, it is highly suspected that the mechanism of PE occurrence involves genetic factors. A previous study by Hill LD in 2011 reported that there was a change in the expression of endoplasmic reticulum aminopeptidase2 (ERAP2) in maternal placenta with PE [9].

Furthermore, Zhang Y et al. found that ERAP2 was expressed on the syncytiotrophoblast cells [10].Meanwhile, ERAP2 itself is a family of oxytocinases where Tsujimoto and Hattori have reported the role of oxytonase in the maintenance of pregnancy to remain physiological [11].Besides, ERAP2 also plays a role in blood pressure regulation, immune response, and production of proinflammatory cytokines [12].

The above findings are supported by Johnson et al. who reported that there was a polymorphism relationship of rs2549782 ERAP2 gene in mothers with PE, supported by Hill study results [13].Thus, polymorphism of rs2549782 gene ERAP2 is associated and contributes to the mechanism of occurrence of PE. Placental Hypoperfusion results in trophoblast damage where the release of the destruction of placental trophoblast cells is extracellular fetal DNA and enters the maternal bloodstream; acts as a toxin antigen, thus triggering antigen-antibody reactions.

Examination of maternal serum extracellular fetal DNA is a non-invasive diagnostic method in the study of the mechanisms of PE. In addition, extracellular fetal DNA can also be used as an indicator to determine the well-being of the fetus and the risk of pathologic fetal development itself [14]. The high amount of extracellular fetal DNA in maternal plasma was significantly correlated with PE.

In addition, it is also associated with the occurrence of HELLP syndrome and intrauterine inhibited fetal growth [15].Placental hypoperfusion is associated with elevated levels of cystatin C protein where it is thought to play a role in the mechanism of occurrence of PE. Cystatin C levels cause the high number of extracellular fetal DNA and result in decreased renal function against extracellular cellular fetal DNA excretion resulting in the accumulation of these proteins in the maternal circulation [16, 17].

The high amount of fetal DNA will cause an increase of pro-inflammation substance that will affect the occurrence of endothelial damage. Systemic endothelial damage may result in complications of PE [16, 17].Based on those mentioned above, this study aims to determine the role of rs2549782 ERAP2 gene polymorphism, extracellular fetal DNA protein, and cystatin C as risk factors in the mechanism of PE occurrence.

Method

A matched case-control study conducted in Department of Obstetrics and Gynecology, Faculty of Medicine, Sanglah General Hospital, Biomolecular Laboratory of Medical Faculty, Universitas Udayana, Bali,

Indonesia and Prodia Laboratory Denpasar during August 1st 2016-2017. Polymorphism examination of RS2549782 ERAP 2 gene, extracellular fetal DNA content, and C cystatin respectively with a sample of 62 maternal over 20 weeks consisting of 31 PE as case and 31 as control. The inclusion criteria were preeclamptic pregnant women (PE) and pregnant women without preeclampsia with more than 20 weeks gestational age, who examined at the Obstetrics and Gynecology Polyclinic Outpatient Installation, Emergency Installation, and Inpatient Installation Sanglah General Hospital who were willing to involve in this study by sign informed consent.

Also, the exclusion criteria were fetal anomalies through ultrasound examination, twin pregnancy, chronic hypertension, congestive heart disease, diabetes, and history of kidney disease. Data were analyzed using SPSS version 16 for Windows to determine the association of rs2549782 ERAP2 gene polymorphism, extracellular fetal DNA level, and maternal serum cystatin C level using Chi-square Test and Odds Ratio (OR).

Discriminant tests with logistic regression was displayed in table and narrative form.

Results

Data regarding age, number of parities, gestational week, and body mass index (BMI) as the baseline characteristic of respondents were depicted in Table 1. Our study found that the average age was tended to be higher in the case group (31.35±6.71 years) compared with the control group (28.52±6.43 years) although not significant (P=0.94) (Table 1). Some parities, gestational age, and BMI score were tended to be higher in the case group (1.29±0.81, 36.61±3.31 weeks, and 31.49±4.39 kg/m^2, respectively) compared with control group although not significantly different (P>0.05) (Table 1).

Table 1: Baseline characteristic of respondents between groups in age, number of parities, gestational age, and BMI

Variables (Mean± SD)	Respondents (N=62)		P
	Case Group (N=31)	Control Group (N=31)	
Age (Year)	31.35±6.71	28.52±6.43	0.940
Number of Parities	1.29±0.81	0.94±0.89	0.183
Gestational Age (Weeks)	36.61±3.31	36.06±3.24	0.512
BMI (kg/m^2)	31.49±4.39	30.56±5.60	0.467

BMI: Body Mass Index; SD: Standard Deviation; kg: kilogram; m: metres; P=Independent T-Test, considered significant if less than 0.05

In order to determine the risk of PE into several parameters assessed, Chi-square analysis was used in this study. The positive ERAP2 gene polymorphism on maternal has 7.20 times higher risk for PE significantly (95%CI: 2.18-23.75; P=0.001) compared with negative polymorphism. In addition, high fetal DNA content also statistically significant to have 8.38 times higher risk for PE on maternal (95%CI: 2.67-26.33; P=0.001). Besides, high Cystatin C levels also considered having 7.03 times higher risk for PE among maternal (95%CI: 2.29-21.48; P=0.001) significantly (Table 2).

Table 2: The risk of PE based on polymorphism of rs2549782 ERAP2 gene, Fetal DNA Content, and Cystatin C levels

Parameters	Groups (N=62)		OR	95% CI	P
	Case (N=31)	Control (N=31)			
ERAP2 gene polymorphism					
Positive	26	13	7.20	2.18-23.75	0.001
Negative	5	18			
Cff DNA Levels					
High	22	7	8.38	2.67-26.33	0.001
Low	9	24			
Cystatin C Levels					
High	23	9	7.03	2.29-21.48	0.001
Low	8	22			

OR: Odds-ratio; ERAP2: endoplasmic reticulum aminopeptidase-2; Cff: cell-free fetal; CI: confidence interval; P: statistically significant if less than 0.05

Discussion

In this study, the mean of maternal age in case of group 31, 35 years and 28, 52 years in the control group. By using t-independent test against maternal age variable, there is no statistical difference between the two groups. The occurrence of rs2549782 polymorphism of ERAP2 gene in the case group was significantly higher than in the

control group. Maternal with polymorphism rs2549782 gene ERAP2 had a risk of having PE 7.20 times higher than maternal without polymorphism rs2549782 gene ERAP2 The results of this study in accordance with some previous research. Johnson et al. was the first investigator to report that the ERAP2 gene was associated with PE in the Australian and Norwegian populations [13].

They also reported about the genetic association with pre-eclampsia occurrence of 2q, 5q and 13q chromosomes in the cohort in the Australian / New Zealand population (Aust/NZ) [13].In addition, the Australia/New Zealand and the Norwegian population have shown that the SNPs ERAP2 (p.392 K> N and p.669 L> Qs, rs2549782 and rs17408150, resp) are associated with PE risk [13].

Other research groups conducted in various countries reported the genotype of ERAP2 SNP rs2549782 was associated with a higher PE risk in African American women (P¼ 0.009) [9].This finding is supported by a recent study, which reveals that elevated levels of transcript ERAP2 in decidual tissue of PE patients, however, contradict the initial observation of this gene expression that decreased in placenta of one-trimester pregnancy in women who progressed to PE [18].One possible explanation for this gene observation is related to when and which ERAP2 transcription variants are expressed and translated to influence the maternal immune response.

Determination of gene expression that deviates before the development of maternal symptoms indicates that ERAP2 is involved in the process of the occurrence of this disease early (early-onset).Therefore, it is essential to determine how and when the presentation of the paternal HLA antigen underwent interference by the ERAP2 variant and the subsequent immune modulation that can evaluate the outcomes of pregnancy. The extracellular DNA fetal levels in the case group were significantly higher when compared with the control group. Maternal with high levels of extracellular fetal DNA had an increased risk of PE 8.38 times higher than maternal with low extracellular fetal DNA.

The results of this study in accordance with some previous research. An investigation has been conducted in the UK to predict PE by examining extracellular fetal DNA in 11-13 weeks and its association with an increase in the pulsatility index of the uterine artery [19].The results obtained were quite interesting, the researchers found that the median extracellular fetal DNA in patients with early-onset PE (median, 95.5 genome equivalents/mL, interquartile range, 72.7-140.9 genome equivalents/mL), but not on the onset of PE when compared with control [19].

When compared with the pulsatility index of the uterine artery, there were significant results between extracellular fetal DNA and PI of the uterine artery ($p = 0.038$) but not in the control group ($p = 0.174$) [19].The finding of this relationship is consistent with the hypothesis that the invasion of trophoblast in the spiral artery causes placental ischemia and malfunction, and consequently is the apoptotic material of the syncytiotrophoblast that computes the fetal DNA fragment into the maternal circulation.

Whether extracellular fetal DNA is merely a marker of placental impairment or extracellular fetal DNA has a role in the pathogenesis of PE by causing endothelial dysfunction and the appearance of clinical symptoms unexplained in this study [19]. Although extracellular fetal DNA levels are elevated in pregnancy with PE, they are not correlated with most laboratory parameters of patients with PE, except for aspartate aminotransferase and alanine aminotransferase activities based on the previous study [20].

In terms of clinical characteristics including patient BMI, there was also no significant association. This elevation of the liver enzyme may indicate the role of hepatocellular necrosis in total circulating DNA fetal in PE [20]. In contrast to the study conducted by Rolnik et al., the extracellular fetal DNA and the fetal fraction was found at 11-13 weeks gestational age and 20-24 weeks in pregnancy with PE, it was concluded that measurement of extracellular fetal DNA content was not a predictor of PE [21].Based on the previous hypothesis which states that extracellular fetal DNA is a result of the release of necrotic tissue of Syncytiotrophoblast that has ischemia and necrosis and is merely a marker of PE, another study should be undertaken.

Extracellular fetal DNA is most likely not just a product of ischemia and necrosis alone but also contributes to the pathogenesis of

PE. The results of this study found that fetal DNA activates NF-κB, which is indicated by the degradation of IκBα resulting in the production of IL-6 via the TLR9 pathway, and this pro-inflammatory response can lead to both PE and preterm delivery [22].Konecna et al. wrote a study and postulated the possibility of the role of extracellular fetal DNA in the pathogenesis of PE [23].In maternal circulation, extracellular fetal DNA and cytosolic DNA are recognized by cell receptors that will trigger cell reactions.

Extracellular fetal DNA circulating in the mother's blood is hypomethylated, meaning that the methyl group is added to the DNA molecule in the cytosine-guanine reside and forming a CpG [23].This addition has a blockade effect of transcription factors followed by gene silencing and downregulation. Then extracellular fetal DNA circulating as a result of apoptosis in the placenta will result in extracellular fetal DNA containing mitochondrial DNA (mtDNA). Toll-like receptors (TLRs) are susceptible to this type of molecule as it is sensitive to bacteria or viruses. This is because mtDNA structurally resembles bacterial DNA and covers the CpG site. The TLR9 receptor will activate the immune system and initiate the inflammatory process [23].

In certain situations, tumour necrosis factor-α (TNF-α) and lipopolysaccharides stimulate cellular cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), which is responsible for DNA synthesis [23].In pregnancy with PE, the formation of cGAMP is the result of high circulation of natriuretic peptide. When cytosolic DNA undergoes transfection, cGAMP is triggered and binds to the endoplasmic reticulum of the STING protein, followed by activation of interferon 3 and interferon ß [23].

Based on what has been described above, it can be hypothesized that extracellular fetal DNA in pregnancy with PE has the same properties as bacterial DNA or mtDNA, and is likely to activate cGAMP and trigger inflammation; thus the possibility of extracellular fetal DNA may be the cause of PE and not only as a marker of apoptosis and placental ischemia. In experimental animals, giving autoantibodies causes increased blood pressure, proteinuria and soluble factor formation from the placenta. As the study of extracellular fetal DNA develops new insights, it is likely that this extracellular fetal DNA that plays a role in triggering the immune and inflammatory response that causes a syndrome in PE, certainly needs further study.

In this study, extracellular fetal DNA content in the case group was significantly higher when compared with the control group. This proves that the findings in this study show that the same is true for populations in Indonesia, that extracellular fetal DNA is elevated in PE cases that are likely to play either as markers or in the pathogenesis of PE [23].This study did not directly address the role of extracellular fetal DNA in the pathogenesis of PE, the conclusion drawn from this study was that there was a significant increase in the concentration of extracellular fetal DNA on maternal serum in PE when compared with non-PE control.

This may answer the role of extracellular fetal DNA as a marker of PE but has not been able to answer the role of extracellular fetal DNA in the pathogenesis of PE. This research can be the basis for the development of further research that can study the path or role of extracellular fetal DNA in the pathogenesis of PE. Based on Cystatin C evaluation, it was found that Cystatin C level in the case group was significantly higher when compared with the control group.

Maternal with high levels of cystatin C had a risk of having PE higher 7.03 times than maternal with low cystatin C levels. At the time of pregnancy, serum cystatin C levels usually increase in the third trimester, due to decreased excretion and increased synthesis by the fetoplacental unit [24].In a study by Sharma S et al., an average measurement of serum cystatin C level was performed in 197 women with a healthy pregnancy. The result was a mean serum cystatin C in all patients of 0.82 + 0.18 mg / l.

Where serum cystatin C is high about 0.89 + 0.12 mg / l during the first trimester, then decreases significantly to 0.65 + 0.14 mg / l during the second trimester compared to the first trimester and then increased again by 0.82 + 0.19 mg / l in the third trimester [24].Strevens H et al. found that cystatin C levels rise proportionally throughout pregnancy [25].The cystatin C may be used

as a marker, not only in renal dysfunction, but also in glomerular endotheliosis and increased glomerular volume in pregnant women, and significantly significant in monitoring pregnancy with PE [25].Another study by Kristensen et al. also found that cystatin C and beta-2-microglobulin in plasma increased considerably in PE.[26] High levels of cystatin C in PE are a reflection of placental ischemia [26].

Conclusion

Based on the results, it can be concluded that the maternal with rs2549782 polymorphism ERAP2 gene increased the risk of PE 7.20 times. The high levels serum of extracellular fetal DNA and Cystatin C in maternal also increased the risk of having PE 8.38 and 7.03 times, respectively, compared with maternal without rs2549782 polymorphism ERAP2 gene.

Ethics Consideration

Ethics approval has been obtained from the Ethics Committee, Faculty of Medicine, Universitas Udayana, Sanglah General Hospital, Bali, Indonesia, prior to the study being conducted.

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