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Original Article

Association between HMGB1 genetic variants and ischemic stroke susceptibility, onset age, and recurrence risk among Chinese Han individuals

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Abstract: Objectives: Ischemic stroke has long been a global health threat. Genetic factors, a looming risk for ischemic stroke, remain unexplored. The high-mobility group box 1 (HMGB1) protein showed a connection with the occurrence and development of ischemic stroke. This study was conducted to find whether frequent *HMGB1* polymorphisms (rs1045411, rs1412125, and rs2249825) play a role in ischemic stroke susceptibility and recurrence risk. Methods: Our study was carried out in a Chinese Han population with a sample size of 871 patients and 858 age-matched healthy controls. Tag single nucleotide polymorphisms (tagSNPs) were selected by conventional protocols and DNA was extracted for genotype analysis after the participants had signed an informed consent. Comprehensive statistical analyses were conducted. Results: It was found that the C allele of the *HMGB1* rs1412125 (OR = 1.263, 95% CI = 1.075-1.483, P = 0.004) and *HMGB1* rs2249825 (adjusted OR = 2.464, 95% CI = 1.215-4.996, P = 0.012) variants was associated with a high risk of ischemic stroke, with the male subgroup carrying the TT allele of the *HMGB1* rs1045411 variant tended to suffer more from the disease (adjusted OR = 3.600, 95% CI = 1.272-10.193, P = 0.016). A haplotype study also showed significant results (OR = 1.554, 95% CI = 1.246-1.938, P = 0.001). The rs1412125 polymorphism was highly associated with the chance of recurrence but not with the onset age (TC vs. TT: P = 0.034; CC vs. TT: P < 0.001). Cox regression analysis and stratified analysis were carried out with notable conclusions. Conclusions: Our study provided evidence for the association between *HMGB1* polymorphisms and ischemic stroke susceptibility and recurrence, indicating that *HMGB1* gene variants may be potential markers for first and secondary stroke prevention.

Keywords: Single nucleotide polymorphism, high-mobility group box 1, ischemic stroke, ischemic stroke recurrence, onset age

Introduction

Ischemic stroke is a major health threat and one of the leading causes of long-term disability and mortality among adults worldwide [1]. Globally, the ischemic stroke burden is increasing as a result of population aging, and the American Heart Association projects that stroke-related medical costs will reach $183 billion by 2030 [2]. Due to rapid advances in medical technology, stroke mortality has decreased, and most patients can survive after the first stroke. However, there is a high risk of recurrent stroke for those who survive an ischemic stroke, which can be costly, disabling, and potentially fatal [3]. For this reason, it is urgent to identify novel biomarkers that can be used to determine if stroke patients are at imminent risk of recurrence.

It is well known that conventional risk factors such as hypertension, diabetes, and smoking may influence the susceptibility and prognosis of ischemic stroke [4]. However, a great deal remains unexplored. Genetic factors, as evidenced by case-control, twin, and family-based studies, may contribute to ischemic stroke development [5]. As part of the pathophysiology of ischemic stroke, inflammation is an essential component [6, 7]. It has been shown that the

HMGB1 protein plays a key role in enhancing inflammation and tissue damage in cerebrovascular diseases such as ischemic stroke [6, 8, 9]. Further, HMGB1 is involved in the immune response that leads to ischemic stroke, and anti-inflammatory interventions may improve stroke outcomes in experimental stroke models [10-12]. As a result, genetic variants linked to *HMGB1* transcription, regulation, or splicing are studied to determine their relationship to the susceptibility and recurrence of ischemic stroke.

In fact, according to Li et al, ischemic stroke risk was reduced by the G allele of *HMGB1* rs2249825 [13], and a significant difference was also found between those carrying the G allele of the *HMGB1* rs2249825 variant and those bearing the major CC genotype. However, since this study was a retrospective case-control study, we cannot exclude the presence of potential bias, such as information bias. Additionally, it only included the southern Chinese Han population, and thus, did cover the entire Chinese Han population. Despite this, other variants in the *HMGB1* gene (rs1412125 and rs1045411) did not show a statistically significant association with ischemic stroke risk. Noticeably, the results came to different conclusions in a very recent study in Caucasians. In this study, genotype data for *HMGB1* polymorphisms and haplotypes (rs1360485, rs1045411, rs3742305, rs2249825, rs1412125) were compared between ischemic stroke cases and controls [14], but genotype variations in *HMGB1* were not associated with the overall risk of ischemic stroke or specific stroke subtypes. It is possible that the differences in ethnicity among the study populations may account for the inconsistent results between this and our study. Therefore, relational investigations should be applied for results confirmation.

Considering the limitations in the previous studies, this study designed a follow-up period to examine whether frequent *HMGB1* polymorphisms (rs1045411, rs1412125, and rs2249825) play a role in ischemic stroke susceptibility and recurrence risk in a northern Chinese Han population in light of the increasing interest in genotype variations as potential biomarkers.

Materials and method

Study subjects

A total of 871 patients and 858 age-matched healthy controls were included in our study. In the period between December 2013 and December 2015, patient data were collected at the First Affiliated Hospital of China Medical University. We applied the same rules to the patients and controls as described in our previous publications [15, 16].

Inclusion criteria: (1) The participants were of Han nationality aged 40-80 years and lived in the Liaoning province in northern China; (2) Patients who were diagnosed for the first time with an acute ischemic stroke based on brain imaging of the corresponding infarction (The diagnosis of an acute ischemic stroke follows the standard criteria including acute onset, focal nerve function defects and imaging lesions or symptoms/signs lasting more than 24 hours according to the guideline [17]); (3) Controls showed no evidence of stroke or other neurological diseases.

Exclusion criteria: Those who had a transient ischemic attack, cardio-embolism, cerebral trauma, cerebral vascular malformations, coagulopathy dysfunction, autoimmune diseases, tumors, kidney diseases, blood diseases, or chronic infectious diseases.

Subsequent follow-ups were conducted every three months until an ischemic stroke recurred, while subjects who died from accidents or other diseases, lost contact out of any reason or due to unexpected events until the end of the follow-up period (December 2018) were excluded from the study. The primary outcome was to observe the recurrence of ischemic stroke during the follow-up period, and the recurrence time was considered to be the secondary outcome. Once diagnosed, the patients were treated during the acute phase with platelet therapy using anti-aspirin and clopidogrel, as well as with lipid-lowering therapy using statins. Following discharge, the patients were asked to continue the medication and during the subsequent follow-ups, they were reminded of their prescriptions. Finally, 784 of the 871 patients with ischemic stroke completed their follow-up with an average time of 44.5 months, while 87 (10.0%) were lost to follow-up, and

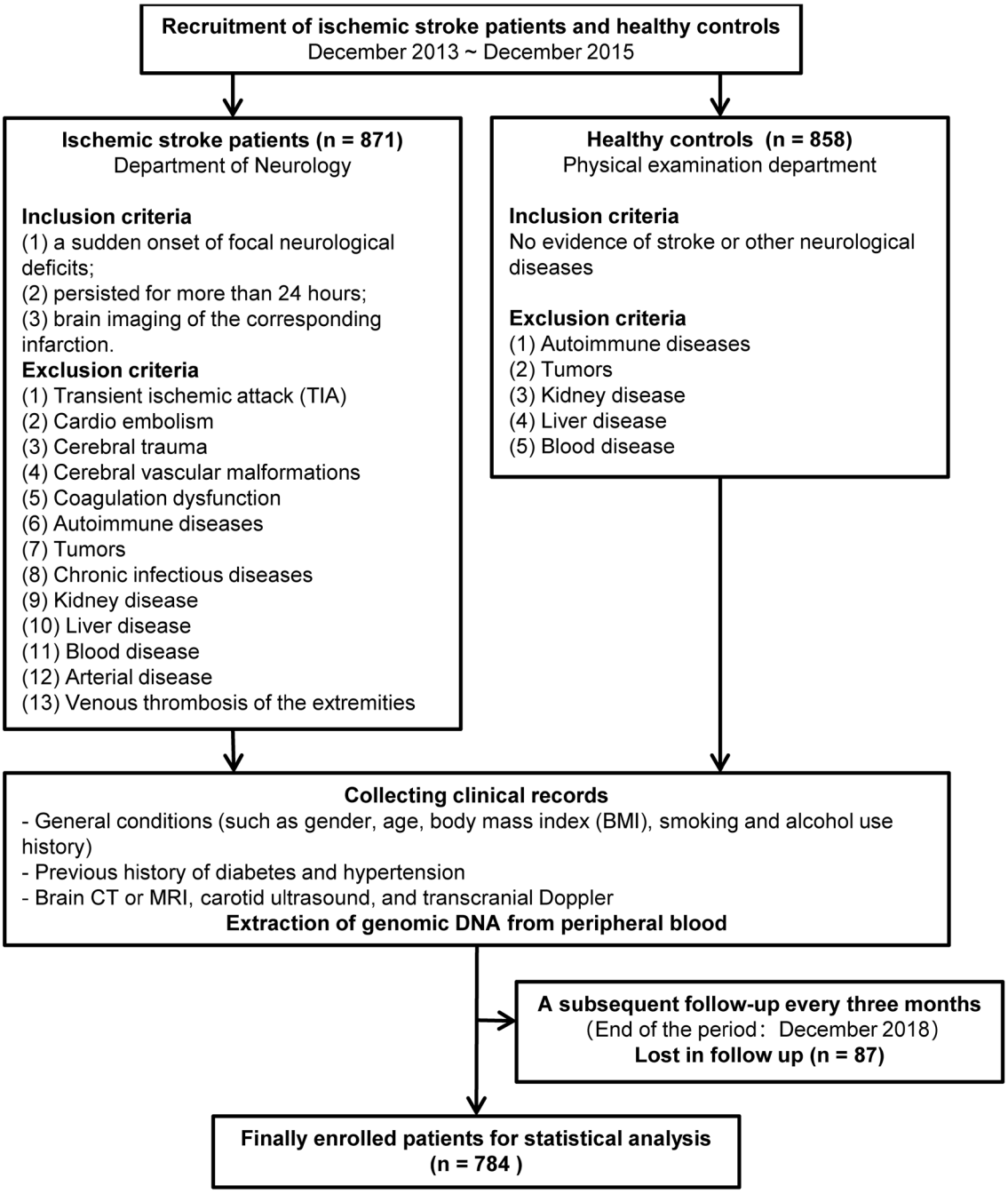

Figure 1. Flowchart of this study.

208 suffered a recurrence. Informed consent was obtained from each participant in the study. On February 20, 2012, the Institutional Ethical Committee of the First Affiliated Hospital of China Medical University approved this study (No. 2012-38-1) in accordance with the World Medical Association's Code of Ethics (Declaration of Helsinki). A protocol for the conduct of this study has been submitted to the Chinese Clinical Trials Registry (registration number: ChiCTR-COC-17013559). The flow chart shown in **Figure 1** illustrates the process.

Tag SNPs selection

HMGB1 (Gene ID: 3146) is located on chromosome 13 q12, with a length of 9006 base pairs.

Table 1. Characteristics of the SNPs selected for the study

SNP ID	Genomic Location (Chr.13)	Test for HWE (*P* Value)	MAF in Hapmap-CHB (minor allele)	Molecular consequences	Function prediction
rs1412125	31041595	0.266	0.241 (C)	5'-Upstream	Enhancer
rs2249825	31037903	0.382	0.147 (C)	intron 1	Transcriptional factor binding site
rs1045411	31033233	0.381	0.183 (T)	3'-UTR	miRNA binding site

Abbreviations: SNP, single nucleotide polymorphism; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency.

For identifying single nucleotide polymorphisms, the phase II database of the International HapMap Project of Chinese population (http://www.hapmap.org/) was used. We considered all polymorphisms occurring in the gene region to be candidate SNPs. This included those occurring 5 kb upstream of the first exon and 5 kb downstream of the last exon. According to the linkage disequilibrium (LD) values, three tagSNPs with minor allele frequency (MAF) > 0.05 and pairwise linkage disequilibrium squared correlation coefficient (r^2) > 0.8 were selected. We also employed a functional candidate strategy to identify functional SNPs that are involved in transcription, regulation, or splicing. For this, we used a selection tool for SNP that is available online (FASTSNP, http://fastsnp.ibms.sinica.edu.tw). To enforce the implementation of tagSNPs, it was necessary to rerun SNPs with predictive biological effects. A final set of tagSNPs in *HMGB1* gene was selected, including rs1045411, rs1412125, and rs2249825. The biological information analysis for regions, alleles, MAFs, and predicted functions of the three SNPs is shown in **Table 1**.

Genotyping

A commercially available DNA purification kit (Promega, Madison, WI, USA) was used to extract genomic DNA from peripheral blood, and the SNaPshot reaction was performed as previously described [15, 16]. Genotyping analysis was conducted using the SNaPshot Multiplex kit (Applied Biosystems Co., Ltd., Foster City, CA, USA). ABI 3130XL DNA sequence detector and GeneMapper 4.0 were used to analyze the experimental results (Applied Biosystems Co., Ltd.).

Statistical analysis

Statistical analysis was carried out using the Statistical Product and Service Solutions (SPSS, v23.0), unless otherwise stated. A two-tailed test was applied, and significance was defined as a $P < 0.05$. The distribution of demographic variables was compared using the Pearson's χ^2 test, and differences between risk factors and genotypes were examined between patients and controls. A goodness-of-fit χ^2-test was used to test the Hardy-Weinberg equilibrium (HWE) for each genotype. We calculated odds ratios (ORs) and 95% confidence intervals (CIs) to estimate the association between ischemic stroke and a particular genotype based on the results of an unconditional logistic regression. Based on the observed frequencies of the three SNPs, we used the SHEsis analysis platform to calculate the linkage disequilibrium (LD) index (D' and r^2) and infer haplotype frequencies (those with frequency < 0.03 were ignored in the subsequent analysis) [18, 19]. The recurrence time was calculated from the date of diagnosis of ischemic stroke to the date of stroke recurrence or the last time a patient was followed up. A Kaplan-Meier recurrence curve was constructed, and the difference between recurrence times was assessed with a log-rank test. A Cox proportional hazards regression model with adjustments for age, gender, diabetes, hypertension, hyperlipidemia, history of drinking, and history of smoking was used to calculate the hazard ratios (HRs) and 95% CIs. The treatment methods were not included for the reason that the thrombolytic treatment rate of the patients was less than 3% until the cutoff of the collection (2013-2015), which did not affect the COX regression model analysis. Additionally, a Bonferroni adjustment was applied to correct for multiple comparisons.

Results

Clinical features of study subjects

Table 2 summarizes the essential characteristics of selected cases and controls, as well as risk factors for ischemic stroke. There were no significant differences in age (P = 0.312) and

Table 2. Characteristics and risk factors for stroke in cases and controls

Variable	Cases (%)	Controls (%)	*P* value
Age (years)	64.26±8.54	63.72±6.57	0.132
Age (≤ 60/> 60)	500 (63.8)/284 (36.2)	578 (67.4)/280 (32.6)	0.158
Gender (male/female)	460 (58.7)/324 (41.3)	454 (52.9)/404 (47.1)	0.019
BMI (kg/m^2)	23.72±2.38	23.49±1.84	0.027
BMI (≤ 22.9/> 22.9)	386 (49.2)/398 (50.8)	308 (35.9)/550 (64.1)	< 0.001
Diabetes mellitus	212 (27.0)	66 (7.7)	< 0.001
Hypertension	484 (61.7)	170 (19.8)	< 0.001
History of smoking	274 (34.9)	134 (15.6)	< 0.001
History of alcohol use	142 (18.1)	94 (11.0)	< 0.001
TG (mmol/L)	1.68±1.01	1.59±0.60	0.02
TG (≤ 1.7/> 1.7)	288 (36.7)/496 (63.3)	268 (31.2)/590 (68.8)	0.19
TC (mmol/L)	4.96±1.30	4.78±0.77	0.001
TC (≤ 5.72/> 5.72)	180 (23.0)/604 (77.0)	116 (13.5)/642 (86.5)	< 0.001
HDL (mmol/L)	1.36±0.38	1.37±0.30	0.573
HDL (> 0.91/≤ 0.91)	60 (7.7)/724 (92.3)	26 (3.0)/832 (97.0)	< 0.001
LDL (mmol/L)	2.85±0.91	2.72±0.82	0.004
LDL (≤ 3.64/> 3.64)	150 (19.1)/634 (80.9)	98 (11.4)/760 (88.6)	< 0.001
Hyperlipidemia	122 (15.6)	50 (5.8)	< 0.001
Fasting blood glucose (< 7.0/≥ 7.0)	308 (39.3)/476 (60.7)	98 (11.4)/760 (88.6)	< 0.001
Fasting glucose (mmol/L)	6.61±2.25	5.1±1.23	0.726

Abbreviations: BMI, body mass index; TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

gender (P = 0.158) between cases and controls, whereas significant differences were identified in body mass index (BMI), diabetes, hypertension, smoking history, alcohol-using history, and hyperlipidemia, which is explicit when compared to conventional risk factors. Generally, patients had higher levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL), and fasting blood glucose (FBG), but lower levels of high-density lipoprotein cholesterol (HDL).

HMGB1 polymorphisms and ischemic stroke susceptibility

In both ischemic stroke cases and controls, the genotype distributions of the three tag-SNPs were in agreement with those predicted by the HWE (*P* values were 0.266 for rs1412-125, 0.382 for rs2249825, and 0.381 for 1045411). It can be seen in **Table 3** that there was a significant difference between the frequency of the genotype CT between patients (42.6%) and controls (32.2%). Further analysis of the dominant model (adjusted OR = 1.399, 95% CI = 1.112-1.758, P = 0.004) indicated that the C allele may be associated with an elevated risk. Allele analysis indicated a significant difference for the allele C (OR = 1.263, 95% CI = 1.075-1.483, P = 0.004), suggesting that it may act as a risk factor. The results for rs2249825 are shown in **Table 4**, and are discussed below. There was an increased risk of IS among individuals carrying the wild-type CC genotype compared to individuals carrying the homozygous GG genotype (adjusted OR = 2.464, 95% CI = 1.215-4.996, P = 0.012). As a result of the recessive model, the rs2249825 CC variant was associated with a significantly increased risk of ischemic stroke compared to the CG/GG variants (adjusted OR = 2.508, 95% CI = 1.240-5.073, P = 0.011). Nevertheless, we did not find any significant association between the rs1045411 polymorphism and ischemic stroke risk in any of the models we analyzed (**Table 5**). For the rs1045411 allele and genotype frequencies, further analyses were conducted in male and female groups and significant results were found in the male group (**Table 6**), but not in the female group (**Table 7**). The TT homozygote was significantly more susceptible to stroke (adjusted OR =

Table 3. rs1412125 allele and genotype frequencies of genetic polymorphisms in cases and controls as well as their main effects on stroke risks

SNP	Cases	Percent (%)	Controls	Percent (%)	OR (95% CI)*,a	*P* value[b]
rs1412125 genotype						
TT (ref)	414	52.8	534	62.2	1.00 (ref)	
CT	334	42.6	276	32.3	1.445 (1.140-1.833)	0.002
CC	36	4.6	48	5.6	1.093 (0.640-1.866)	0.746
Dominant effect						
TT (ref)	414	52.8	534	62.2	1.00 (ref)	
CT+CC	370	47.2	324	37.8	1.399 (1.112-1.758)	0.004
Recessive effect						
CT+TT (ref)	748	95.4	610	94.4	1.00 (ref)	
CC	36	4.6	48	5.6	0.950 (0.561-1.611)	0.850
rs1412125 allele						
T (ref)	1162	74.1	1344	0.783	1.00 (ref)	
C	406	25.9	372	0.217	1.263 (1.075-1.483)	0.004

Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval. *ORs and 95% CIs were calculated by logistic regression. [a,b]Adjusted OR (95% CI) and *P* value, adjusted for age, gender, BMI, diabetes mellitus, hypertension, history of smoking, history of alcohol use, and hyperlipidemia.

Table 4. rs2249825 allele and genotype frequencies of genetic polymorphisms in cases and controls as well as their main effects on stroke risks

SNP	Cases	Percent (%)	Controls	Percent (%)	OR (95% CI)*,a	*P* value[b]
rs2249825 genotype						
GG (ref)	560	71.4	628	73.2	1.00 (ref)	
CG	200	35.5	212	24.7	0.926 (0.713-1.204)	0.566
CC	24	3.1	18	2.1	2.464 (1.215-4.996)	0.012
Dominant effect						
GG (ref)	560	71.4	628	73.2	1.00 (ref)	
CG+CC	224	28.6	230	26.8	1.017 (0.791-1.308)	0.895
Recessive effect						
CG+GG (ref)	760	96.9	840	97.9	1.00 (ref)	
CC	24	3.1	18	2.1	2.508 (1.240-5.073)	0.011
rs2249825 allele						
G (ref)	1320	84.2	1468	85.5	1.00 (ref)	
C	248	15.8	248	14.5	1.112 (0.919-1.346)	0.276

Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval. *ORs and 95% CIs were calculated by logistic regression. [a,b]Adjusted OR (95% CI) and *P* value, adjusted for age, gender, BMI, diabetes mellitus, hypertension, history of smoking, history of alcohol use, and hyperlipidemia.

3.600, 95% CI = 1.272-10.193, P = 0.016) and to ischemic stroke (adjusted OR = 3.790, 95% CI = 1.345-1.667, P = 0.012) in the recessive model.

Haplotypes analysis for the HMGB1 polymorphisms

We further characterized *HMGB1* polymorphisms using linkage disequilibrium (LD) and haplotype analyses. The loci chosen for haplotype analysis were rs1412125, rs2249825, and rs1045411. In this study population, there was LD at three SNPs (D' > 0.8 or r^2 > 0.4, **Figure 2**). Only four haplotypes were found to be significant in both cases and controls, which were further analyzed (**Table 8**). Statistical analysis of the C-G-C haplotype revealed a significant difference between patients and controls (OR = 1.554, 95% CI = 1.246-1.938, P =

Table 5. rs1045411 allele and genotype frequencies of genetic polymorphisms in cases and controls as well as their main effects on stroke risks

SNP	Cases	Percent (%)	Controls	Percent (%)	OR (95% CI)*,a	*P* value[b]
rs1045411 genotype						
CC (ref)	512	65.3	568	66.2	1.00 (ref)	
CT	246	31.4	264	30/8	0.912 (0.713-1.168)	0.466
TT	26	3.3	26	3.0	1.576 (0.831-2.988)	0.164
Dominant effect						
CC (ref)	512	65.3	568	66.2	1.00 (ref)	
CT+TT	272	34.7	290	33.8	0.961 (0.757-1.219)	0.741
Recessive effect						
CT+CC (ref)	758	96.7	832	97.0	1.00 (ref)	
TT	26	3.3	26	3.0	1.621 (0.859-3.061)	0.136
rs1045411 allele						
C (ref)	1270	81.0	1400	81.6	1.00 (ref)	
T	298	19.0	316	18.4	1.040 (0.872-1.239)	0.665

Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval. *ORs and 95% CIs were calculated by logistic regression. [a,b]Adjusted OR (95% CI) and *P* value, adjusted for age, gender, BMI, diabetes mellitus, hypertension, history of smoking, history of alcohol use, and hyperlipidemia.

Table 6. rs1045411 allele and genotype frequencies of genetic polymorphisms in cases and controls as well as their main effects on stroke risks in the male population

SNP	Cases	Percent (%)	Controls	Percent (%)	OR (95% CI)*,a	*P* value[b]
rs1045411 genotype						
CC (ref)	310	67.4	310	68.3	1.00 (ref)	
CT	134	29.1	138	30.4	0.839 (0.604-1.166)	0.296
TT	16	3.5	6	1.3	3.600 (1.272-10.193)	0.016
Dominant effect						
CC (ref)	310	67.4	310	68.3	1.00 (ref)	
CT+TT	150	32.6	144	31.7	0.936 (0.680-1.287)	0.684
Recessive effect						
CT+CC (ref)	444	96.5	448	98.7	1.00 (ref)	
TT	16	3.5	6	1.3	3.790 (1.345-10.677)	0.012
rs1045411 allele						
C (ref)	750	81.9	758	83.6	1.00 (ref)	
T	166	18.1	150	16.5	1.118 (0.877-1.426)	0.366

Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval. *ORs and 95% CIs were calculated by logistic regression. [a,b]Adjusted OR (95% CI) and *P* value, adjusted for age, gender, BMI, diabetes mellitus, hypertension, history of smoking, history of alcohol use, and hyperlipidemia.

0.001). Conversely, one haplotype in the ischemic stroke cases, "T-G-C", was significantly associated with lower susceptibility to ischemic stroke (OR = 0.792, 95% CI = 0.679-0.925, P = 0.003).

HMGB1 polymorphisms and age at ischemic stroke onset

In the current study, 784 patients with ischemic stroke were included in a case group. *HMGB1* polymorphisms were not associated with the age at ischemic stroke onset (P > 0.05), suggesting that these variants cannot be used to predict stroke onset time (**Figure 3**).

HMGB1 polymorphisms and ischemic stroke recurrence

In **Table 9**, the clinical characteristics of the 784 patients with ischemic stroke for the prognosis analysis are summarized. Following the

Table 7. rs1045411 allele and genotype frequencies of genetic polymorphisms in cases and controls as well as their main effects on stroke risks in the female population

SNP	Cases	Percent (%)	Controls	Percent (%)	OR (95% CI)*,a	*P* value[b]
rs1045411 genotype						
CC (ref)	202	62.3	258	63.9	1.00 (ref)	
CT	112	34.6	126	31.2	0.995 (0.682-1.451)	0.979
TT	10	3.1	20	5.0	0.995 (0.339-2.243)	0.777
Dominant effect						
CC (ref)	202	62.3	258	63.9	1.00 (ref)	
CT+TT	122	37.3	146	36.1	0.982 (0.682-1.415)	0.922
Recessive effect						
CT+CC (ref)	314	96.9	384	95.0	1.00 (ref)	
TT	10	3.1	20	5.0	0.874 (0.343-2.226)	0.777
rs1045411 allele						
C (ref)	514	79.6	642	79.5	1.00 (ref)	
T	132	20.4	166	20.5	0.993 (0.769-1.283)	0.958

Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval. *ORs and 95% CIs were calculated by logistic regression. [a,b]Adjusted OR (95% CI) and *P* value, adjusted for age, gender, BMI, diabetes mellitus, hypertension, history of smoking, history of alcohol use, and hyperlipidemia.

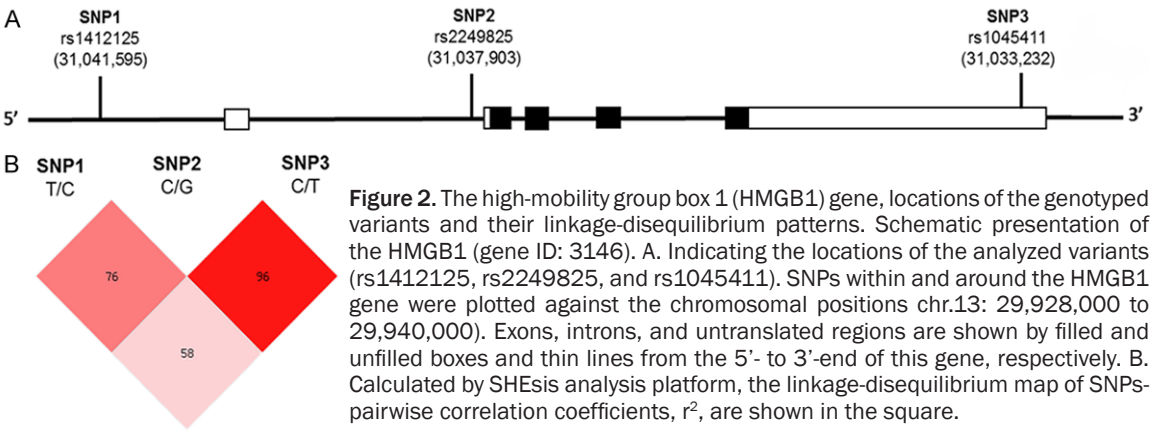

Figure 2. The high-mobility group box 1 (HMGB1) gene, locations of the genotyped variants and their linkage-disequilibrium patterns. Schematic presentation of the HMGB1 (gene ID: 3146). A. Indicating the locations of the analyzed variants (rs1412125, rs2249825, and rs1045411). SNPs within and around the HMGB1 gene were plotted against the chromosomal positions chr.13: 29,928,000 to 29,940,000). Exons, introns, and untranslated regions are shown by filled and unfilled boxes and thin lines from the 5'- to 3'-end of this gene, respectively. B. Calculated by SHEsis analysis platform, the linkage-disequilibrium map of SNPs-pairwise correlation coefficients, r^2, are shown in the square.

Table 8. Haplotype frequencies in cases and controls as well as their relationship with stroke risks

Haplotype	Cases (%)	Controls (%)	OR (95% CI)	*P* value
C-C-T	195.46 (12.5)	208.18 (12.1)	1.041 (0.645-1.283)	0.707
C-C-C	1.21 (0.1)	2.52 (0.1)	-	-
C-G-T	1.60 (0.1)	6.30 (0.4)	-	-
C-G-C	207.74 (13.2)	155.00 (9.0)	1.554 (1.246-1.938)	< 0.001
T-C-T	44.26 (2.8)	35.64 (2.1)	-	-
T-C-C	7.07 (0.5)	1.66 (0.1)	-	-
T-G-T	56.68 (3.6)	65.88 (3.8)	0.947 (0.659-1.360)	0.769
T-G-C	1053.98 (67.2)	1240.82 (72.3)	0.792 (0.679-0.925)	0.003

Abbreviations: OR, odds ratio; CI, confidence interval.

follow-up period, 208 individuals experienced stroke recurrence. Age was found to be an extremely significant factor in increasing the risk of ischemic stroke recurrence ($P < 0.001$). There were no other risk factors associated with ischemic stroke recurrence (including gen-

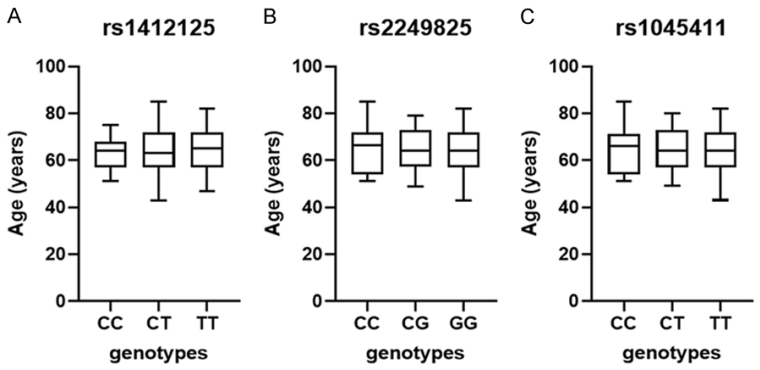

Figure 3. Association between the HMGB1 SNPs and age at onset of ischemic stroke (A. rs1414125; B. rs2249825; C. 1045411). high-mobility group box 1 (HMGB1), single nucleotide polymorphisms (SNPs).

Table 9. Clinical characteristics of ischemic stroke patients

Variable	Patients (N = 576)	Recurrence (N = 208)	Log-rank *P*
Age (> 60)	363	137	< 0.001
Gender (male/female)	335/241	125/83	0.788
BMI (> 22.9)	281	105	0.388
Diabetes mellitus	160	52	0.917
Hypertension	358	126	0.558
History of smoking	205	69	0.390
History of alcohol use	107	35	0.917
Hyperlipidemia	90	32	0.280

Abbreviations: BMI, body mass index.

der, hypertension, diabetes, smoking history, alcohol-using history, and hyperlipidemia). **Table 10** illustrates the association between *HMGB1* polymorphisms and ischemic stroke recurrence. Only rs1412125 was significantly associated with stroke recurrence (TC vs. TT: P = 0.034; CC vs. TT: P < 0.001), whereas all other tagSNPs showed no association (**Figure 4**). To calculate the association between the rs1412125 polymorphism and stroke recurrence risk for various genetic models, Cox regression analysis was used, with Bonferroni correction for multiple comparisons. The statistical significance level was determined to be 0.0125 (0.05/4 genetic models = 0.0125). According to Cox regression analysis, the association of rs1412125 with ischemic stroke recurrence was similar under homozygous, heterozygous dominant, and recessive models, with the corresponding HRs (95% CIs) at 3.097 (1.761-5.446), 1.544 (1.155-2.064), 1.648 (1.243-2.184), and 2.495 (1.453-4.285), respectively (**Table 11**). A stratified analysis of the rs1412125 polymorphism by age, gender, diabetes, hypertension, hyperlipidemia, smoking history, and alcohol-using history status is presented in **Table 12**. It was still evident that rs1412125 increased recurrence risk in both dominant and recessive models.

Discussion

To determine whether three well-identified *HMGB1* gene variants were associated with ischemic stroke in a large northern Chinese population, an in-hospital, case-control study was conducted. It was determined that the correlation between *HMGB1* polymorphisms and ischemic stroke risk was statistically significant. A high risk of ischemic stroke was associated with people carrying the variant genotypes (CT and CC) of *HMGB1* rs1412125 and *HMGB1* rs2249825. In addition, stratification further revealed that the male subgroup was more likely to develop ischemic stroke when the TT allele was present in *HMGB1* rs1045411. According to a haplotype study, the C-G-C haplotype of *HMGB1* (corresponding to rs1412125-rs2249825-rs1045411) negatively impacted ischemic stroke susceptibility.

Additionally, according to our data, the rs1412125 polymorphism was highly associated with the chance of recurrence but not with the age at which an ischemic stroke first occurred. Patients with the CT/CC genotype within rs1412125 had a 1.648-fold higher risk of recurrence than those with the TT genotype, indicating that rs1412125 may be detrimental for stroke recurrence. Even after Bonferroni correction, multivariate Cox regression analysis revealed that the rs1412125 CT/CC genotype was independently related to stroke recurrence. We did a stratified analysis based on several risk factors and, interestingly, discovered that the effect of the rs1412125 CT/CC genotype was still noticeable. The *HMGB1*

Table 10. Association of selected SNPs with ischemic stroke recurrence

Genotype	All the cases				
	Patients	Percent (%)	Recurrence	Percent (%)	Log-rank P
rs1412125 genotype					
TT	326	55.6	88	42.3	
CT	229	39.8	105	50.5	0.034
CC	21	3.6	15	7.2	< 0.001
rs2249825 genotype					
GG	415	72.0	145	69.7	
CG	144	25.0	56	26.9	0.06
CC	17	3.0	7	3.4	0.222
rs1045411 genotype					
TT	386	67.0	126	60.6	
CT	174	30.2	72	34.6	0.420
CC	16	2.8	10	4.8	0.490

rs1412125 polymorphism may be a new genetic biomarker and potential detective target for ischemic stroke susceptibility and recurrence, according to the findings summarized above. To the best of our knowledge, this is the first study to investigate the association between the risk, onset age, and recurrence of ischemic stroke and functional tagSNPs in the *HMGB1* genes (rs1412125, rs2249825, and rs1045411) in a northern Chinese population.

Through the activation of signaling pathways associated with Toll-like receptors (TLRs), specifically TLR4 and TLR2, HMGB1, a conserved chromatin protein, controls the expression of other genes [20, 21]. A number of immune cells, including macrophages and monocytes, as well as necrotic or injured cells, may release HMGB1, which has proinflammatory properties [22, 23]. Studies have indicated that the serum levels of HMGB1 are significantly increased in patients who suffer from inflammatory diseases, such as endotoxemia [24], sepsis [25], acute lung injury [26], autoimmune disease [27], and ischemia/reperfusion injury [12, 28]. In line with the aforementioned findings, we have earlier discovered that cerebral ischemia/reperfusion caused the release of HMGB1, which triggered TLR4 [24]. Concurrently, the level of expression of HMGB1 protein was observed to be similar to the level of death and apoptosis of penumbral neurons. This suggests that HMGB1 can induce downstream signals that result in cytokine production and, therefore, initiate an inflammatory response. The above evidence suggests that HMGB1 might be an important candidate gene for ischemic stroke risk and might play a key role in its occurrence and development.

It has previously been shown that *HMGB1* polymorphisms are associated with a number of diseases [29-32], but for the first time, we found an association between the C allele of the *HMGB1* rs2249825 variant and IS susceptibility. A bioinformatics analysis suggested that the C to G change at rs2249825 may influence v-Myb binding to HMGB1 binding sites (FASTSNP, http://fastsnp.ibms.sinica.edu.tw), thereby regulating *HMGB1* expression. Thus, *HMGB1* rs2249825 variants may alter binding to v-Myb, which could influence *HMGB1* expression.

It should be noted that a gender-specific analysis indicated that males with the rs1045411 TT genotype were at a significantly higher risk of ischemic stroke than females with either the rs1045411 CC or CT genotype. There is an *HMGB* domain on the Y gene in the gender-determining region of the Y chromosome, which is involved in male sexual differentiation [33]. In light of these findings, it would appear that the *HMGB1* variant may be a risk factor for the development of ischemic stroke specifically for men. In view of the relationship between the *HMGB1* rs1045411 polymorphism and altered microRNA (miRNA)-505-5P binding in the 3'-UTR of messenger RNA (mRNA) transcripts [34, 35], *HMGB1* gene polymorphisms may play an influential role in ischemic stroke development through a post-transcriptional mecha-

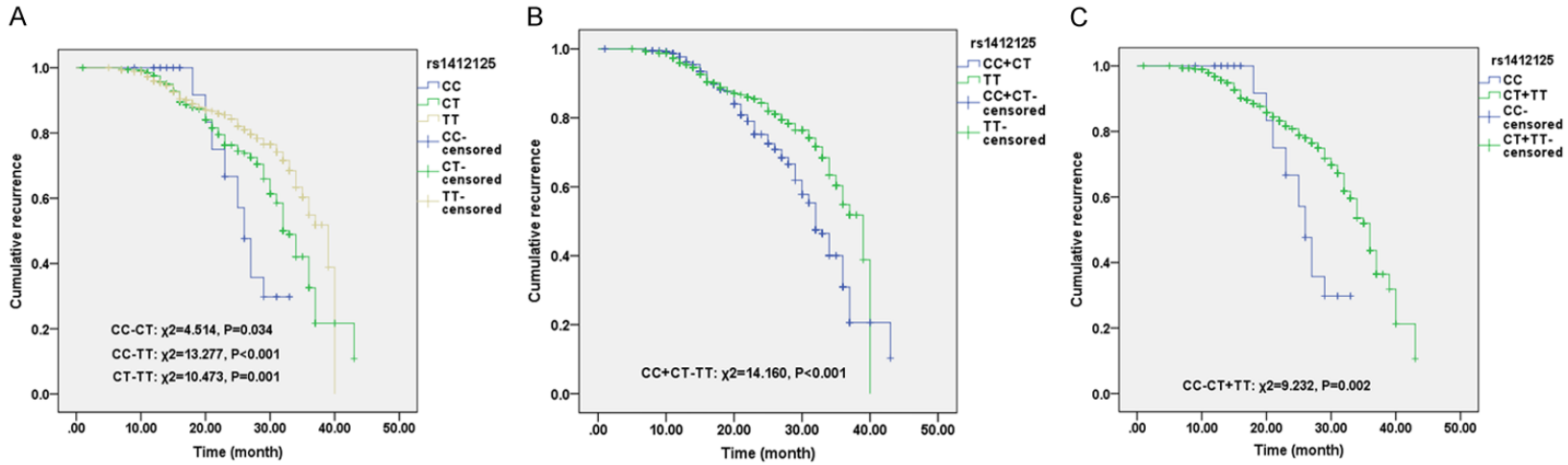

Figure 4. Kaplan-Meier survival curves for recurrence among ischemic stroke cases based on rs1414125 genotypes. A. Additive model; B. Dominant model; C. Recessive model.

Table 11. Association between rs1412125 and recurrence of ischemic stroke

SNP	Patients (%)	Recurrence (%)	HR (95% CI)*,a	*P*b
rs1412125 genotype				
TT (ref)	326 (55.6)	88 (42.3)		
CT	229 (39.8)	105 (50.5)	1.544 (1.155-2.064)	0.003
CC	21 (3.6)	15 (7.2)	3.097 (1.761-5.446)	< 0.001
Dominant effect				
CT+CC vs. TT			1.648 (1.243-2.184)	0.001
Recessive effect				
CC vs. CT+TT			2.495 (1.453-4.285)	0.001

Abbreviations: SNP, single nucleotide polymorphism; HR, hazard ratio; CI, confidence interval. *HRs and 95% CIs were calculated by Multivariate Cox regression. a,bAdjusted HR (95% CI) and *P* value, adjusted for age, gender, BMI, diabetes mellitus, hypertension, history of smoking, history of alcohol use, and hyperlipidemia.

Table 12. Stratified analysis of rs1412125 associated with the recurrence of ischemic stroke

Variable	CT+CC vs. TT		CC vs. CT+TT	
	HR (95% CI)*,a	*P*b	HR (95% CI)*,a	*P*b
Age (> 60)	1.575 (1.189-2.087)	0.002	2.459 (1.435-4.213)	0.001
Gender (male/female)	1.695 (1.277-2.250)	< 0.001	2.438 (1.418-4.192)	0.001
BMI (> 22.9)	1.705 (2.284-2.263)	< 0.001	2.560 (1.486-4.411)	0.001
Diabetes mellitus	1.591 (1.197-2.116)	0.001	2.657 (1.539-4.590)	< 0.001
Hypertension	1.668 (1.257-2.213)	< 0.001	2.463 (1.433-4.235)	0.001
History of smoking	1.693 (1.273-2.253)	< 0.001	2.453 (1.428-4.212)	0.001
History of alcohol use	1.647 (1.241-2.186)	0.001	2.512 (1.459-4.324)	0.001
Hyperlipidemia	1.649 (1.242-2.189)	0.001	2.583 (1.502-4.443)	0.001

*HRs and 95% CIs were calculated by Multivariate Cox regression. a,bAdjusted HR (95% CI) and *P* value, adjusted for age, gender, BMI, diabetes mellitus, hypertension, history of smoking, history of alcohol use, and hyperlipidemia.

nism. In addition, because the rs1045411 polymorphism is located in the 3'-flanking region, it may play a role in mRNA stability since miRNAs can bind to the 3'-UTR region of mRNA transcripts and inhibit *HMGB1* expression after transcription [36, 37]. The information presented above may suggest that rs1045411 is an active expression quantitative trait locus (e-QTL) for *HMGB1*. Recent research has revealed that e-QTLs, which are variants that regulate gene expression, can influence the risk of disease under different circumstances and have high population heterogeneity [38, 39]. Based on the results of our study, rs1045411 significantly contributes to the risk for ischemic stroke in male subjects, indicating that this variant is likely the e-QTL for *HMGB1* in males.

It is interesting to note that our study showed that patients carrying the CC or TC genotype of the rs1412125 polymorphism are more likely to experience ischemic stroke recurrence than those carrying the TT genotype. Furthermore, our findings suggest that the rs1412125 locus is an independent predictor of ischemic stroke recurrence. Importantly, stratified analyses did not affect the association between rs1412125 and the recurrence risk. This was based on age, gender, BMI, diabetes mellitus, hypertension, history of smoking, history of alcohol use, or hyperlipidemia. Why is there an association between *HMGB1* rs1412125 polymorphism and stroke recurrence? A growing body of evidence indicates that the rs1412125 allele is located in the core of the CCAAT box, which is a common element in eukaryotic promoters [40, 41]. The details of the rs1412125 gene have not been discussed in the current study. The role of *HMGB1* polymorphism in ischemic stroke recurrence should be elucidated by further experiments.

One of the strengths of our study is the follow-up period of an average of 44.5 months, which may enhance the robustness of the results.

Further supporting our conclusions, every patient in this study was newly diagnosed with ischemic stroke, preventing prevalence-incidence bias. Finally, both stratified and pooled analyses were performed in order to examine the relationship between the SNP in *HMGB1* and ischemic stroke risk. However, our study has some limitations. First, the cases and controls were drawn from a single hospital with a homogeneous ethnic background. Our findings need to be replicated with larger sample sizes, including additional ethnicities. Nonetheless, the hospital-based Berkson's bias has been minimized to some extent possible by recruiting control subjects from medical examination centers within the same hospital [42, 43]. The second limitation of this study is that it was carried out only in a Han Chinese population. As a result, it is necessary to extrapolate our findings to other races or ethnicities, since strong genetic heterogeneity is widely recognized to underlie susceptibility to ischemic stroke [44, 45]. The third issue was the lack of some clinical rating scales for stroke prognosis, such as the Barthel index and the Modified Rankin Score (mRS), as well as consideration of the factor of treatment methods in COX regression. Furthermore, drug and lifestyle interventions were not detailed, thus leaving some room for further exploration, especially from the gene-environment interaction perspective. As a final note, we did not conduct functional studies as part of our study. To overcome these limitations and further support the evidence presented here, a large-scale study with an improved design is needed. A more detailed assessment of personal information and clinical scales will be essential in order to obtain more precise adjusted estimates. As part of the following study, wet bench experiments will be designed to investigate the underlying molecular mechanisms. If the results confirm the observations of the current study, *HMGB1* genetic markers may be utilized in the future to predict ischemic stroke vulnerability and recurrence risk.

According to our findings, *HMGB1* polymorphisms are associated with susceptibility to ischemic stroke and recurrence. We also provide insights into naturally occurring haplotype-based variants. We believe that *HMGB1* gene variants may be useful as potential markers for first and secondary stroke prevention, although further confirmation by larger studies is required. Developing optimal preventive approaches aimed at reducing the adverse effects of ischemic stroke requires accurate biomarkers for such types of variants.

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Informed consent was obtained from all individual participants included in the study.

Disclosure of conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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