Study on the Association between protein Z gene polymorphisms and the risk of ischemic stroke

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Abstract: Background: The vitamin K-dependent protein Z (PZ) has been shown to possess anticoagulant as well as procoagulant properties, but its exact physiological function remains unclear. We aimed to study the possible genetic predisposition and especially the role of PZ allele as a risk factor for ischemic stroke (IS) in the Chinese Henan Han population. **Materials and methods:** Polymerase chain reaction and restriction fragment length polymorphism was performed to determine the genotypes of PZ gene A-13G and G79A in 964 unrelated subjects (IS, 492; and healthy controls, 472). Meanwhile, relevant variables of all subjects were measured, including blood lipid and body mass index (BMI). **Results:** The genotypic and allelic frequencies of PZ gene G79A were significantly different between the IS patients and controls (p=0.047). The GG genotype and G allele were associated with an increased risk of IS (p=0.017 for GG vs. AA, p=0.022 for G vs. A). However, no association between PZ gene A-13G polymorphism and IS risk was found. **Conclusion:** The gene polymorphism of PZ G79A is correlated to IS. The G allele might be a IS-susceptible gene and might be associated with the increased risk in Chinese Henan Han IS patients.

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1. Introduction

Protein Z (PZ) is a member of the family of vitamin K-dependent plasma coagulation factors, which includes procoagulant proteins such as factors II, VII, IX, and X, as well as the anticoagulant protein C [1,2]. PZ was first purified from bovine plasma and later found in humans [3]. The exact physiological function of PZ remains unclear.

In patients with low PZ levels, a bleeding tendency has been described [4-6]. In contrast, the combination of PZ deficiency and factor V Leiden results in a strong thrombophilia phenotype in mice and in humans [7]. Thus, PZ seems to play a paradoxical role in thrombosis [2]. Moreover, PZ was found to act as a cofactor in the inhibition of factor Xa by a plasma protein called Z-dependent protease inhibitor (ZPI) [1]. Vasse et al. [8] were the first to report a significant association between PZ deficiency and ischemic stroke. Further support for a role of low PZ levels in arterial thrombosis comes from two additional studies in patients with ischemic stroke and acute coronary syndromes [9,10]. Several other clinical case-control studies, mainly among younger patients, have yielded contradictory results both for ischemic stroke [11-14] and coronary heart disease [15].

The gene encoding for PZ has been identified and several common single nucleotide polymorphisms in the gene have been characterized [16,17]. Intron F polymorphism G79A and promoter polymorphism A-13G of the PZ gene have been found to influence PZ plasma levels [18-21]. In healthy carriers of the A allele of the intron F polymorphism protein Z plasma levels were lower than in carriers of the G allele [18-21]. G allele of the promoter polymorphism was also found to be associated with lower levels of PZ in healthy subjects [20,21]. Therefore, genetically determined low levels of circulating PZ could be implicated in promoting thrombophilia in ischemic stroke (IS).

To further determine to what extent this role is genetically determined, we studied the association of the PZ A-13G promoter and G79A intron gene polymorphism with first-ever IS. Moreover, to our knowledge, the genetic evidence on the association between PZ variants and IS in humans is poor, especially in Chinese Han population. Therefore, the present study aimed to clarify whether the PZ A-13G and G79A gene polymorphism is associated with the risk of IS.

2. Material and Methods:

2.1. Subjects:

A total of 492 patients with IS were recruited from hospitalized patients in the Second Affiliated

Hospital of Zhengzhou University. All the enrolled IS patients received a strict neurological examination and brain magnetic resonance imaging. The diagnosis of IS was according to the International Classification of Diseases (9th revision). Patients with

a transit ischemic attack, embolic brain infarction, stroke caused by inflammatory disease, cardioembolic stroke, autoimmune disease, or serious chronic diseases were excluded from this study.

A total of 472 healthy controls matched by age, gender, and geographical area were included. The controls were judged to be free of IS by questionnaires, medical history, and clinical examination. All individuals enrolled were from the Han population in Henan, China. A standard questionnaire was used to ascertain general information and medical history from all participants. The study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Zhengzhou University. Informed consent was obtained from all subjects after receiving a full explanation of the study.

2.2. Genotyping Analysis:

All the genotyping analyses in IS patients were performed after hospitalization, and all the venous blood samples were obtained from the patients and controls after at least 12 h of fasting. Genomic DNA was isolated from peripheral blood leukocytes using the phenol-chloroform method. The detection of the protein Z A-13G polymorphism and G79A polymorphism was performed using a based polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method using the PCR primers described by Lichy et al. [18]. For analysis of the protein Z A-13G polymorphism in the promoter region, a 272-bp fragment was amplified by polymerase chain reaction (PCR) using the following the primers: 5'-GGGTCCTCTGAGCCTTCACCGTTCATTT-3' and 5'-CAGGCACAACAGACAGGTAAGCCAGATG-3'. Polymerase chain reaction was carried out in a P.E. 9600 (Perkin Elmer Cetus) T.C. in 25 µl reaction volume containing 100 ng template DNA, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 200 µM each of dATP, dCTP, dGTP, dTTP (Amersham Pharmacia Biotech), 2.5 mM $MgCl_2$, 0.5 µM each primer and 1 U Taq DNA Polymerase. Following an initial denaturation step (2 min at 95℃), samples were subjected to 35 cycles of 95℃ for 20 sec, 64℃ for 20 sec, 72℃ for 20 sec with a final extension time of 3 min at 72℃. After incubation for 2 hours at 37℃ with HinfI, an isoschizomere of HhaI (MBI Fermentas), the G allele yielded 2 DNA fragments of 157 and 115 bp on a 2% agarose gel after ethidium bromide staining. The A allele was not digestible.

The G79A polymorphism of intron F of the PZ gene was analyzed by amplification of a 320-bp sequence using the primers 5'-TAACACCATAGACAGAGTCCGATATTCGC-3' and 5'-ATGAACTCGGCATTAGAACATGGTTGGAA-3'. Polymerase chain reaction was performed with the same condition of the A-13G polymorphism except that annealing temperature was 66℃. Digestion for 2 hours at 37℃ with an isoschizomere of HpaI, BstHPI (MBI Fermentas), yielded 2 products of 221 and 99 bp in length in the presence of the A allele, whereas the G allele was not digestible.

2.3. Statistical Analysis:

Data analyses were implemented using the SPSS17.0 statistical software (SPSS Inc., Chicago, IL, USA), with quantitative data represented as mean±standard deviation. A standard goodness-of-fit test was used to test the Hardy-Weinberg equilibrium. A chi-square analysis was used to evaluate the difference in genotype distribution and sex ratio between the groups. The general characteristics between the cases and controls were tested using Student's unpaired t-test. The correlation between the genotype and IS occurrence was analyzed by logistic regression, and the odds' ratio (OR) and 95% CI were used to report the degree of correlation. $p<0.05$ was statistically significant for any difference.

3. Results:

Baseline characteristics of the patients with IS and the controls are given in Table 1. The mean age, male to female ratio, serum LDL-C and the percentages of subjects who smoked were similar between the controls and IS patients. The average body mass index (BMI), systolic blood pressure, pulse pressure, and serum triglyceride (TG) levels were significantly higher, but serum total cholesterol (TC), HDL-C and the percentages of subjects who consumed alcohol were markedly lower in the IS patients than those in the controls.

For PZ A-13G gene polymorphism, the frequency of the G and A alleles was 53.6% and 46.4% in the IS patients, and 55.8% and 44.2% in the controls respectively (Table 2). The frequency of the GG, AG and AA genotypes was 27.2%, 52.6% and 20.2% in the IS patients, and 29.4%, 52.7% and 17.9% in the controls respectively. There were not statistical differences in the genotypic and allelic frequencies distribution between IS patients and controls ($p>0.05$). The genotypic and allelic frequencies were concordant with those predicted by the Hardy-Weinberg proportions in both IS group and controls ($p>0.05$). The A allele was not found to be associated with the risk of IS (OR=0.915; 95% CI=0.763-1.097; p=0.355), as well as AA (OR=1.213; 95% CI=0.83-1.772; p=0.355), AG (OR=1.08; 95% CI=0.802-1.454; p=0.649) and AG/AA (OR=1.114; 95% CI=0.839-1.479; p=0.47) genotypes.

For PZ G79A gene polymorphism, the frequency of the A and G alleles was 48.9% and 51.1% in the IS patients, and 59.3% and 40.7% in the controls

respectively (Table 2). The frequency of the AA, AG and GG genotypes was 29.1%, 50% and 20.9% in the IS patients, and 35.4%, 50.8% and 13.8% in the controls respectively. There were statistical differences in the genotypic and allelic frequencies distribution between IS patients and controls ($p<0.05$). The genotypic and allelic frequencies were concordant with those predicted by the Hardy-Weinberg proportions in both IS group and controls ($p>0.05$). Moreover, the G allele was associated with increased risk of IS (OR=1.239; 95% CI=1.034-1.484; p=0.022). The GG genotype was also associated with an increased risk of IS (OR=1.601; 95% CI=1.099-2.332; p=0.017). No significant association was found in other genotypes.

Table 1. Comparison of general clinical data between the patients and the controls

Parameters	**IS group (n=492)**	**Control group (n=472)**	***p* value**
Age (years)	67.1±7.9	66.4±8.6	0.1882
Gender (male/female)	250/242	245/227	0.748
BMI (kg/m^2)	23.39±3.27	22.37±2.85	<0.001
TC (mmol/L)	4.41±1.06	4.95±1.02	<0.001
TG (mmol/L)	1.78±1.16	1.61±1.05	0.0174
LDL-C (mmol/L)	2.87±0.89	2.93±0.83	0.2798
HDL-C (mmol/L)	1.33±0.49	1.92±0.52	<0.001
Current smoking	41.3%	43.9%	0.435
Alcohol consumption	30.2%	45.1%	<0.001
Systolic blood pressure (mmHg)	148.24±23.02	130.45±20.15	<0.001
Diastolic blood pressure (mmHg)	83.65±12.83	82.24±13.25	0.0936
Pulse pressure (mmHg)	64.27±17.25	49.79±14.93	<0.001

Notice IS, ischemic stroke; BMI, body mass index; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

Table 2. Frequencies of the Protein Z A-13G, G79A gene polymorphism genotypes and alleles in the ischemic stroke group and the control group

Gene	**Control group (n=472) (n, %)**	**IS group (n=492) (n, %)**	**OR**	**95% CI**	***p* value**
PZ A-13G Genotype [a]					
GG	133 (29.4%)	134 (47.1%)	1.000 (Reference)		
AG	238 (52.7%)	259 (43.9%)	1.08	0.802-1.454	0.649
AA	81 (17.9%)	99 (9.0%)	1.213	0.83-1.772	0.335
AG+AA	319	358	1.114	0.839-1.479	0.470
Allele [b]					
G	504 (55.8%)	527 (53.6%)	1.000 (Reference)		
A	400 (44.2%)	457 (46.4%)	0.915	0.763-1.097	0.355
PZ G79A Genotype [c]					
AA	160 (35.4%)	143 (29.1%)	1.000 (Reference)		
AG	240 (50.8%)	246 (50%)	1.147	0.861-1.528	0.38
GG	72 (13.8%)	103 (20.9%)	1.601	1.099-2.332	0.017
AG+GG	312	118	1.252	0.953-1.643	0.111
Allele [d]					
A	560 (59.3%)	532 (48.9%)	1.000 (Reference)		
G	384 (40.7%)	452 (51.1%)	1.239	1.034-1.484	0.022

Note: [a] $x^2=0.998$, $p=0.607$; [b] $x^2=0.916$, $p=0.355$; [c] $x^2=6.107$, $p=0.047$; [d] $x^2=5.422$, $p=0.022$
IS, ischemic stroke; OR, odds ratio; CI, confidence interval; PZ, protein Z.

With IS as the dependent variable and the A allele (genotype AA+AG for PZ A-13G gene), the G allele (genotype GG+AG for PZ G79A gene), gender, age, smoking history, BMI, serum TC, TG, LDL-C, HDL-C, alcohol consume and hypertension history as the independent variables, multivariate logistic regression analysis was implemented. According to the results, A allele and G allele were not found to be an independent variable for IS (Table 3). Serum TG, HDL-C, BMI and hypertension history were identified to be the risk factors for IS.

Table 3. Logical regression analysis on the risk factors of ischemic stroke disease

Variables	Partial regression coefficient	*p* value	OR	95% CI
Carrying A allele	0.412	0.052	1.512	0.983-2.221
Carrying G allele	0.523	0.063	1.568	0.976-2.248
Gender	0.147	0.55	0.861	0.533-1.401
Age	0.013	0.269	1.014	0.991-1.037
TC	-0.254	0.069	0.778	0.597-1.019
TG	0.335	0.004	1.398	1.109-1.698
LDL-C	0.161	0.335	1.176	0.852-1.621
HDL-C	-1.541	0.002	0.218	0.108-0.436
Smoking history	0.276	0.166	1.317	0.892-1.936
BMI	0.386	0.027	1.624	1.208-1.878
Alcohol consume	0.187	0.124	1.258	0.878-1.823
Hypertension history	0.349	0.016	1.427	1.079-1.923

TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; BMI, body mass index; OR, odds ratio; CI, confidence interval

4. Discussion

The gene for human PZ is localized to chromosome 13q34, where the genes for factors VII and X exist side by side, and it spans approximately 14kb, consisting of 9 exons, including 1 alternative exon [16]. The complete amino acid sequence has been described by Ichinose et al. [22] in 1990 and Sejima et al. [23]. Two major mechanisms have been described to represent its role in coagulation. First, PZ promotes the assembly of thrombin with phospholipid surfaces, thus enhancing coagulation [24]. Second, it is responsible for the binding of a specific PZ-dependent protease inhibitor to factor Xa and therefore indirectly acts as a natural anticoagulant [1,2]. Furthermore, the presence of PZ in atherosclerotic lesions suggests a role in the development of atherosclerotic disease [25].

More recent clinical case-control studies on PZ levels and the risk of cerebral ischemia yielded contradictory results. Kobelt and colleagues [11] described a significant association of high plasma levels of PZ with cryptogenic stroke, Vasse and colleagues [8] found decreased levels in juvenile patients with cerebral ischemia. Another study by Heeb et al. [26] performed in an elderly population also depicted an association of low PZ and risk of stroke, but this finding was restricted to nondiabetic males. The study by Hankey et al. [27] was the only one that investigated PZ levels in the acute stage of cerebral ischemia. It found increased levels of PZ in acute stroke, especially of atherosclerotic etiology. Interestingly, this association was no longer detectable in the same population 3 months after the ischemic event, thus indicating that PZ either plays a role in the acute stage of ischemia or is an acute phase reactant. Moreover, Lopaciuk et al. [12] did not detect any association between PZ levels and juvenile stroke in their study. Finally, Staton et al. [21] found increased blood concentration of PZ concentrations were associated causally with an increased risk of ischemic stroke, but in 2008 van Goor et al. [28] demonstrated that low PZ levels are independently associated with an increased risk of ischemic stroke. It is unclear from these data whether the association between PZ levels and the risk of cerebral ischemia is causal, confounded, or a consequence of the acute stroke event.

It has been demonstrated that common genetic polymorphisms of the PZ gene influence blood concentrations of PZ [16,17,19]. Because PZ genotypes are determined by random assortment of maternal and paternal alleles at the time of gamete formation, the association between PZ genotype and ischemic stroke should be largely free from confounding by other determinants of PZ levels in the blood or risk factors for stroke [29]. Therefore, examining the consistency of the association between PZ polymorphisms and IS may help to clarify whether the association between PZ and stroke is causal or confounded. To our knowledge, this is the first study that has examined the association between PZ polymorphisms and cerebral vascular events among unselected cases of first-ever IS compared with community-based controls in Henan Han Chinese population. In the present study, we showed that the frequencies of the AA, AG and GG genotypes of PZ A-13G gene polymorphism were 19.1%, 52.6% and 28.3%, respectively. The frequencies of A allele and G allele were 45.4% and 54.6%. Moreover, we found that the frequencies of the GG, AG and AA genotypes of PZ G79A gene polymorphism were 18.5%, 51.5% and 30%, respectively. The frequencies of G allele and A allele were 43.4% and 56.6%. The prevalence of both polymorphisms in our control group was significantly different from those found in other healthy European population [18,30]. These results suggest that ethnic variations may exist between these two PZ gene polymorphisms. All participating

subjects were of the Asian race and the allele frequencies of the polymorphism in the control group were in Hardy-Weinberg equilibrium, indicating that we studied an unselected group. Moreover, our results further demonstrated that a G allele at intron F position 79 was associated with increased risk of IS, especially GG genotype. A protective effect of PZ A-13G G allele was not confirmed, but it is most likely because of lack of statistical power. Our findings are in agreement with those of Lichy et al. [18] who reported a significant association between PZ G79A genotypes and IS risk in young German stroke patients, as well as the study by Staton et al [21].

Our study cannot directly clarify the pathophysiological mechanism of the association between PZ genotype and stroke, and it remains possible that the PZ polymorphisms studied are simply markers of an enhanced acute phase response in patients with an acute atherothrombotic vascular event. A strength of our study was that we included a broad range of patients with stroke who were compared with randomly selected community based controls, matched for age, sex, and postal code. Our study also has limitations. First, in the present study, we did not measure the PZ concentrations in stroke patients and controls. The mechanism of the association between PZ genotype and PZ concentrations or ischemic stroke has not been established and there may be linkage disequilibrium with polymorphic variants of loci that predispose to conventional or novel risk factors for stroke. Second, we decided to study the PZ A-13G and G79 Apolymorphisms, because they are both common and have been reported to be important determinants of PZ concentrations. However, we did not determine linkage disequilibrium between the 2 polymorphisms and did not study the numerous other PZ polymorphisms that have also been described [16,17,19].

5. Conclusion

In conclusion, our study may suggest an independent association between PZ G79A polymorphism and an increased risk of ischemic stroke in Asian patients. Further studies are necessary to confirm this association and to elucidate its possible causal nature. Such studies should more focus on PZ gene polymorphisms and PZ level simultaneously in stroke populations of different ethnicity and age.

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