Acta Pharmacologica Sinica (2011) 32: 1357–1363
 1671-4083/11 $32.00
www.nature.com/aps

Original Article

Polymorphisms of genes in nitric oxide-forming pathway associated with ischemic stroke in Chinese Han population

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Aim: To investigate the association of polymorphisms in four critical genes implicated in the NO-forming pathway with ischemic stroke (IS) in a Chinese Han population.
Methods: DNA samples of 558 IS patients and 557 healthy controls from Chinese Han population were genotyped using the Taqman™ 7900HT Sequence Detection System. Six SNPs (rs841, rs1049255, rs2297518, rs1799983, rs2020744, rs4673) of the 4 related genes (eNOS, iNOS, GCH1, and CYBA) in the NO forming pathway were analyzed using the SPSS 13.0 software package for Windows.
Results: One SNP located in the intron of GCH1 (rs841) was associated with IS independent of the traditional cardiovascular risk factors in co-dominant and dominant models (P=0.003, q=0.027; P=0.00006, q=0.0108; respectively). Moreover, the combination of rs1049255 CC+CT and rs841 GA+AA genotypes was associated with significantly higher risk for IS after adjustments (OR=1.73, 95% CI: 1.27–2.35, P<0.0001, q<0.0001).
Conclusion: The data suggest that genetic variants within the NO-forming pathway alter susceptibility to IS in Chinese Han population. Replication of the present results in other independent cohorts is warranted.

Keywords: ischemic stroke; nitric oxide; polymorphism; genetics; Chinese Han population

Acta Pharmacologica Sinica (2011) 32: 1357–1363; doi: 10.1038/aps.2011.114; published online 3 Oct 2011

Introduction

Ischemic stroke (IS), known to be a multifactorial disorder, is the leading cause of disability and the second leading cause of death in China[1]. IS usually results from hypertension, atherosclerosis, diabetes, smoking, vasculitis or other etiologies. Beyond the conventional risk factors, evidence is accumulating that genetic factors may also contribute to the risk of stroke development[2].

Nitric oxide (NO) is synthesized by the enzyme nitric oxide synthase (NOS) from *L*-arginine and oxygen in endothelial cells, neurons, glia and macrophages[3]. NO plays an important role in the control of cerebral blood flow, thrombogenesis, and the modulation of neuronal activity[4]. High concentrations of NO originating from cerebral ischemia mediate inflammatory and cytotoxic pathways leading to neuronal death[5]. NO is also important to protect vessels against atherosclerosis[6]. NO bioavailability is tightly regulated by a balance between its production and detoxification or degradation, and therefore, cerebral ischemia could be related to abnormalities in the expression and activity of NOS.

Several potential functional polymorphisms in the nitric oxide-forming pathway have recently been discovered, including (1) Leu608Ser (rs2297518) in inducible [*iNOS*][7], (2, 3) Glu298Asp (rs1799983) and T-786C (rs2070744) in the promoter region of endothelial [*eNOS*][8–12], (4, 5) Tyr72His (rs4673) and C+640T (rs1049255) in the 3′-untranslated region (UTR) of the cytochrome b-245, alpha polypeptide gene (*CYBA*)[13–16], which encodes the p22*phox* subunit of the NADPH oxidase, and (6) G+243A (rs841) in the 3′-UTR of the GTP cyclohydrolase 1 gene (*GCH1*)[17]. With special attention to the biological process of cerebral ischemia regulation, we investigated whether polymorphisms in these genes implicated in the pathway of NO formation are associated with IS in a large cohort in the Chinese Han population.

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Received 2010-11-11 Accepted 2011-07-17

Materials and methods

Study population and data collection

This was a multicenter, collaborative study for assessment of risk factors for stroke sponsored by the Ministry of Science and Technology of China. The study protocol was approved by the review board of Tongji Medical College at Huazhong University of Science and Technology and the ethics committees at all participating hospitals. An informed consent form was obtained from all participants.

A total of 558 IS patients were recruited between November 2004 and June 2006 from five hospitals in Wuhan, China. Only 2 subtypes of stroke — cerebral thrombosis (atherothrombosis) and lacunar infarction (lacunar) —were included. Subjects with subarachnoid hemorrhage, embolic brain infarction, brain tumors and cerebrovascular malformation were excluded from the study, as were those with severe systemic diseases such as pulmonary fibrosis, endocrine and metabolic disease (except diabetes mellitus), severe inflammatory diseases, autoimmune disease, tumors and serious chronic diseases (*eg*, hepatic cirrhosis and renal failure). Subjects with cardioembolic stroke and documented atrial fibrillation were also excluded from our study. Stroke diagnosis was based on the results of neurological examination and CT or MRI according to the International Classification of Diseases, ninth edition. Five hundred fifty-seven ethnically and geographically matched controls were randomly selected either from normal individuals of nearby community-based residents (89.6%) or inpatients (10.4%) with minor illnesses. All control subjects were free of neurological diseases following the same exclusion criteria as cases. They were also asked for a detailed medical history and received a physical examination of neurological systems, including an evaluation of body mass index.

DNA isolation and genotyping

DNA was extracted from leukocytes as previously described[18]. All samples were genotyped using the Taqman™ 7900HT Sequence Detection System according to the manufacturer's instructions. Each assay was conducted using 10 ng DNA in a 5 μL reaction consisting of TaqMan™ universal PCR master mix (Applied Biosystems, Foster City, CA, USA), forward and reverse primers and FAM and VIC labeled probes designed by Applied Biosystems [ABI Assays-on-Demand (rs841, C_9866639_10; rs1049255, C_7516913_10; rs2297518, C_11889257_10; rs1799983, C_3219460_20; rs4673, C_2038_20) and Assays-on-Design (rs2070744)]. Allelic discrimination was measured automatically using the Sequence Detection Systems 2.1 software (autocaller confidence level 95%). A total of 10% of all genotypes were repeated in independent PCRs to check for consistency and to ensure intraplate and interplate genotype quality control. No genotyping discrepancies were detected between the repeated samples. In addition, all the DNA samples for cases and controls were run in the same batches.

Statistical analysis

Statistical analysis were performed with the SPSS 13.0 software package for Windows (SPSS Inc, Chicago, IL, USA). The normality of quantitative variable distribution was assessed using the 1-sample Kolmogorov-Smirnov test, and a transformation was applied to non-normal variables when necessary. Summary statistics were expressed as the mean±standard error or as percentages. The χ^2 test was used to assess the deviation from Hardy-Weinberg equilibrium for genotype frequencies in both cases and controls. Continuous variables were compared between cases of stroke and controls using Student's *t*-test. Frequencies of categorical variables were compared by χ^2 test or Fisher's exact test. The potential independent role of each single-nucleotide polymorphism (SNP) on stroke was investigated with multiple unconditional logistic regression analysis adjusted for age, sex, body mass index, hypertension, hyperlipidemia, diabetes mellitus and smoking status. To minimize the false positive results generated from the multiple statistical tests used in our analysis, we adopted a method proposed by Story and Tibshirani to estimate the FDR (false discovery rate)-based *q* value using QVALUE software (setting [lambda]=0, false discovery rate level<0.05)[19]. All association analyses were conducted in three genetic models: co-dominant, dominant and recessive. Power calculations were performed using the QUANTO software program[20] (Version 1.2.3).

Results

Baseline characteristics of the subjects

Table 1 summarizes the clinical characteristics of individuals enrolled in the study. The mean age, gender ratio and total cholesterol level were similar in cases and controls. Expectedly, there were significantly higher percentages of hypertension, diabetes mellitus, hyperlipidemia, and smoking in overall IS and subtype groups versus the controls (P<0.05). Body mass index, systolic blood pressure, and diastolic blood pressure were also higher in cases than controls. Compared to the control group, patients in both the overall IS and subtype groups had significantly lower HDL cholesterol (P<0.05).

Genotypes in relation to ischemic stroke and its subtypes

Next, we assessed associations between six SNPs from four related genes (three at two isoforms of NOS, one at GCH1, and two at CYBA) and IS in the Chinese Han population. All genotype distributions were consistent with Hardy-Weinberg equilibrium (P>0.05). Table 2 lists single SNP allelic frequencies of the four genes among IS subjects (n=558) and control subjects (n=557). Mutiplicative-type corrections such as Bonferroni corrections for correlated genetic factors and tests are highly conservative. Therefore, we present the *q* value, a measure of false discovery rate expected for a given *P* value in the follow-up analysis. Notably, rs841 in the intron region of *GCH1* demonstrated allelic frequency differences between overall IS and lacunar stroke subjects compared with controls (P=0.001 and 0.002, respectively), which maintained statistical significance after multiple comparison correction (q=0.018 for both) (Table 2).

To further investigate how each of the SNP alleles interact in conferring genetic risk for IS, we conducted a genotypic

Table 1. Baseline characteristics of patients.

Variable	Control	Ischemic stroke	Atherothrombosis	Lacunar infarction
n	557	558	410	148
Age, year	62.2±9.3	61.0±9.8	60.0±10.0[b]	64.1±8.2[b]
Men, %	62.1	64.7	65.4	62.8
BMI, kg/m^2	23.7±3.2	24.5±3.7	24.4±3.4	24.7±4.4
SBP, mmHg	131.3±20.8	146.6±23.3[b]	147.1±24[b]	144.9±20.9[b]
DBP, mmHg	78.8±11.1	86.4±13.9[b]	87±14.2[b]	84.9±12.9[b]
TCH, mmol/L	4.6±1.7	4.6±1.1	4.6±1.1	4.6±1.1
HDL-C, mmol/L	1.3±0.4	1.0±0.5[b]	1.0±0.3[b]	1.2±0.9
Hypertension, %	19.2	69.7[b]	69.0[b]	71.6[b]
Diabetes, %	3.2	18.0[b]	19.8[b]	12.8[b]
Hyperlipidemia, %	21.0	35.0[b]	35.5[b]	33.8[b]
Smoking, %	37.3	46.9[b]	45.8[b]	50.0[b]

n, number of individuals; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TCH, total cholesterol; HDL-C, high-density lipoprotein cholesterol. [b]*P*<0.05 *vs* control.

Table 2. Allele distribution of each polymorphism.

SNP ID	Function (M>m)	Gene	Position	Population	MAF	P_{allele}
rs1799983	(D298E) G>T	eNOS	Chr7:150327044	Control	0.108	
				Ischemic stroke	0.119	NS
				Atherothrombosis	0.116	NS
				Lacunar infarction	0.128	NS
rs2070744	intron T>C	eNOS	Chr7:150321012	Control	0.104	
				Ischemic stroke	0.113	NS
				Atherothrombosis	0.120	NS
				Lacunar infarction	0.095	NS
rs2297518	(L608S) G>A	iNOS	Chr17:23120724	Control	0.169	
				Ischemic stroke	0.151	NS
				Atherothrombosis	0.132	0.025
				Lacunar infarction	0.206	NS
rs841	intron G>A	GCH1	Chr14:54380242	Control	0.311	
				Ischemic stroke	0.377	0.001[b]
				Atherothrombosis	0.367	0.009
				Lacunar infarction	0.405	0.002[b]
rs1049255	3′-UTR C>T	CYBA	Chr16:87237238	Control	0.428	
				Ischemic stroke	0.389	NS
				Atherothrombosis	0.399	NS
				Lacunar infarction	0.361	0.038
rs4673	(Y72H) G>A	CYBA	Chr16:87240737	Control	0.078	
				Ischemic stroke	0.064	NS
				Atherothrombosis	0.068	NS
				Lacunar infarction	0.051	0.012

MAF, minor allele frequency; P_{allele}, value of allele was determined by χ^2 test; NS, not significant. [b]FDR *q* value<0.05.

association analysis assuming 3 common genetic models (co-dominant, dominant and recessive). Interestingly, SNP rs841 showed consistent effects using both a co-dominant (without adjustment for covariates, *P*=0.001, *q*=0.009; after adjustment

for traditional risk factors, P=0.003, q=0.027) and a dominant model (without adjustment for covariates, P=0.0003, q=0.0054; after adjustment for traditional risk factors, P=0.0006, q=0.0108) (Table 3).

To test the possible effect of the IS subtypes in detecting an association, we then re-assessed the association between rs841 and the cerebral thrombosis and lacunar infarction groups (Supplementary Tables 1 and 2). It is of interest to note that rs841 was significantly associated with both subtypes (nominal P<0.05). However, none of these results pass the significance threshold after multiple corrections. These negative results could be due to reduced sample size and statistical power for subtype analysis.

Association of genotype combinations with ischemic stroke

We applied the logistic regression analyses to test potential interactions among polymorphisms within genes involved in the NO-forming pathway that may confer IS risk and identified significant interactions between rs1049255 and rs841 (P<0.001). In comparison with the reference combination of rs1049255 CC+CT and rs841 GG wild type genotypes, the combination of the rs1049255 CC+CT genotype together with the rs841 GA+AA genotype was found to be significantly associated with IS (P=0.002, q=0.008; Table 4). The distribution combinations of other genotypes did not differ from the wild-type reference in overall IS. The combination of rs1049255 CC+CT and rs841 GA+AA genotypes was associated with significantly higher risk of IS even after adjustment for sex, age, and multiple cardiovascular risk factors (OR=1.73, 95% CI: 1.27–2.35; P<0.0001, q<0.0001) (Table 5). Given that the reduced sample size for IS subtypes resulted in largely insufficient power to test potential interactions, subtype analyses were not performed. Taken together, our data suggest an interaction of NO-forming pathway genes in the risk of IS.

Discussion

This study is to investigate the association between genetic polymorphisms in the genes implicated in NO production and risk for IS development in the Chinese Han population. The present results indicate that polymorphisms in *GCH1* (rs841) are independently associated with an increased risk for IS. In contrast, we failed to detect significant independent association with the rest of SNPs even though they have been suggested to be associated with cardiovascular diseases such as hypertension, coronary heart disease or stroke[21–23].

Nitric oxide, produced by nitric oxide synthase, is an important bioregulatory molecule and displays diverse biological activities. Tetrahydrobiopterin (BH4) is an essential cofactor for all three NOS isoforms, and basal enzyme activity correlates with the amount of BH4 bound tightly to NOS[24]. BH4 deficiency is proposed to lead to NOS uncoupling associated with decreased NO bioavailability and increased production of superoxide radicals from the uncoupled enzymatic form[25]. GTP cyclohydrolase 1 (GCH1) is the first-step and rate-limiting enzyme for BH4 biosynthesis in its *de novo* pathway[26]. Experimental mouse models with alterations in systemic or vascular-specific *GCH1* expression have shown that GCH1 is a key regulator of vascular BH4 levels *in vivo*[27, 28]. Low brain levels of BH4 have been shown in the mouse model for dominantly inherited GCH1 deficiency[29]. Recent evidence suggests that one SNP (rs841), located in the 3'-UTR of the *GCH1* gene, is also associated with reduced biopterin-dependent effects[17]. The *CYBA* gene, located on the long arm of chromosome 16 at position 24, encodes human p22*phox,* which is an essential subunit for the functionality of the NADPH oxidase[30]. NADPH oxidase is an important enzymatic source of oxidative stress as well as uncoupled NOS caused by BH4 deficiency and plays a key role in the pathophysiology of several major cardiovascular diseases, including stroke[31]. Previous studies have shown the association between CYBA polymorphisms and vascular diseases[30]. Our combined analysis identified *GCH1* (rs841) and *CYBA* (rs1049255) interactions, indicating that these two functional polymorphisms may confer risk for IS through biological interactions with each other. Further studies might be required to ascertain whether *CYBA* (rs1049255) affects *GCH1* expression and how such an effect might be mediated.

To control for potential false-positive results, we took several factors into consideration and carefully designed our study. First, all selected candidate SNPs have substantial functional effects that are likely involved in the development of IS. Second, assuming disease prevalence between 0.5% and 1%, our combined sample size can reach >98% power to detect a susceptibility locus with a genotypic relative risk >1.65 at the nominal type I error rate of <0.05 for SNPs with minor allele frequencies >0.31 under the dominant model. Third, we recruited only ethnically and geographically matched subjects from Chinese Han cohorts. Given the homogenous study population, we expect population substructure to be minimal. However, additional replication of the association signals in other independent cohorts is warranted.

In summary, genetic variants in the genes implicated in NO formation could have potentially important effects on the pathogenesis of vascular diseases, and genotyping of these variants may provide an additional tool to predict the risk for ischemic stroke in the Chinese Han population.

Acknowledgements

This study was supported by China 973 program (No 2007CB512004), and 863 program (No 2006AA02A406).

Author contribution

Jiang-tao YAN, Lan ZHANG and Yu-jun XU performed the research; Xiao-jing WANG and Cong-yi WANG contributed new analytical tools and reagents; Jiang-tao YAN and Lan ZHANG analyzed the data; and Jiang-tao YAN, Lan ZHANG and Dao-wen WANG wrote the paper.

Supplementary information

Supplementary tables are available at Acta Pharmacologica Sinica website of NPG.

Table 3. Genotype distribution in three models in ischemic stroke.

SNP	Genotype	Controls *n*=557	*n*=558	Crude odd ratio	95% CI	*P* value	Ischemic stroke Adjusted odd ratio	95% CI	*P* value
rs1799983	Co-dominant					0.139			0.219
	GG	446	417	1.00			1.00		
	GT	102	123	1.29	0.96–1.73		1.29	0.91–1.85	
	TT	9	5	0.59	0.20–1.79		0.53	0.14–2.05	
	Dominant					0.165			0.242
	GG	446	417	1.00			1.00		
	GT+TT	111	128	1.23	0.93–1.64		1.23	0.87–1.74	
	Recessive					0.421			0.312
	GG+GT	548	540	1.00			1.00		
	TT	9	5	0.54	0.12–1.71		0.51	0.13–1.93	
rs2070744	Co-dominant					0.009			0.126
	TT	451	434	1.00			1.00		
	TC	96	122	1.32	0.98–1.78		1.30	0.91–1.87	
	CC	10	2	0.21	0.05–0.95		0.31	0.05–2.03	
	Dominant					0.187			0.269
	TT	451	434	1.00			1.00		
	TC+CC	106	124	1.22	0.91–1.63		1.22	0.86–1.74	
	Recessive					0.015			0.146
	TT+CT	547	556	1.00			1.00		
	CC	10	2	0.20	0.04–0.9		0.29	0.04–1.93	
rs2297518	Co-dominant								
	GG	382	400	1.00		0.520	1.00		0.985
	GA	162	147	0.87	0.67–1.13		0.99	0.72–1.36	
	AA	13	11	0.81	0.36–1.83		1.08	0.41–2.86	
	Dominant					0.258			0.98
	GG	382	400	1.00			1.00		
	GA+AA	175	158	0.86	0.67–1.11		1.00	0.73–1.36	
	Recessive					0.676			0.869
	GG+GA	544	547	1.00			1.00		
	AA	13	11	0.84	0.37–1.89		1.09	0.41–2.86	
rs841	Co-dominant					0.001[b]			0.003[b]
	GG	267	208	1.00			1.00		
	GA	234	279	1.53	1.19–1.97		1.67	1.23–2.27	
	AA	56	71	1.63	1.10–2.41		1.60	0.99–2.59	
	Dominant					0.0003[b]			0.0006[b]
	GG	267	208	1.00			1.00		
	GA+AA	290	350	1.55	1.22–1.97		1.65	1.24–2.21	
	Recessive					0.160			0.381
	GG+GA	501	487	1.00			1.00		
	AA	56	71	1.30	0.90–1.89		1.23	0.78–1.93	
rs1049255	Co-dominant					0.103			0.102
	CC	188	206	1.00			1.00		
	CT	261	270	0.94	0.73–1.23		0.96	0.70–1.31	
	TT	108	82	0.69	0.49–0.98		0.65	0.42–0.99	
	Dominant					0.269			0.329
	CC	188	206	1.00			1.00		
	CT+TT	369	352	0.87	0.68–1.11		0.86	0.64–1.16	
	Recessive					0.037			0.034
	CC+CT	449	476	1.00			1.00		
	TT	108	82	0.72	0.52–0.98		0.66	0.45–0.97	

(To be continued)

SNP	Genotype	Controls *n*=557	Ischemic stroke *n*=558	Crude odd ratio	95% CI	*P* value	Adjusted odd ratio	95% CI	*P* value
rs4673	Co-dominant					0.211			0.37
	GG	471	487	1.00			1.00		
	GA	85	71	0.81	0.58–1.13		0.77	0.50–1.16	
	AA	1	0	/	/		/	/	
	Dominant					0.192			0.199
	GG	471	487	1.00			1.00	0.50–1.16	
	GA+AA	86	71	0.80	0.57–1.12		0.76		
	Recessive					0.499			0.516
	GG+GA	556	558	1.00			1.00		
	AA	1	0	/	/		/	/	

CI, confidence interval; SNP, single-nucleotide polymorphism. Adjusted odd ratios were adjusted for gender, age, body mass index, hypertension, diabetes, hyperlipidemia and smoking status. [b]FDR *q* value<0.05.

Table 4. Distribution of combined genotypes in overall ischemic stroke.

Genotype		Ischemic stroke	Control	*P* value
rs1049255	rs841	(*n*=558)	(*n*=557)	
CC+CT	GG	175	210	reference
CC+CT	GA+AA	301	239	**0.002**
TT	GG	33	57	0.130
TT	GA+AA	49	51	0.526

Table 5. Analysis of multiple logistic regression model for synergism genotypes in ischemic stroke group.

Variable	Adjusted OR	95% CI		*P* value
		Lower	Upper	
Gender (m/f)	0.58	0.39	0.87	0.01
Age (*n*)	0.96	0.95	0.98	<0.0001
Hypertension (y/*n*)	7.20	5.11	10.16	<0.0001
Diabetes (y/*n*)	3.36	1.79	6.32	<0.0001
Hyperlipidemia (y/*n*)	1.48	1.02	2.15	0.04
Smoking (y/*n*)	2.18	1.49	3.18	<0.0001
rs1049255(CC+CT)* rs841(GA+AA)	1.73	1.27	2.35	<0.0001

OR, odd ratio; Variables included in the model were gender, age, body mass index, hypertension, diabetes, hyperlipidemia and smoking status. Nonsignificant variables (*P*>0.05) have not been shown in the table.

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