Original Paper

The rs9932581 and rs1049255 Polymorphisms in *CYBA* is not Associated with Preeclampsia in Chinese Han Women

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Key Words

CYBA • Polymorphism • Preeclampsia • NADPH oxidase

Abstract

Background/Aims: Several lines of evidence have been reported that oxidative stress plays an important role in the pathogenesis of Preeclampsia (PE). Therefore, this research is aimed to investigate whether polymorphisms of *CYBA* are related to susceptibility to PE in Chinese Han women. **Methods:** We studied the genetic frequency of the rs9932581 and 1049255 polymorphisms in *CYBA* in 1029 PE patients and 1400 controls of later pregnant women by the TaqMan allelic discrimination real-time PCR and a case-control model. **Results:** Our research indicated that no significant differences were found for the genotypic or allelic frequencies at the two polymorphic sites in *CYBA* between PE patients and controls. To further study the relationship between the polymorphic sites and PE, we also found that there is no significant difference in the genetic distributions identified between the mild or severe PE and early or the late-onset PE and controls. **Conclusion:** The study demonstrated that the genetic variants of rs9932581 and rs1049255 in *CYBA* might not be associated with PE. However, investigations of genetic variability that influence on the disease outcome are needed in other large prospective populations or regions, so the complicated interconnection of genetic and environmental elements can be emulated for better understanding.

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Introduction

PE is a complication of pregnancy affecting about 3%-7% of all pregnancies [1]. According to the guidelines for the diagnosis and treatment of hypertensive disorder complicating pregnancy, S. Liu and X. Li are the co-first authors of this paper.

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pregnancy (2015), PE, characterized by the hypertension and proteinuria after 20th week of gestation [2], is a leading cause of maternal and perinatal morbidity and mortality nationwide and seriously influences the health of maternal-child [3, 4]. Although a series of studies have tried to illustrate the etiology and pathogenesis of PE, such as abnormal placentation and vasculogenesis [5-7], placental and vascular endothelial dysfunction [8], immune factors and the systemic inflammatory response [9, 10], endoplasmic reticulum stress [11], inadequate trophoblast invasion [12, 13], the exact mechanisms remain undefined. Recently, oxidative stress is becoming an increasing attention as one of the pathogenesis of PE [1, 14] and may impact the vasoactive pathways to alter the endothelial cell function in PE [15].

As the main sources in the vascular production of reactive oxygen species (ROS), especially superoxide anion (O_2^-), NADPH oxidase could be activated by the signal stimulation, such as vasoactive factors, growth factors, hemodynamic forces and genetic factors, to enhance the production of O_2^- and cause endothelial dysfunction in cardiovascular diseases [16]. P22^{phox}, a crucial subunit for the functionality of NADPH oxidase, has an effect on enzyme activation and maintains the structure of NADPH oxidase and is necessary to the stimulation of O_2^- production [17]. The P22^{phox} protein, localized to Ch16. 24.3 and encoded by *CYBA* (light chain of cytochrome b558, cytochrome b245 alpha), spans 8.5kb and includes 5 introns and 6 exons which encode an approximately 600bp open reading frame [16]. The -930^{A/G} polymorphism (rs9932581) is located in the nucleotide position -930 from the ATG codon [18] and can affect the transcriptional activity of *CYBA* [19]. Moreno et al. found a higher *CYBA* expression in the -930G allelic promoter than -930A allelic promoter in hypertension by a functional analysis [18]. As another functional variant, A640G polymorphism (rs1049255) situated at 3'-untranslated region (3'-UTR) in *CYBA* [16], which was associated with an increased risk of coronary heart disease (CHD) in Chinese Han population [20] and was potential usefulness to the analysis of Chronic granulomatous disease (CGD) families [21].

Based on the above discussion, it is obviously noted that *CYBA* may be related to the hypertension and oxidative stress. Therefore, we tried to analyze whether polymorphisms of *CYBA* are associated with PE and to analyze the potential interactions of *CYBA* alleles in the Chinese Han population in this study.

Materials and Methods

Subjects

We recruited 1029 PE patients and 1400 healthy later pregnant women from the Affiliated Hospital of Qingdao University, Binzhou Medical University Hospital, the People's Hospital of Linyi, the Maternal and Child Care Service Centre of ZaoZhuang and the Anyang Maternal and Child Health Care Hospital between the January 2012 and November 2014. All collected participants were ethnically Chinese Han women and the healthy controls were selected at random. The maternal age, gestational age, age of menarche, times of gravidity, number of abortion, blood pressure, and the results of laboratory tests are included in the demographic and clinical characteristics. After the research was approved by the ethics committee of the Affiliated Hospital of Qingdao University, controls and PE patients have signed the informed consent.

According to the guidelines for the diagnosis of hypertensive disorder complicating pregnancy (2015): PE was defined as blood pressure ($\geq 140/90$) go with proteinuria ($\geq 0.3g$ in 24h or $\geq 1+$ by dipstick) after 20 weeks of gestation [2]. The controls matched with the cases according to the ages (age ≥ 26 years, gestational age ≥ 30 weeks). And both PE patients and healthy controls have no primary hypertension, acute or chronic nephritis, coronary heart disease, diabetes, thyroid disease, cancer and other serious organic disease, and there is no history of blood transfusion and immune therapy, without severe obstetric complication like placenta previa, artificial insemination, premature rupture of membranes, threatened abortion, twin/multiple pregnancy and macrosomia. In addition, the two groups have no the history of smoking.

Genetic studies

Genomic DNA were extracted from 300 ul peripheral venous blood by the Qiagen DNA extraction kit in accordance with manufacturer's instructions (Qiagen, Hilden, Germany). The genetic

contribution of rs9932581 and rs1049255 in *CYBA* were evaluated by TaqMan allelic discrimination real-time PCR. According to the PCR reaction system, Taqman probes were designed and synthesized by Applied Biosystems of Life Technologies (ABI, New York, USA). The rs9932581 primer sequences were 5'-GAAAAACGGCGGAGGCCGAGGCAG-3' (forward) and 5'-AATGCTGGTTTATCCCCATGGCCA-3' (reverse) and rs1049255 primer sequences were 5'-GACCTCGCCCCGGACCTGCCCTCCC-3' (forward) and 5'-CCAGGTGCACCCACCTGCAATAAAT-3' (reverse). The PCR cocktail contained 25 µl total reaction volume: 1.25 µl 20×SNP Genotyping Assay, 12.5 µl 2×PCR Master Mix, 11.25 µl DNA and DNase-free water. Amplifications were applied to in a C1000™ thermal cycler system and CFX96™ real-time system (Bio-Rad, California, USA) with the following conditions: 95°C for 3 min; 45 cycles of 95°C for 15 s and 60°C for 1 min. The genotypes were distinguished according to the strength of the fluorescent signals from VIC/FAM-labeled probes.

Statistical analysis

All statistical analysis were performed by the Statistical software package SPSS19.0. Demographic and clinical data such as age, pregnant times, gestational age, abortion times and blood pressure were analyzed by student's t-test or nonparametric test to compare differences between the cases and controls. Differences of the genotypic and allelic frequencies between two groups were compared by chi-square test (when the expected values were below 5, Fisher's exact test was performed). The Hardy-Weinberg equilibrium was examined by chi-square t-test in the control group to insure whether it was representative. The odds ratio (ORs) and 95% confidence intervals (CIs) were used to show the relative risk degree. When bilateral P value < 0.05, the results have statistical significance.

Results

Demographic and Clinical Characteristics

The comparison of demographic and clinical characteristics between the cases and controls were detailed in Table 1. No significant differences between the PE patients and healthy women group in maternal age, times of gravidity and number of abortion ($p > 0.05$); However, PE patients had an earlier admission and delivery gestational weeks ($p < 0.001$), higher blood pressure ($p < 0.001$), relatively high white blood cell ($p < 0.001$) and elevated levels of neutrophil ($p < 0.05$) compared with the controls.

Genotypic and Allelic Frequencies

The Hardy-Weinberg equilibrium was examined by chi-square test in control groups and there were no significant differences (rs9932581: $\chi^2 = 0.269$, $P = 0.604$; rs1049255: $\chi^2 = 0.198$, $P = 0.656$). So, the genotypic frequency distribution is representative. Table 2 showed the results of genotypic and allelic frequencies of rs9932581 and rs1049255 in

Table 1. Demographic and clinical characteristics of the two groups

Characteristics	PE(1029)	Controls(1400)	t	P-value
Maternal age(years)	30.24±3.90	30.14±5.43	0.472	0.637
Gestational age admission (weeks)	35.22±3.49	39.17±1.38	-34.28	<0.001
Gestational age at delivery (weeks)	35.81±3.13	39.42±1.15	-34.09	<0.001
Times of gravidity	2.21±1.18	2.25±1.27	-0.774	0.439
Number of abortion	1.42±0.71	1.55±0.85	-2.493	0.13
Systolic blood pressure (mmHg)	161.01±18.7	114.87±9.95	-72.03	<0.001
Diastolic blood pressure (mmHg)	104.63±13.73	73.51±7.72	-65.52	<0.001
White blood cell($\times 10^9/L$)	9.70±3.53	9.01±2.43	-5.44	<0.001
Neutrophil($\times 10^9/L$)	7.34±4.08	6.99±3.28	-2.26	<0.05

Table 2. Genotypic and allelic frequencies in PE and control groups

Genotype	PE(1029)	Control(1400)	χ^2	P	OR	95%CI
Allele						
rs9932581						
CC	194	272	1.444	0.486		
CT	540	701				
TT	295	427				
TT	295	427	0.952	0.329	0.996	0.768-1.093
CC+CT	734	973				
C	928	1245	0.164	0.686	1.024	0.913-1.148
T	1130	1555				
rs1049255						
AA	178	228	4.637	0.098		
AG	443	664				
GG	408	508				
AA	178	228	0.437	0.509	1.075	0.867-1.333
GG+AG	851	1172				
A	799	1120	0.686	0.407	0.952	0.847-1.070
G	1259	1680				

Table 3. Genotypic and allelic frequencies between mild or severe PE and control groups.

Groups	N	rs9932581					rs1049255				
		CC	CT	TT	C	T	AA	AG	GG	A	G
Mild PE	158	25	82	51	132	184	35	61	62	131	185
Control	1400	272	701	427	1245	1555	228	664	508	1120	1680
χ^2		1.209			0.834		5.6			0.25	
P-value		0.546			0.361		0.1			0.617	
OR					0.896					1.062	
95%CI					0.708-1.134					0.839-1.345	
Severe PE	850	166	448	236	780	920	140	377	333	657	1043
Control	1400	272	701	427	1245	1555	228	664	508	1120	1680
χ^2		2.06			0.859		2.3			0.81	
P-value		0.36			0.354		0.3			0.368	
OR					1.059					0.945	
95%CI					0.938-1.195					0.835-1.069	

PE and control groups. There were no significant differences at the two SNPs between two groups (rs9932581, genotype $\chi^2 = 1.444$, $P = 0.486$ and allele $\chi^2 = 0.164$, $P = 0.686$, OR = 1.024, 95% = 0.913-1.148; rs1049255, genotype $\chi^2 = 4.637$, $P = 0.098$ and allele $\chi^2 = 0.686$, $P = 0.407$, OR = 0.952, 95%CI = 0.847-1.070). Moreover, there was no relation between rs9932581TT genotype and CC+CT genotypes in the two groups ($X^2=0.952$, $P=0.329$, OR=0.996, 95%CI=0.768-1.093); And the rs1049255 also no significant difference between AA genotype and GG+AG genotypes in two group ($\chi^2=0.437$, $P=0.509$, OR=1.075, 95%CI=0.867-1.333).

To further discuss the association between the SNPs of CYBA and PE, PE can be divided into mild and severe PE according to the obstetrics and gynecology of American society of (ACOG) in 2002 [22]. Then genotypic and allelic frequencies of rs9932581 and rs1049255 polymorphisms were compared between mild or severe PE subgroups and controls. No significant differences were presented in Table 3 between mild or severe PE subgroups and controls(mild PE vs. control, rs9932581, genotype $\chi^2 = 1.209$, $p = 0.546$ and allele $\chi^2 = 0.834$,

Table 4. Genotypic and allelic frequencies between early-onset or late-onset PE and control groups

Groups	N	rs9932581					rs1049255				
		CC	CT	TT	C	T	AA	AG	GG	A	G
Early-onset PE	336	54	181	101	289	383	58	136	142	252	420
Control	1400	272	701	427	1245	1555	228	664	508	1120	1680
χ^2		2.41			0.47		5.58			1.42	
P		0.3			0.49		0.06			0.23	
OR					0.94					0.9	
95%CI					0.795-1.117					0.757-1.071	
Late-onset PE	693	140	359	194	639	747	120	307	266	547	839
Control	1400	272	701	427	1245	1555	228	664	508	1120	1680
χ^2		1.4			1.01		1.83			0.11	
P		0.5			0.32		0.4			0.74	
OR					1.07					0.98	
95%CI					0.939-1.216					0.857-1.115	

p = 0.361, OR = 0.896, 95%CI = 0.708-1.134; rs1049255, genotype χ^2 = 5.607, p = 0.061 and allele χ^2 = 0.250, p = 0.617, OR = 1.062, 95%CI = 0.839-1.345; severe PE subgroup vs. control, rs9932581: genotype χ^2 = 2.064, p = 0.356 and allele χ^2 = 0.859, p = 0.354, OR = 1.059, 95%CI = 0.938-1.195; rs1049255, genotype χ^2 = 2.275, p = 0.321 and allele χ^2 = 0.810, p = 0.368, OR = 0.945, 95%CI = 0.835-1.069).

On the basis of Alvarez-Fernandez [23], before 34 weeks of gestation is defined as the early-onset PE, and more than 34 weeks of PE is called late-onset PE. Table 4 showed the comparison of genotypic and allelic frequencies distribution between the PE and controls, and no significant differences were found between the early-onset PE or late-onset PE and controls (early-onset PE vs. control, rs9932581: genotype χ^2 = 2.413, p = 0.299 and allele χ^2 = 0.467, p = 0.494, OR = 0.942, 95%CI = 0.795-1.117; rs1049255: genotype χ^2 = 5.584, p = 0.061 and allele χ^2 = 1.417, p = 0.234, OR = 0.90, 95%CI = 0.757-1.071; late-onset PE subgroup vs. control, rs9932581: genotype χ^2 = 1.397, p = 0.497 and allele χ^2 = 1.007, p = 0.316, OR = 1.068, 95%CI = 0.939-1.216; rs1049255: genotype χ^2 = 1.826, p = 0.401 and allele χ^2 = 0.110, p = 0.740, OR = 0.978, 95%CI = 0.857-1.115).

Discussion

Oxidative stress refers to the imbalance between oxidants and antioxidants that the pro-oxidants capacity overwhelms the antioxidants under the condition of harmful stimulus of internal and external environment and results in the increased generation of ROS which contains superoxide anion (O_2^-), hydroxyl free radical (OH), nitric oxide (NO) and hydrogen peroxide (H_2O_2) etc. As their stronger oxidizing properties, they can cause the pathophysiological changes, such as the damage of cellular DNA, proteins and lipids [1].

Oxidative stress was involved in PE both in the placenta and in the mother's circulation. Compared with the normal placentas, the placentas of PE patients could decrease antioxidant capacity [24]. Moreover, the antioxidant levels was decreased in the blood of PE, so do the levels of protein and lipid oxidation [25]. Sankaralingam et al. reported oxidative stress as one of the pathogenesis of PE [26, 27], which was characterized by systemic small vasospasm, decreased visceral blood flow perfusion, hypoxia-ischemia and oxygen free radical generation. The combination of polyunsaturated fatty acids on the cell membrane and the oxygen free radical could promote oxidation reaction, resulting in destruction of biological membrane (protein oxidation, DNA mutations and fracture), vascular endothelial injury. Due to the oxidation reaction, structure of antioxidant enzyme protein was changed,

the enzymatic activity was reduced and free radical was further increased. Eventually, they caused a series of pathophysiological changes, like the extensive endothelial cell damage, release of inflammatory media, platelet aggregation, increased proteinuria, to develop disease.

NADPH oxidase, located on the cell membrane of endothelial and neutrophils cells, is the major source of O_2^- in the cardiovascular system [19] and consists of membrane-bound elements (catalytic subunit gp91phox and regulatory subunit p22phox), cytosolic subunits (p40phox, p47phox and p67phox) and guanosinetriphosphatase(GTPase), which indicates the NADPH oxidase has an effect on the physiology and pathology in diseases in which oxidative stress is participated [28]. Huang et al. demonstrated that thrombin could increase the expression of sFlt-1 in trophoblasts by the activation of PAR-1/NADPH oxidase/ROS signaling pathways and might be participated in the pathogenesis of PE [29]. Raijmakers et al. showed that the enzyme released O_2^- production that could partly control the blood pressure and result in the vasoconstriction and a rise in blood pressure [28]. It is generally that hypertension is the one of characteristics in PE, and report has confirmed that NADPH oxidase is taken part in elevated $\cdot O_2^-$ production in spontaneously hypertensive rats, which may be conducive to the endothelial dysfunction in hypertension [30].

It is acknowledged that PE is a complex disease and many factors are associated with it. Just as Bagci B and his colleagues have indicated that renase gene polymorphism (rs10887800 and rs2576178) may increase the susceptibility to PE in 110 PE patients and 102 normotensive controls [31]. And Savas et al. assessed that the serum levels of ET-1, M30, Ang-1, and Ang-2 increased in the PE patients or HELLP syndrome in 74 pregnant women (37 normal controls, 25 PE, 12 HELLP syndrome) [32]. In our study, we discussed mainly the correlation of CYBA polymorphisms and PE. No significant differences in genotypic and allelic frequencies of rs9932581 and rs1049255 in CYBA were found between 1029 PE patients and 1400 normal pregnant women. To further explore the relationship between them, it was divided into mild or severe, early-onset or late-onset PE. Compared with the control group, there were also no significant differences in genetic distributions at the two SNPs respectively. So this study suggests that these two SNPs in CYBA may not be associated with the mechanism of PE in Chinese Han women. Previous study have reported the C242T-polymorphism (rs4673) of the p22phox subunit is not related to PE between 95 patients and 78 control women in a Caucasian population [28], which is line with our results. However, these two SNPs of CYBA may play vital roles in other diseases. So far, the G allele of rs9932581 was regarded as a genetic marker related to hypertension in the Spanish people [18]. And some studies have been indicated that rs1049255 polymorphism affected the risk of ischemic stroke and coronary heart disease [20, 33]. In addition, these SNPs were associated with diverse cardiovascular diseases, like coronary artery disease, myocardial infarction and diabetic [16].

In summary, there were many reasons to explain the results of our study. PE is a complex and polygenetic hereditary disease, determined by both genetic and environmental factors and also their interactions [34]. However, we ignored the influence of environmental risk factors for PE in our study. Moreover, the results might also be affected by the racial and regional differences, and most subjects of this study were Chinese Han women. Additionally, there were different SNPs in CYBA and we just detected two polymorphisms of CYBA. So we should enlarge the scale of subjects and explore more susceptibility genes of PE from multiple regions, to provide the basis for the pathogenesis of PE from perspective of genetics analysis.

Acknowledgements

We are obliged to all participants who have made efforts for the study. This project was offered support by a grant from the National Natural Science Foundation of China (81371499 and 30971586).

Disclosure Statement

The authors declare that they have no conflict of interest and it has been approved by all named authors.

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