Original Research

Interaction between *CONNEXIN37* and *PDE4D* gene polymorphisms with susceptibility to ischemic stroke in Chinese population

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Impact statement

Till now, no study investigated the interaction between *CONNEXIN37* and *PDE4D* gene, and the gene–environment interaction. Therefore, in the current study, we aimed to evaluate the impact of interactions between *CONNEXIN37* and *PDE4D* gene, and its interaction with environmental risk factors on susceptibility to ischemic stroke (IS).

Abstract

The objective of this study was to test the relationship of several single nucleotide polymorphisms (SNPs) within phosphodiesterase 4D (*PDE4D*) and connexin 37 (*CONNEXIN37*) gene additional interactions with ischemic stroke (IS) risk. The online software SNPstats was used for Hardy–Weinberg equilibrium testing. Generalized multifactor dimensionality reduction (GMDR) was employed to detect the potential interactions among *CONNEXIN37* gene, *PDE4D* gene, and smoking. The results indicated that the rs1764391-T and rs966221-G were correlated with higher IS risk, the corresponding ORs (95% CI) were 1.66 (1.21–2.03) and 1.48 (1.11–1.92), respectively. We also found that the first two loci including rs1764391 and rs918592, and the other two-loci including rs1764391 and smoking were significant in the GMDR model. Participants with rs1764391-CT/TT and rs918592-CT/TT genotype have the highest IS risk, compared to subjects with rs1764391-CC and rs918592-CC genotype, OR (95%CI) = 3.16 (1.83–4.45); smokers with rs1764391-CT/TT genotype also have the highest IS risk, compared to never smokers with rs1764391-CC genotype, OR (95%CI) = 2.82 (1.53–4.15), but no significant interaction combinations were found between gene and alcohol drinking. So in this study, the rs1764391-T and rs966221-G, rs1764391–rs918592 interaction, rs1764391–smoking interaction were all associated with higher IS susceptibility.

Keywords: Ischemic stroke, single nucleotide polymorphisms, *CONNEXIN37*, *PDE4D*, interaction, smoking

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Introduction

Ischemic stroke (IS) is an important cause of death and disability for adults around the world.[1] In China, with the development of the economy, nearly 1.6 million persons died from stroke per year, and 157 out of every 100,000 die from stroke.[2,3] To date, several risk factors of IS were reported, including age, hypertension, diabetes mellitus (DM), smoking, and cardiac arrhythmias.[4] It has been shown that genetic factor was a significant risk factor for stroke susceptibility, and 30% of stroke risk can be attributed to genetic factors.[5] Previously, several genes have been reported in different populations, such as phosphodiesterase 4D (*PDE4D*) and connexin 37 (*CONNEXIN37*) gene.

CONNEXIN37 gene (GJA4, also known as Cx37) mainly participates in the regeneration of damaged tissues and endothelial cell aging. Previously, several *CONNEXIN37* gene single nucleotide polymorphisms (SNPs) were reported with some common diseases, including hypertension,[6] coronary heart disease,[7] polycystic ovarian syndrome[8] and so on. A recent study[9] also indicated that the minor alleles of *CONNEXIN37* gene are associated with higher IS risk in Northern Han Chinese. *PDE4D* gene has 8 splice variants, consisted of 22 exons and hundreds of SNPs.[10] Compared to *CONNEXIN37* gene, *PDE4D* gene has been studied more in different case–controls studies[11,12] and meta-analysis.[13,14] But these studies concluded conflicting results. In addition, it was also considered that IS

susceptibility was resulting from both genetic, environmental factors and their interactions.[4] However, till now, no study investigated the impact of interactions, such as interaction between *CONNEXIN37* and *PDE4D* gene, and the gene–environment interaction on IS risk. Therefore, we aimed to detect the interactions between *CONNEXIN37* and *PDE4D* gene, and its interaction with environmental risk factors on susceptibility to IS.

Materials and methods

Subjects

A total of 1773 participants, who were hospitalized in Huangshi Maternity and Child Health Hospital or Huangshi Fifth Hospital were selected, including 892 normal controls and 881 patients with IS. All IS cases were diagnosed by at least two related senior neurologists, using magnetic resonance imaging or/and computed tomography scanning. Those IS patients with family history of some diseases including myocardial infarction, tumor, hemorrhagic stroke, atrial arrhythmia or trauma were excluded from the case group. Age (±3 years) and gender-matched control subjects, in nearly 1:1 ratio to IS patients were selected. Those have IS, any history, or symptoms of cerebrovascular disease were excluded from the control group. At the beginning of the investigation, all enrolled participants have signed informed consent.

Questionnaire

Data on general demography, lifestyle information, physical measurement, history, and duration of the main disease for all participants were obtained. Some physical indicators were measured, such as weight and height. Cigarette smoking was defined according to Doll and Hill.[15] Alcohol drinkers were those who drink alcohol every week in one year.

SNP selection and genotyping

NCBI SNPs-database was used for SNPs selection. The genotyping for the selected four SNPs was conducted using PCR-based restriction fragment length polymorphism. Table 1 shows the primers and restriction endonuclease used for genotyping in current study. All participants were genotyped in a randomized, blinded fashion. A 20-μL reaction volume was used for PCR in the genotyping process. The amplification conditions for rs1764391 and rs1764390 of *CONNEXIN37* gene: amplification for 35 cycles, 94°C for denaturation (1 min), 58°C for annealing (1 min), 72°C for extension (1 min) and final extension (10 min). The amplification conditions for the rs918592 and rs966221 of *PDE4D* gene were: 95°C for initial denaturation (5 min), 94°C for 38 cycles (60 s), 72°C for about 60 s and final extension (10 min). PCR products were digested by the specific restriction endonuclease for about 4 h (37°C), which were shown in Table 1.

Statistical analysis

The percentages were calculated for categorical variables and a chi-squared test was used to test the difference between groups; and means ± standard deviations were calculated for the continuous variables with normal distribution, and *t*-test was used for comparison. The online software SNPstats was used to test the Hardy–Weinberg equilibrium (HWE) in control participants. Logistic regression was used to calculate the ORs (95% CI) for subjects with different genotypes. All *P* values are two-tailed, and considering the multiple tests performed,[16,17] the threshold of significance after Bonferroni correction was set and shown in Table 3. The best interaction combinations were assessed by generalized multifactor dimensionality reduction (GMDR). Some parameters were also calculated to verify which combination was the best one, such as the testing balanced accuracy, the sign test, and cross-validation consistency.

Results

Table 2 shows the description for patients and normal controls on demographic and general clinical characteristics. A total of 1773 participants were selected, including 881 patients with IS and 892 normal controls. The average age for all participants was 65.0 ± 14.2 years. The percentages of participants who consumed alcohol, smoked cigarettes, and with hypertension, T2DM and means durations of hypertension and T2DM were higher in IS patients than in controls.

Table 1. Description and primer sequences designed for sequencing four SNPs.

SNPs	Chromosome	Restriction endonuclease	Major/minor alleles	Primer (5′→3′)
CONNEXIN37 gene				
rs1764391 C1019T	1:34795168	*PspFI*	C/T	Forward: 5′-GTCTTCTTCTACCTCCCCGTG-3′ Reverse: 5′-TTCTCAGGACCCCTCTGTTGG-3′
rs1764390	1:34794360	*BseYI*	A/G	Forward: 5′-TGACGGTGCTCTTCATCTTCC-3′ Reverse: 5′-GGTGTGCTGACGAAGAGGAAC-3′
PDE4D gene				
rs918592 SNP87	5:60401476	*BpmI*	C/T	Forward: 5′-ACGTTGGATGCTCTAACCAAGTGCTTGCTG-3′ Reverse: 5′-ACGTTGGATGTGAGGAAGAATAATGGATGC-3′
rs966221 SNP83	5:60206693	*BstYI*	A/G	Forward: 5′-ACGTTGGATGGTCCTGCTATTAATAGAAAC-3′ Reverse: 5′-ACGTTGGATGTTGGAAGGATCTGCTGCTGG-3′

CONNEXIN37: connexin 37; *PDE4D*: phosphodiesterase 4D; SNP: single nucleotide polymorphism.

Table 2. General characteristics of 1773 study participants in case and control group.

Variables	IS patients ($n = 881$)	Normal controls ($n = 892$)	*P* values
Age (year), mean ± SD	64.5 ± 14.7	65.6 ± 15.3	0.123
Males, *N* (%)	613 (69.6)	608 (68.2)	0.519
Smoking, *N* (%)	323 (36.7)	228 (25.6)	0.000001
Alcohol drinking, *N* (%)	340 (38.6)	274 (30.7)	0.000493
BMI (kg/m^2), mean ± SD	24.3 ± 8.6	23.8 ± 9.1	0.235
Hypertension, *N* (%)	345 (39.2)	287 (32.2)	0.002138
T2DM, *N* (%)	183 (20.5)	100 (11.2)	<0.0001
Duration of hypertension (years), mean ± SD	14.1 ± 5.7	10.6 ± 6.1	<0.0001
Duration of T2DM (years), mean ± SD	11.5 ± 4.6	9.2 ± 5.2	<0.0001

BMI: body mass index; IS: ischemic stroke; SD: standard deviation; T2DM: type 2 diabetes mellitus.
Mean ± standard deviation for age, BMI, duration of hypertension, and T2DM; number and percentages for males, smokers, drinkers, T2DM, and hypertension cases.

Table 3. Association analysis for four target SNPs within *CONNEXIN37* and *PDE4D* gene and IS risk.

SNPs	Genotypes or alleles	Frequencies, *N* (%)		OR (95% CI)*	*P* values	HWE test for controls
		Controls ($n = 892$)	IS cases ($n = 881$)			
CONNEXIN37–rs1764391						
	CC genotype	582 (65.2)	444 (50.4)	1.00 (ref)		0.600
	CT genotype	274 (30.7)	364 (41.3)	1.55 (1.16–1.93)	0.108	
	TT genotype	36 (4.0)	73 (8.3)	2.00 (1.31–2.69)	0.0002	
	C allele	1438 (80.6)	1252 (71.1)	1.00 (ref)		
	T allele	346 (19.4)	510 (28.9)	1.66 (1.21–2.03)	0.0013	
CONNEXIN37–rs1764390						
	AA genotype	546 (61.2)	508 (57.7)	1.00 (ref)		0.469
	AG genotype	299 (33.5)	306 (34.7)	1.20 (0.80–1.81)	0.426	
	GG genotype	47 (5.3)	67 (7.6)	1.51 (0.70–2.32)	0.625	
	A allele	1391 (78.0)	1322 (75.0)	1.00 (ref)	0.538	
	G allele	393 (22.0)	440 (25.0)	1.24 (0.77–1.95)		
PDE4D gene–rs966221 (SNP83)						
	AA genotype	560 (63.6)	454 (51.5)	1.00 (ref)		0.267
	AG genotype	278 (31.6)	341 (38.7)	1.40 (1.08–1.82)	0.0120	
	GG genotype	43 (4.9)	86 (9.8)	1.91 (1.22–2.83)	0.0003	
	A allele	1398 (79.3)	1249 (70.9)	1.00 (ref)		
	G allele	364 (20.7)	513 (29.1)	1.48 (1.11–1.92)	0.0021	
PDE4D gene–rs918592 (SNP87)						
	CC genotype	531 (59.5)	474 (53.8)	1.00 (ref)		0.224
	CT genotype	306 (34.3)	324 (36.8)	1.24 (0.96–1.64)	0.283	
	TT genotype	55 (6.2)	83 (9.4)	1.35 (0.88–1.89)	0.562	
	C allele	1368 (76.7)	1272 (72.2)	1.00 (ref)		
	T allele	416 (23.3)	490 (27.8)	1.29 (0.90–1.68)	0.437	

CONNEXIN37: connexin 37; IS: ischemic stroke; *PDE4D*: phosphodiesterase 4D; SNP: single nucleotide polymorphism.
*Adjusted for age, gender, BMI, smoking, and alcohol drinking; the threshold of significance after Bonferroni correction was set at approximately 0.0125.

The genotype frequencies in the control of the current study were all distributed accordingly to HWE. The frequencies of the *CONNEXIN37*–rs1764391 T allele were 28.9% in patients and 19.4% in controls, which indicating a statistically significant difference. The frequencies of the *PDE4D* gene–rs966221 G allele was 29.1% in IS patients and 20.7% in controls. Logistic regression indicated that rs1764391-T and rs966221-G alleles were correlated with higher IS risk, adjusted ORs (95% CI) were 1.66 (1.21–2.03) and 1.48 (1.11–1.92), respectively (Table 3). We also found that *CONNEXIN37*–rs1764390 and *PDE4D*–rs918592 were not correlated with IS risk significantly.

The SNP–SNP and gene–environment interaction model were determined by GMDR analysis (Table 4). We found significant interaction combinations involving rs1764391 and rs918592, rs1764391, and smoking. We also conducted stratified analysis for interaction effects using logistic regression. We found that participants with rs1764391-CT/TT and rs918592-CT/TT genotype have the highest IS risk, compared to subjects with rs1764391-CC and rs918592-CC genotype, OR (95%CI) = 3.16 (1.83–4.45; Figure 1); in addition, smokers with rs1764391-CT/TT genotype also have the highest IS risk, compared to never smokers with rs1764391-CC genotype, OR (95%CI) = 2.82

Table 4. GMDR analysis for the best interaction combination models.

Locus no.	Best combination	Cross-validation consistency	Testing balanced accuracy	*P* values*
Gene–gene interactions*				
2	1, 3	10/10	0.632	0.010
3	1, 2, 3	7/10	0.496	0.256
4	1, 2, 3, 4	7/10	0.515	0.359
Gene–alcohol drinking interactions**				
2	1, 5	8/10	0.496	0.624
3	1, 2, 5	7/10	0.521	0.746
4	1, 2, 3, 5	5/10	0.524	0.857
5	1, 2, 3, 4, 5	6/10	0.512	0.425
Gene–smoking interactions***				
2	1, 6	10/10	0.607	0.018
3	1, 2, 6	8/10	0.532	0.172
4	1, 2, 3, 6	7/10	0.515	0.324
5	1, 2, 3, 4, 6	8/10	0.512	0.532

BMI: body mass index; GMDR: generalized multifactor dimensionality reduction; T2DM: type 2 diabetes mellitus.
Variables numbered as 1–6 were rs1764391, rs1764390, rs918592, rs966221, alcohol drinking, and smoking, respectively.
*Adjusted for age, gender, BMI, T2DM, hypertension, alcohol drinking, and smoking.
**Adjusted for age, gender, BMI, hypertension, T2DM, and smoking.
***Adjusted for age, gender, BMI, hypertension, T2DM, and alcohol drinking.

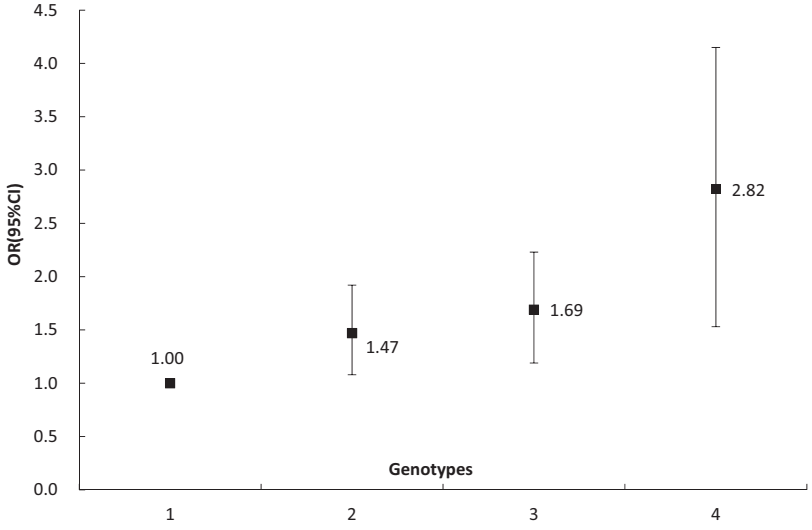

Figure 1. Stratified analysis for gene–gene interaction on IS risk using logistic regression. (1) rs1764391-CC and rs918592-CC; (2) rs1764391-CT/TT and rs918592-CC; (3) rs1764391-CC and rs918592-CT/TT; (4) rs1764391-CT/TT and rs918592-CT/TT.

(1.53–4.15), adjusting for age, gender, and alcohol consumption status (Figure 2).

Discussion

The current study shown that *CONNEXIN37*–rs1764391-T and *PDE4D*–rs966221-T alleles were correlated with higher IS risk. We also found that rs1764390 and rs918592 were not significantly related with the susceptibility to IS; the *CONNEXIN37* gene is located in chromosome lp35.1 and mainly expressed in the vascular endothelium.[18] To date, limited number of studies involved in the relationship between *CONNEXIN37* gene SNPs and IS risk. A 10.7-year cohort study for Chinese Taiwan population indicated that rs1764391 (C1019T) within *CONNEXIN37* gene was associated with increased IS risk,[19] while another case–control study also performed for Chinese Taiwan population showed that rs1764391 (C1019T) polymorphism within *CONNEXIN37* gene was not associated with IS susceptibility.[20] Therefore, the relationship between SNPs within *CONNEXIN37* gene and IS risk remains conflicted and need to be determined in the other studies in Chinese populations. Recently, Li *et al*.[9] concluded that rs1764390-AG or GG and rs1764391-CC genotype of *CONNEXIN37* gene was related with higher IS susceptibility. This article was the first study to investigate the relationship between *CONNEXIN37*–rs1764390 or rs1764391 and IS risk in Chinese mainland population. However, the study by Li *et al*.[9] was just performed in the Chinese Northern Han population; in addition, just 385 IS patients and 362 control patients were selected; the sample size was relatively small. Thirdly, the control group consisted of 362 hypertension

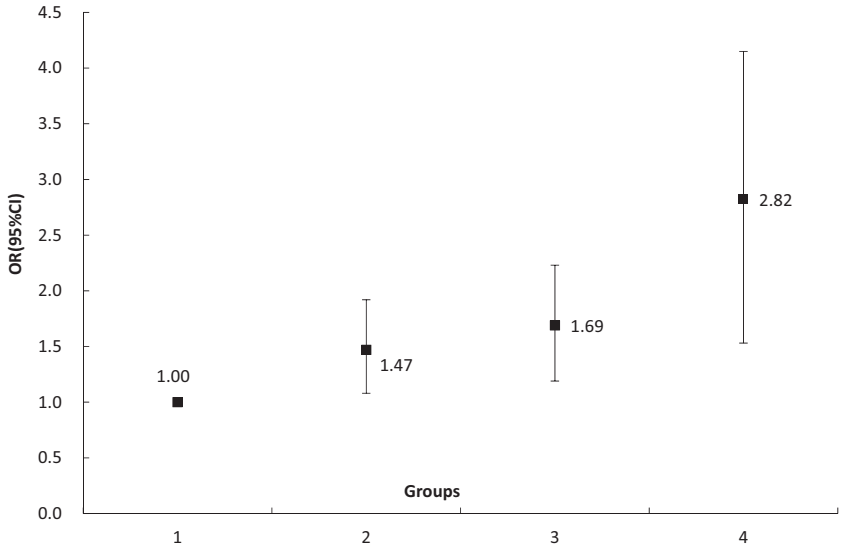

Figure 2. Stratified analysis for gene–smoking interaction on IS risk using logistic regression. (1) Never smokers with rs1764391-CC; (2) never smokers with rs1764391-CT/ TT; (3) smokers with rs1764391-CC; (4) Smokers with rs1764391-CT/TT.

patients; as we all know that hypertension was an important risk factor for IS, which may lead to false positive results. In our study, the normal controls were those without IS and any type of diseases related to IS risk, including hypertension, and the sample size of our study was larger than the study by Li *et al*.[9]

PDE4D gene is located on chromosomal region 5q12, and consists of many SNPs,[21] among which the SNP 83 (rs966221) and SNP 87 (rs2910829) of *PDE4D* gene have been extensively investigated previously. However, these studies[22–27] have not concluded consistent result. In terms of SNP83, some studies[22,23] indicated that SNP83 was not associated with IS risk, but some studies[24,25] also concluded conflict results. A meta-analysis[14] suggested that the *PDE4D* gene was related with IS susceptibility, perhaps in Asian populations, however, this fore-mentioned relationship was still controversial in Caucasian populations. In terms of SNP87, most studies[26,27] have indicated that this SNP was not associated with IS risk, except for one study conducted by He *et al*.[28] in Chinese young populations.

The IS incident could be caused not only by both the genetic and environmental factors, but also by the gene–environment interactions.[4] Previous studies have concluded that gene–gene,[29,30] gene–environment[29] interaction could influence the IS risk in different populations and involved different gene. Our study was the first to test the impact of interaction between *CONNEXIN37* and *PDE4D* gene, in addition gene–environmental factors interaction on IS risk. This study concluded not only a significant rs1764391 and rs918592 interaction, although the relationship between rs918592 and IS was not significant, but also an rs1764391 and smoking interaction. Previously, *CONNEXIN37* gene–smoking interaction just was reported in relation to ischemic heart disease,[31] to the best of our knowledge, the current study firstly investigated the impact of *CONNEXIN37* gene–smoking interaction on susceptibility to IS.

The limitations of this study were: firstly, we only examined the Han Chinese patients in Western China, different ethnic or social and economical background should be checked. Secondly, just four SNPs were studied, these four SNPs were normal and more investigated previously, especially for *PDE4D* gene, so more SNPs should be verified in the future studies and in the different populations.

In conclusion, we suggested that the rs1764391-T and rs966221-G alleles, rs1764391–rs918592 interaction, rs1764391–smoking interaction were all correlated with higher IS risk. The main finding of this study was that the minor alleles of rs1764391 and rs966221 were risk factors for IS incidence; in addition, the association between rs1764391 and ID risk could be influenced and modified by some others SNPs, such as rs918592, and by smoking status. In future, the results obtained in current study should be verified in different population and races, and more interacted SNPs within different genes and more interacted environmental factors.

Author contributions: All authors participated in the design, analysis of the data and review of the manuscript; LZ, HJ, PK, and LW performed the experiments, HD contributed the manuscript errors revision. LZ and HJ wrote the manuscript, and QX contributed data analysis and statistical analysis.

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DECLARATION OF CONFLICTING INTERESTS

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