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Interaction between *ALOX5AP-SG13S114A/T* and *COX-2-765G/C* increases susceptibility to cerebral infarction in a Chinese population

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ABSTRACT. We made a case-control study to investigate a possible association between *ALOX5AP-SG13S114A/T*, *COX-2-765G/C*, and *COX-1-50C/T* polymorphisms with cerebral infarction in a Chinese population. A total of 411 cases with cerebral infarction were included; 411 controls matched for age, gender, and risk factors were also selected. The *ALOX5AP-SG13S114A/T* (*rs10507391*), *COX-2-765G/C* (*rs20417*), and *COX-1-50C/T* (*rs3842787*) polymorphisms were determined using PCR-RFLP. The generalized multifactor dimensionality reduction method was employed to detect gene-gene interactions. Based on single-gene analysis, there were no significant differences in the genotype and allele frequency distributions of *ALOX5AP-SG13S114A/T*, *COX-2-765G/C*, and *COX-1-50C/T* between the cerebral infarction group and controls. However, in those cases carrying *ALOX5AP-SG13S114AA* as well as *COX-2-765CC*, the risk of cerebral infarction increased significantly by 2.84 times (95%CI = 1.324-6.543). The single-gene *ALOX5AP-SG13S114A/T*, *COX-2-765G/C*, and *COX-1-50C/T* polymorphisms appear not to be associated with the development of cerebral infarction in Chinese populations. However, the interaction between *ALOX5AP-*

SG13S114AA and *COX-2-765CC* apparently increases susceptibility to cerebral infarction.

Key words: Cerebral infarction; 5-Lipoxygenase activating protein; Genetics; Polymorphism; Cyclooxygenase

INTRODUCTION

Cerebral infarction is a leading cause of death and disability in the world (Feigin, 2005; Domingues-Montanari et al., 2008), and it occurs on the basis of genetic and environmental risk factors. Atherosclerosis, a chronic inflammatory disease, plays an important role in the development of cerebral infarction. Arachidonic acid is a precursor of multiple important cardiovascular active and vasoactive substances in humans, and their major metabolites such as leukotrienes, prostaglandin (PG) E2, and thromboxane 2 (TXA2) are involved in promoting the development of atherosclerosis, thrombosis, and platelet adhesion and aggregation (Davi and Patrono, 2007; Gross et al., 2007; Peters-Golden and Henderson, 2007).

Arachidonic acid is transferred to leukotrienes by 5-lipoxygenase with the participation of 5-lipoxygenase-activating protein (ALOX5AP or FLAP). The DeCODE investigators were the first to suggest that common allelic variants of the *ALOX5AP* gene increase the risk of myocardial infarction and stroke (Helgadottir et al., 2004, 2005). Subsequent studies by others in several non-Icelandic populations have since yielded conflicting results (Meschia et al., 2005; Zee et al., 2006).

Cyclooxygenase (COX) converts arachidonic acid to PGH2, which is then metabolized to thromboxane by thromboxane synthase in platelets and to PGE by PGE synthase in several different body tissues. There are 2 subtypes of COX, COX-1 and COX-2. COX-derived PGE has been shown to increase in subclinical atherosclerosis, contributing to plaque rupture and cerebrovascular disease events (Cipollone et al., 2004a). The possible role of genetic variation in COX-associated cerebral infarction has seen limited research, and results vary greatly. Cipollone et al. (2004b) found the *COX-2-765G>C* promoter polymorphism to be an inherited protective factor against myocardial infarction and stroke in 1728 unrelated Italian individuals. However, the same polymorphism was shown in the Atherosclerosis Risk in Communities Study to increase the risk of incident stroke in African-Americans (Kohsaka et al., 2008; Lee et al., 2008).

Stroke is a common complex trait that does not follow a Mendelian pattern of inheritance. Gene-gene or gene-environment interactions may be responsible for the complex trait. How the interactions contribute to stroke remains unclear. In general, gene-gene interactions in complex diseases have been investigated by logistic regression and multilocus linkage disequilibrium tests, but these data have limitations in their general applications. Ritchie et al. (2001) proposed an algorithm called the "multifactor dimensionality reduction" (MDR) method for balanced case-control or discordant sib-pair designs. MDR is a constructive induction algorithm and a flexible computational framework for collapsing high-dimensional genetic data into a single dimension, thus permitting the detection of interactions in relatively small sample sizes (Moore et al., 2006). To improve the adjustment of covariables, Lou et al. (2007) extended the MDR method and proposed a generalized multifactor dimensionality reduction (GMDR) framework based on the score of a generalized linear model. GMDR

has several additional advantages, including: 1) offering a unified framework for coherently handling both dichotomous and quantitative phenotypes and 2) applicability to a variety of flexible population-based study designs.

The purpose of our study was to determine the polymorphisms of *ALOX5AP-SG13S114A*/*T*, *COX-2-765G*/*C*, and *COX-1-50C*/*T* using the polymerase chain reaction-restriction fragment length polymorphism (PCR-PFLP) method in cases of cerebral infarction and controls. We also aimed to characterize the association between the polymorphisms and the development of cerebral infarction in a Chinese population, and to explore whether gene-gene interactions increase susceptibility to cerebral infarction.

MATERIAL AND METHODS

Subjects

Cases with first onset of cerebral infarction who were admitted to the Department of Neurology of our hospital from May 2010 through December 2011 and identified using cranial computed tomography (CT) and magnetic resonance imaging (MRI) were included in the present study. They were divided into 2 types of cerebral infarction - atherosclerosis thrombosis type and small artery disease type - according to the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification (Han et al., 2007). Cases were excluded if they met the following exclusion criteria: 1) cardiogenic cerebral embolism, cerebral infarction caused by other factors or without clear causes; 2) medical or family history of apoplexy; 3) transient ischemic attack and/or cerebral hemorrhage; and 4) reluctance to participate. In total, 411 cases were involved in the analysis, including 231 males and 180 females with a mean age of 69.3 ± 10.3 years. Of the 411 subjects, 270 had the atherosclerosis thrombosis type of cerebral infarction, and 141 had the small artery disease type.

Patients who were hospitalized in the Department of Cardiology or Department of Endocrinology of the same hospital during the study period and those in whom a physical examination was performed served as controls. No intracranial lesions were detected in medical histories, physical examination, cranial CT scan and/or MRI, and controls had no medical or family history of apoplexy, atrial fibrillation or other heart diseases. A 1:1 case-control match was employed, and 411 controls were matched for age, gender, and risk factors (hypertension, diabetes, hyperlipidemia, smoking, etc.), including 231 males and 180 females with a mean age of 68.9 ± 10.2 years old.

All subjects had no medical or family history of hereditary diseases, and blood relationships were observed. Participants enrolled in the present study were free of arteritis, infection, tumor, blood disease, severe heart, lung, liver, kidney and thyroid disease, and autoimmune diseases.

This study was approved by the Ethics Review Committee of the hospital, and informed consent was obtained from all participants following a detailed description of the purpose and potential benefits of the study.

Genotype determination

Three milliliters of blood was collected from the anticubital vein of each subject and

then anticoagulated with ethylenediaminetetraacetic acid. Genomic DNA was isolated with the AxyPrep™ Blood Genomic DNA Maxiprep kit (Axygen Biosciences, USA) using cell lysis and a hemoglobin/protein precipitation technique in combination with selective DNA adsorption to the membrane. Double-distilled water was added to dissolve the isolated DNA, and the concentration was determined using a nucleic acid spectrometer. The DNA was stored at -80°C.

The single nucleotide polymorphisms (SNPs) of the 3 newly added *ALOX5AP*, *COX-2*, and *COX-1* genes were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/SNP). Three TagSNPs of the 3 above-mentioned genes were found in the human HapMap project database (http://www.hapmap.org) with minor allele frequency ≥0.10, which were *ALOX5AP-SG13S114A/T* (*rs10507391*), *COX-2-765G/C* (*rs20417*), and *COX-1-50C/T* (*rs3842787*).

PCR was performed using 3 pairs of primers, which were synthesized by Sangon Biotech Co., Ltd. (Shanghai). The forward primer for COX-1-C50T was 5'-GGTGCCCGGTG GGGAATTTTC-3', and the reverse primer was 5'-GAGGGGAAAGGAGGGGGTTG-3'. The forward primer for COX-2-G765C was 5'-CCGCTTCCTTTGTCCATCAG-3', and the reverse primer was 5'-GGCTGTATATCTGCTCTATATGC-3'. The forward primer for ALOX5AP-SG13S114A/T was 5'-GTGTTCAGGAAGGGAGTTTCTGT-3', and the reverse primer was 5'-GTCTATGGTTGCAACATTGAGATTA-3'. PCR amplification was performed in a final reaction volume of 50 µL containing 0.1 µg DNA, 12.5 µL Premix Ex Taq™, 0.5 µL ROX Reference Dye II, 2 µL each primer, 8 µL template DNA and 25 µL double-distilled water. The following protocol was used for PCR: pre-denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s and 72°C for 45 s, and a final extension at 72°C for 10 min. The amplification product was identified with 2% agarose gel electrophoresis, stained with ethidium bromide, and observed with ultraviolet light.

Five microliters of PCR amplification product was sampled and digested at 37°C for 16 h in a final reaction volume of 15 µL, containing 1 µL 1 U *Smu*I (Fermentas, Glen Burnie, MD, USA) for COX-1-C50T and COX-2-G765C or *Vsp*I (Fermentas) for ALOX5AP-SG13S114, 1 µL 10X Buffer1 Tango™, and 8 µL sterile, deionized, double-distilled water. The digestive product was identified using 2% agarose gel electrophoresis at 100 V, stained with ethidium bromide, and observed and photographed under ultraviolet light. The PCR products of some gene loci were identified using sequencing with the ABI3730 DNA Analyzer (PE Applied Biosystems, Carlsbad, CA, USA).

Statistical analysis

All statistical analyses were performed using Statistical Package for the Social Sciences v. 16.0 (SPSS 16.0, SPSS Inc., Chicago, IL, USA). The goodness-of-fit test was performed to test the Hardy-Weinberg equilibrium of the genotype distributions of the subjects. The differences in measurement data that followed an approximately normal distribution including body mass index, levels of fasting blood glucose, and serum cholesterol, triglyceride, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol were compared for statistical significance using *t*-tests. Differences in proportions of the count data such as hypertension, diabetes, and history of smoking, and differences in genotype and allele frequencies were tested for statistical significance with chi-square tests. The interaction of multiple factors was investigated using the statistical software GMDR Beta program version 0.7 (www.healthsystem.virginia.edu/internet/addiction-genomics/Software) and multiple logistic regres-

sion. The relative risk of genotype and prevalence of cerebral infarction was expressed with odds ratios (OR) and 95% confidence intervals (CI). $P < 0.05$ was considered to be statistically significant.

RESULTS

Clinical characteristics

Major clinical characteristics of controls and subjects with cerebral infarction are shown in Table 1. No significant differences were observed ($P > 0.05$).

Table 1. Comparison of major clinical characteristics of subjects and controls.

Characteristic	Cerebral infarction group (N = 411)	Control (N = 411)	P value
Age (years)	69.30 ± 10.30	68.90 ± 10.20	0.35
Male cases	231	231	0.99
Body mass index (kg/m^2)	24.20 ± 2.33	24.30 ± 2.13	0.44
Smokers	104	102	0.94
Total cholesterol (mM)	5.53 ± 1.35	5.42 ± 1.26	0.15
High-density lipoprotein cholesterol (mM)	1.52 ± 0.56	1.55 ± 0.28	0.46
Low-density lipoprotein cholesterol (mM)	2.87 ± 0.62	2.85 ± 0.68	0.24
Triglyceride (mM)	1.86 ± 1.13	1.82 ± 1.12	0.65
Cases with hypertension	322	312	0.88
Cases with type 2 diabetes	126	125	0.97

Hardy-Weinberg equilibrium

The Hardy-Weinberg equilibrium test of the SNP genotypes of the cases with cerebral infarction and the controls showed that the genotype frequency distribution of *ALOX5AP-SG13S114A*/*T* and *COX-2-765G*/*C* did not deviate from Hardy-Weinberg equilibrium (χ^2 = 2.137 and 3.172, respectively, both $P > 0.05$). This indicates that the gene frequency of the subjects included in the current study could represent the gene distribution of the normal populations. For *COX-1-50C/T*, the CT genotype was detected in only 6 cases in each group and no TT genotype was found, and therefore, a Hardy-Weinberg equilibrium test was not necessary.

Comparison of genotype and allele frequency distributions of *ALOX5AP-SG13S114A/T*

The genotype and allele frequency distributions of cases with cerebral infarction and comparison with controls are shown in Table 2. There were no significant differences detected ($P > 0.05$).

Table 2. Comparison of genotype and allele frequency distribution of *ALOX5AP-SG13S114A/T* between subjects and controls.

Group	N	Genotype frequency (%)			Allele frequency (%)	
		TT	TA	AA	T	A
Cerebral infarction	411	145 (35.3)	194 (47.2)	72 (17.5)	484 (58.9)	338 (41.1)
Control	411	165 (40.1)	197 (47.9)	49 (11.9)	527 (64.1)	295 (35.9)
P value		0.38			0.071	

Genotype distribution of *COX-1-50C/T* in cases with cerebral infarction and controls

The homozygous CC genotype was observed in both the cases with cerebral infarction and controls, while the CT genotype was detected in only 6 cases in each group. No TT genotype was found.

Comparison of genotype and allele frequency distributions of *COX-2-765G/C*

The genotype and allele frequency distributions of *COX-2-765G/C* in cases with cerebral infarction and comparison with controls are presented in Table 3. No significant differences were observed (P > 0.05)

Table 3. Comparison of genotype and allele frequency distribution of *COX-2-765G/C* between subjects and controls.

Group	N	Genotype frequency (%)			Allele frequency (%)	
		GG	GC	CC	G	C
Cerebral infarction	411	229 (55.7)	124 (30.2)	58 (14.1)	582 (70.8)	240 (29.2)
Control	411	247 (60.1)	129 (31.4)	35 (8.5)	623 (75.8)	199 (24.2)
P value		0.37			0.23	

GMDR analysis

High-order interactions were investigated for cerebral infarction using the GMDR method, and significant high-order interactions were detected (Table 4). With covariable adjustments, the best model for cerebral infarction included *ALOX5AP-SG13S114A/T* and *COX-2-765G/C*, scoring 10 for cross-validation consistency and 9 for sign test (P = 0.0107).

Table 4. Comparison of best models, prediction accuracies, cross-validation consistencies, and P values for cerebral infarction identified by GMDR.

Number of loci	Best model*	Training balanced accuracy	Testing balanced accuracy	Cross-validation consistency	Sign test (P value)
1	3	0.5249	0.5251	9	7 (0.1719)
2	1,2	0.5617	0.5313	10	9 (0.0107)
3	1,2,3	0.5414	0.5266	8	8 (0.0647)

**ALOX5AP-SG13S114A/T*, *COX-2-765G/C* and *COX-1-50C/T* are symbolized as 1-3, respectively.

Logistic regression analysis

Table 5 demonstrates the associations between cerebral infarction and different combinations of genotypes compared with *ALOX5AP-SG13S114TT* and *COX-2-765GG*. The estimated risk of cerebral infarction was significantly higher in individuals with *ALOX5AP-SG13S114AA* and *COX-2-765CC* than those with *ALOX5AP-SG13S114TT* and *COX-2-765GG* (OR = 2.842, 95%CI = 1.324-6.543, P = 0.023). A higher risk was also observed in the cases with *ALOX5AP-SG13S114AA* and *COX-2-765CC/GC* than those with *ALOX5AP-SG13S114TT* and *COX-2-765GG* (OR = 2.124, 95%CI = 1.101-5.142, P = 0.045). However, a lower risk was detected in the individuals with the combinations of *ALOX5AP-SG13S114AA/*

TA and *COX-2-765CC* as well as *ALOX5AP-SG13S114TA* combined with *COX-2-765GC* than those with *ALOX5AP-SG13S114AA* in combination with *COX-2-765CC* (OR = 1.445, 1.172 *vs* 2.842). However, the P value of these combinations did not reach the cut-off significance level of 0.05. The results indicate that the interaction of multiple genes conferred higher risk for stroke than did a single susceptibility gene.

Table 5. Associations between cerebral infarction and different combinations of genotypes.

ALOX5AP-SG13S114A/T	TT	AA	AA, TA	AA	TA	AA, TA
COX-2-765G/C	GG	CC	CC	CC, GC	GC	CC, GC
OR	1*	2.842	1.445	2.124	1.172	1.081
95%CI	-	1.324-6.543	0.752-2.856	1.101-5.142	0.664-2.014	0.654-1.582
P value	-	0.023	0.262	0.045	0.534	0.742

*Non-risk genotype for each genetic factor was used as the reference OR.

DISCUSSION

Helgadottir et al. (2004, 2005) found that the SNP haplotype of the gene *ALOX5AP* was associated with twice the risk of myocardial infarction and cardiovascular diseases in Icelandic and Scottish subjects, and this genetic variant was named HapA. *ALOX5AP* encodes 5-lipoxygenase activating protein (FLAP), and there are 5 cerebral infarction-associated SNP loci that have been identified, *SNPSG13S25*, *SG13S32*, *SG13S89*, *SG13S100*, and *SG13S114*. The latter 2 are particularly important (Lohmussaar et al., 2005). The present study showed, however, that there were no significant differences in the genotype and allele frequency distributions of *ALOX5AP-SG13S114* between the cases with cerebral infarction and the controls, which may be due to many factors responsible for the development of cerebral infarction at molecular levels. Due to the abundance of neurotransmitters in brain tissue along with complex signal transduction and gene expression, there are many factors influencing *ALOX5AP* expression, which leads to a lack of effect of the *ALOX5AP* polymorphism on the development of cerebral infarction. In addition, race is thought to contribute to the present findings.

COX is an important enzyme that inhibits TXA2 synthesis. Aspirin leads to the inactivation of *COX-1* and a decrease in production of TXA2, achieving the goal of antithrombotic therapy (Lee et al., 2005). One study revealed that the minor allele frequencies in *COX-1-50C>T* and *COX-2-765G>C* were 8.6 and 21.3% in Caucasians, suggesting that a variation of either gene would alter the effectiveness of aspirin (Halushka et al., 2003). The present study showed that the homozygous CC genotype of *COX-1-50C>T* was dominant in a Chinese population, and that no TT genotype was found. This is in agreement with other studies in Japanese and Chinese populations (Fujiwara et al., 2007; Li et al., 2007). Few *COX-1-50C>T* polymorphisms have been examined in Asian populations.

COX-2 is rarely expressed under normal physiological conditions. However, under many pathological conditions, various stimuli in the internal and external environment could lead to an increase in *COX-2* expression. The association between *COX-2* polymorphism and cerebral infarction has gradually gained research attention. However, most subjects in the literature are sampled from European and American countries, and the results vary greatly. Colaizzo et al. (2006) revealed an association between the *COX-2G-765C* gene polymorphism and cerebrovascular ischemia, suggesting that the *COX-2* gene is a susceptibility locus for the

risk of cerebrovascular ischemic disease. Cipollone et al. (2004b) found that the *-765G>C* polymorphism of the *COX-2* gene was a protective factor against myocardial infarction and ischemic stroke. Another study found the *COX-2G-765C* polymorphism to be a risk factor for incident stroke in African-Americans (Kohsaka et al., 2008), while no association between the *765C* allele and cerebral infarction was observed in Korean and American populations (Lee and Kong, 2007; Lemaitre et al., 2009). Ultimately, the association between the *765G/C* polymorphism and cerebral infarction remains unclear. Our study was designed to investigate the association between them, and our findings revealed no significant association between the *COX-2-765G/C* polymorphism and cerebral infarction in a Chinese Han population.

It is known that cerebral infarction is a complicated disease caused by multiple genes and multiple risk factors, which do not follow a Mendelian pattern of inheritance. Generally, the occurrence and development of the disease is determined by several variations (polymorphisms) with minor genetic effect, without major gene effects observed (Schork et al., 2009). As a result of gene-gene and gene-environment interactions, linkage analysis, which is used to investigate single-gene disorders, is not suitable for the genetic study of cerebral infarction. Therefore, the present study employed association analyses (Liu et al., 2009). The results demonstrated that the polymorphism of either *ALOX5AP-SG13S114* or *COX-2-765G/C* was not associated with cerebral infarction in Chinese Han individuals. However, GMDR analysis revealed that the risk for cerebral infarction increased by 2.842 times (95%CI = 1.32-6.54, P = 0.023) in the individuals carrying both *ALOX5AP-SG13S114AA* and *COX-2-765CC* genotypes, indicating that the interaction between *ALOX5AP* and *COX-2* increased the susceptibility to cerebral infarction in our Chinese population. Arachidonic acid is a precursor of multiple important cardiovascular active and vasoactive substances in humans. ALOX5AP and COX-2 are important enzymes in the metabolism of arachidonic acid, and their activities are encoded and regulated by the *ALOX5AP* and *COX-2* genes. Therefore, the interactions between the *ALOX5AP* and *COX-2* genes may lead to over-production of arachidonic acid metabolites such as leukotrienes, PGE2, and TXA2, initiation of the arachidonic acid cascade, and effect on the pathogenesis of cerebral infarction (Cimino et al., 2008; Rink and Khanna, 2011; Ward et al., 2011).

To our knowledge, the present study is the first to identify the interaction between *ALOX5AP* and *COX-2* genes, suggesting that this interaction may lead to increased susceptibility to cerebral infarction in Chinese individuals.

LIMITATIONS

However, due to the limited sample size used and single-center study, these results may not represent the full disease status in China. Many candidate gene association studies should be performed for a number of different diseases. Our results should be validated in larger, multi-center studies. Therefore, future studies should focus on a larger sample size and study area.

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Conflict of interests

The authors declare no conflict of interest.

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