

# Association between Histone Deacetylase 9 Gene Polymorphism and Stroke in Chinese Han Population

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**Objective :** To explore the correlation between the polymorphism of histone deacetylase 9 gene (rs1060499865, rs723296, rs957960) and ischemic stroke (IS) in Chinese Han population in Dali region.

**Methods :** This study included 155 IS patients and 128 healthy physical examinees. TaqMan-polymerase chain reaction technology and multivariate logistic regression were performed.

**Results :** In the case group, there was no polymorphism of rs1060499865 observed in the two groups; whereas on the rs723296 locus the frequencies of C allele and TC genotype were significantly higher than that in the control group, alleles C and T were associated with a 2.158-fold increase in IS risk, and genotypes TC and TT were associated with a 2.269-fold increase in IS risk. The locus rs957960 exhibited no significant difference between the two groups.

**Conclusion :** An association between rs723296 and the risk of IS was found in the Chinese Han population in Dali region. No significant association was found between rs1060499865, rs957960 and IS in the Chinese Han population in Dali region.

**Key Words :** Stroke · HDAC9 protein · Polymorphism.

## INTRODUCTION

Stroke is a multifactor disease, the interaction between environmental and genetic factors plays a significant role in the pathogenesis of stroke. The prevalence of stroke is on the rise as the aging process of the global population is constantly increasing<sup>9,22</sup>. About 15 million people suffer stroke worldwide every year, of whom 20% leads to fatality, more than 50% lose

their ability to take care of themselves and to perform their daily activities<sup>30</sup>. Ischemic stroke (IS) accounts for more than 85% of strokes, which is increasing every year in China, and shows a trend of younger population suffering from IS<sup>28</sup>. IS being a complex disease is caused by multiple factors; family and twins' studies have indicated that genetic factors play an important role in the pathogenesis of IS<sup>1</sup>.

Histone deacetylase 9 (*HDAC9*) is located on human chro-

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mosome 7p21, the region associated with neurological diseases and various types of tumors. The *HDAC9* gene contains 32 exons with an overall length of about 500 KB. The 3' end is about 150 KB away from the downstream *TWIST* gene, mainly encoding *HDAC9* and *HDRP* enzyme protein subtypes<sup>16</sup>. *HDAC9* is primarily found in the cytoplasm, shuttling between the nucleus and cytoplasm, while regulating acetylation of histones and nonhistones<sup>11</sup>. *HDAC9* acts as an important regulator of histone acetylation to control gene transcription, rendering the gene promoter inaccessible to the transcriptional regulatory elements, thus inhibiting transcription. Disturbances in the balance of nonhistone acetylation and deacetylation affect many aspects of normal cellular functioning. To date, the *HDAC9* gene has been identified as the strongest risk locus for large-vessel strokes<sup>14</sup>. Hypoacetylation at gene promoters usually result in transcriptional inhibition by restraining accessibility of transcriptional machinery to chromatin<sup>24</sup>. Monocytes, macrophages, T lymphocytes, vascular endothelial, and smooth muscle cells express *HDAC9*<sup>15</sup>. *HDAC9* was upregulated in human carotid and aortic atherosclerotic plaques<sup>17</sup> and the plasma *HDAC9* expression was found to be significantly higher in patients with coronary artery disease<sup>25</sup>.

Genome-wide association study revealed a new IS susceptibility *HDAC9* gene. The loci rs11984041 and rs2107595 of *HDAC9* polymorphism were found to significantly increase the risk of atherosclerotic IS<sup>8,21</sup>. *HDAC9* has been reported to regulate gene expression by modifying the chromatin structure, though DNA sequence remains unchanged. *HDAC9* was revealed in whole genome sequencing studies that was found associate closely with IS; the *HDAC9* gene was identified as a highly risky gene responsible for IS. *HDAC9* zymoprotein is found abundantly in brain, muscles, myocardium, and vascular endothelial cells. The *HDAC9* gene has been reported to have a significant role in atherosclerosis, inflammatory response, lipid metabolism, and vascular regeneration<sup>23</sup>. The expression of *HDAC9* was found to be upregulated in the case of human atherosclerotic plaques<sup>20</sup>.

Presently, few studies have already reported a correlation between *HDAC* polymorphism and IS; however, this study explores the relationship between the polymorphism of three single nucleotide polymorphisms (SNP) loci in *HDAC9* (rs1060499865, rs723296, and rs957960) and stroke patients in Dali region of China. Here TaqMan-polymerase chain reaction (PCR) technology was used to investigate the association

between the *HDAC9* polymorphism and IS in the Chinese Han population, aiming to supplement data for further exploring the genetic basis of IS.

## MATERIALS AND METHODS

### Study subjects

The study protocol was approved by the Medical Ethics Committee of the First Affiliated Hospital of Dali University (No. 81360206), and signed informed consent was obtained from all the study participants. From December 2017 to August 2018, 155 IS patients were admitted to the neurology department of the First Affiliated Hospital of Dali University. One hundred and twenty-eight healthy subjects from the outpatient department of our hospital were included in this study as the control group. Following inclusion criteria were considered for forming the IS group : 1) conformed diagnosis of IS based on the diagnostic criteria for cerebrovascular diseases provided in the fourth national conference<sup>3</sup> and 2) existing IS patients in Dali whose is living there for more than three generations. Exclusion criteria are as follows : 1) subjects with severe diseases such as heart, kidney, liver, bone diseases and cancer as well as serious infections such as acquired immunodeficiency syndrome and syphilis; 2) people with severe neurological deficits; and 3) no consanguinity was reported between the selected healthy subjects and IS patients.

The control group members were free from cardiovascular and cerebrovascular diseases, autoimmune disorders, malignant tumor, immunological disease, neurological deficits, and severe hepatic and renal dysfunction. No sib ship existed between the selected individuals and IS patients, who were hailed from Dali region whose more than three generations have been living there. Certain demographic and clinical data were collected, such as age, sex, fasting blood glucose (FBG), red blood cells (RBC), white blood cells (WBC), cholesterol, triglycerides (TG), low-density lipoprotein (LDL), systolic pressure, diastolic pressure (DBP), and body mass index (BMI).

### DNA extraction

A portion of 5 mL of venous blood collected from each subject was stored in tubes containing ethylene diaminetetraacetic acid. Sample DNA was extracted using the QIAGEN DNA

extraction kit. Genomic DNA extraction was performed according to manufacturer's protocols (Bomaide Technology, Beijing, China), and the extract was stored finally at  $-80^{\circ}\text{C}$  until analysis.

### Design and synthesis of primer probes

Based on NCBI database (<https://www.ncbi.nlm.nih.gov/snp/>), rs1060499865, rs723296, and rs957960 loci of the gene *HDAC9* were selected. The amplification was performed in 25  $\mu\text{L}$  volume, 12.5  $\mu\text{L}$  2 $\times$ Taq enzyme mixture, 0.5  $\mu\text{L}$  CF forward primer, 0.5  $\mu\text{L}$  TF forward primer, 1  $\mu\text{L}$  R reverse primer, 8.5  $\mu\text{L}$  ddH<sub>2</sub>O, and 2  $\mu\text{L}$  DNA sample.

#### Reaction conditions

The reaction conditions followed during the experiments are as follows: predegeneration at  $95^{\circ}\text{C}$  for 5 minutes, denaturing at  $95^{\circ}\text{C}$  for 10 seconds, annealing at  $60^{\circ}\text{C}$  for 30 seconds, followed by 30 cycles, extending at  $72^{\circ}\text{C}$  for 2 minutes, and conservation at  $16^{\circ}\text{C}$  for 5 minutes were followed. Primers and probes were designed using Primer 5 software (Premier, Quebec, Canada). The sequences of primers and probes were procured from Anhui General Biosystems, Inc. (Chuzhou, Anhui, China) and are listed in Table 1.

### Statistical analysis

The statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA, USA) and SPSS version 19.0 software (SPSS, IBM, Armonk, NY, USA). Mean $\pm$ standard deviation or standard error values of the continuous variables are presented. Count data were performed using *t* test; quanti-

tative data analyses were performed using the chi-squared test. Univariate and multivariate logistic regression analyses were used to derive the odds ratio (OR). Hardy-Weinberg equilibrium (HWE) was assessed using the SPSS software and Microsoft Excelget corresponding value for determining *HDAC9* SNP genotypes distribution in controls. Statistical significance was defined as two-sided  $p < 0.05$ .

## RESULTS

### Clinical characteristics

The IS patients' group comprising 155 patients included 90 males and 65 females, with an average age of  $58.63 \pm 12.97$  years. The control group included 128 subjects with 76 males and 50 females, their age on average being  $57.59 \pm 14.01$ . No significant differences in factors such as age, sex, DBP, and BMI values were recorded between controls and IS but were recorded in the values of FBG, WBC, RBC, TC, TG, LDL, and SBP, and the observed differences are shown in Table 2.

### HWE test

In both the IS and control groups, the locus rs1060499865 exhibited no polymorphism; whereas for rs723296, the  $\chi^2$  and *p* values were 0.211 and 0.646 for the control group and 1.19 and 0.27 for the IS group, respectively; and for rs957960, the  $\chi^2$  and *p* values were 0.289 and 0.59 for control group and 1.041 and 0.308 for the IS group, respectively. All  $p > 0.05$  values indicate that the distribution of the data in this study represents the gene distribution of the population.

**Table 1.** PCR primer sequences used in this study

SNP	Polymorphism	Probe name	Sequence
rs1060499865	C/T	CC	5'-ACGCATGGCTGTTGGCTGTGTCATC-3'
		TT	5'-ACGCATGGCTGTTGGCTGTGTCATT-3'
		Reverse	5'-CTGAGGCCACTTTGGAAGCCAGCTC-3'
rs723296	C/T	CC	5'-ACATTTCTTTTGTCTATGCATAC-3'
		TT	5'-ACATTTCTTTTGTCTATGCATAT-3'
		Reverse	5'-GGGAAGGAATTTATGTATGTCAAGT-3'
rs957960	A/C	AA	5'-TCTTTGAACACAACCATTCCATTA-3'
		CC	5'-TCTTTGAACACAACCATTCCATTAC-3'
		Reverse	5'-CACTTAATATCTGCGTTTACATTAT-3'

PCR: polymerase chain reaction, SNP: single nucleotide polymorphism

### Comparison of the association of rs1060499865, rs723296, and rs957960 polymorphisms with IS

No polymorphism in rs1060499865 was observed when a comparison of polymorphism was performed among the three loci rs1060499865, rs723296, and rs957960. The locus rs723296 genotype TC/CC was found to be associated with a

2.269-fold increase in IS risk (OR, 2.269; 95% confidence interval [CI], 1.046–4.923;  $\chi^2=4.474$ ;  $p=0.034$ ); allele C/T was observed to be associated with a 2.158-fold increase in IS risk (OR, 2.158; 95% CI, 1.016–4.582,  $\chi^2=4.179$ ;  $p=0.041$ ), indicating that individuals with C allele and TC genotype are at a higher risk for IS. The locus rs957960 did not exhibit any significant association between allele C/A and genotypes AC/AA and CC/AA, and IS, as indicated in Table 3.

**Table 2.** Characteristics of IS patients and controls

Characteristic	IS patient	Control	p-value
Age (years)	58.63±12.97	57.59±14.01	0.516
Male gender	90 (58.0)	76 (59.3)	0.824
FBG (mmol/L)	6.25±2.73	4.75±0.87	<0.001
WBC (10 <sup>12</sup> /L)	7.79±2.32	6.27±1.57	<0.001
RBC (10 <sup>12</sup> /L)	4.81±0.72	4.97±0.55	0.004
TC (mmol/L)	5.01±1.29	4.33±0.42	<0.001
TG (mmol/L)	1.72±1.40	1.35±0.75	0.008
LDL (mmol/L)	3.05±1.06	2.82±0.72	0.033
SBP (mmHg)	144.92±25.88	133.85±15.34	<0.001
DBP (mmHg)	86.46±13.02	84.66±10.40	0.205
BMI (kg/m <sup>2</sup> )	23.15±3.28	22.84±3.91	0.478

Values are presented as mean±standard deviation or number (%). IS : ischemic stroke, FBG : fasting blood glucose, WBC : white blood cells, RBC : red blood cells, TC : total cholesterol, TG : triglycerides, LDL : low-density lipoprotein, SBP : systolic pressure, DBP : diastolic pressure, BMI : body mass index

### DISCUSSION

Our results indicate that polymorphism of *HDAC9* gene rs723296 significantly influences the risk of IS, whereas rs1060499865 and rs957960 did not have any association with IS. Studies have shown that *HDAC9* gene stimulates angiogenesis by targeting the microRNA-17-92 group that is antiangiogenic in vascular endothelium<sup>10</sup>. Therefore, the effect of *HDAC9* activity on angiogenesis may be associated with susceptibility to IS.

IS is a complex disease caused as a result of atherosclerosis, which affects the interaction of genomic abnormalities with various environmental factors, and is a major public health burden in China<sup>26</sup>. Age, diabetes, hypertension, and hypercholesterolemia have been identified as risk factors for IS.

**Table 3.** Genotype frequencies of *HDAC9* gene polymorphisms in IS and control groups and their associations with IS

Genotype	Case group	Control group	$\chi^2$	p-value	OR	95% CI
rs723296						
TT	130 (83.87)	118 (92.19)	Reference	Reference		
TC	25 (16.13)	10 (7.81)	4.474	0.034	2.269	1.046–4.923
CC	0 (0.00)	0 (0.00)				
T	285 (91.94)	246 (96.09)	Reference	Reference		
C	25 (8.04)	10 (3.91)	4.179	0.041	2.158	1.016–4.582
rs957960						
AA	37 (23.87)	39 (30.47)	Reference	Reference		
AC	74 (47.74)	58 (45.31)	1.054	0.304	1.115	0.904–1.375
CC	44 (28.39)	31 (24.22)	1.012	0.314	1.187	0.847–1.662
AA/AC+CC			1.319	0.251	1.088	0.940–1.259
CC/AA+AC			0.276	0.599	1.114	0.744–1.666
A	148 (47.74)	136 (53.13)	Reference	Reference		
C	162 (52.26)	120 (46.87)	1.096	0.295	1.095	0.923–1.298

Values are presented as number (%). IS : ischemic stroke, OR : odds ratio, CI : confidence interval

Moreover, genetic factors have been found to be particularly involved in the pathogenesis of IS, but certain associations between genetic factors and IS are replicated<sup>19</sup>. *HDAC9* polymorphisms affect lipid homeostasis, cholesterol efflux, platelet activation, and genes involved in inflammatory responses. Several studies have shown that genetic variation in 7p21.1 chromosome is closely linked to IS caused by arterial disease<sup>27</sup>. The *HDAC9* gene encodes proteins responsible for deacetylation of histones and in turn regulating the chromatin structure and gene transcription. Several studies have replicated that *HDAC9* gene polymorphism is linked to susceptibility of IS. By way of Markus Carotid Artery Ultrasound (MCAO) found that a genetic variant of *HDAC9* stimulates carotid plaque formation and increases the risk of stroke, and through isotope labeling found that the *HDAC9* gene is highly expressed in the endothelial cells of blood vessels in the brain and myocardium<sup>29</sup>. The MCAO results indicated that *HDAC9* expression was found to be significantly increased in a rat model of cerebral ischemia reperfusion injury<sup>7</sup>. Studies have shown that the *HDAC9* SNP allele of rs3757720 is associated with coronary heart disease, which is a marker of atherosclerosis<sup>2</sup>. In the mice knockout *HDAC9* gene, the extent of atherosclerosis lesions was decreased significantly<sup>4</sup>. This indicates that the absence of *HDAC9* expression can result in the down-regulation of inflammation-related genes and upregulation of lipid-related genes<sup>5,13</sup>. As per several other related studies, the *HDAC9* gene variant is associated with the risk of stroke, but results may vary with different genes.

A study conducted on 262 Chinese Han stroke patients, investigated the loci rs2389995 (A/G), rs2240419 (C/T), and rs1984041 (T/C) of the *HDAC9* gene. No polymorphism of rs1984041 (C/T) locus was found in the Chinese Han population; the alleles A and T of genes rs2389995, rs2240419 were, respectively, found to increase the risk of stroke<sup>18</sup>. In a case-control study performed on 279 Han stroke and 984 healthy population from the Second Military Medical University of Shanghai, alleles A and T of rs2389995 and rs2240419 were, respectively, observed to have no correlation with stroke risk. Subcomponent type was performed for stroke and alleles A and T of rs2389995, rs2240419 were, respectively observed to increase the risk of macrovascular stroke<sup>6</sup>. Liu et al.<sup>12</sup> conducted a study on SNP sites which included 45 Han Chinese patients of Beijing, and found that the binding of the *HDAC9* gene rs2526630 to micro-RNA might be related to the inci-

dence of atherosclerotic IS. From this it can be deduced that *HDAC9* may contain risk susceptibility loci for stroke, and polymorphism of *HDAC9* gene has racial differences among various ethnic populations.

In this study, TaqMan-PCR was performed to analyze 155 IS patients and 128 healthy Han individuals residing in Dali, and the correlation between rs1060499865, rs723296, and rs957960 polymorphisms of *HDAC9* and stroke was confirmed. The rs1060499865 gene was located on Chromosome:7:18793416, genic downstream transcript variant, synonyms variant, and coding sequence variant. The rs723296 gene was located on Chromosome:7:18389685, intron variant, and genic upstream transcript variant. The rs957960 gene was located on Chromosome:7:18837785, intron variant, and genic downstream transcript variant. In this study, no polymorphism of the rs1060499865 gene was observed; both rs723296 and rs957960 appeared to be gene mutants. There was no homozygous CC at the rs723296 locus, hence, only select alleles (C/T) and additive (TC/TT) genetic models were analyzed. In the C/T allele model ( $p=0.041$ ; OR, 2.158; 95% CI, 1.016–4.582), showed in rs723296 sites in patients with C allele were proved to be 2.158-folds more IS risk associated than that of T allele. In additive genetic model TC/TT ( $p=0.034$ ; OR, 2.269; 95% CI, 1.046–4.923), illustrated in rs723296 sites in patients with TC gene was found to be 2.269-folds more IS risk associated than that of T allele. The results indicated that the rs723296 locus was associated with the risk of stroke in individuals carrying the C allele and CT genotype that was higher than that in individuals carrying the T allele and TT genotype, which may be attributed to rs723296 polymorphisms that alter the acetylation-deacetylation balance and increase the risk of stroke. At the rs957960 site, in alleles, additive, dominant, or recessive models, gene mutations were not found to be associated with stroke, indicating no significant association between rs957960 polymorphism and the incidence of stroke in patients.

This study had some limitations : 1) our sample was selected from the Han Chinese population of Dali city, which renders the study inapplicable to other ethnicities; 2) regional disparity resulted in the possible inconsistencies in the role of the same SNP locus in case of the same disease occurring in different ethnic groups and different diseases occurring in the same ethnic group; and 3) inclusion and exclusion criteria of IS vary for different studies.

Altogether, the results of this study demonstrate that polymorphism of the *HDAC9* gene locus rs723296 increases the risk of IS in Han populations of Dali. Nevertheless, there was no polymorphism in locus rs1060499865; rs957960 polymorphism was not found to be significantly associated with the IS patients from the Dali region. Larger sample sizes need to be investigated for the analysis of the correlation between *HDAC9* gene polymorphism and IS susceptibility in different regions and ethnic groups; moreover, the mechanism of gene polymorphism in IS pathogenesis needs to be further studied.

## CONCLUSION

To conclude, the polymorphism of the gene *HDAC9*, rs723296 locus increases the risk of IS. TC genotype was observed to be associated significantly with the risk of IS in Han population of Dali. This finding of the study provides new insight for future explorations regarding IS pathogenesis.

## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

## INFORMED CONSENT

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

## AUTHOR CONTRIBUTIONS

Conceptualization : XY

Data curation : XY

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