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Associations of platelet-activating factor acetylhydrolase gene polymorphisms with risk of ischemic stroke

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Abstract. Platelet-activating factor acetylhydrolase (PAF-AH) has an important function in the pathogenesis of ischemic stroke. The aim of the present study was to investigate the correlation between the variation of polymorphisms (R92H and V279F) in PAF-AH and ischemic stroke. A total of 375 patients with ischemic stroke and 370 healthy controls were recruited into the study. Polymorphisms of V279F and R92H in PAF-AH were detected by polymerase chain reaction and DNA direct sequencing method. No significant association was observed between V279F and ischemic stroke. However, the RH+HH genotype, RH genotype and H allele of R92H were significantly associated with an increased risk of ischemic stroke (P=0.02, P=0.03 and P=0.02, respectively), In addition, these correlations remained following adjustment for confounding risk factors of stroke. Furthermore, subgroup analysis showed that a significant association with R92H was identified in the large-artery atherosclerotic stroke subgroup. These findings indicated that variation of R92H in the PAF-AH gene may contribute to ischemic stroke susceptibility in the population studied.

Introduction

Stroke is the second cause of fatality and the leading cause of disability worldwide. Currently, in developing countries its prevalence is also increasing rapidly. In China, stroke is a major cause of fatality (1). Presently, ischemic stroke is the most common type of stroke in China (2). Numerous independent and major risk factors, such as hypertension, diabetes mellitus, hypercholesterolemia, smoking, alcohol consumption, family history, lack of exercise, diet and lifestyle, are known causes of ischemic stroke (3).

Previous studies have proved that the heritable elements can influence the pathogenesis of ischemic stroke. A significant number of genomic alleles and variants have been discovered to increase the risk of ischemic stroke (4); however, these only explain a minor part of the assumed heritability. Exploring the genetic polymorphisms contributing to the susceptibility of ischemic stroke is important. Recent studies have shown that inflammation is one of the key risk factors and has an important role in the development of ischemic stroke (5). Therefore, genes involved in inflammatory responses are under investigation to identify the variants predisposing to ischemic stroke.

Platelet-activating factor acetylhydrolase (PAF-AH), also known as lipoprotein-associated phospholipase A2 (Lp-PLA2), is a newly identified inflammatory enzyme involved in lipoprotein metabolism and inflammatory pathways (6). PAF-AH may have an important role in the pathophysiology of inflammation. Clinical and epidemiological studies have indicated that elevated PAF-AH concentrations are associated with incident and recurrent stroke events, as this enzyme exhibits proinflammatory and oxidative activities (7). Several other studies have examined the association of PAF-AH gene polymorphisms with coronary artery disease. V279F in exon 9 of the PAF-AH gene is associated with coronary artery disease and carotid atherosclerosis in the Japanese population. R92H in exon 4 is associated with coronary artery disease in the USA (8). According to this information, we hypothesized that the genetic variants in PAF-AH may have a critical role in the susceptibility to ischemic stroke. To confirm this hypothesis, a case-control study was conducted to investigate the association between two PAF-AH polymorphisms (V279F and R92H) with ischemic stroke in an eastern Chinese population.

Materials and methods

Study population. The present study was a hospital-based, case-control study. A total of 375 patients with ischemic stroke and 370 healthy controls were diagnosed at Weifang People's Hospital (Weifang, Shandong, China) were recruited between January 2011 and February 2015. The ischemic stroke diagnoses were carried out according to the World Health Organization guidelines (9) and the reported procedures. According to the TOAST classification, ischemic stroke can be divided into five subtypes: i) Large-artery atherosclerosis (LAA), ii) small-vessel occlusion (SVO), iii) cardioembolism (CE), iv) stroke of other determined etiology, and v) stroke of undetermined etiology (10). Patients with LAA and SVO, two of the most common subtypes of ischemic stroke, were

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included, while the other subtypes were excluded. All the subjects in the control group were free of clinical or radiological evidence of stroke and other neurological diseases. Those having mental or significant physical diseases, as well as familial or self-psychiatric history, were excluded.

The completion of questionnaires for each subject was performed by trained interviewers, and their clinical assay markers were measured using standard laboratory procedures. The study was approved by the Ethics Committee of the Weifang People's Hospital and written informed consent was obtained from every recruited subject. This study was conducted in accordance with the Declaration of Helsinki.

DNA extraction and genotyping. Genomic DNA extraction was executed using commercial kits designed for extracting blood DNA (Qiagen, Valencia, CA, USA) and following the manufacturer's protocol. TE buffer was used to dissolve the extracted DNA. The resulted DNA solution was stored at -20˚C until further use, and was also used as a template for the following polymerase chain reaction (PCR). The sequences of the primers for PCR are shown in Table I. PCR amplification was performed in a total volume of 10 μl that contained 1X GC buffer I (Takara, Otsu, Japan), 3.0 mM Mg^{2+} (Takara), 0.3 mM deoxyribonucleotide triphosphate (dNTP) (Generay Biotech, Shanghai, China), 1 unit HotStarTaq polymerase (Qiagen, Hilden, Germany), 1 μl each primer (Sangon, Shanghai, China) and 1 μl genomic DNA. The PCR cycling program was set at 95˚C for 2 min, followed by 11 cycles of 94˚C for 20 sec, 65˚C (decreased 0.5˚C per cycle) for 40 sec, 72˚C for 1.5 min, and subsequently 24 cycles of 94˚C for 20 sec, 59˚C for 30 sec, and 72˚C for 1.5 min, and a final extension at 72˚C for 2 min. Multiplex PCR products were checked for quality and yield by running 5 μl in 2% agarose-TBE gels. PCR products (15 μl) were treated with 5 units of shrimp alkaline phosphatase and 2 units of exonuclease I to remove excess dNTPs and primers, respectively. The results of electrophoresis are shown in Figs. 1 and 2.

Statistical analysis. Continuous variables are presented as mean ± standard deviation and categorical variables as percentages. Continuous variables were compared using student's t-test. Categorical variables were compared by χ^2 test. Differences of the distributions of alleles and genotypes between cases and controls were analyzed using χ^2 test. All the genotype frequencies were checked for Hardy-Weinberg analysis in two groups with the χ^2 test.

The association of the PAF-AH gene polymorphisms with ischemic stroke was evaluated by computing the odds ratios (OR) and 95% confidence intervals (CI) from logistic regression analyses following adjustment for confounding risk factors. P<0.05 was considered to indicate a statistically significant difference for all statistical analyses. All the statistical analyses were carried out with Stata 12.0 software (StataCorp, College Station, TX, USA).

Results

Characteristics. The clinical characteristics of the control subjects and ischemic stroke patients are shown in Table II. There were no significant differences in age, gender or body mass index. The genotype and allele frequencies of the two PAF-AH polymorphisms in patients and control subjects are shown in Tables III and IV. All the genotype distributions in the patients and controls were in the Hardy-Weinberg equilibrium. As shown in Tables III and IV, there was no significant difference in the distributions of genotypes and alleles of V279F between ischemic stroke patients and controls. By contrast, the significant association was observed for R92H in a dominant model. A higher frequency of the HH+RH genotype for R92H was observed in all the participants with ischemic stroke (OR=1.44; 95% CI, 1.06-2.01; P=0.02). The RH genotype (28.8%) of R92H was represented at an increased frequency in the group of patients (OR=1.42; 95% CI, 1.02-1.97; P=0.03). The frequency of R92H H allele was significantly higher in patients with ischemic stroke compared to the control group (OR=1.41; 95% CI, 1.06-1.73; P=0.02).

Table I. Primer sequences of V279F and R92H.

SNPs	Primer sequences
R92H	Forward: 5' CAATCACCACAGCAGCCTAA3'
	Reverse: 5' TCCCATCCAACTCAGAATGG3'
V279F	Forward: 5' TTTATGGGGGCAAAAGAATAGCC3'
	Reverse: 5' AACCATCCCCATGAAATSAACAAT3'

SNP, single nucleotide polymorphism.

Table II. Clinical characteristics of the study subjects.

Characteristics	Cases	Control	P-value
Age[a], years	63.4±4.73	60.1±8.09	0.81
Males, n (%)	254 (67.7)	243 (65.7)	0.74
BMI[a], kg/m^2	27.2±2.91	26.8±2.03	0.23
Hypertension, n (%)	263 (70.1)	218 (58.9)	<0.01
Diabetes, n (%)	131 (34.9)	69 (18.6)	<0.01
Hyperlipidemia, n (%)	167 (44.5)	129 (34.9)	<0.01
Smoking, n (%)	131 (34.9)	67 (18.1)	<0.01

[a]Data are mean ± standard deviation. BMI, body mass index.

Association between polymorphisms and ischemic stroke. The association between the polymorphisms and the disease was assessed using univariate or multivariate logistic regression analyses. Logistic regression analyses revealed that following adjustment for the confounding factors (hypertension, diabetes mellitus, hyperlipidemia and smoking), the presence of the RH+HH genotype and the RH genotype of single nucleotide polymorphism R92H was associated with a higher risk of ischemic stroke (OR=1.42; 95% CI, 1.02-2.00; P=0.04; and OR=1.41; 95% CI, 1.01-2.08; P=0.04, respectively).

The statistical differences between the PAF-AH gene and different subtypes of ischemic stroke were analyzed. The HH+RH genotype and the RH genotype of the R92H gene were significantly associated with large-artery atherosclerotic

Table III. Genotype and allele distributions of R92H in patients with ischemic stroke and the controls.

R92H	Cases, n (%)	Control, n (%)	OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
Genotype						
RR	259 (69.1)	287 (77.6)	Reference	Reference		
RH	104 (27.7)	79 (21.4)	1.42 (1.02-1.97)	0.03	1.41 (1.01-2.08)	0.04
HH	12 (3.2)	4 (1.1)	2.12 (0.62-7.41)	0.19	1.72 (0.48-6.15)	0.40
(HH+RH)/RR	116/259	83/287	1.44 (1.06-2.01)	0.02	1.42 (1.02-2.00)	0.04
HH/(RH+RR)	12/363	4/386	2.11 (0.54-6.39)	0.25	1.55 (0.44-5.59)	0.48
Allele						
R	622 (82.9)	653 (87.8)	Reference			
H	128 (17.1)	87 (12.2)	1.41 (1.06-1.73)	0.02		

OR, odds ratio; CI, confidence interval.

Table IV. Genotype and allele distributions of V279F in patients with ischemic stroke and the controls.

V279F	Cases, n (%)	Control, n (%)	OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
Genotype						
VV	338 (90.1)	334 (90.2)	Reference	Reference		
VF	35 (9.3)	33 (8.9)	0.93 (0.57-1.46)	0.77	1.02 (0.63-1.62)	0.88
FF	2 (0.5)	3 (0.8)	0.23 (0.02-2.12)	0.35	0.21 (0.02-2.01)	0.17
(FF+VF)/VV	37/338	36/334	0.86 (0.54-1.27)	0.54	0.92 (0.61-1.51)	0.83
FF/(VF+VV)	2/373	3/367	0.24 (0.02-2.22)	0.36	0.21 (0.02-2.10)	0.18
Allele						
V	711 (94.8)	701 (94.7)	Reference			
F	39 (5.2)	39 (5.3)	0.82 (0.44-1.24)	0.35		

OR, odds ratio; CI, confidence interval.

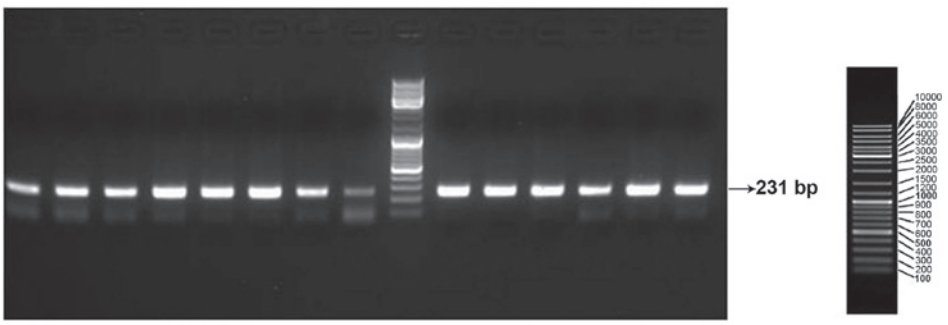

Figure 1. Electrophoresis of R92H.

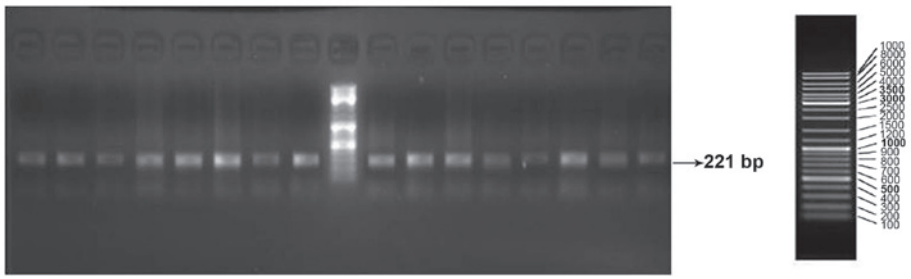

Figure 2. Electrophoresis of V279F.

Table V. Genotype and allele distributions of R92H and its associations with the stroke subtypes.

R92H	Cases, n (%)	Control, n (%)	OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
LAA subgroup						
RR	133 (64.4)	151 (73.7)	Reference			
RH	70 (32.4)	42 (20.5)	1.73 (1.19-2.52)	<0.01	1.67 (1.13-2.48)	0.01
HH	6 (3.2)	12 (5.9)	3.67 (1.06-12.76)	0.06	3.02 (0.81-11.27)	0.10
Dominant model						
RR	133 (64.4)	151 (73.7)	Reference			
HH+RH	76 (36.4)	54 (26.3)	1.82 (1.27-2.62)	<0.01	1.73 (1.18-2.55)	<0.01
Recessive model						
RR+RH	203 (97.1)	193 (94.1)	Reference			
HH	6 (2.9)	12 (11.4)	0.75 (0.21-1.90)	0.11	0.63 (0.14-1.72)	0.15
Allele						
R	336 (80.4)	344 (83.9)	Reference			
H	82 (19.6)	66 (16.1)	1.74 (1.26-2.39)	0.23		
SVO subgroup						
RR	126 (75.9)	106 (64.2)	Reference			
RH	39 (23.5)	56 (33.9)	1.09 (0.71-1.68)	0.69	1.11 (0.71-1.73)	0.65
HH	1 (0.6)	3 (1.8)	0.59 (0.07-5.31)	1.00	0.33 (0.03-3.36)	0.35
Dominant model						
RR	126 (75.9)	106 (64.2)	Reference			
HH+RH	40 (24.1)	59 (35.8)	1.07 (0.70-1.64)	0.75	1.07 (0.69-1.66)	0.78
Recessive model						
RR+RH	165 (99.4)	162 (98.2)	Reference			
HH	1 (0.6)	3 (1.8)	0.58 (0.06-5.19)	0.99	0.32 (0.03-3.23)	0.33
Allele						
R	291 (87.7)	268 (81.2)	Reference			
H	41 (12.3)	62 (18.7)	1.04 (0.70-1.53)	0.85		

OR, odds ratio; CI, confidence interval.

stroke, even after adjusting for the confounding factors (OR=1.73; 95% CI, 1.18-2.55; P<0.01; and OR=1.67; 95% CI, 1.13-2.48; P=0.01, respectively). However, the same differences were not observed between the remaining small vessel occlusive stroke subgroup and all genetic models (P>0.05), as shown in Table V.

Discussion

The present study investigated the association between the PAF-AH genotypes and the risk of ischemic stroke in the eastern Chinese Han population. The major finding of this study is that in the Chinese Han population the frequencies of the minor alleles of the R92H polymorphisms of the PAF-AH gene were significantly higher in ischemic stroke patients. This difference remained following all the factor-related adjustments. However, no association of V279F with the risk of ischemic stroke was identified.

Several previous studies have explored the association between the PAF-AH polymorphism and cardiovascular disease. However, the results of these studies were inconsistent. Li *et al* (11) reported that the V279F variant was associated with coronary artery disease in the Chinese Han population. However, the association between the V279F variant and cardiovascular disease was not found in the South Korean population (12). With regards to ischemic stroke, Hiramoto *et al* (13) identified an association with the FF+VF genotype and the F allele. In the present study, this association has not been replicated in the population of eastern China. Two points should be considered with regards to these findings. Genetic heterogeneity may be an important factor. Firstly, genotype frequencies of the V279F in the control subjects (88% VV, 11% VF, and 1% FF) differ from those in the Japanese populations (75% VV, 22% VF, and 3% FF). Secondly, the study by Hiramoto *et al* (13) was conducted with a relatively small sample size and there was a low frequency of homozygosity of the minor allele. It may be more likely to produce false positive results.

The R92H is a polymorphism, with a non-synonymous change located within the coding region of the Lp-pla2 gene in exon 4, which should result in an arginine-to-histidine substitution at position 92. The association of R92H with cardiovascular disease is also contradictory. Sutton *et al* (8) reported that the R92H polymorphism was significantly

associated with the risk of CHD, with the R92H variant allele observed more frequently in the experiment group compared to the control group. Zheng *et al* (14) identified the significant association of the R92H variant with premature myocardial infarction in the Chinese population. In the present study, the focus was on the association between R92H in the PAF-AH gene and ischemic stroke. The HH+RH genotype, the RH genotype and the H allele of R92H were significantly associated with the increased risk of ischemic stroke in the Chinese Han population.

The association between R92H in the PAF-AH gene and ischemic stroke in the Chinese population can possibly be explained as follows: i) Several previous studies have investigated the association between the PAF-AH polymorphism and the risk of ischemic stroke. A study of older individuals by Rosso *et al* (15) found that PAF-AH was associated with ischemic and hemorrhagic strokes. In the Bruneck study (16), PAF-AH activity was identified as a top hit associated with ischemic stroke. ii) A few studies have investigated the association between the R92H polymorphism and plasma PAF-AH activity. A meta-analysis including a total of 14 studies showed that the R92H variant shows the strongest association with PAF-AH activity among the variants in PAF-AH gene (17).

When evaluating the association of R92H with stroke subtypes, a significant association was found for this polymorphism with the LAA subgroup, but not the SVO subgroup. This can be explained by the role of PAF-AH in the process of atherosclerosis. Lyso-PC, the important bioproduct of PAF-AH, is involved in the inflammatory cytokine production and in the induction of the expression of adhesion molecules and cytokines. It has a chemoattractant property for macrophages, and it induces vascular smooth muscle migration (18). Additionally, Lyso-PC can upregulate PAF-AH activity, resulting in a vicious cycle in which pro-inflammatory mediators are upregulated, contributing to plaque progression and destabilization (19). In addition, oxNEFAs, another detrimental substrate of PAF-AH, can promote atherosclerosis by increasing oxidative stress and the presence of oxidized LDL and other lipoproteins in the plasma and arterial walls, thereby initiating fatty streak formation (20).

There are several limitations in the present study that require discussion, such as the relatively small sample size and the lack of PAF-AH activity measurements. Therefore, further studies including functional evaluations are warranted to elucidate the potential mechanism of these polymorphisms in ischemic stroke.

In conclusion, the present study demonstrated that the PAF-AH gene polymorphism R92H may modify the risk of ischemic stroke in the eastern Chinese Han population. Further studies involving functional evaluations are warranted to clarify the potential mechanism of these polymorphisms in ischemic stroke.

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