Original Paper

Association of Polymorphisms of the Matrix Metalloproteinase 9 Gene with Ischaemic Stroke in a Southern Chinese Population

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Abstract

Background/Aims: Matrix metalloproteinase 9 (MMP9), a potent endopeptidase degrading extracellular matrix, plays a pivotal role in the pathogenesis of ischaemic stroke (IS). The present study was undertaken to determine the association of *MMP9* gene polymorphisms and the risk of IS in a southern Chinese population. ***Methods:*** A cohort of 1274 patients and 1258 age-matched healthy controls were genotyped to detect the four *MMP9* polymorphisms (rs17156, rs3787268, rs3918241 and rs3918242) using SNaPshot. ***Results:*** Our study demonstrated a significant difference in the genotype and allele frequencies of the *MMP9* rs3918242 polymorphism between the IS patients and the controls (P = 0.012 for the genotype and P = 0.0092 for the allele). Stratification by smoking status showed statistically significant differences in the frequency and allele of the rs3918242 polymorphism between IS patients and the controls (P = 0.0052 for the genotype and P = 0.0019 for the allele). Further stratification by IS subtypes revealed that the presence of the T allele of the *MMP9* rs3918242 polymorphism confers a higher risk of the large artery atherosclerosis subtype of IS (P = 0.017). Moreover, IS patients with the rs3918242 T allele of *MMP9* presented with increased serum MMP9 production, and this increase was more significant in smokers with IS (P = 0.022). Patients carrying the variant T allele of the *MMP9* rs3918242 polymorphism exhibited significantly higher infarct volumes than those with the major CC genotype (P = 0.036). ***Conclusion:*** Our study provides preliminary evidence that the *MMP9* rs3918242 polymorphism is linked to a

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higher risk of IS, confirming the role of MMP9 in the pathophysiology of IS, with potentially important therapeutic implications.

Introduction

Stroke is the second most common cause of death worldwide and is the leading cause of long-term disability in developing and developed countries [1]. Approximately 80% of strokes are ischaemic in origin. In China, there are more than one million people who die from stroke-related diseases every year. Multiple factors, including hypertension, diabetes, smoking, hyperlipidaemia, and hyperhomocysteinemia, are associated with a higher risk of stroke [2]. However, these risk factors can only explain a small part of the aetiology. A large body of scientific research has indicated that gene polymorphisms modulate the pathophysiological processes of IS and confer a small to moderate risk [3-5].

Matrix metalloproteinases (MMPs) are a family of proteolytic zinc-dependent enzymes whose main function is to degrade and deposit structural proteins within the extracellular matrix (ECM) in both physiological and pathological process [6]. MMPs play a pivotal role in early atherosclerosis, vascular remodelling, and development of arterial plaque rupture [7, 8]. Among MMPs, MMP9 is the most widely investigated enzyme in acute ischaemic stroke (IS), and its expression is rapidly upregulated after cerebral ischaemia [9, 10]. MMP9 is highly expressed in the vulnerable regions of atherosclerotic plaques, contributing to the weakening of the plaques' fibrous cap and to the development of acute ischaemic events resulting from plaque rupture and thrombus formation. This appeared to play a pivotal role in vascular remodelling, development of atherosclerotic lesions and arterial plaque rupture [6, 11, 12]. A very recent prospective study reported that higher serum MMP9 levels in the acute phase of IS were associated with increased risk of mortality and major disability, suggesting that serum MMP9 could be an important prognostic factor for IS [13].

The human *MMP9* gene is located on the long arm of chromosome 20 (20q11.1-13.1), and some important functional polymorphisms have been reported [14, 15]. The -1562C/T polymorphism (rs3918242) located in the *MMP9* gene promoter has been reported to be associated with higher MMP9 circulating levels [16]. The -1562C/T polymorphism of *MMP9* has been reported to play a role in the development of a number of diseases, including coronary heart disease (CHD) [17], myocardial infarction (MI) [18] and IS [19]; however, the results are inconclusive. A meta-analysis suggests that the *MMP9* -1562T allele is a risk factor for CHD [17]. Another meta-analysis performed by Juan *et al*. indicates that the *MMP9* -1562 C/T polymorphism is a risk factor associated with increased risk of MI in white ethnicity, but not in Asian population [18]. Contrarily, Wang *et al*. reported that *MMP9* -1562 C/T was significantly associated with increased risk for MI in Asian population, but not in Western population [20]. This discrepancy might result from demographic and profound ethnic differences. Moreover, the association between other *MMP9* polymorphisms and the risk of IS is insufficiently investigated. Therefore, we carried out a case-control study to determine the role of *MMP9* polymorphisms in the development of IS in a southern Chinese population.

Materials and Methods

Study population

In this case-control study, 1274 IS patients (mean age, 66.87 ± 10.60 years) were recruited from the Department of Neurology, Affiliated Hospital of Guangdong Medical University, between 2013 and 2017. All the patients were identified as having newly diagnosed IS by at least two independent neurologists, according to the clinical signs and symptoms. All patients underwent computed tomography (CT) scans and/or magnetic resonance imaging (MRI) as well as standardized blood tests. Based on the clinical manifestations and neuroimaging data, two neurologists classified all ischaemic strokes into subtypes based on the Chinese IS subclassification (CISS) system [21]. Patients with a history of transient ischemic attacks,

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peripheral vascular diseases, coronary artery diseases, autoimmune diseases, systemic inflammatory diseases, blood diseases, or malignant tumours were excluded from this study. One subject diagnosed with recurrent stroke was also excluded.

The healthy control group consisted of 1258 individuals (65.55 ± 9.11 years) who were recruited from the Health Examination Center of the Affiliated Hospital of Guangdong Medical University during the same time period, and these control subjects were comparable to the IS subjects in terms of age and race. Control subjects did not have a recent history of cerebrovascular disease or MI. The same exclusion criteria were used as above.

Smokers were patients who smoked more than 10 cigarettes per day for five years, and hypertension was defined as having a systolic pressure >130 mm Hg and a diastolic pressure > 80 mm Hg (stage 1) on more than one occasion [22] and included patients currently taking antihypertensive medications. Diabetes mellitus was defined as a fasting plasma glucose level >126 mg/dL (7.0 mmol/L) and included patients taking antidiabetic medications. Written informed consent was obtained from each participant prior to enrollment in the study. The study was approved by the Ethics Committee of Guangdong Medical University.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the EZ-10 Spin Column Whole Blood Genomic DNA Isolation Kit (Sangon Biotech®, Shanghai, China) according to the manufacturer's instructions. The DNA concentration was determined using a DNA spectrophotometer (ND-1000, NanoDrop, Wilmington, USA).

The four *MMP9* SNPs (rs17576, rs3787268, rs3918241 and rs3918242) were selected based on previous studies [19, 23, 24]. The selected *MMP9* SNPs were genotyped using the SNaPshot Multiplex Kit (Applied Biosystems Co., Ltd., Foster City, CA, USA), and the primers for PCR amplification and SNaPshot extension were designed based on the GenBank database. The primers used in the SNaPshot were as follows:
rs17576F: ACGTTGGATGTGGGGGTTATAATGTGCTGT,
rs17576R: ACGTTGGATGTGGAAGTGAATGGAAACTG,
rs3787268F: ACGTTGGATGATCCTGGGCCATAGAGGATG,
rs3787268R: ACGTTGGATGTCCTCACTCAGCCTCCCTT,
rs3918241F: ACGTTGGATGTCGTGACTGCAAAGCAGATG,
rs3918241R: ACGTTGGATGAATGAGACTGTGAGATGGAG;
rs3918242F: ACGTTGGATGCCTCCCGAGTAGCTGGTATT,
rs3918242R: ACGTTGGATGCCTGGTCAACGTAGTGAAAC. The SNaPshot reactions and PCR procedures were performed as previously described [25].

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected as soon as the diagnosis was established. Blood specimens were drawn in EDTA-containing tubes and centrifuged at low speed, and the serum aliquots were stored at -20°C. The serum MMP9 levels were determined in duplicate using the Quantikine sandwich ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Infarct volume quantification

Infarct volumes indicated by diffusion-weighted magnetic resonance imaging (DWI) were measured with MIPAV software (Medical Image-Processing, Analysis, and Visualization, version 3.0; NIH, Bethesda, MD) [26]. Acute diffusion lesions were defined on a slice-by-slice basis using a semiautomatic segmentation approach, consulting apparent diffusion coefficient and fluid-attenuated inversion recovery imaging sequences to distinguish acute from non-acute diffusion change. DWI infarct volumes were calculated by multiplying the slice thickness by the total lesion area.

Statistical Analyses

All statistical tests were performed using the SPSS 19.0 software program (IBM, Armonk, NY, USA). The Hardy-Weinberg equilibrium of the SNPs was examined using a chi-squared test to compare the observed genotype frequencies with the expected frequencies among the control subjects. The baseline characteristics are expressed as the means ± standard deviation (SD) for the continuous variables, and as the medians and percentage for the quantitative variables. A chi-squared test and Student's t-test were used to compare the variables between the two groups.

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The allele and genotype frequency differences between the patients and healthy controls were assessed using the χ2 test or Fisher's exact test. The odds ratio (OR) and 95% confidence interval (CI) were calculated to assess the correlation between the *MMP9* genotype and IS. The comparisons of the serum MMP9 levels among the different *MMP9* polymorphisms between the patients and controls were evaluated using Student's t-test for the normally distributed data or a Mann-Whitney-U test for the non-parametric data. Correlations between the genotypes of the *MMP9* polymorphisms and the DWI infarct volume were assessed using analysis of variance (ANOVA). Association between the polymorphism and the risk of IS was evaluated using logistic regression analysis and was adjusted by age, gender, smoking, hypertension, diabetes mellitus and hyperlipidaemia. Benjamini-Hochberg (BH) multiple testing correction was applied for multiple comparisons with control type 1 error. The criterion for significance was set at P < 0.05 for all of the tests.

Results

Demographic characteristics

The demographic characteristics of all of the participants in the study are presented in Table 1. Of the 2532 participants, 1274 were patients with IS and 1258 were healthy controls. There were no significant differences between the patients and controls in terms of age. The mean age was 66.87 years old (±10.60 years) for the IS subjects and 65.55 years old (±9.11 years) for the control subjects. Significant differences were also found in risk factors, such as gender, smoking status, hypertension, diabetes and hyperlipidaemia. The triglycerides and homocysteine levels tended to be higher in the IS patients than in controls, whereas the high-density lipoprotein levels were lower at admission in the IS patients. The total cholesterol and low-density lipoprotein levels in the IS patients were not significantly different from those noted in the healthy control subjects.

MMP9 gene polymorphisms and the risk of IS

The genotype and allele frequencies of the *MMP9* polymorphisms are shown in Table 2. No deviation from the Hardy-Weinberg equilibrium for the polymorphisms examined was observed in the genotype distributions of the IS patients and controls (data not shown).

The comparison of genotype distributions between the IS patients and control subjects with the χ2-test revealed that there was a statistical association (P = 0.012) between the rs3918242 polymorphism of *MMP9* and the risk of IS. In a dominant model (CT+TT versus CC), a significant difference was detected between the IS group and controls (OR = 1.30, 95% CI: 1.07–1.59, P = 0.012). In a recessive model (TT versus CC+CT), a significant difference was observed in the IS group compared with the controls (OR = 2.07, 95% CI: 1.11–3.85, P = 0.019). The frequency of the T allele at the rs3918242 polymorphism was significantly different in the IS group compared with the controls (OR = 1.32, 95% CI: 1.11–1.59, P=0.0092), after BH correction. However, we failed to find any statistical association between other polymorphisms and the risk of IS.

Haplotype analysis

Four haplotypes in which the frequency of the haplotype was greater than 5% in

Table 1. Characteristics of ischemic stroke cases and controls. Continuous data are presented as the mean ± SD, median (range) or n (%) [a] P < 0.05 is indicated in bold font

Variables	IS (n=1274)	Control (n=1258)	P value
Mean age (years)	66.87±10.60	65.55±9.11	0.22
Male/female	890/384	823/435	0.017
Smokers, n (%)	620	382	< 0.001
Hypertension, n (%)	783	528	< 0.001
Diabetes, n (%)	257	125	< 0.001
Hyperlipidaemia, n (%)	194	152	0.0003
Total cholesterol (mg/dL)	4.94±1.16	5.01±1.06	< 0.001
Triglycerides (mmol/L)	1.64±0.98	1.40±0.81	< 0.001
HDL (mmol/L)	1.24±0.36	1.47±0.39	< 0.001
LDL (mmol/L)	3.01±0.98	3.04±0.93	0.095
HCY (mmol/L)	13.52±5.67	10.84±3.05	< 0.001

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Table 2. Genotype and allele frequencies of MMP9 polymorphisms between IS patients and controls, and corresponding ORs for IS. Adjusted for age, gender, smoking, hypertension, diabetes mellitus and hyperlipidaemia. *False discovery rate-adjusted P value for multiple hypotheses testing using the Benjamini-Hochberg method. P < 0.05 is indicated in bold font

Genotype & Allele	IS patients n=1274 (%)	Controls n=1258 (%)	AOR (95%CI)	P value
rs17576				
GG	751(58.9)	730(58.0)		0.89
GA	457(35.9)	461(36.7)		
AA	66(5.2)	67(5.3)		
Dominant model GG vs. GA/AA	523(41.1)	528(42.0)	0.96(0.82-1.13)	0.64
Recessive model GG/GA vs. AA	1208(94.8)	1191(94.7)	0.97(0.68-1.38)	0.86
G allele	1959(76.9)	1921(76.4)	1.000	
A allele	589(23.1)	595(23.6)	0.97(0.85-1.11)	0.65
rs3787268				
GG	437(34.3)	431(34.3)		0.80
GA	630(49.5)	634(50.4)		
AA	207(16.2)	193(15.3)		
Dominant model GG vs. GA/AA	837(65.7)	827(65.7)	1.00(0.85-1.18)	1.00
Recessive model GG/GA vs. AA	1067(83.8)	1065(84.7)	1.07(0.86-1.33)	0.53
G allele	1504(59.0)	1496(59.5)	1.000	
A allele	1044(41.0)	1020(40.5)	1.02(0.91-1.14)	0.75
rs3918241				
TT	986(77.4)	988(78.5)		0.42
TA	269(21.1)	258(20.5)		
AA	19(1.5)	12(1.0)		
Dominant model TT vs. TA/AA	288(22.6)	270(21.5)	1.07(0.89-1.29)	0.49
Recessive model TT/TA vs. AA	1255(98.5)	1246(99.0)	1.57(0.76-3.25)	0.22
T allele	2241(88.0)	2236(88.8)	1.000	
A allele	307(12.0)	282(11.2)	1.09(0.92-1.29)	0.35
rs3918242				
CC	1002(78.7)	1041(82.8)		0.012*
CT	241(18.9)	202(16.0)		
TT	31(2.4)	15(1.2)		
Dominant model CC vs. CT/TT	272(19.2)	217(17.2)	1.30(1.07-1.59)	0.012*
Recessive model CC/CT vs. TT	1243(97.6)	1243(98.8)	2.07(1.11-3.85)	0.019*
C allele	2245(88.1)	2284(90.8)	1.000	
T allele	303(11.9)	232(9.2)	1.32(1.11-1.59)	0.0092*

IS patients and controls were included in the haplotype analysis. However, no significant associations were observed between these haplotypes and IS (Table 3).

The association of MMP9 rs3918242 gene polymorphism with demographic characteristics

Table 3. The frequencies of haplotypes of MMP9 gene in patients and controls. [a] adjusted for age, gender, smoking, hypertension, diabetes mellitus and hyperlipidaemia. All those frequency<0.05 will be ignored in analysis

Haplotypes	Case (freq)	Control (freq)	OR (95%)	P value
	MMP9 (rs17576, rs3787268, rs3918241, rs3918242)			
A-G-T-C	459.01(0.182)	527.84(0.212)	0.871(0.756-1.003)	0.0557
G-A-T-C	919.93(0.365)	888.53(0.356)	1.120(0.993-1.262)	0.0645
G-G-A-C	242.41(0.096)	239.73(0.096)	1.050(0.869-1.268)	0.615
G-G-T-C	554.29(0.220)	590.56(0.237)	0.959(0.838-1.097)	0.539

The associations of *MMP9* rs3918242 gene polymorphisms with demographic characteristics are shown in Table 4. In an analysis stratified by smoking status, an increased risk of IS was associated with the variant genotypes CT and TT at the rs3918242 polymorphism in smoking patients (P = 0.0052 for the genotype, and P = 0.0019 for the allele). However, no significant interaction was found between *MMP9* rs3918242 polymorphism and age, gender, diabetes and hypertension in conferring the risk of IS.

Associations between MMP9 rs3918242 gene polymorphism and stroke subtypes

To explore whether the effects of *MMP9* rs3918242 gene polymorphism are confined to a specific subtype or related to overall risk, we further separated the IS patient groups into stroke subgroups based on the CISS system [21]. As shown in Table 5, when the population was stratified according to the CISS classification system, the carriers of the T allele at the

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Table 4. A comparison between the baseline characteristics of the MMP9 rs3918242 genotypes and alleles in the IS patient and control groups. P_G: P value of the difference in alleles between the case and control groups; P_A: P value of the difference in genotype between the case and control groups [a] adjusted for age, gender, smoking, hypertension, diabetes mellitus and hyperlipidaemia. *False discovery rate-adjusted P value for multiple hypotheses testing using the Benjamini-Hochberg method. [b] P < 0.05 is indicated in bold font

Characteristics	IS patient group					Control group					P_G[a] value	P_A[a] value
	Genotype n (%)			Allele n (%)		Genotype n (%)			Allele n (%)			
	CC	CT	TT	C	T	CC	CT	TT	C	T		
Age												
≥65 years	509(78.3)	121(18.6)	20(3.1)	1139(87.6)	161 (12.4)	525 (81.8)	108(16.8)	9 (1.4)	1158(90.2)	126(9.8)	0.076	0.039
<65 years	493(79.0)	120(19.2)	11(1.8)	1106(88.6)	142(11.4)	516(83.8)	94(15.2)	6(1.0)	1126(91.4)	106(8.6)	0.075	0.023
Gender												
Male	711(79.9)	156(17.5)	23(2.6)	1578(88.7)	202(11.3)	684(83.1)	128(15.6)	11(1.3)	1496(90.9)	150(9.1)	0.087	0.032
Female	291(75.8)	85(22.1)	8(2.1)	667(86.8)	101(13.2)	357(82.1)	74(17.0)	4(0.9)	788(90.6)	82(9.4)	0.058	0.023
Smoking												
Yes	469(75.6)	129(20.8)	22(3.5)	1067(83.1)	173(16.9)	316(82.7)	61(16.0)	5(1.3)	693(90.7)	71(9.3)	0.0052*	0.0019*
No	533(81.5)	112(17.1)	9(1.4)	1178(90.1)	130(9.9)	725(82.8)	141(16.1)	10(1.1)	1591 (90.8)	161(9.2)	0.77	0.49
Diabetes												
Yes	191(74.3)	58(22.6)	8(3.1)	440(85.6)	74(14.4)	105(84.0)	18(14.4)	2(1.6)	228(91.2)	22(8.8)	0.11	0.036
No	811(79.7)	183(18.0)	23(2.3)	1805(88.7)	229(11.3)	936(82.6)	184(16.2)	13(1.2)	2056(90.7)	210(9.3)	0.068	0.034
Hypertension												
Yes	595(76.0)	165(21.1)	23(2.9)	1355(86.5)	211(13.5)	421(79.7)	100(18.9)	7(1.4)	942(89.2)	114(10.8)	0.092	0.046
No	407(82.9)	76(15.5)	8(1.6)	890(90.6)	92(9.4)	620(84.9)	102(14.0)	8(1.1)	1342(91.9)	118(8.1)	0.53	0.27

Table 5. The relationship between MMP9 genotypes and IS stratified by CISS classification in IS patients LAA: Large-artery atherosclerosis; PAD: Penetrating artery disease; CS: Cardioembolic Stroke; UE: Undetermined aetiology [a] adjusted for age, gender, smoking, hypertension, diabetes mellitus and hyperlipidaemia. *False discovery rate-adjusted P value for multiple hypotheses testing using the Benjamini-Hochberg method

Value	MMP9 rs3918242						
	Genotype				Allele		
	CC	CT	TT	P value[a]	C	T	P value[a]
Controls	1041 (82.8)	202(16.0)	15 (1.2)		2284(90.8)	232(9.2)	
Cases							
LAA (n=887)	692(78.6)	173 (18.9)	22 (2.5)	0.028*	1557(87.8)	217(12.2)	0.0068*
PAD (n=266)	219 (80.5)	40(16.9)	7 (2.6)	0.264	478(89.8)	54(10.1)	0.51
CS (n=54)	40 (74.0)	13 (24.1)	1 (1.9)	0.264	93(86.1)	15(13.9)	0.227
UE (n=67)	51 (76.1)	15 (22.4)	1 (1.5)	0.272	117(87.3)	17(12.7)	0.227

MMP9 rs3918242 gene polymorphism appeared to have a higher risk of stroke of the large artery atherosclerosis (LAA) subtype compared with the controls (P = 0.0068). No statistical associations were observed between the *MMP9* rs3918242 gene polymorphism and other stroke subtypes in the healthy controls.

The serum levels of MMP9 based on the MMP9 rs3918242 polymorphisms

The serum levels of MMP9 measured in 124 IS patients and 120 controls are presented in Fig. 1. Generally, the serum MMP9 levels were significantly higher in the IS patients than in the controls (P<0.05) (Fig. 1). Moreover, when the samples were stratified according to the *MMP9* rs3918242 genotypes, the serum MMP9 levels were significantly higher in patients with the CT and TT genotypes than in those with the CC genotypes at the rs3918242 polymorphism (P = 0.031) (Fig. 1A). However, in the healthy controls, no significant differences in serum MMP9 levels were detected among the controls with different genotypes (Fig. 1A). We also determined the serum MMP9 levels in the IS patients and controls who were stratified according to smoking status. A significant increase in the serum MMP9 levels was found in the smoking patients (P = 0.022).

Association of the MMP9 rs3918242 polymorphisms with the infarct volume

Associations of the *MMP9* rs3918242 polymorphisms with the actual volumetric measurements of infarct volume by DWI in 115 IS patients were explored, and the results
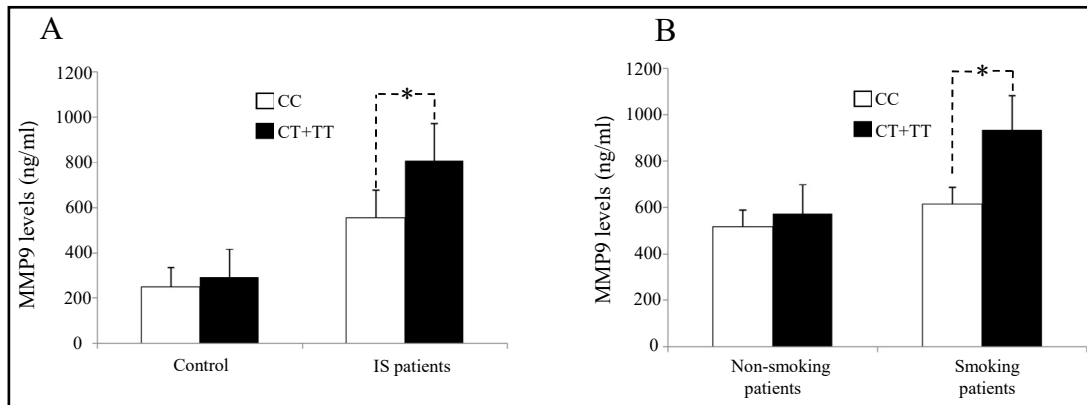
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Fig. 1. (A) The serum MMP9 levels in the IS patients (n=124) and the controls (n=120) stratified based on the MMP9 rs3918242 genotypes. The blank box represents the rs3918242 CC genotype (n=98 for the IS patients and n=99 for the controls) and the black box represents the rs3918242 CT + TT genotype (n=26 for the IS patients and n=21 for the controls).*P = 0.031 when comparing the serum MMP9 levels in IS patients between the CC genotype and the CT + TT genotype. (B) The serum MMP9 levels in the IS patients stratified according to smoking status and the rs3918242 genotype. The blank box represents the rs3918242 CC genotype (n=45 for the smoking patients and n=52 for the non-smoking patients) and the black box represents the rs3918242 CT + TT genotype (n=15 for the smoking patients and n=12 for the non-smoking patients). *P = 0.022. The serum MMP9 levels in the IS patients and the healthy individuals were measured using ELISA. The data are shown as the mean ± SD. An asterisk indicates P<0.05.
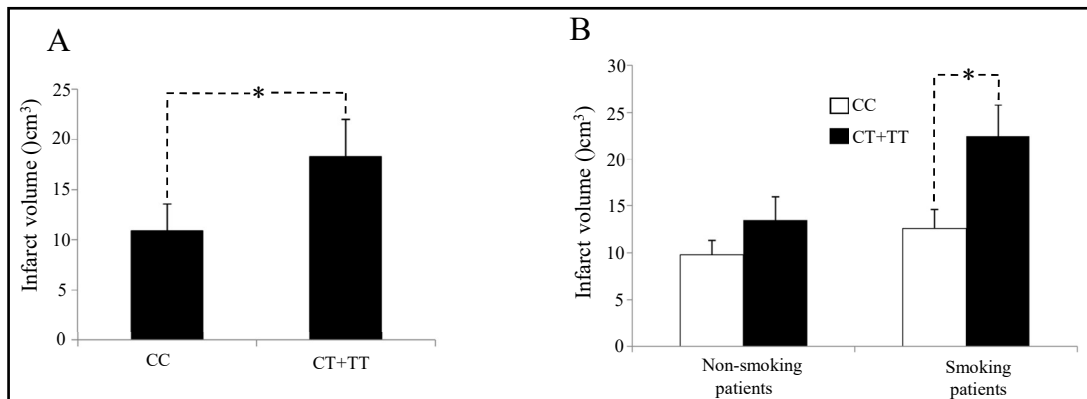

Fig. 2. (A)The mean infarct volumes ±SD in the IS patients stratified based on the MMP9 rs3918242 genotypes (n=91 for the patients with CC genotypes and n=24 for the patients with CT + TT genotypes).*P = 0.036. (B) The mean infarct volumes ±SD in the IS patients stratified according to smoking status and the presence of the mutated allele. The blank box represents the rs3918242 CC genotype (n=42 for the smoking patients and n=49 for the non-smoking patients) and the black box represents the rs3918242 CT + TT genotype (n=14 for the smoking patients and n=10 for the non-smoking patients). *P = 0.027.The data are shown as the mean ± SD. Correlations between the genotypes of the MMP9 rs3918242 polymorphism and the DWI infarct volume were assessed using analysis of variance (ANOVA). An asterisk indicates P<0.05.

are shown in Fig. 2. The mean infarct volumes in the IS patients with the *MMP9* rs3918242 genotypes (CT + TT) were significantly higher than those in patients with the major CC genotype (P = 0.036) (Fig. 2A). When the IS patients were stratified by smoking status, a significant increase in the infarct volumes was found in the smoking patients with the variant rs3918242 genotypes (CT + TT) (P = 0.027) (Fig. 2B).

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Discussion

In this hospital-based case-control study, we investigated the association of four important polymorphisms of the *MMP9* gene, including rs17576, rs3787268, rs3918241 and rs3918242, with the risk of IS in a relatively large southern Chinese population, and we observed a significant association between the *MMP9* rs3918242 polymorphism and the risk of IS. The presence of the *MMP9* rs3918242 T allele conferred a higher risk of developing IS, which may hav e resulted from the interaction with smoking. Further stratification revealed that the variant T allele of the *MMP9* rs3918242 polymorphism was associated with a higher risk of IS of the LAA subtype. The serum MMP9 levels were significantly higher in IS patients with the variant CT and TT alleles of the *MMP9* rs3918242 polymorphism than in those carrying the major CC genotype. Additionally, patients with the variant genotypes (CT + TT) of the *MMP9* polymorphism had higher infarct volumes than those carrying the major CC genotype.

MMPs regulate many aspects of cellular activity, with functions ranging from ECM degradation, cell proliferation, adhesion, and migration to release of ECM-sequestered molecules by proteolysis, shedding of cell-surface proteins that transduce signals from the ECM [27], and activation of pro-inflammatory cytokines [28]. MMP9 has been documented to play critical roles in blood–brain barrier integrity, tissue repair and remodelling following stroke [29]. Following stroke, increased levels of MMP9 have been detected in both peripheral and central cells, including neurons, glia, endothelial cells and neutrophils [30, 31]. Animal studies indicated that increased expression of pro/active MMP9 was detected within hours to days following stroke in non-human primates [32], rats [33], and mice [34]. Clinical studies also demonstrated that MMP9 is elevated in the serum of stroke patients and is correlated with a worsened outcome [35, 36]. These lines of evidence have led us to formulate the hypothesis that MMP9 could be of pathogenic importance in IS.

Several previous studies have examined the role of *MMP9* genetic polymorphisms and the susceptibility to stroke, but the results are inconclusive. Buraczynska *et al.* [19] and Zhang *et al.* [37] indicated that *MMP9* -1562 C/T polymorphism was significantly associated with risk of stroke in the population studied. Kaplan *et al.* [38] suggested that *MMP9* haplotypes or SNPs were not associated with myocardial infarction or stroke. Pollanen et al. confirmed the correlation between the -1562C/T polymorphism of the *MMP9* gene and the instability and rupture of atherosclerotic plaques [39]. In our present study, we found that the variant T allele of *MMP9* rs3918242 was associated with the risk of developing IS in a Chinese population. Carriers of the *MMP9* rs3918242 T allele showed a significantly greater prevalence of IS than subjects with the CC genotype. Further large-scale studies are needed to verify the results of our findings.

The pathogenesis of IS is the result of interactions between genetic predispositions and environmental factors. As an independent modifiable risk factor for cardiovascular disease, cigarette smoke doubles the risk of stroke [40]. Smoking is estimated to be responsible for 12% to 37% of all stroke events. Individuals who smoke approximately 20 cigarettes per day are six times more likely to have a stroke than non-smokers [41]. Smoking increases the risk for vascular disease by induction of a procoagulant state and acceleration of atherosclerosis [41]. In our present study, when the *MMP9* rs3918242 genotype and allele frequency were further stratified by age, gender, smoking status, diabetes, and hypertension, an increased risk of IS was found in smoking subgroups of the IS patients compared to the controls. Therefore, it is highly speculated that environmental risk factors and smoking may interplay with the *MMP9* genetic predisposition in the present study.

The upregulation of MMP9 was revealed to be involved in the development and progression of a number of vascular diseases [13, 36, 42]. The -1562C/T polymorphism in the promoter region of the *MMP9* gene abolishes the DNA-protein interaction, resulting in higher activity of the T-allelic promoter [14]. Thus, the -1562C/T polymorphism may affect the expression of MMP9 and contribute to the occurrence of IS. In the present study, we found that individuals carrying the mutated rs3918242 T allele expressed higher MMP9

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levels when compared with populations carrying the CC genotype. Given the key role of MMP9 in vascular damage, it is conceivable that in the individuals with the rs3918242 T allele, increased serum MMP9 levels may result in degradation of a number of components of the extracellular matrix, which may cause subsequent vascular remodelling. Our findings also indicated a significant increase in the serum MMP9 levels in smoking patients with the variant rs3918242 T allele. Cigarette smoke extract exposure induced increased MMP2 and MMP9 both *in vitro* and *in vivo* [43, 44]. Snitker *et al.* reported that MMP9 level in the blood was significantly higher in current smokers compared to never and former smokers [45]. Wang *et al.* described that a smoker with -1562C/T or TT genotype was associated with a 1.31-fold risk of MI compared with a nonsmoker with -1562CC genotype, and the multiple logistic regression analysis showed that the interaction between smoking and the -1562 T allele resulted in a significant 4.42-fold increased risk of MI [46]. From the observation of this evidence and our findings, it is highly likely that smokers with the rs3918242 T allele will be more susceptible to increased MMP9 release, thereby contributing to the development of IS.

Accumulating evidence confirmed that the plasma level of MMP9 was related to neurological worsening, infarct volumes, and haemorrhagic transformation [47, 48].In animal studies, it was shown that plasma MMP9 release increased the size of the brain infarct area [49]. In a study related to IS, a strong correlation was detected between plasma MMP9 level and size of infarct area in patients treated with t-PA [50]. Montaner *et al.* confirmed a positive correlation between plasma MMP9 level and infarct volume [51]. In the present study, we found that patients carrying the variant genotypes (CT and TT) of the rs3918242 polymorphism have higher infarct volumes than those carrying the major CC genotype. In addition, we confirmed that individuals with the variant genotypes (CT and TT) of the rs3918242 polymorphism had significantly higher infarct volumes than those carrying the major CC genotype in smoking patients. Considering the effects of increased MMP9 expression on IS development, it is conceivable that smokers with T allele of *MMP9* rs3918242 may have increased MMP9 expression, thereby contributing to higher infarct volumes.

This case–control study had some limitations that should be accounted for when interpreting the results. First, potential bias, including information bias, selection bias and confounding bias, cannot be entirely excluded. Specifically, control subjects were determined free of IS by medical history, or a lack of examination by CT or MRI. Without confirmation of imaging examinations, some control subjects may have been affected by silent stroke, which may reduce the statistical power. Second, some other functional polymorphisms may influence the expression of MMP9 and contribute to the development of IS, and their combined effects must be studied to better predict the occurrence, severity, and outcome of IS. Third, the other risk factors in the study group, such as age, gender, smoking, hypertension, diabetes or hyperlipidaemia, may have complicated the association between *MMP9* polymorphisms and IS. Larger prospective studies are necessary to fully elucidate the role of these polymorphisms in IS.

Conclusion

In conclusion, our findings support the existence of an association between the *MMP9* rs3918242 polymorphism and the risk of developing IS in a southern Chinese population. In particular, smokers carrying the rs3918242 T allele of *MMP9*, which is associated with increased MMP9 levels, may run a higher risk of developing IS. Our study may provide clues for the evaluation of individual susceptibility to IS and for the development of effective measures to control and prevent IS.

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Disclosure Statement

The authors have no actual or potential conflicts of interest related to this manuscript. Appropriate approval was obtained, and appropriate procedures were followed concerning human subjects.

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