Original Article

Association of ALOX5AP with ischemic stroke in eastern Chinese

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BACKGROUND: 5-lipoxygenase protein (ALOX5AP) has been recognized as a susceptibility gene for stroke and coronary artery diseases. The present study was to explore the role of this gene in the eastern Chinese patients with ischemic stroke.

METHODS: Using a case-control design, we studied 658 patients with ischemic stroke and 704 unrelated population-based controls who were age- and sex-matched. The 658 patients were classified by the Trial of Org 10172 in Acute Stroke Treatment (TOAST). Two single-nucleotide polymorphisms (SNPs) covering ALOX5AP were genotyped.

RESULTS: The genotype frequencies of TG of the SNPs rs17222919 located in the promoter of the ALOX5AP gene were significantly higher in patients with ischemic stroke than in controls ($OR=1.34$, $95\%CI=1.02-1.75$), especially in patients with ischemic stroke caused by small-artery occlusion (SAO) ($OR=1.40$, $95\%CI=1.02-1.93$). Meanwhile, the genotype frequencies of TG and TG/GG were higher in female patients than in the controls. After specification, the genotype frequencies of TG and TG/GG were higher in the patients than in controls with hypertension. The genotype frequencies of AG and AG/GG of the SNPs rs9579646 located in the intron of the ALOX5AP gene were higher in the controls than in the patients. After specification, the genotype frequencies of TG were higher in the controls than patients without hypertension.

CONCLUSION: The present study suggests that sequence variants in the ALOX5AP gene are significantly associated with ischemic stroke.

KEY WORDS: 5-lipoxygenase activating protein (ALOX5AP); Leukotrienes (LTs); Trial of Org 10172 in Acute Stroke Treatment (TOAST); Single nucleotide polymorphisms (SNPs); Ischemic stroke

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INTRODUCTION

Stroke is the leading cause of morbidity and mortality worldwide.^[1,2] In China, over 2.5 million people are affected by stroke, and more than 1 million die of stroke each year (<http://www.moh.gov.cn>). Nearly 85%-90% of patients with stroke suffer from ischemic stroke.^[3] Over the last decade, studies such as twin studies, family studies, and case-control studies have demonstrated that the interaction between genetic and environmental risk factors lead to ischemic stroke.^[4-6]

In 2004, the Iceland DeCODE study revealed the linkage of the 13q12-13 region and that the gene encoding arachidonate 5-lipoxygenase activating protein (ALOX5AP) was associated with myocardial infarction (MI) and ischemic stroke.^[7] ALOX5AP encodes 5-lipoxygenase-activating protein, which is required for the synthesis of leukotriene A₄ (LTA₄), a pro-inflammatory mediator implicated in the pathogenesis and progression of atherosclerosis.^[8-13] Subsequent studies on non-Icelandic populations yielded conflicting results.^[14-22]

In 2008, we investigated the association of the SNPs of ALOX5AP with stroke in the Han population in east China. The rs9579646 AG genotype of ALOX5AP was found to be associated with a marginally decreased risk for stroke (adjusted odds ratio, 0.65; 95% CI, 0.45–0.96), compared with the AA genotype.^[23] Since the number of patients with ischemic stroke was not large enough and could not be classified by TOAST, we took ALOX5AP as a susceptibility gene for ischemic stroke in a larger number of stroke patients and matched controls from east China.

METHODS

Study population

The study population was composed of 658 patients and 704 age- and sex-frequency-matched controls. The patients were consecutively recruited from 3 hospitals between 2008 and 2011: the First Affiliated Hospital of Nanjing Medical University, Brain Hospital Affiliated to Nanjing Medical University, and Gulou Hospital in Nanjing. Stroke was confirmed by neurological examination, CT, or MRI according to the International Classification of Diseases (9th revised edition), which was usually performed within 3 days after admission. Other types of stroke (transient ischemic attack, intracerebral hemorrhage, subarachnoid hemorrhage, brain tumors, and cerebrovascular malformation) and severe systemic diseases such as pulmonary fibrosis, endocrine and metabolic diseases (except diabetes mellitus), severe inflammatory diseases, autoimmune diseases, tumors, and serious chronic diseases (e.g., hepatic cirrhosis, renal failure) were excluded from the study.

The patients were further classified into stroke subtypes using the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification.^[24] Altogether 189 patients had large vessel disease (LAA), 69 had cardioembolic stroke (CE), and 354 had lacunar stroke (SAO). In 14 patients, stroke with other determined causes was diagnosed (OC), and in 32, the cause of stroke remained unclear despite diagnostic efforts were made.

Seven hundred and four ethnically and geographically matched controls were randomly selected either from healthy residents in the community or inpatients with minor illnesses. The controls were free of neurological diseases according to the exclusion criteria. They were asked for a detailed medical history and subjected to a physical examination of cardiovascular and neurological systems, including evaluation of body mass index.

The study was conducted to assess the risk factors

of stroke with the approval from the institutional review board of Nanjing Medical University. Informed consent was obtained from each subject (or their next of kin) who donated 5 mL of blood for DNA extraction.

Diagnostic criteria

Alcohol consumption was defined as a dichotomous variable, where individuals consuming more than 3 drinks per week were considered drinkers. Current smokers were those who reported smoking regularly during the 6 months preceding the ischemic stroke; former smokers were characterized as individuals who had smoked regularly for at least 6 months, but not during the year preceding the stroke. Never and former smokers were grouped into single category of nonsmokers for statistical analysis. Family history was obtained through self-reported on first-degree relatives (parents, siblings, or children). Individuals were classified as hypertensive when their systolic pressure was ≥ 140 mmHg and/or their diastolic pressure ≥ 90 mmHg, on at least two separate occasions. In addition, any individual using antihypertensive agents was classified as hypertensive. A patient was classified as diabetic if he or she had a previous diagnosis, a history of anti-diabetic medication use, or fasting levels of plasma glucose ≥ 7 mmol/L.

SNP selection

According to significant association with stroke in the previously reported study,^[17-23] we captured two SNPs (rs9579646 and rs17222919) on the ALOX5AP gene for analysis, which were selected from the genotyped SNPs in the Chinese Han population of the Hap Map project (the Phase II database) using Haploview 4.0.^[25] SNP rs9579646 had shown a marginal association with the eastern Chinese Han population in our previous study,^[23] but not been analyzed with TOAST. In this study, we firstly explored the SNP rs17222919 in the Chinese population, which is the promoter of the ALOX5AP gene and then shown a significance association with intracerebral hemorrhage in the Korean population^[26] But there are no studies on the relationship between rs17222919 and ischemic stroke.

DNA isolation and genotyping

Genomic DNA was extracted from the peripheral white blood cells using the phenol/chloroform method. DNA was quantified and diluted to a final concentration of 10 ng/L. All samples were genotyped by the Taqman 7900HT Sequence Detection System, Applied Biosystems, Foster City, California, according to the

manufacturer's instructions. Each assay was carried out using 10 ng DNA in a 5-L reaction consisting of TaqMan universal polymerase chain reaction master mix, forward and reverse primers, and 6-carboxyfluorescein (FAM) and 4, 7, 2-trichloro-7-phenyl-6-carboxyfluorescein (VIC) labeled probes. Allelic discrimination was measured automatically using the Sequence Detection System 2.3 software (autocaller confidence level 95%). A total of 10% of all genotypes were repeated in independent polymerase chain reactions to check for consistency. The results were 100% concordant.

Statistical analysis

Statistical analyses were performed with SPSS 15.0 and SAS 9.3 for Windows. The presence of Hardy-Weinberg equilibrium per SNP was tested using Haploview 4.0, which is based on the Chi-square test and the goodness-of-fit test. All quantitative variables were generally described as means with SD. For comparison of the baseline characteristics among different groups, Student's *t* test was performed on quantitative variables, such as age, body mass index (BMI), high-density lipoprotein-cholesterol, and so on. The Chi-square test was used for qualitative variables. For each SNP, differences of allelic and genotype frequencies between the patients and controls were determined by the Chi-square or Fisher's exact test. Stratified analyses were conducted to observe the modification effect by genetic polymorphisms on the association between environment factors and the risk of ischemic stroke. A logistic regression model was used to evaluate potential multiplicative interaction effects. Conditional univariate and multivariate logistic regression analyses

were performed to obtain the crude and adjusted odds ratios (ORs) for risk of stroke and their 95% confidence intervals (CIs). The departures from multiplicative results were assessed by main effect variables and their product terms in the logistic regression model when adjusting for potential confounding factors. We considered smoking, alcohol consumption, BMI (continuous), hypertension, diabetes, age and gender as potential confounding factors. *P* value <0.05 (two-tailed) was considered statistically significant.

RESULTS

Clinical characteristics of studied subjects

The clinical characteristics of the patients and controls are summarized in Table 1. As expected, when compared with the control group, the ischemic stroke group had a higher level of BMI, a higher percentage of patients with hypertension, diabetes, coronary artery disease (CAD), and transient ischemic attack (TIA), and high levels of glucose, TG, and TC (*P*<0.05). However, the differences in smoking, alcohol intake, and low level of HDL between the two groups were not statistically significant (*P*>0.05).

Association of polymorphisms with stroke and stroke subtypes

The successful genotyping rates of the two genotyped SNPs were both 99%. The observed genotype frequencies of the 2 SNPs on ALOX5AP were in Hardy-Weinberg equilibrium among the controls (rs17222919 ($\chi^2=0.28$, *P*=0.60) and rs9579646 ($\chi^2=0.80$, *P*=0.37), respectively). The genotype and allele frequencies of the two SNPs for

Table 1. Clinical characteristics of the two groups

Parameters	Patients (<i>n</i> =658)		Controls (<i>n</i> =704)		<i>P</i> *
	Number	%	Number	%	
Age (average $\pm$ SD, year)	69.42 $\pm$ 10.58		69.22 $\pm$ 9.04		0.070
Gender					
Male	392	59.97	388	55.11	0.096
Female	266	40.03	316	44.89	
BMI (kg/m ²)	24.20 $\pm$ 3.18		23.27 $\pm$ 2.38		<0.001
Smoking	164	24.92	179	25.43	0.831
Drinking	111	16.87	136	19.32	0.241
Coronary artery disease	128	19.45	17	2.41	<0.001
Hypertension	514	78.12	218	30.97	<0.001
Diabetes	223	33.89	89	12.64	<0.001
TIA	168	25.53	8	1.14	<0.001
Blood glucose (mmol/L)	6.42 $\pm$ 2.64		5.90 $\pm$ 2.41		0.000
TC (mmol/L)	4.67 $\pm$ 1.17		4.44 $\pm$ 1.18		0.001
TG (mmol/L)	1.68 $\pm$ 1.25		1.37 $\pm$ 0.92		<0.001
HDL (mmol/L)	1.28 $\pm$ 3.04		1.26 $\pm$ 0.35		0.893

*P**: Chi-square test.

the patients and controls are listed in Table 2. Of the two SNPs, only rs17222919 showed a significant association with ischemic stroke. In multivariate analyses, only the TG genotype of rs17222919 had a 1.34-fold (95%CI 1.02–1.75) increased risk of ischemic stroke, compared with those with the TT genotype, after adjusting for conventional risk factors such as age, gender, smoking, alcohol intake, BMI, hypertension, and diabetes. In addition, we found a significant association of rs17222919 with small vessel subtype (SAO), respectively. The TG genotype of rs17222919 had a 1.40-fold (95%CI 1.02–1.93) increased risk of small vessel stroke, respectively, compared with those with the TT genotype, after adjusting for conventional risk factors (Table 3). However, we found no association between rs9579646 and ischemic stroke.

Combined effects of polymorphism and environment exposure

We also explored combined effects of the genetic variants and the environmental exposures in relation to ischemic stroke. As shown in Table 4, elevation of ischemic stroke risk was obvious among TG and TG/GG genotype carriers who are female or have hypertension, with adjusted combined ORs of 1.88(1.24–2.85), 1.71(1.13–2.57), and 1.61(1.11–2.32), 1.45(1.02–2.06), respectively, compared with low-risk individuals for rs17222919 (TT carriers who were male and not hypertensive) after adjusting conventional risk factors.

Descent of ischemic stroke risk was obvious among AG and AG/GG genotype carriers who are male, with adjusted combined ORs of 0.69(0.49–0.97), 0.65 (0.44–0.96), respectively, compared with low-risk individuals

Table 2. Association of two SNPs on ALOX5AP with ischemic stroke patients and controls in the eastern Chinese Han population

SNP	Patients		Controls		<i>P</i> value	<i>OR</i> *	95% CI*
	<i>n</i>	%	<i>n</i>	%			
rs17222919 ^a							
TT	417	63.37	469	66.81		1.00	—
TG	219	33.28	207	29.49		1.34	1.02–1.75
GG	22	3.34	26	3.70		0.83	0.42–1.66
rs9579646 ^a							
TG/GG	241	36.63	233	33.19	0.184	1.28	0.98–1.65
AA	171	26.03	169	24.25	0.681	1.00	—
AG	325	49.47	360	51.65		0.78	0.58–1.05
GG	161	24.51	168	24.10		0.95	0.67–1.36
AG/GG	486	73.97	528	75.75	0.450	0.83	0.62–1.11

^a: Genotype failed in 1 patient and 7 controls; *P* value: The Chi-square test; *OR** and 95% CI*: Adjusted for conventional risk factors such as age, gender, smoking, alcohol intake, BMI, hypertension, and diabetes.

Table 3. Single polymorphism association for ischemic stroke subtypes in the eastern Chinese Han population

SNP	LAA		CE		SAO		OC		SUD		<i>P</i> value
	<i>OR</i>	95% CI*	<i>OR</i>	95% CI*	<i>OR</i>	95% CI*	<i>OR</i>	95% CI*	<i>OR</i>	95% CI*	
TT	1.00		1.00		1.00		1.00		1.00		
TG	1.36	(0.91–2.02)	1.17	(0.64–2.12)	1.40	(1.02–1.93)	2.57	(0.87–7.57)	0.66	(0.31–1.38)	0.80
GG	0.39	(0.13–1.20)	0.84	(0.18–3.98)	0.89	(0.41–1.96)			0.85	(0.11–6.67)	
TG/GG	1.17	(0.79–1.72)	1.31	(0.64–2.02)	1.35	(0.99–1.84)	2.07	(0.70–6.10)	1.78	(0.85–3.74)	0.49

P value: The Chi-square test; *OR** and 95% CI*: Adjusted for conventional risk factors such as age, gender, smoking, alcohol intake, BMI, hypertension, and diabetes.

Table 4. Combined effects between rs17222919 and rs9579646 with environment risk factors on ischemic stroke risk

Variables	rs17222919 <i>OR</i> * (95% CI*) ^a				rs9579646 <i>OR</i> * (95% CI*) ^b			
	TT	TG	GG	TG/GG	AA	AG	GG	AG/GG
Gender								
Male	1.00	1.08 (0.75–1.55)	0.86 (0.38–1.95)	1.04 (0.74–1.48)	1.00	0.69 (0.49–0.97)	1.07 (0.72–1.60)	0.65 (0.44–0.96)
Female	1.00	1.88 (1.24–2.85)	0.46 (0.12–1.84)	1.71 (1.13–2.57)	1.00	0.95 (0.65–1.39)	1.26 (0.82–1.94)	1.19 (0.76–1.87)
Hypertension								
Yes	1.00	1.61 (1.11–2.32)	0.60 (0.25–1.45)	1.45 (1.02–2.06)	1.00	0.93 (0.67–1.29)	0.99 (0.67–1.45)	0.89 (0.60–1.31)
No	1.00	1.08 (0.71–1.62)	0.88 (0.36–2.37)	1.05 (0.70–1.56)	1.00	0.64 (0.44–0.95)	1.32 (0.86–2.03)	0.76 (0.50–1.16)

^a: Adjusting conventional risk factors such as age, smoking, alcohol, BMI, hypertension, and diabetes; ^b: Adjusting conventional risk factors such as age, gender, smoking, alcohol, BMI, and diabetes.

for rs9579646 (AA carriers who were female) after adjusting conventional risk factors (age, gender, smoking, alcohol, BMI, and diabetes).

DISCUSSION

The ALOX5AP gene (GenBank Accession: NT_024524) mapped to chromosome 13q 12.3 consists of five exons and four introns, and encodes FLAP. FLAP plays an important role in the initial steps of leukotriene biosynthesis, which is largely confined to leukocytes and can be triggered by a variety of stimuli. Firstly, unesterified arachidonic acid is converted to LTA4 by the action of 5-LO and its activating protein FLAP in this biosynthetic pathway. Then the unstable epoxide LTA4 is further metabolized to LTB4 or LTC4 by LTA4 hydrolase and LTC4 synthase, respectively. In addition, LTA4 can be exported to neighboring cells that are devoid of 5-LO activity and are subjected to transcellular leukotriene biosynthesis, which can lead to lots of proinflammatory effects. LTB4 could activate leukocytes, leading to chemotaxis and increased adhesion of leukocytes to vascular endothelium, release of lysosomal enzymes such as myeloperoxidase and production of superoxide anions. The cysteinyl-containing leukotrienes (LTC4 and its metabolites LTD4 and LTE4) increase vascular permeability in postcapillary venules and are potent vasoconstrictors of coronary arteries. Lots of studies have shown that the greater activity of the 5-LO pathway plays a role in predisposition to atherosclerosis, which can lead to CAD and stroke.^[8-12]

Helgadottir et al^[7] reported two at-risk haplotypes (HapA, HapB) in the ALOX5AP gene that were associated with stroke firstly. Then, in 2005 Löhmußaar et al^[18] reported that sequence variants in the ALOX5AP gene are significantly associated with stroke, particularly in males, in a Central European sample population. In 2007, Kaushal and coworkers^[20] showed that the ALOX5AP SNPs (rs9579646 and rs4769874) were significantly associated with ischemic stroke among whites but not blacks. In 2009, Domingues-Montanari et al^[22] also showed that the rs10507391 SNP was an independent risk factor for ischemic stroke in a Iberian population and was associated with ALOX5AP expression levels. However, in 2006 Zee^[19] reported that there was no evidence for an association of the specific Icelandic ALOX5AP variants tested with risk of incident ischemic stroke in white males. In 2011, Bondarenko et al^[27] reported no statistically significant associations between acute stroke and ALOX5AP gene

polymorphisms examined in a Russian population. Moreover, Dong et al^[26] revealed that a promoter SNP (rs17222919) of ALOX5AP contributes to ICH susceptibility not ischemic stroke in a Korean population in 2011. Because there were lots of conflicting results in ALOX5AP, we decided to investigate the association between this gene and stroke in the eastern Chinese population from 2008.

In this study we found that the G allele of rs17222919 in the ALOX5AP gene was associated with an increased risk of ischemic stroke, especially with the subtype—small vessel disease in eastern Chinese Han people. The polymorphism rs17222919 was located in the promoter of the ALOX5AP gene. But the functional significance of ALOX5AP is still unknown. This polymorphism may lead to the change in the location of transcription factor binding which may enhance or silence the expression of ALOX5AP.

In our study we explored the genetic contribution to the subtype of ischemic stroke and the gene-environment interaction effect on the etiology of ischemic stroke. We found the most important association with rs17222919 genotype, which increases the risk of stroke in female subjects with a history of hypertension. However, the rs9579646AG genotype significantly decreases the risk of stroke in male subjects. The results were consistent with those reported previously showing a significant association of SNPs rs9579646AG genotype with stroke risk among whites^[20] and our previous study.^[23]

The results of the present study showed that variants of ALOX5AP were associated with greater risk of ischemic stroke in the eastern Chinese population. The rs17222919 TG in genotype was associated with a significantly increased risk of ischemic stroke, compared with the TT genotype, especially associated with SAO. To our knowledge, this is the first study that correlates polymorphism in the promoter on the ALOX5AP encoding FLAP with the prevalence of stroke in Chinese.

There are still several limitations in our study. Firstly, since the patients and controls were recruited from the hospital, there was potential selection bias. Secondly, the sample size (658 cases and 704 controls) of this study may not be large enough to detect a small effect from very low-penetrance SNPs, and to evaluate gene-environment interaction. Especially the number of subtypes of ischemic stroke may not be adequate, there is potential bias.

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Conflicts of interest: The authors have no competing interests relevant to the present study.

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