Original Article

Association of *ALOX5*, *LTA4H* and *LTC4S* gene polymorphisms with ischemic stroke risk in a cohort of Chinese in east China

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BACKGROUND: Genetic variations of the 5-lipoxygenase activating protein and leukotriene A4 hydrolase genes that confer an increased risk of ischemic stroke have implicated the family of leukotrienes as potential mediators of ischemic stroke. This study aimed to explore the association of *ALOX5*, *LTA4H* and *LTC4S* gene polymorphisms with ischemic stroke risk in a cohort of Chinese in east China.

METHODS: This case-control study consisted of 690 patients with ischemic stroke and 690 controls. Polymorphisms of *ALOX5* rs2029253 A/G, *LTA4H* rs6538697 T/C, and *LTC4S* rs730012 A/C were genotyped by the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis. The multivariate logistic regression model was used to exclude the effects of conventional risk factors on ischemic stroke.

RESULTS: Carriers of C allele in rs730012 were more susceptible to ischemic stroke (OR: 1.37; 95%CI: 1.08–1.73; $P=0.009$). The rs2029253 GG genotype showed a risk-reducing effect on ischemic stroke (OR: 0.72; 95%CI: 0.55–0.93; $P=0.013$) while the rs6538697 CC genotype had an increased risk of ischemic stroke (OR: 1.77; 95%CI: 1.09–2.89; $P=0.022$). The rs730012 variant was not associated with ischemic stroke risk after adjusting confounding factors ($P>0.05$).

CONCLUSION: The present study suggested that gene polymorphisms in the leukotrienes pathway may exert influences, with independent genetic effects, on ischemic stroke susceptibility in a cohort of Chinese in east China.

KEY WORDS: Leukotrienes; Gene polymorphisms; Risk factors; Ischemic stroke

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INTRODUCTION

Stroke is the most serious neurological disease and one of the leading causes of severe disability and mortality in China.^[1] The majority of strokes are of ischemic origin, accounting for 80% of all strokes.^[2] Ischemic stroke (IS) is a heterogeneous, complex multifactorial and polygenic disorder that is thought to result from interactions between individuals' genetic background and various environmental factors. Atherothrombosis is considered the main cause.^[3] Studies demonstrated many IS risk

predictors like age, sex, obesity, cigarette smoking, hypertension, diabetes and dyslipidemia.^[4] However, these risk factors cannot fully account for the overall risk of IS.^[5] Huge amounts of genetic epidemiological studies have consistently suggested genetic contributions to the susceptibility to IS and have already identified many predisposing genes that are associated with IS risk including those in the leukotrienes (LTs) biosynthesis pathway.^[6]

It is known that the processes of atherosclerotic

plaque formation and rupture are driven by inflammation. Plaque rupture correlates with increased inflammation within the plaque, implicating the genes involved in inflammatory processes as excellent candidates for study.^[7] LTs are a group of potent proinflammatory lipid mediators and involved in the pathogenesis and progress of atherosclerosis.^[8] Lipooxygenation of arachidonic acid by 5-lipoxygenase (5-LO or *ALOX5*) and its activating protein (*ALOX5AP*) yields the unstable product LTA4. Subsequently, the LTs synthesis follows two distinct pathways. Hydrolysis of LTA4 by LTA4 hydrolase (*LTA4H*) leads to formation of LTB4, a potent chemoattractant and leukocyte activator. On the other arm of the pathway, LTA4 is conjugated with glutathione by the action of LTC4 synthase (*LTC4S*). The latter enzymatic step leads to the formation of LTC4, D4 and E4 (collectively referred as the cysteinyl LTs), which are associated with vasoconstriction.^[9] LTs induce proinflammatory actions through activation of special receptors. Thus, genetic effects in the LTs biosynthesis pathway could be a potential contributor to an increased risk of IS.^[10]

In order to investigate the contribution of genetic variations in LTs-related genes (i.e. genes encoding key enzymes in this pathway) to IS in a cohort of Chinese in east China, a case-control study was carried out to clarify the involvement of *ALOX5*, *LTA4H* and *LTC4S* single nucleotide polymorphisms (SNPs) as risk factors for the pathogenesis of IS.

METHODS

Study subjects

A total of 690 unrelated patients with IS were recruited from the First Affiliated Hospital of Nanjing Medical University, Nanjing, China, between January 2009 and January 2011. All the patients were genetically unrelated Han ethnicity Chinese from Jiangsu province and surrounding regions in east China. Stroke was defined by the presence of a new focal neurological deficit, with an acute onset and with symptoms and signs persisting for more than 24 hours.^[11] IS was confirmed in all patients by computed tomography and/or magnetic resonance imaging. The control group consisted of 690 unrelated individuals who were recruited simultaneously from the same geographical area as the patients. They had no clinical evidence of neurological diseases nor a neurovascular or cardiovascular history of stroke or a family history of stroke. Patients with inflammatory, autoimmune or malignant diseases were excluded from

the study.

Demographic characteristics and other risk factors of the patients and controls were collected using the same structured questionnaire regarding body mass index (BMI), smoking, hypertension, and diabetes. Smoking was defined as smoking at least one cigarette per day for one year or over, and former smokers with more than three-year cessation of smoking were not included.^[12] Hypertension was defined as a systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg based on the average of the two measurements of blood pressure, a patient's self-reported history of hypertension, or the use of antihypertensive drugs. Diabetes was diagnosed if fasting plasma glucose was ≥ 7.0 mmol/L, plasma glucose was ≥ 11.1 mmol/L 2 hours after oral administration of 75 g glucose, random plasma glucose was ≥ 11.1 mmol/L or the patient was on anti-diabetic medication.^[13]

The study protocol was approved by the Institutional Review Board for Human Studies of Nanjing Medical University. Informed consent was obtained from all study participants.

Clinical laboratory measurements

Samples of venous blood were collected after at least 12 hours of fasting. Total plasma cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured by an automatic biochemical analyzer (AU5400, Olympus, Tokyo, Japan).

SNPs selection and genotyping

Genomic DNA was extracted from the peripheral white blood cells using the phenol/chloroform method. DNA samples were stored at -20°C . According to the information from the NCBI SNP and HapMap database, SNPs which had a minor allele frequency (MAF) >0.05 in the cohort or those with a significant association with IS risk were chosen.^[14] Therefore, the present study selected three SNPs, including *ALOX5* gene rs2029253, *LTA4H* gene rs6538697, and *LTC4S* gene rs730012. Characteristics of the investigated SNPs are shown in Table 1. Genotypes of the three polymorphisms were determined in all subjects by the polymerase chain reaction (PCR) - restriction fragment length polymorphism (RFLP) analysis. Briefly, the SNPs were genotyped by PCR followed by restriction enzyme digestion and agarose gel electrophoresis of the amplified fragments. The primers and restriction enzymes are provided in Table 2. For quality control, 10% of the

Table 1. Characteristics of the investigated SNPs

SNPs	Position in chromosome	Minor allele frequency (MAF)			HWE <i>P</i> value in controls
		Database*	Case	Control	
rs2029253	45891484	0.433	0.394	0.372	0.711
rs6538697	96403610	0.367	0.282	0.256	0.219
rs730012	179220638	0.156	0.131	0.099	0.106

* MAF for Chinese in HapMap.

Table 2. Primers and restriction enzymes for PCR-RFLP genotyping

SNPs	Sequence of primers (5'-3')*	T _{anneal} (°C)	Length of PCR (bp)	Restriction enzymes	Length of fragment (bp)
rs2029253 A>G	F: TTCCACAGTGTATGGCCTGG(T→G)C R: ACTCTCCTCTCCATCTCTGTCTGGA	60.0	120	<i>Hin6I</i>	AA: 120 AG: 120, 100, 20 GG: 100, 20 TT: 144
rs6538697 T>C	F: TCTCCGTAAATCATGCTTGCTG(T→G)GTAC R: ACGGAGTCCCAGTCACAACTCT	59.4	144	<i>Acc65I</i>	TC: 144, 122, 22 CC: 122, 22 AA: 112
rs730012 A>C	F: CAGGAACAGCCTGGATGGGT(G→T)AC R: ACTTCTCCAGGGCCTTGACAG	62.8	112	<i>KpnI</i>	AC: 112, 90, 22 CC: 90, 22

* the underlined nucleotide represented the mismatch base introduced to create a restriction enzyme site using a PCR-PIRA (primer introduced restriction analysis) method.

samples were employed to repeat PCR and genotyping, and no discrepancies were detected.

Statistical analysis

EpiData 3.0 was used to establish the database. For comparison of the clinical characteristics, quantitative variables were expressed as mean±SD and compared using unpaired Student's *t* test. Qualitative variables were compared using the Chi-square test. Deviation from the Hardy-Weinberg equilibrium (HWE) was tested by comparing the observed and expected genotype frequencies of the controls. Genotypes were assessed according to recessive genetic models (variant homozygotes versus the combined group of wild-type homozygotes and heterozygotes). The Chi-square test was used to compare the distribution of genotypes and alleles of SNPs between the patients and controls. The multivariate logistic regression model was used to exclude the effects of possible confounding factors (including sex, age, BMI, smoking, hypertension, and diabetes) on the association between genetic variants and IS. The odds ratios (ORs) and 95% confidence intervals (95% CIs) for the effects of genotypes on IS risk were uncorrected for confounding variables in the Chi-square test and unadjusted logistic regression, and were corrected for covariates in adjusted regression models. All the statistical tests were performed in SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). A two-tailed *P* value of less than 0.05 was considered statistically significant.

Table 3. Clinical characteristics of the controls and IS patients

Variables	Controls (n=690)	Cases (n=690)	<i>P</i>
Age (y)	67.01±9.59	67.87±9.52	0.097
Male (%)	54.6	59.7	0.057
BMI (kg/m ²)	23.32±2.39	24.20±3.21	<0.001
Smoking (%)	22.8	30.3	0.002
Hypertension (%)	26.8	77.1	<0.001
Diabetes (%)	11.6	34.1	<0.001
SBP (mmHg)	124.98±17.47	144.09±22.17	<0.001
DBP (mmHg)	79.38±31.58	83.69±11.80	0.001
TC (mmol/L)	4.48±1.22	4.67±1.17	0.003
TG (mmol/L)	1.38±0.98	1.67±1.19	<0.001
HDL-C (mmol/L)	1.28±0.36	1.15±0.34	<0.001
LDL-C (mmol/L)	2.55±0.76	2.79±0.84	<0.001

RESULTS

Clinical characteristics of subjects

The general characteristics of the IS patients and controls are shown in Table 3. The mean age of the patients and controls was 67.87±9.52 years and 67.01±9.59 years respectively; 59.7% of the patients and 54.6% of the controls were male. The IS group showed more conventional risk factors such as male, smoking, history of hypertension and diabetes (*P*<0.05), higher BMI, systolic and diastolic blood pressure, TC, TG, LDL-C, and lower HDL-C (*P*<0.01) than the control group.

Association study

The genotype distributions of SNPs in the controls

Table 4. Genotypic distributions and allelic frequencies of SNPs

Gene	SNPs	Alleles [*] (1/2)	Group	Genotypes (n, %)			<i>P</i> [▲]	2 vs. 1	
				1/1	1/2	2/2		<i>P</i>	OR (95%CI)
<i>ALOX5</i>	rs2029253	G/A	Control	274 (39.7)	318 (46.1)	98 (14.2)	0.001	—	1.00 (Ref.)
			Patient	224 (32.5)	388 (56.2)	78 (11.3)		0.240	1.10 (0.94–1.28)
<i>LTA4H</i>	rs6538697	T/C	Control	376 (54.5)	275 (39.9)	39 (5.7)	0.073	—	1.00 (Ref.)
			Patient	362 (52.5)	267 (38.7)	61 (8.8)		0.122	1.14 (0.97–1.35)
<i>LTC4S</i>	rs730012	A/C	Control	556 (80.6)	131 (19.0)	3 (0.4)	0.009	—	1.00 (Ref.)
			Patient	522 (75.7)	155 (22.5)	13 (1.9)		0.009	1.37 (1.08–1.73)

Ref.: reference group; ^{*}: major allele was referred to as allele 1 and minor allele as allele 2. [▲]: χ^2 test for genotypic distributions between the controls and patients.

Table 5. Association results of SNPs with ischemic stroke risk according to the recessive genetic model

SNPs	Genotype	Controls		Patients		Unadjusted		Adjusted	
		<i>n</i>	%	<i>n</i>	%	OR (95%CI)	<i>P</i>	OR (95%CI)	<i>P</i>
<i>ALOX5</i>									
rs2029253	AA, AG	416	60.3	466	67.5	1.00 (Ref.)	—	1.00 (Ref.)	—
	GG	274	39.7	224	32.5	0.73 (0.59–0.91)	0.005	0.72 (0.55–0.93)	0.013
<i>LTA4H</i>									
rs6538697	TT, TC	651	94.3	629	91.2	1.00 (Ref.)	—	1.00 (Ref.)	—
	CC	39	5.7	61	8.8	1.62 (1.07–2.46)	0.022	1.77 (1.09–2.89)	0.022
<i>LTC4S</i>									
rs730012	AA, AC	687	99.6	677	98.1	1.00 (Ref.)	—	1.00 (Ref.)	—
	CC	3	0.4	13	1.9	4.40 (1.25–15.50)	0.012	3.77 (0.92–15.50)	0.066

Ref.: reference group; unadjusted (without covariates) and adjusted (for age, sex, body mass index, smoking, hypertension and diabetes) multivariable Logistic regression analysis was performed using the recessive genetic model between the controls and patients.

were consistent with the results of the Hardy-Weinberg equilibrium ($P>0.05$) (Table 4). There were significant differences in the genotypic distributions of *ALOX5* rs2029253 and *LTC4S* rs730012 between the IS patients and controls ($P=0.009$ and $P=0.001$, respectively). The C allelic frequency of rs730012 was significantly higher in the IS patients than in the controls (13.3% versus 9.9%; OR: 1.37; 95%CI: 1.08–1.73; $P=0.009$). There were no differences in *LTA4H* rs6538697 between the patients and controls.

Compared with AA/AG genotypes, GG genotype of *ALOX5* rs2029253 had a risk-reducing effect on IS (unadjusted OR: 0.73; 95%CI: 0.59–0.91; $P=0.005$) (Table 5); similarly it was also found by logistic regression analysis while adjusting age, sex, BMI, smoking, hypertension and diabetes (adjusted OR: 0.72; 95%CI: 0.55–0.93; $P=0.013$). Moreover, CC genotype of *LTA4H* rs6538697 was significantly associated with IS risk (unadjusted OR: 1.62; 95%CI: 1.07–2.46; $P=0.022$ and adjusted OR: 1.77; 95%CI: 1.09–2.89; $P=0.022$). Variants of *LTC4S* rs730012 also contributed to an increased risk of IS (CC versus AA/AC, unadjusted OR: 4.40; 95%CI: 1.25–15.50; $P=0.012$). However, no significant associations were observed after excluding effects of the confounding factors ($P>0.05$).

DISCUSSION

Inflammatory activation has emerged as a key element in all critical steps of atherosclerosis, including in the development of atherosclerotic lesions and in the progression to mature atheroma. Moreover, this activation promotes thrombosis, the most dreadful complication of atherosclerosis, which can result in IS.^[15] The LTs biosynthesis pathway has recently garnered attention for its potential role in atherosclerosis-related traits. This stems from a series of biochemical, genetic, human, and pharmacological studies over the last few years that have provided evidence for the pro-atherogenic role of LTs.^[16] LTs participate in atherosclerotic progresses, implicating LTs-related genes involved in inflammatory processes as excellent candidates for study and their variants might enhance the susceptibility to IS.^[17]

The rate-limited step in the LTs biosynthesis pathway is catalyzed by the enzyme *ALOX5* and protein *ALOX5AP*. The biologically active LTs are synthesized by subsequent conversion to LTB₄ and cysLTs via enzymatic reactions of *LTA4H* and *LTC4S*, respectively. LTs then affect the function of target cells through receptor-mediated signal transduction.^[18] Genetic variation in members of the LTs biosynthesis pathway

could be an important contributor to the development of atherosclerosis and to an increased risk of IS through the formation of the proinflammatory LTB₄ and/or through an increase in vascular permeability caused by cysLTs.^[19]

Dwyer et al^[20] have implicated that the LTs biosynthesis pathway worked at several different stages of the atherosclerosis process. LTs have been suggested to be involved in the initiation of atherosclerosis, through both lipid retention and thickening of the vascular wall, as well as in the changes of the endothelial homeostasis that is characterized by early atherosclerosis.^[21] Thus, the potent LTs-induced immunostimulatory actions indicate a key role in the development of atheroma. Besides, some studies^[10,11] have indicated a role for the LTs pathway in plaque rupture, causing thrombosis and vessel occlusion. The plaque rupture induces ischemia in distal organs (such as the brain), and the contribution of LTs in stroke has also been studied in models of ischemia and reperfusion.^[22]

A study^[23] of gene polymorphisms showed a significant association of the LTs pathway with early signs of atherosclerosis and the development of IS. Since a genome-wide linkage analysis conducted by the deCODE group in an Icelandic population suggested a four-SNP haplotype in the *ALOX5AP* gene conferring a nearly two times greater risk of stroke,^[24] other groups examined the association of IS risk with variants of LTs-related genes. Genetic association studies in different populations were subsequently carried out. In particular, a significant association of IS with *LTC4S* variants was found in Danish^[25] and British^[14] populations, whereas no association was identified with variants in the *ALOX5* and *LTA4H* genes in the British population, and replication in a German cohort revealed a similar IS risk.^[14] No evidence of a significant association was reported in *LTA4H* gene polymorphisms with IS in Japanese individuals with metabolic syndrome.^[26] The present study is the first time to explore gene polymorphisms in members of the LTs biosynthesis pathway and IS risk in the Chinese in east China. The study suggested that carriers of C allele of rs730012 in the *LTC4S* gene were more susceptible to IS than those of A allele. The *ALOX5* rs2029253 A/G variant bore a risk-reducing effect on IS while *LTA4H* rs6538697 T/C and *LTC4S* rs730012 A/C variants had an increased risk of IS.

In addition, the present study suggested that the genetic variations of *LTC4S* rs730012 lost the 'independent main effects' ($P > 0.05$) after the correction for confounding factors, i.e., age, sex, BMI, smoking, hypertension, diabetes, etc. The results might be due to

the etiology of IS, which is influenced by multiple genes, and each single susceptibility gene may exert marginal effects.^[27] Since IS is a complex disease involving multiple genetic variations and gene-environment interactions, a single locus cannot fully explain their genetic susceptibility.^[28] Therefore, analysis of the combined effects of multiple genes and multi-locus could capture more information about IS risk than analysis of a single susceptibility gene or locus.^[29] Further studies are needed to demonstrate the gene-gene interactions in members of the LTs biosynthesis pathway affecting the susceptibility to IS.

In conclusion, we found an association between IS risk in a Chinese cohort in east China and genetic variations of *ALOX5*, *LTA4H* and *LTC4S* genes in the LTs biosynthesis pathway. The finding needs further investigation in other populations and the molecular mechanism of these variants in IS should be detected.

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Ethical approval: The study was approved by the Institutional Review Board for Human Studies of Nanjing Medical University.

Conflicts of interest: There is no conflict of interest related to this study.

Contributors: Wang GN proposed the study, analyzed the data and wrote the first drafts. All authors contributed to the design and interpretation of the study and to further drafts.

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