RESEARCH ARTICLE

Association of G-protein coupled purinergic receptor P2Y2 with ischemic stroke in a Han Chinese population of North China

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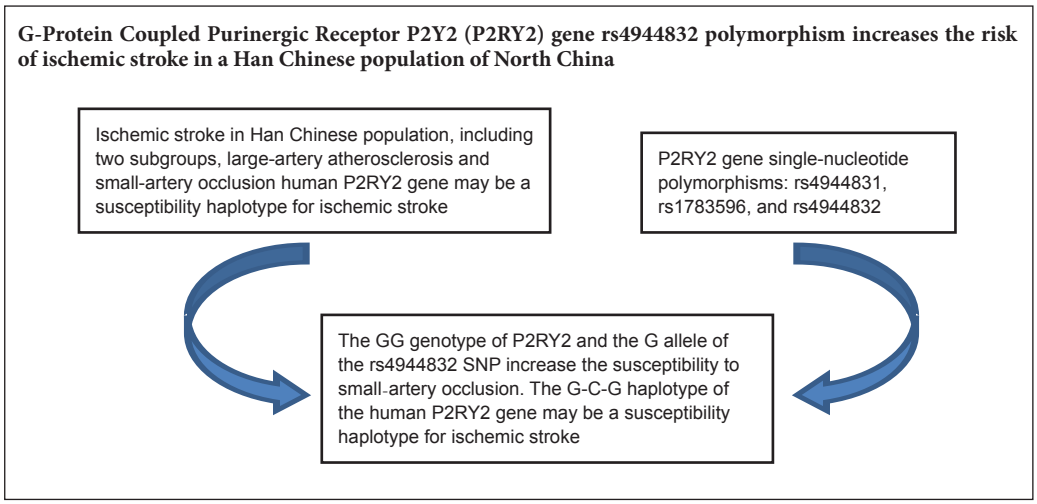
Graphical Abstract



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Abstract

The G-protein-coupled purinergic receptor P2Y2 (P2RY2) plays an important role in the mechanism of atherosclerosis, which is relevant to ischemic stroke. This retrospective case-control study aimed to assess the relationship between *P2RY2* gene polymorphisms and ischemic stroke risk in the northern Han Chinese population. In this study, clinical data and peripheral blood specimens were collected from 378 ischemic stroke patients and 344 controls. The ischemic stroke participants were recruited from the First Affiliated Hospital of China Medical University and the First Affiliated Hospital of Liaoning Medical University. The controls were recruited from the Health Check Center at the First Affiliated Hospital of China Medical University. Ischemic stroke patients were divided into two subgroups according to the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification: large-artery atherosclerosis (n = 178) and small-artery occlusion (n = 200) strokes. All subjects were genotyped for three single nucleotide polymorphisms (rs4944831, rs1783596, and rs4944832) in the *P2RY2* gene using peripheral venous blood samples. The distribution of the dominant rs4944832 phenotype (GG *vs.* GA+AA) differed significantly between small-artery occlusion patients and control subjects (odds ratio (*OR*) = 1.720, 95% confidence interval (*CI*): 1.203–2.458, $P < 0.01$). Multivariable logistic regression analysis revealed that the GG genotype of rs4944832 was significantly more prevalent in small-artery occlusion patients than in control subjects (*OR* = 1.807, 95% *CI*: 1.215–2.687, $P < 0.01$). The overall distribution of the haplotype established by rs4944831-rs1783596-rs4944832 was significantly different between ischemic stroke patients and controls ($P < 0.01$). In ischemic stroke patients, the frequency of the G-C-G haplotype was significantly higher than in control subjects ($P = 0.028$), whereas the frequency of the T-C-A haplotype was lower than in control subjects ($P = 0.047$). These results indicate that the G-C-G haplotype of *P2RY2* is a susceptibility haplotype for ischemic stroke. In addition, the GG genotype of rs4944832 may be associated with the development of small-artery occlusion in the northern Han Chinese population. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University on February 20, 2012 (No. 2012-38-1) and the First Affiliated Hospital of Liaoning Medical University, China, on March 1, 2013 (No. 2013-03-1). All participants gave their informed consent. This trial was registered with the ISRCTN Registry (ISRCTN11439124) on October 24, 2018. Protocol version (1.0).

Key Words: *nerve regeneration; P2RY2 gene; ischemic stroke; single nucleotide polymorphism; case-control study; haplotype; northern Han Chinese population; large-artery atherosclerosis; small-artery occlusion; hypertension; candidate gene; neural regeneration*

Chinese Library Classification No. *R446; R741*

Introduction

Ischemic stroke is a leading cause of disability and death, both in China and worldwide. The incidence of ischemic stroke is higher in northern China (486 per 100,000 person-years) than elsewhere (Wu et al., 2013). The Han ethnicity is the main ethnic group in northern China. Many previous studies have confirmed a relationship between various genetic polymorphisms and the incidence of ischemic stroke

in northern Han Chinese (Li et al., 2018b; Ye et al., 2018). Some studies have demonstrated that many polymorphisms in candidate genes are associated with atherosclerosis and hypertension in certain populations, and these studies have contributed to the identification of ischemic stroke susceptibility loci (Kaneko et al., 2006; Naganuma et al., 2009; Wang et al., 2009, 2010; Stepanyan et al., 2018).

A total of eight P2Y receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) have been identified (Webb et al., 1996; Von et al., 2000). Dasari et al. (1996) first mapped the human *P2RY2* gene to chromosome 11q13.5-q14.1. *P2RY2* plays an important role in the progression of hypertension and atherosclerosis, and it is involved in platelet aggregation, coagulation, and inflammation (Gachet et al., 2006). Furthermore, *P2RY2* has been implicated in the development of vascular diseases (Ralevic et al., 1991; Di et al., 2002; Dissmore et al., 2016). The role of *P2RY2* in endothelial dysfunction has been reported in many studies, which have also shown that this receptor plays an important role in the mechanism of atherosclerosis, which is related to ischemic stroke (Seye et al., 2004, 2006; Guns et al., 2006). Apolipoprotein E null ($ApoE^{-/-}$) mice deficient in P2Y2R exhibit low VCAM-1 levels in endothelial cells and have a delayed onset of atherosclerosis; the results of this study suggest that targeting this nucleotide receptor may be a new therapeutic approach for atherosclerosis (Qian et al., 2016).

Ischemic stroke is known to be a multifactorial and multigenic disorder (Gao et al., 2017; Hachiya et al., 2017; Li et al., 2018a, c). Many studies have attempted to identify gene variants that are associated with the risk of ischemic stroke (Wang et al., 2009; Matsushita et al., 2010). Although a previous study revealed some *P2RY2* polymorphisms that may contribute to ischemic stroke (Wang et al., 2009), no studies have yet examined the association between human *P2RY2* gene polymorphisms and ischemic stroke in the northern Han Chinese population. We performed this case-control study to investigate the association between *P2RY2* single nucleotide polymorphisms (SNPs: rs4944831, rs1783596, and rs4944832) and the incidence of ischemic stroke in the northern Han Chinese population. In addition, we divided the ischemic stroke subjects into two subgroups, large-artery atherosclerosis (LAA) and small-artery occlusion (SAO), according to the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification. This study aimed to confirm the relationships between ischemic stroke of different etiologies and gene polymorphisms in the northern Han Chinese population.

Participants and Methods

Participants

This retrospective case-control study was carried out using 378 ischemic stroke patients (178 with LAA, 200 with SAO) and 344 unrelated healthy controls. All subjects were Han Chinese individuals from Liaoning Province, China. The ischemic stroke patients were enrolled from the First Affiliated Hospital of China Medical University, Shenyang, and the First Affiliated Hospital of Liaoning Medical University, Jinzhou, from September 1, 2010 to May 30, 2011. The healthy controls were enrolled from the Health Check Center of the First Affiliated Hospital during the same period. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University on February 20, 2012 (No. 2012-38-1) and the First Affiliated Hospital of Liaoning Medical University in China on March 1, 2013 (No. 2013-03-1) (**Additional file 1**). This study was performed in strict accordance with the *Declaration of Helsinki* developed by the World Medical Association. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (**Additional file 2**). All participants provided written informed consent (**Additional file 3**).

Inclusion criteria for the ischemic stroke group

According to the TOAST classification, patients in the ischemic stroke group were diagnosed depending on clinical features and neuroimaging criteria that included the sudden onset of a global or focal neurological deficit, with corresponding brain infarction as seen on computed tomography (CT) imaging or magnetic resonance imaging (MRI) (Adams et al., 1993). Examinations of cerebral blood vessels, such as computed tomography angiography and magnetic resonance angiography, were performed when necessary.

Exclusion criteria for the ischemic stroke group

Patients were excluded if they had cancer, hemorrhagic stroke, transient ischemic attack, or severe cardiac, hepatic, or renal diseases.

Inclusion criteria for the control group

Unrelated healthy controls presented no clinical or radiological evidence of stroke or cerebrovascular diseases and were matched with ischemic stroke patients in sex, age, ethnic origin, and area of residence. Some healthy controls had vascular risk factors such as hypertension, diabetes, smoking, drinking, and hypercholesterolemia.

Exclusion criteria for control subjects

Control subjects were excluded if they had cancer, autoimmune disease, chronic inflammation, renal insufficiency, liver insufficiency, or hematopathy.

Data collection

All participant interviews were administered by our investigators, who followed a Chinese structured questionnaire of patients (**Additional file 4**). Any history of vascular risk factors, such as smoking, drinking, hypertension, and diabetes, was recorded. Body weight, height, blood pressure, fasting plasma glucose, total plasma cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) measures were also measured. To obtain body mass index (BMI) scores, body weight (kg) was divided by height squared (m^2).

After the overall collection and evaluation of the clinical

data, ischemic stroke patients with cardioembolic stroke, stroke of other determined etiology, or stroke of undetermined etiology according to TOAST classification, were excluded from the analysis (Adams et al., 1993). In our study, ischemic stroke patients included two subgroups: LAA and SAO. LAA patients had clinical and brain imaging findings of either significant (> 50%) stenosis or occlusion of a major brain artery or branch cortical artery, presumably due to atherosclerosis. The SAO subgroup included patients whose strokes were often labeled lacunar infarcts in other classifications. The patients had a normal CT/MRI examination or a relevant brainstem or subcortical hemispheric lesion with a diameter of less than 1.5 cm.

Polymorphism genotyping

Three *P2RY2* SNPs (rs4944831, rs1783596, and rs4944832) were obtained from the public dbSNP database (http://www.ncbi.nlm.nih.gov/SNP). All three SNPs represent minor allele frequencies of at least 5% in the Han Chinese population. SNP1 (rs4944831) is located in intron 1, SNP2 (rs1783596) is located in the coding regions of exon 3, and SNP3 (rs4944832) is located in the 3 flanking region of the *P2RY2* gene.

Peripheral blood samples were collected from the median cubital vein of each subject. Genomic DNA was extracted from peripheral blood leukocytes using a DNA extraction kit (Wizard Genomic DNA purification kit; Promega, Sunnyvale, CA, USA) and quantified with a spectrophotometer (Thermo Scientific, Waltham, MA, USA). All DNA samples were stored at −20°C (Nakayama et al., 2001).

Genotyping of *P2RY2* SNPs was performed using Multiplex SNaPshot sequencing. Specifically, genomic DNA was first amplified by multiplex polymerase chain reaction (PCR) using the primers shown in **Table 1**.

Table 1 Primer sequences for *P2RY2* gene variants rs4944831, rs1783596, and rs4944832 amplification

Single-nucleotide polymorphism	Primer sequences (5′–3′)	Product size (bp)
rs4944831	Forward: CTG TCC CAT GCA GCA GTG ATT C	250
	Reverse: ACC CAG CAT AGC CCA GAA CAG G	
rs1783596	Forward: CTG GCT GTC TTC GCC CTC TG	174
	Reverse: CAG GAA GTA GAG CAC GGG GTC A	
rs4944832	Forward: TGC AGA CTC AAG GCC AGA GAT G	245
	Reverse: CTG CAG GCA CAT CAG CAG ACA T	

PCR samples (10 μL/tube) consisted of 10 ng genomic template, 1 μM forward primer, 1 μM reverse primer, 10 μL of 1× GC buffer I (Takara, Otsu, Shiga, Japan), 3 mM Mg^{2+} (Takara), 0.3 mM dNTPs (Generay Biotech, Shanghai, China), and 1 U of HotStar Taq polymerase (Qiagen, Hilden, Germany). PCR was performed in duplicate. The cycling conditions consisted of 95°C for 15 minutes; 11 cycles of 94°C for 20 seconds, 67.5°C for 40 seconds, and 72°C for 90 seconds; 24 cycles of 94°C for 20 seconds, 63°C for 30 seconds, and 72°C for 110 seconds; and an extension step at 72°C for 120 seconds.

PCR products were characterized via SNaPshot Multiplex sequencing and GeneMapper 4.0 (Applied Biosystems, Princeton, NJ, USA). Following characterization, 10% of positive samples were randomly selected for repeated genotyping to assess the experimental quality, and the same results were obtained.

Primary outcome measures

The primary outcome measures of this study were the genotype frequencies of the three *P2RY2* SNPs (rs4944831, rs1783596, and rs4944832) in ischemic stroke patients and controls.

Secondary outcome measures

Secondary outcome measures were as follows:

(1) Allele frequencies of the three *P2RY2* SNPs (rs4944831, rs1783596, and rs4944832) in ischemic stroke patients and controls.

(2) Inferred haplotypes of these three *P2RY2* SNPs (rs4944831, rs1783596, and rs4944832).

Statistical analysis

Statistical analyses were performed using SPSS 19.0 software (IBM Corporation, Armonk, NY, USA). Continuous and categorical data are presented as the mean ± SD and percentages, respectively. Differences in vascular risk factors between ischemic stroke patients and healthy controls were assessed using Pearson's chi-squared test and Student's *t* test. The allele frequencies of the *P2RY2* SNPs were calculated based on the genotypes of all participants. Differences in the genotype and allele frequencies of *P2RY2* SNPs between ischemic stroke patients and control subjects were evaluated with the chi-squared test and reported as odds ratios (*OR*s) and 95% confidence intervals (95% *CI*s). The Hardy-Weinberg equilibrium was used to examine genotype distributions. Multivariable logistic regression analysis was selected to assess the relationships between *P2RY2* polymorphisms and ischemic stroke by adjusting for confounding variables.

The linkage disequilibrium index (D-prime and r^2) and the inferred haplotypes of these three SNPs (rs4944831, rs1783596, and rs4944832) were assessed using the SHEsis analysis platform (http://analysis.bio-x.cn/myAnalysis.php) (Shi and He, 2005). A difference was considered statistically significant if $P < 0.05$.

Results

Clinical characteristics of study subjects

A total of 378 ischemic stroke patients (227 males and 151 females) and 344 control subjects (208 males and 136 females) were enrolled to determine the potential association of *P2RY2* SNPs with ischemic stroke risk. There were no significant differences in age, BMI, or serum HDL-C lev-

els between ischemic stroke patients and control subjects. However, the percentages of individuals with hypertension, diabetes, current smoking status, or current alcohol consumption were higher in ischemic stroke patients (LAA: 178, SAO: 200) than in controls (**Table 2**). Levels of serum fasting plasma glucose, total cholesterol, and LDL-C were also higher in ischemic stroke patients than in controls.

Genotype analysis

The genotype distributions of the three SNPs (rs4944831, rs1783596, and rs4944832) were in Hardy–Weinberg equilibrium in both patients and controls ($P > 0.05$). There was no significant difference in the distributions of each SNP between ischemic stroke patients and controls ($P > 0.05$). The genotype and allele frequencies of the *P2RY2* SNPs in ischemic stroke patients (including in the LAA and SAO subgroups) and controls are shown in **Table 3**.

There were no significant differences in the rs4944831 and rs1783596 genotypes or allele distributions between ischemic stroke patients and controls. In contrast, there were significant differences in the distributions of the GG genotype and of the G allele of rs4944832 between SAO patients and control subjects ($P < 0.01$). However, this difference was not observed in patients with LAA. Multivariable logistic regression analysis was used to assess the relationship between the rs4944832 polymorphism and SAO by adjusting for confounding variables. The GG genotype of rs4944832 remained significantly associated with an increased risk of SAO (*OR* = 1.807, 95% *CI*: 1.215–2.687, $P < 0.01$; **Table 4**).

Using the SHEsis program platform, these *P2RY2* SNPs (rs4944831, rs1783596, and rs4944832) were found to be in linkage disequilibrium in the Chinese population. Six haplotypes with frequencies greater than 3% among both ischemic stroke patients and control subjects were selected in the haplotype analysis. **Table 5** presents the distribution of the individual haplotypes constructed with rs4944831-rs1783596-rs4944832. The overall haplotype distributions were significantly different between the ischemic stroke patients and controls (global test, $P < 0.01$). The frequency of the G-C-G haplotype constructed with rs4944831-rs1783596-rs4944832 in ischemic stroke patients was significantly higher than that in controls (*OR* = 1.772, 95% *CI*: 1.057–2.971, $P = 0.028$), while the frequency of the T-C-A haplotype in ischemic stroke patients was lower than that in control subjects (*OR* = 0.754, 95% *CI*: 0.570–0.997, $P = 0.047$).

Discussion

P2RY2 belongs to a family of G-protein coupled receptors and participates in various physiological responses upon adenosine triphosphate binding and releasing; these responses include shifts in membrane potential, secretion, platelet aggregation, and neurotransmission (Dubyak et al., 1993; Parr et al., 1994; Foresta et al., 1996; Kunapuli et al., 1998; Kunzelmann et al., 2005; Rieg et al., 2007; Orriss et al., 2017).

The *P2RY2* gene is considered to be critical in the mechanism of atherosclerosis and hypertension (Seye et al., 2002, 2003; Wang et al., 2010; Dissmore et al., 2016). Marrelli et al. (1999) found that P2Y2 receptors have a significant influence on middle cerebral artery dilation after ischemia reperfusion in rats. *P2RY2* also has a significant effect on blood pressure regulation in response to adenosine triphosphate binding (Von et al., 2000; Ruppert et al., 2003). In addition, recent studies have reported that endothelial cell-specific P2Y2R deficiency promotes plaque stability in $ApoE^{-/-}$ mice (Chen et al., 2017). The *P2RY2* gene may also play a causative role in hypertension (Rieg et al., 2007, 2011; Erlinge et al., 2008; Wang et al., 2015) and myocardial infarction (Wihlborg et al., 2006). In a recent study in Japan, rs4944831 and the T-A-G haplotype of the *P2RY2* gene were genetic

Table 2 Clinical characteristics of study participants

Item	Controls (n = 344)	Ischemic stroke (n = 378)	LAA (n = 178)	SAO (n = 200)
Age (year)	62.73±8.44	62.52±8.71	62.40±8.56	62.57±8.71
BMI (kg/m^2)	24.34±2.38	24.53±2.13	24.51±1.91	24.54±2.31
Hypertension	92(26.7)	209(55.3)**	97(54.5)**	112(56.0)**
SBP (mmHg)	132.41±14.68	144.43±19.25**	145.51±19.36**	143.47±19.15**
DBP (mmHg)	79.00±8.85	87.39±12.27**	88.05±12.43**	86.81±12.13**
Diabetes	33(9.6)	101(26.7)**	47(26.4)**	54(27.0)**
Smoking	44(12.8)	138(36.5)**	64(36.0)**	74(37.0)**
Alcohol consumption	31(9.0)	92(24.3)**	46(25.8)**	46(23.0)**
FPG (mM)	5.73±1.16	6.66±2.31**	6.74±2.46**	6.59±2.16**
Total cholesterol (mM)	4.46±0.89	4.71±1.04**	4.73±1.13**	4.69±0.95**
Triglycerides (mM)	1.33±0.66	1.59±1.02**	1.64±0.97**	1.56±1.07**
HDL-C (mM)	1.23±0.25	1.22±0.29	1.20±0.28	1.23±0.29
LDL-C (mM)	2.83±0.77	3.19±0.98**	3.27±1.05**	3.12±0.91**

Hypertension, diabetes, smoking, and alcohol consumption were assessed using Pearson's chi-square test and are expressed as n(%). Age, BMI, SBP, DBP, FPG, total cholesterol, triglycerides, HDL-C and LDL-C were assessed using Student's t-tests and are expressed as the mean ± SD. **$P < 0.01$, *vs*. controls. There was no significant difference in age, BMI, or HDL-C levels between the patients and controls. Hypertension, diabetes, smoking, alcohol consumption, and higher serum levels of FPG, total cholesterol, and LDL-C were associated with ischemic stroke. 1 mmHg = 0.133 kPa. BMI: Body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FPG: fasting plasma glucose; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; LAA: large-artery atherosclerosis; SAO: small-artery occlusion.

Table 3 Genotype and allele distributions in ischemic stroke patients and controls

	Controls (n = 344)	Ischemic stroke (n = 378)	*OR* (95% *CI*), *P*	LAA (n = 178)	*OR* (95% *CI*), *P*	SAO (n = 200)	*OR* (95% *CI*), *P*
rs4944831 genotype							
T/T	171 (49.7)	199 (52.6)	Reference	94 (52.8)	Reference	105 (52.5)	Reference
T/G	139 (40.4)	145 (38.4)	1.12 (0.82–1.52), 0.49	66 (37.1)	0.86 (0.59–1.27), 0.46	79 (39.5)	1.08 (0.75–1.56) ,0.68
G/G	34 (9.9)	34 (9.0)	1.16 (0.69–1.95), 0.57	18 (10.1)	0.96 (0.52–1.80), 0.91	16 (8.0)	1.31 (0.69–2.48), 0.42
Dominant model							
TT	171 (49.7)	199 (52.6)	Reference	94 (52.8)	Reference	105 (52.5)	Reference
TG+GG	173 (50.3)	179 (47.4)	1.13 (0.84–1.51), 0.43	84 (47.2)	0.88 (0.62–1.27), 0.50	95 (47.5)	1.12 (0.79–1.59), 0.53
Recessive model							
GG	34 (9.9)	34 (9.0)	Reference	18 (10.1)	Reference	16 (8.0)	Reference
TG+TT	310 (90.1)	344 (9I.0)	1.11 (0.67–1.83), 0.68	160 (89.9)	1.03 (0.56–1.87), 0.93	184 (92.0)	1.26 (0.68–2.35), 0.46
rs4944831 allele							
T	481 (69.9)	543 (71.8)	Reference	254 (71.3)	Reference	289 (72.3)	Reference
G	207 (30.1)	213 (28.2)	0.91 (0.73–1.11), 0.42	102 (28.7)	0.93 (0.70–1.24), 0.63	111 (27.7)	1.12 (0.85–1.47), 0.41
rs1783596 genotype							
T/T	130 (37.8)	159 (42.1)	Reference	78 (43.8)	Reference	81 (40.5)	Reference
T/C	159 (46.2)	168 (44.4)	1.16 (0.84–1.59), 0.37	78 (43.8)	0.82 (0.55–1.21), 0.31	90 (45.0)	1.10 (0.75–1.61), 0.62
C/C	55 (16.0)	51 (13.5)	1.32 (0.84–2.06), 0.22	22 (12.4)	0.67 (0.38–1.12), 0.16	29 (14.5)	1.18 (0.70–2.01), 0.54
Dominant model							
TT	130 (37.8)	159 (42.1)	Reference	78 (43.8)	Reference	81 (40.5)	Reference
TC+CC	214 (62.2)	219 (57.9)	1.20 (0.89–1.61), 0.24	100 (56.2)	0.78 (0.54–1.13), 0.18	119 (59.5)	1.12 (0.78–1.60), 0.53
Recessive model							
CC	55 (16.0)	51 (13.5)	Reference	22 (12.4)	Reference	29 (14.5)	Reference
TC+TT	289 (84.0)	327 (86.5)	1.22 (0.81–1.84), 0.34	156 (87.6)	0.74 (0.44–1.26), 0.27	171 (85.5)	1.12 (0.69–1.83), 0.64
rs1783596 allele							
T	419 (60.9)	486 (64.3)	Reference	234 (65.7)	Reference	252 (63.0)	Reference
C	269 (39.1)	270 (35.7)	0.87 (0.70–1.07), 0.18	122 (34.3)	0.81 (0.62–1.06), 0.13	148 (37.0)	1.09 (0.85–1.41), 0.49
rs4944832 genotype							
G/G	173 (50.3)	210 (55.6)	Reference	83 (46.6)	Reference	127 (63.5)	Reference
G/A	142 (41.3)	143 (37.8)	1.21 (0.89–1.64), 0.23	81 (45.5)	0.84 (0.58–1.23), 0.37	62 (31.0)	1.68 (1.15–2.45), < 0.01**
A/A	29 (8.4)	25 (6.6)	1.41 (0.80–2.50), 0.24	14 (7.9)	0.99 (0.50–1.98), 0.99	11 (5.5)	1.94 (0.93–4.02), 0.07
Dominant model							
GG	173 (50.3)	210 (55.6)	Reference	83 (46.6)	Reference	127 (63.5)	Reference
GA+AA	171 (49.7)	168 (44.4)	1.24 (0.92–1.67), 0.16	95 (53.4)	0.86 (0.60–1.24), 0.43	73 (36.5)	1.72 (1.20–2.46), < 0.01**
Recessive model							
AA	29 (8.4)	25 (6.6)	Reference	14 (7.9)	Reference	11 (5.5)	Reference
GA+GG	315 (91.6)	353 (93.4)	1.30 (0.75–2.27), 0.35	164 (92.1)	1.08 (0.56–2.10), 0.82	189 (94.5)	1.58 (0.77–3.24), 0.21
rs4944832 allele							
G	488 (70.9)	563 (74.5)	Reference	247 (69.4)	Reference	316 (79.0)	Reference
A	200 (29.1)	193 (25.5)	0.84 (0.66–1.06), 0.13	109 (30.6)	0.93 (0.70–1.23), 0.60	84 (21.0)	1.54 (1.15–2.06), < 0.01**

ORs and 95% *CIs* were calculated using Pearson's chi-square tests and are expressed as n(%). $^{**}P < 0.01$, *vs*. controls. The GG genotype frequency and G allele frequency of rs4944832 in the patients with small-artery occlusion were markedly higher than those in the controls. There were no significant differences in the rs4944831 and rs1783596 genotype or the allele distributions between ischemic stroke patients and controls. *OR*: Odds ratio; *CI*: confidence interval; LAA: large-artery atherosclerosis; SAO: small-artery occlusion.

markers for essential hypertension (Wang et al., 2010). These studies show that the *P2RY2* gene has a strong association with ischemic stroke.

Our haplotype-based case–control study aimed to assess the association between the *P2RY2* gene and ischemic stroke in the northern Han Chinese population. The ischemic stroke group was also divided into two subgroups for further analysis. The results revealed that the genotypic distribution of the G allele of rs4944832 and the dominant model (GG *vs*. GA+AA) of rs4944832 significantly differed between SAO stroke patients and control subjects. Logistic regression analyses showed that SAO stroke patients had a significantly different GG genotype distribution of rs4944832 compared with control subjects. This difference illustrates that the rs4944832 SNP is most likely associated with SAO stroke in the northern Han Chinese population.

Haplotype-based analyses have several well-known advantages over those based on individual SNPs (Morris et al., 2002). In this haplotype-based case-control study, we established *P2RY2* haplotypes from different combinations of the selected SNPs (rs4944831, rs1783596, and rs4944832). The haplotype analysis showed that the overall haplotype distri-

Table 4 Multivariable logistic regression analysis of the small-artery occlusion stroke subtype

Variable	*B*	*OR* (95% *CI*)	*P* value
Hypertension	1.12	3.05 (2.05–4.54)	< 0.01
Smoking	1.32	3.72 (2.06–6.75)	< 0.01
Alcohol consumption	0.29	1.34 (0.69–2.62)	0.39
FPG	0.34	1.41 (1.23–1.61)	< 0.01
Genotype (GG)	0.59	1.81 (1.22–2.69)	< 0.01

ORs and 95% *CIs* were calculated based on multivariable logistic regression. Dependent variable: whether SAO occured. The GG genotype of rs4944832 was significantly associated with an increased risk of small-artery occlusion adjusted for history of hypertension, smoking, alcohol consumption and FPG. FPG: fasting plasma glucose; *OR*: odds ratio; *CI*: confidence interval; SAO: small-artery occlusion.

Table 5 An rs4944831-rs1783596-rs4944832 haplotype analysis [*n* (%)] of the *P2RY2* gene in ischemic stroke patients and controls

Haplotype	Controls (*n* = 344)	Ischemic stroke (*n* = 378)	*OR* (95% *CI*)	*P* value
G-C-A	64.4 (9.5)	52.2 (6.9)	0.71 (0.49–1.04)	0.08
G-C-G	23.0 (3.3)	43.4 (5.7)	1.77 (1.06–2.97)	< 0.05
G-T-G	118.5 (17.2)	111.5 (14.8)	0.84 (0.63–1.11)	0.22
T-C-A	126.7 (18.4)	109.1 (14.4)	0.75 (0.57–1.00)	< 0.05
T-C-G	53.9 (7.8)	65.4 (8.7)	1.13 (0.77–1.64)	0.54
T-T-G	292.6 (42.5)	342.7 (45.3)	1.14 (0.92–1.40)	0.23

ORs and 95% *CIs* were calculated by SHEsis program (http:// analysis. Bio-x.cn/my analysis. php). The G-C-G haplotype constructed with rs4944831, rs1783596 and rs4944832 in patients with ischemic stroke was greater than that in controls. The T-C-A haplotype in controls was greater than that in patients with ischemic stroke. *OR*: Odds ratio; *CI*: confidence interval.

bution (rs4944831-rs1783596-rs4944832) significantly differed between ischemic stroke patients and control subjects ($P < 0.01$). Significant differences were also observed in the frequency of the G-C-G haplotype between ischemic stroke patients and control subjects. The G-C-G haplotype can be regarded as a susceptibility haplotype for ischemic stroke in northern Han Chinese individuals, because this haplotype was significantly more common among ischemic stroke patients in our study. Furthermore, the frequency of the T-C-A haplotype was lower for ischemic stroke patients than for control subjects; this was therefore regarded as a resistant haplotype for ischemic stroke in the northern Han Chinese population.

In summary, the GG genotype of *P2RY2* and the G allele of the rs4944832 SNP increase susceptibility to SAO stroke in the northern Han Chinese population. The G-C-G haplotype of the *P2RY2* gene may be a susceptibility haplotype for ischemic stroke in this population. The results of this study also suggest that the T-C-A haplotype is a resistance marker for ischemic stroke.

No previous studies have linked the *P2RY2* gene to the risk of SAO; they may be linked *via* the mechanism by which *P2RY2* increases the risk of primary hypertension. However, our study has a relatively small sample size. In the future, further studies in different ethnic groups should be carried out using larger samples to further investigate detailed genotyping and to isolate the functional mutations in the *P2RY2* gene that can influence the occurrence of ischemic stroke.

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Additional file 2: *STROBE checklist.*
Additional file 3: *Informed consent (Chinese).*
Additional file 4: *Chinese structured questionnaire of patients (Chinese).*
Additional file 5: *Open peer review report 1.*

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