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Protein Z and anti-protein Z IgG levels, but not the promotor A13G polymorphism, are associated with preeclampsia

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Summary

The present study was designed to determine the association between PZ promotor A13G polymorphism, PZ levels, anti-PZ antibodies levels, and the occurrence of preeclampsia (PE). A case-control study including normal pregnant women (control group, n=75) and pregnant women with PE (PE group, n=125) was performed. PZ levels (mg/L) were significantly lower in PE group (1.45 ± 0.21) than control group (2.07 ± 0.29, $p < 0.05$). The plasma anti-PZ IgG concentrations (AU/ml) in PE group (5.2 ± 0.62) were significantly higher than that in control group (3.3 ± 0.61, $p < 0.05$), while there were no significant differences of anti-PZ IgM concentrations (AU/ml) between two groups (12.2 ± 0.92 *vs.* 12.2 ± 1.18, $p > 0.05$). Multivariate analysis showed that decreased PZ [OR (95% CI) = 200.39 (11.80-3403.91)] and elevated anti-PZ IgG [OR (95% CI) = 0.013 (0.002-0.088)] were independent risk factors of PE. The results suggested that low PZ levels and high anti-PZ IgG levels are associated with the occurrence of PE.

Key words: Preeclampsia; Protein Z; Anti-protein Z antibodies; Gene polymorphism.

Introduction

Preeclampsia (PE) is a disorder of pregnancy characterized by high blood pressure and proteinuria [1]. Women with PE receive higher risk of heart disease and stroke [2]. Therefore, PE is considered as one of the most common causes of death in pregnancy [3]. It is accepted that obesity, older age, hypertension, and diabetes mellitus are risk factors of PE. The underlying mechanism of PE involves abnormal balance of blood coagulation and anticoagulation. The pressure of uterus coiled artery are increased by formation of microthrombosis. Excessive formation of thrombotic lesions in the placental villi [4] and decidual vessels [5, 6], and higher maternal plasma concentrations of thrombin-anti-thrombin complexes were found in patients with PE[7-8].

Protein Z is a vitamin K-dependent glycoprotein that plays a role in activation of factor X [9, 10]. This inhibition of factor X leads to decrease of thrombin generation, thus PZ deficiency is linked with anticoagulation disorder. There are some conflicting reports on association between PZ levels, anti-PZ antibodies levels, gene polymorphisms, and PE. On one hand, some have reported that there is no significant difference in the concentrations of PZ between patients with PE and normal pregnant woman [11]. On the other hand, some researchers found the levels of PZ were lower in the patients with PE than those in normal pregnant woman [12]. However, there was no research on PZ gene promotor A13G polymorphism and anti-PZ antibodies in PE. With the aim, to further clarify the function of PZ in PE, the association between the median plasma concentrations of PZ, PZ promotor A13G polymorphism, the levels of anti-PZ antibodies, and PE was investigated in this study.

Materials and Methods

In this study, all cases were divided into two groups, the patients with PE (PE group, n=125) and normal pregnant women (control group, n=75). PE was diagnosed as pregnant women with previously normal blood pressure with systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg in two separate measurements, which were four to six hours apart, after 20 weeks gestation. Hypertension was associated with one of the following situations: proteinuria ≥ 0.3 grams or more in a 24-hour; a SPOT urinary protein to creatinine ratio ≥ 0.3; urine dipstick test ≥ 1+ [13]. There were no thrombotic events in all cases. No medical treatments including oral contraceptive pills that could affect the measured parameters were given. Informed consent was obtained from all cases. The study was approved by the Institutional Review Board of the 1st Hospital Affiliate of Wannan Medical College and complied with the Declaration of Helsinki.

Blood samples were collected in vacuum tubes containing 0.109 M trisodium citrate anticoagulant solution and EDTA. The samples in 0.109 M trisodium citrate were centrifuged at 2,500 rpm for 10 minutes at 4°C. The samples in EDTA were kept frozen until DNA extraction. The levels of PZ and anti-PZ antibodies were measured by enzyme-linked immunoassay.

Concentrations of PZ were determined by a quantitative immunoenzymatic method. Briefly, the standards and samples were incubated in duplicate wells of a 96-well microtiter plate precoated with a monoclonal antibody specific for PZ. During incubation, repeated washings were performed to remove unbound

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materials. Then a secondary monoclonal antibody coupled with peroxidase directed against another epitope of PZ was added to the assay plates. Additional washes to remove unbound materials were performed. Finally, an equal amount of stabilized chromogen, ortho-phenylenediamine and urea peroxide substrate were added in each well. The development of color was halted at a set time by the addition of sulfuric acid (3M). Optical densities were measured by a programmable microplate reader. PZ concentrations were determined by interpolation from individual standard curves. The calculated inter- and intra-assay coefficients of variation were 3.31% and 1.52%, respectively. The sensitivity of PZ immunoassay in our laboratory was 0.05 μg/ml.

Anti-PZ IgG and IgM were tested by ELISA using Anti-Protein Z kits. Firstly, diluted samples were incubated in wells of the microtiter plates, which precoated with highly purified human PZ. Secondly, samples were washed to remove unbound antibodies. In order to detect IgG isotype, each well was added with a peroxidase conjugated goat anti-human IgG. Similarly, to detect IgM isotype, a peroxidase conjugated goat anti-human IgM were added. After repeated washings, tetramethylbenzidine was added. Finally, the development of color was stopped by the addition of acid solution. The intensity of color was measured by a programmable microtiter plate spectrophotometer. Anti-PZ IgG or IgM in samples were determined by interpolation from individual standard curves. The calculated inter- and intra-assay coefficients of variation for anti-PZ IgG isotype were 5.11% and 4.52%, respectively. The sensitivity for the anti-PZ IgG isotype was 1.1 AU/ml. The calculated inter- and intra-assay coefficients of variation for anti-PZ IgM isotype were 6.35% and 2.42%, respectively. The sensitivity for the anti-PZ IgM isotype was 2.1 AU/ml.

Genomic DNA was extracted from blood leukocytes using a rapid method. The promotor A13G polymorphism of the PZ gene was determined by PCR using of the following oligonucleotides: 5'-GGG TCC TCT GAG CCT TCA CCG TTC ATT T-3, 5′-CAG GCA CAA CAG ACA GGT AAG CCA GAT G -3′ as primers. The procedure of PCR consisted of four-minute denaturation at 95°C, followed by 35 cycles of 30-second denaturation at 95°C, 30-second annealing at 58°C, and 45-second extension at 72°C. Then samples were maintained at 72°C for five minutes. The products of PCR were incubated for at least three hours with a restriction enzyme: HhaI at 37°C. The G allele yielded two DNA fragments of 157 and 115 bp on a 2.5% agarose gel electrophoresis after ethidium bromide coloration, whereas the A allele was not digested.

The age was compared by Student-*t* test, the distribution of risk factors was compared by χ^2 test. A univariate logistic regression to calculate the odds ratio and the 95% confidence interval when distribution were significantly different. As PZ concentrations were not normally distributed, Kruskal-Wallis and Mann-Whitney U tests were used for comparisons. A p-value < 0.05 stands for statistically significance. Statistical analysis was performed using SPSS software (Version 21.0).

Results

The basic demographic and clinical characteristics of the all cases in this study are presented in Table 1. The median gestational age at blood collection of control group was lower than that in the PE group, while the median gestational age at delivery was higher in control group than that of PE group.

The mean PZ level (mg/L) in PE group (mean ± S.D. 1.45 ± 0.21) was significantly lower than that in control group (mean ± S.D. 2.07 ± 0.29, $p < 0.05$) (Figure 1A). As to anti-PZ antibodies, the mean level (AU/ml) of anti-PZ IgG in PE group (mean ± S.D. 5.2 ± 0.62) was significantly higher than that in control group (mean ± S.D. 3.3 ± 0.61, $p < 0.05$), but there was no significant difference in anti-PZ IgM level (AU/ml) between PE and control group (mean ± S.D. 12.2 ± 0.92 *vs.* 12.2 ± 1.18, $p > 0.05$) (Figure 1B, 1C). Through multivariate analysis, PZ [OR (95% CI) = 200.39 (11.80-3403.91)] and anti-PZ IgG [OR (95% CI) = 0.013 (0.002-0.088)] were shown as independent risk factors of PE (Table 2). Receiver operating characteristic curve (ROC) analysis showed the area under the curve for anti-PZ IgG was 0.985 and for anti-PZ IgM was 0.493 (Figure 1D).

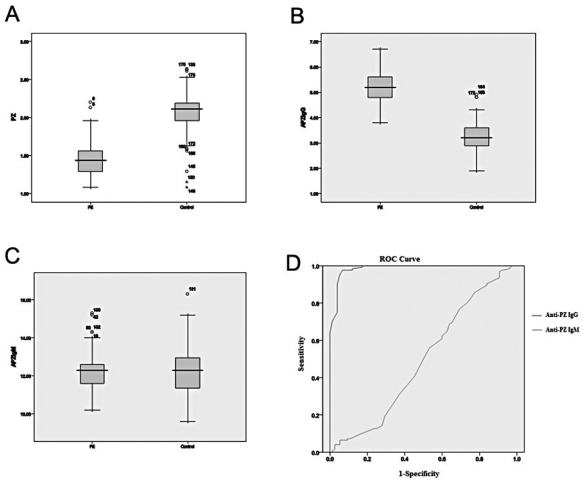

Figure 1. — A) Comparison of the median maternal plasma protein Z concentrations between women with preeclampsia and those with normal pregnancy ($p < 0.05$). B) Maternal plasma concentrations of anti-protein Z IgG ($p < 0.05$) in patients with preeclampsia and normal pregnancies. C) Maternal plasma concentrations of anti-protein Z IgM ($p > 0.05$) in patients with preeclampsia and normal pregnancies. D) ROC curve based on binary logistic regression and discriminant classification analysis for preeclampsia/control groups. The area under the curve is 0.985 for anti-PZ IgG and 0.493 for anti-PZ IgM. The value of specificity is plotted as 1-specificity on the x-axis.

The prevalence of PZ promotor A13G is shown in Table 3. The distributions of the PZ promotor A13G polymorphism were: AA genotype in 33 (26.4%) patients, AG genotype in 53 (42.4%) patients, GG genotype in 39 (31.2%) patients in PE group, AA genotype in 22 (29.3%) patients, AG genotype in 31 (41.3%) patients, and GG genotype in 22 (29.3%) patients in control group. There were no significant differences of frequency of PZ promotor A13G distributions between two groups ($p = 0.668$).

Table 1. — *The basic demographic and clinical characteristics of the study population.*

	Preeclampsia (n=125)	Normal pregnancy (n=75)
Maternal age (years)	26.1 ± 4.3	25.5 ± 3.8
Gravidity		
1	53 (42.4%)	36 (48.0%)
2-3	63 (50.4%)	33 (44.0%)
≥4	9 (7.2%)	6 (8.0%)
Parity		
1	113 (90.4%)	68 (90.7%)
2-3	11 (8.8%)	7 (9.3%)
≥ 4	1 (0.8%)	0 (0.0%)
Gestational age at blood collection (weeks)	34.2 ± 4.1	34.5 ± 4.7
Gestational age at delivery (weeks)	34.5 ± 3.9*	39.8 ± 2.2

*Data are presented as mean±standard deviation or number (%). The test group was compared with the normal pregnancy group: *p < 0.05.*

Table 2. — *Matched odds ratios for preeclampsia Risk by Protein Z and anti-protein antibodies*.*

	Isotype	OR	95%CI	*p*
Mutivariate	PZ	200.391	11.797-3403.905	0.000
level	Anti-PZ IgG	0.013	0.002-0.088	0.000
	Anti-PZ IgM	0.817	0.268-2.487	0.722

**Odds Ratios (OR) and their corresponding 95% confidence intervals (95% CI) for risk of preeclampsia by protein Z and anti-protein antibodies.*

Table 3. — *Comparison of genetic variations between patients with preeclampsia and control.*

	Preeclampsia (n=125)	Controls (n=75)	*p*	Crude OR(95%CI)
PZ A13G N(%)				
Wild type(AA)	33 (26.4)	22 (29.3)		
Heterozygote (AG)	53 (42.4)	31 (41.3)	0.668	
Homozygote (GG)	39 (31.2)	22 (29.3)		
At least 1 G allele	92 (73.6)	53 (70.7)	0.783	1.03 (0.82-1.31)

Discussion

PZ has been proposed to play a critical role in pregnancy. During gestational period, with increased concentration of factor X and thrombin generation, the plasma level of PZ was increased accordingly [14]. PE is characterized by higher rate of thrombotic lesions in the decidua and placental villi [15, 16]. The pathogeny of PE is associated with hypercoagulable state and excessive thrombin generation [17]. PZ deficiency is considered as a risk factor for thrombosis [9]. Bafunno *et al.* demonstrated that patients with PZ deficiency (< 1 mg/L) have a significantly higher incidence of deep venous thrombosis than normal patients [18]. Therefore, it is possible that the lower plasma concentration of PZ could exist in women with PE. Anti-PZ antibodies which are present in the serum, participate in the clearance of catabolic products and respond to foreign antigens [19, 20]. However, the mechanisms how high level of anti-PZ IgG contribute to the development of PE are still not clear. Perhaps increased immune complex formation is associated with inhibition of PZ activity [21]. This procedure would lead a tendency of hypercoagulation in the maternal side of the placenta [22], resulting in PE and other miscarriages.

In this study, the authors investigated the effect of PZ levels, anti-PZ antibodies levels, and the promotor A13G polymorphism on PE in the Han nationality. They found that decreased plasma concentration of PZ was associated with an increased risk of PE. Other researches had also indicated that low levels of PZ were associated with some artery and venous diseases, which is consistent with the present results. However regarding anti-PZ antibodies level and gene polymorphism, there are still controversies. Sater *et al*. found that there was significant elevation of anti-PZ IgG and IgM titers in women with recurrent spontaneous miscarriage [23]. Erez *et al.* have reported that there was no differences of the concentrations of anti-PZ IgG or IgM between patients with PE and normal pregnancy women in

African-Americans, Caucasian, Hispanics, and so on [21]. In the present study, the authors found that the increased levels of anti-PZ IgG, but not IgM, were associated with an increased risk of PE. Demir *et al.* have reported an increased A allele frequency of PZ intron F G79A polymorphism in Turkish Behçet patients [24]. Topalidou *et al.* found no association between PZ intron F G79A polymorphism and unexplained pregnancy loss [25]. In the present study, there was no significant difference of PZ promotor A13G gene polymorphism between PE group and control group. However the present authors found that there were more mutations in gene promotor 13 A→G in ethnic Han than Caucasian. The results of this study indicate that PE is associated with PZ and anti-PZ IgG. The role of the PZ in normal pregnancies and those with complications as a systemic or a local regulator remains unclear. By regulating FX activity, the presence of the PZ complex may provide a local defence mechanism against vascular injury accompanying poor placentation and fetal loss. As such, reduced PZ activity stemming from specific anti-PZ autoantibodies precipitates adverse pregnancy outcomes.

There were still some limitations in this study. The first limitation of this study was the gestational age at blood collection. The samples should have been collected in early and late periods of gestation separately in order to avoid evaluating partially. The second limitation is that this study did not clarify the mechanism of the association between PE and PZ genotype. The gene polymorphism described in this study has been reported to be important determinants of PZ level. However, the authors did not find the difference of the gene polymorphism in this study and did not research on other PZ gene polymorphisms.

In conclusion, the results of this study indicate that low PZ concentrations and high anti-PZ IgG levels are associated with PE, whereas the promotor A13G polymorphism showed no effects in those study. Prospective studies with larger scale are still needed. Along with other factors affecting coagulation and anti-coagulation, understanding PZ might help to develop new therapeutic strategies and more pregnancy women would benefit.

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