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ORIGINAL RESEARCH

A Polymorphism Within the 3′UTR of *NLRP3* is Associated with Susceptibility for Ischemic Stroke in Chinese Population

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Abstract Stroke was regarded as a severe disorder with high morbidity and high mortality worldwide, ischemic stroke (IS) accounts for 85 to 90 % of new increased stroke cases. Partial mechanisms were elucidated by genetic factors including genomic instability such as single nucleotide polymorphism (SNP). Previous reports demonstrated that inflammation was involved in IS, *NLRP3* [nucleotide-binding domain (NOD)-like receptor protein 3], acting as a specific inflammatory gene, however, its function and influence on IS was not well clarified. In this study, a case–control study including 1102 IS patients and 1610 healthy controls was conducted to investigate the association between IS susceptibility with a SNP (rs10754558) in 3′UTR of *NLRP3*. Logistic regression analysis showed that the heterozygote and the homozygote GG confer a significantly increased risk of CRC after controlling for other covariates (adjusted OR = 1.52, 95 % C.I. 1.19–1.97, $P = 0.002$; adjusted OR = 2.22, 95 % C.I. 2.18–3.67, $P < 0.001$, respectively). Carriage of G allele was associated with a greatly increased risk of developing the disease (OR = 1.69, 95 % C.I. 1.31–1.83, $P < 0.001$). Stratification analysis found that hypertension had interaction with rs10754558 to modulate IS risk. Further in vitro assay revealed that rs10754558 can affect mRNA level of *NLRP3*, suggesting its possible functional significance. Our data suggested that genetic polymorphisms in *NLRP3* may influence IS risk in Chinese population. Replication of our studies in other populations and further functional studies are required for complete comprehension of the roles of *NLRP3* polymorphisms in IS risk.

Keywords Ischemic Stroke · *NLRP3* · rs10754558 · 3′UTR

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Introduction

Stroke was regarded as a severe disorder with high morbidity and high mortality, it claims over 6 million deaths each year worldwide, while developing countries such as China contributes the majority of death toll (Donnan et al. 2008; Matarin et al. 2010). As far as we know, the prevalence rate has been growing in China, ischemic stroke (IS) accounts for 85–90 % of new increased stroke cases (Adams et al. 2007; Feigin et al. 2014). Multiple elements are correlated with its pathogenesis, which include environmental factors and genetic profiles (Donnan et al. 2008; Dichgans 2007). Although Genome-wide association study (GWAS) was widely conducted in IS susceptibility-

associated SNPs identification during a decade or two, coverage of the human genome with genome-wide studies is neither equal across regions of the genome nor comprehensive (Kubo et al. 2007; Yamada et al. 2009; Hata et al. 2007; Matarin et al. 2008). Many previously associated candidate genes such as ACE are not adequately covered by the currently commercially available GWAS platforms, which leads to incomplete understanding of the exact genetic susceptibility for IS.

Recently, the correlation between inflammation and IS has attracted much attention (Ahmad et al. 2014; Fann et al. 2013). Inflammation contributes to ischemic events through the promotion of atherosclerosis, Inflammatory genes may thereby influence the incidence and outcome of IS (Tong et al. 2015). Inflammasome pattern recognition receptors, which belong to the family of multimeric proteins, play an important role in innate immunity, including NLRPs, NLRC, and NAIP. Among these receptors, NLRP3 (nucleotide-binding domain (NOD)-like receptor protein 3) inflammasome may activate the inflammation and participate in atherosclerosis (Kristiina et al. Kristiina et al. 2010), pathophysiology of myocardial infarction (Takahashi 2014), result in cardiovascular diseases such as IS. The gene *NLRP3* was involved in human acute immune and inflammatory response, inadequate or excessive reaction will cause damage to the human body. Innate immunity plays an important role in inflammation-related neuronal injury, which is associated with IS. Several investigations showed that the polymorphism within the 3′UTR of *NLRP3* rs10754558 was associated with multiple diseases, and these diseases mainly resulted from inflammation (Zhang et al. 2014; Tan et al. 2013; Kamada et al. 2014). Furthermore, inflammation-associated genes including *NLRP3* were known to influence IS. The polymorphism rs10754558 was located in potentially functional region-3′UTR of *NLRP3*, which may indicate a possible manner for *NLRP3* expression modulation in IS. Thus, previous reports refereed above encouraged us to speculate that the polymorphism modulates IS risk, its function may be based on influence of *NLRP3* expression.

To our knowledge, there were no well-conducted studies on the relationship between *NLRP3* rs10754558 and IS, although the evidence indicated that they may have an association. In our study, polymerase chain reaction with sequence-specific primers (PCR-SSP) was used for *NLRP3* rs10754558 genotyping and that whether the gene variant was associated with IS in a Chinese population was also validated, furthermore, qPCR was conducted in PBMCs of enrolled cases to demonstrate the genotype-phenotype connection. Here, our research group reported that *NLRP3* rs10754558 was associated with the risk of IS, genotyping subgroups also showed dose-dependent effect in transcription level of blood and it may be a potentially promising marker for IS susceptibility. Our investigation may serve to high-risk populations identification and early prevention for IS patients.

Materials and Methods

All subjects recruited were unrelated ethnic Han Chinese. The case series consisted of 597 IS patients diagnosed, hospitalized and treated in ward and outpatient department of Emergency of the Suzhou Kowloon Hospital Shanghai Jiao Tong University School of Medicine from 2013 to 2015, and 505 IS patients diagnosed, hospitalized and treated in ward and outpatient department of Neurology of the Beijing 302 Military Hospital of China. All subjects underwent a standardized clinical evaluation. We included stroke patients from outpatients department as well as from neurology ward (where severely disable patients are more in number) to deal with survival bias. Stroke was classified as per TOAST classification (Adams et al. 1993). A total of 1610 controls were stroke-free individuals selected from community nutritional surveys that were conducted in the same regions during the same period as recruitment of IS patients, which consisted of 843 healthy controls from Suzhou Kowloon Hospital and 767 healthy controls from Beijing 302 Military Hospital of China. Informed consent was obtained from all individual participants each patient/control or from the relatives or a legal representative in the case of critically disabled patients. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Selection of Cases and Controls

Inclusion criteria for stroke patients were (a) diagnosis of stroke as defined by WHO (rapidly developing clinical symptoms and/or signs of focal (at times global) disturbance of cerebral function, with symptoms lasting more than 24 h with no apparent cause other than that of vascular origin; (b) age (18–85 years); (c) NCCT-head consistent with ischemic stroke; (d) birth place of subjects and their ancestors (2 generation) from mainland China. For this study patients and controls were recruited from Xuzhou and Beijing; (e) no evidence of trauma and brain tumor/metastases; and (f) willingness to provide informed consent by self or legal representative. Selection criteria for controls were same as cases except that they were stroke-free determined by means of the questionnaire for verifying stroke-free status (QVSS) (Meschia et al. 2000).

Questionnaire and Definition of Variables

Demographic characteristics and risk factors were collected in standardized data collection form using a structural questionnaire. Risk factors included hypertension, diabetes, dyslipidemia, smoking, first generation family history of stroke, body mass index (BMI).Definitions of variables were modified from the study (Feigin et al. 1998) and are as follows: Hypertension: subjects were considered to have hypertension if they either had the diagnosis of hypertension or were treated for hypertension before the stroke or reference date. Diabetes: if a subject had the diagnosis documented by a physician on the medical record or if fasting blood sugar level was ≥126 mg/dl. Dyslipidemia: if they either had the diagnosis of dyslipidemia or were treated for dyslipidemia before the stroke or reference date. Smoker: a person was defined as regular smoker if smoking ≥1 cigarette(s) daily, and biris and cigar for preceding ≥3 months. Body Mass Index: BMI were calculated by weight in kilograms divided by the square of height in meters (Table 1).

DNA Extraction and Genotyping

A Chelex method was used for extracting genomic DNA of human peripheral blood samples (Walsh et al. 1991). Genotyping was applied by polymerase chain reaction with sequence-specific primers (PCR-SSP). All primers for the PCR-SSP were designed using the genomic sequences in the GenBank (http://www.ncbi.nlm.nih.gov). The primer sequences of the polymorphism are listed in Table 2. PCR amplification was performed with a total of 30 μL reaction mixture containing 20 ng DNA, 0.5 μM of each primer, 2.5 μM dNTP, 2.0 Mm $MgCl_2$ and 1.25 U Taq DNA polymerase with 1 × reaction buffer (Takara). The PCR protocol included an initial denaturation step at 95 °C for 5 min; 30 cycles of 1 min denaturation step at 95 °C, 45 s annealing step at appropriate temperatures and 1 min extension step at 72 °C, and with 10 min final extension step at 72 °C. The amplified products were assessed for the presence/absence of PCR specific to the particular alleles using a standard 2 % agarose gel electrophoresis followed by ethidium bromide staining. 10 % samples were then confirmed by sequencing, and the reproducibility was 100 %.

RNA Extraction and qPCR

PBMCs were separated from human peripheral blood samples by Ficoll/Histopaque (Sigma–Aldrich). Total RNA was extracted using the TaKaRa RNAiso Reagent (Takara)

Table 1 Demographic characteristics of enrolled subjects in two groups

Characteristics	Case		Control		*P* value
	n	Frequencies (%)	*n*	Frequencies (%)	
Suzhou group					
Age (mean ± SD)	53.9 ± 13.4		53.6 ± 12.9		Matched
BMI (kg/m^2)	25.1 ± 3.6		24.9 ± 3.3		0.21[a]
Sex					
Male	488	81.7	689	81.7	Matched
Female	109	18.3	154	18.3	
Smoking status					
Smokers	268	44.9	202	24.0	<0.001[b]
Nonsmokers	329	55.1	641	76.0	
Hypertension status					
Yes	371	62.1	184	21.8	<0.001[b]
No	226	37.9	659	78.2	
Family history of IS					
Yes	141	23.6	65	7.7	<0.001[b]
No	456	76.4	778	92.3	
Diabetes					
Yes	187	31.3	150	17.8	<0.001[b]
No	410	68.7	693	82.2	
Dyslipidemia					
Yes	258	43.2	162	19.2	<0.001[b]
No	339	56.8	681	80.8	
Beijing group					
Age (mean ± SD)	54.1 ± 11.2		53.8 ± 13.1		Matched
BMI (kg/m^2)	25.2 ± 2.9		25.1 ± 3.1		0.31[a]
Sex					
Male	403	79.8	612	79.8	matched
Female	102	20.2	155	20.2	
Smoking status					
Smokers	241	47.7	198	25.8	<0.001[b]
Nonsmokers	264	52.3	569	74.2	
Hypertension status					
Yes	307	60.8	178	23.2	<0.001[b]
No	198	39.2	589	76.8	
Family history of IS					
Yes	113	22.4	50	6.5	<0.001[b]
No	392	77.6	717	93.5	
Diabetes					
Yes	167	33.1	116	15.1	<0.001[b]
No	338	66.9	651	84.9	
Dyslipidemia					
Yes	225	44.6	144	18.8	<0.001[b]
No	280	55.4	623	81.2	

SD standard deviation

[a] Two-sided two-sample *t* test between cases and controls

[b] χ^2 test for differences between cases and controls

Table 2 Primers for *NLRP3* rs10754558 polymorphism genotyping

Primer name	Sequence5′–3′
Internal control reverse primer	TAGTCAACTAGTTCTGT GTTATGGTCA
Common forward primer	ATCCAGGCCAAGACCACAG
Specific primer G (reverse)	AACTGAGGCGCTGTGATGAC
Specific primer C (reverse)	AACTGAGGCGCTGTGATGAG
Forward primer (for real-time PCR)	TTGTGACACAGAGGAGCCTG
Reverse primer (for real-time PCR)	CCTCGTTCTCCTGAATCAGAC

from PBMCs and cDNA was obtained using the Prime-Script II 1st strand cDNA synthesis kit(Takara). qPCR assay was performed to detect the expression of PBMCs in three genotype groups for the relative quantitative analysis, primers for qPCR are listed in Table 2.

Statistical Analysis

Allele frequencies and genotype distributions of *NLRP3* rs10754558 in different groups were compared using Chi-square test. Conditional logistic regression model was used to estimate OR and 95 % confidence intervals (CI) for association of *NLRP3* genotype with ischemic stroke. Multivariate logistic regression analysis was used to exclude the effect of possible confounding factors. Comparison between the mean value of *NLRP3* level and *NLRP3* genotypes was analyzed using the ANOVA test. Odds ratios with 95 % CI were taken to estimate the association of the specific genotype with stroke and were considered significant at $P < 0.05$. Chi-square test was used to determine the frequency distribution of genotypes in accordance with Hardy–Weinberg equilibrium (HWE). Data were analyzed using the SPSS statistical package, version 17.0 (SPSS, Chicago, IL, USA).

Results

Demographic Characteristics of Enrolled Subjects

The statistical analysis of demographic characteristics of the 1102 IS patients and 1610 controls is summarized in Table 1. Subjects of our case–control study were matched with age and sex, there were marked differences between cases and controls in terms of the frequency distribution of hypertension, diabetes, dyslipidemia, smoking status, and family history of stroke in both centers, the trend was similar with recruited subjects from previous reports. Beyond expected, BMI was not shown as a significant risk factor for IS in recruited subjects of Suzhou group.

Correlation Between *NLRP3* rs10754558 and IS Risk

Statistical analysis of NLRP3 rs10754558 and IS risk in two centers is summarized in Table 3. Genotype distributions had no deviation from Hardy–Weinberg equilibrium in Suzhou control group, Beijing control group or pooled control group ($P > 0.05$, data not shown). Our results showed that rs10754558 was significantly associated with IS susceptibility, at both the allele and genotype levels (Table 3). As depicted in the co-dominant model of Suzhou case–control group, compared with the reference (CC), we found that the heterozygote and homozygote GG of rs10754558 were associated with a significantly increased risk of is after controlling for other covariates selected in our study (adjusted OR = 1.52, 95 % C.I. 1.19–1.97, $P = 0.002$; adjusted OR = 2.22, 95 % C.I. 2.18–3.67, $P < 0.001$, respectively). In the dominant model (CG + GG vs CC), significant associations were also observed between cases and controls (adjusted OR = 1.82, 95 % C.I. 1.82(1.47–2.81), $P < 0.001$), data from recessive model displayed similar trend. Meanwhile, frequencies of C or G allele were significantly different between IS and control groups. In the additive model, each additional copy of G allele was associated with a 69 % increased risk of developing cancer (OR = 1.69, 95 % C.I. 1.31–1.83, $P < 0.001$). Analysis of Beijing group and pooled group validated our findings in Suzhou group.

Stratification Analysis of rs10754558 with Risk Factors in IS Cases

NLRP3 rs10754558 and was confirmed as a risk factor for IS risk, we then had stratified analysis in IS cases to detect potential interactions between the polymorphism and candidate risk factors enrolled in present study. After stratification, only one risk factor hypertension status showed positive interaction with polymorphism (Table 4). Other candidate risk factors such as age, sex, BMI, diabetes, dyslipidemia, smoking status, and family history of stroke showed no significantly statistical difference (data not shown).

The Influence of rs10754558 on *NLRP3* Expression

To further investigate whether rs10754558 would likely to affect the level of transcriptional activity of *NLRP3*, qPCR was conducted in PBMCs of enrolled cases to demonstrate the genotype-phenotype connection. As shown in Fig. 1, we found that IS cases carried with G allele drove a 1.36–1.88-fold increased transcription compared with the CC genotyped counterparts. Control genotyped groups showed similar propensity, but expression activities were

Table 3 Associations between rs10754558 genotype and IS susceptibility

Genetic Model	Genotype	Cases *n* (%)		Control *n* (%)		OR (95 % c.i.)[a]	*P*
Suzhou group							
Co-dominant model	CC	119	19.9	263	31.2	1.00 (Reference)	
	CG	304	51.0	439	52.1	1.52 (1.19–1.97)	0.002
	GG	174	29.1	141	16.7	2.22 (2.18–3.67)	<0.001
Dominant model	CC	119	19.9	263	31.2	1.00 (Reference)	
	CG+GG	478	80.1	580	68.8	1.82 (1.47–2.81)	<0.001
Recessive model	CC+CG	423	76.7	702	83.3	1.00 (Reference)	
	GG	174	29.1	141	16.7	2.01 (1.55–2.85)	<0.001
Additive model[b]	C allele	542	45.4	965	57.2	1.00 (Reference)	
	G allele	652	54.6	721	42.8	1.69 (1.31–1.83)	<0.001
	P_{trend}						<0.001
Beijing group							
Co-dominant model	CC	98	19.4	229	29.9	1.00 (Reference)	
	CG	250	49.5	395	51.5	1.49 (1.11–1.92)	0.007
	GG	157	31.1	143	18.6	2.59 (1.81–3.67)	<0.001
Dominant model	CC	98	19.4	229	29.9	1.00 (Reference)	
	CG+GG	407	80.6	538	70.1	1.81 (1.37–2.36)	<0.001
Recessive model	CC+CG	348	68.9	624	81.4	1.00 (Reference)	
	GG	157	31.1	143	18.6	1.97 (1.55–2.55)	<0.001
Additive model[b]	C allele	446	44.2	853	55.6	1.00 (Reference)	
	G allele	564	55.8	681	44.4	1.59 (1.41–1.83)	<0.001
	P_{trend}						<0.001
Pooled group							
Co-dominant model	CC	217	19.7	492	30.6	1.00 (Reference)	
	CG	554	50.3	834	51.8	1.52 (1.21–1.72)	<0.001
	GG	331	30.0	284	17.6	2.65 (2.18–3.27)	<0.001
Dominant model	CC	217	19.7	492	30.6	1.00 (Reference)	
	CG+GG	885	80.3	1118	69.4	1.81 (1.57–2.11)	<0.001
Recessive model	CC+CG	771	70.0	1326	82.4	1.00 (Reference)	
	GG	331	30.0	284	17.6	2.01 (1.65–2.45)	<0.001
Additive model[b]	C allele	988	44.8	1818	56.5	1.00 (Reference)	
	G allele	1216	55.2	1402	43.5	1.60 (1.41–1.73)	<0.001
	P_{trend}						<0.001

[a] Adjusted for proper confounders when appropriate

[b] Assuming an additive effect of the G allele

slightly lower when compared with IS cases counterparts, the expression comparison showed no obvious difference ($P > 0.05$, data not shown).

Discussion

As far as we knew, this was the first molecular epidemiological report to investigate the correlation of *NLRP3* polymorphism with risk of IS in Chinese Han population. The genotyping of 1102 IS patients and 1610 healthy control individuals showed significant associations with the SNP variant within 3′UTR of *NLRP3* with IS susceptibility. Stratified analysis found that hypertension could interact with the polymorphism to upregulate IS risk in the present study. Furthermore, *NLRP3* transcription in blood analysis showed that the G variant allele significantly upregulated the transcription activity of the *NLRP3* gene compared with the C allele; control groups also showed similar propensity.

Polymorphisms within 3′UTR are possibly the most potential factors for gene expression inhibition/enhancement. The presence of the rs10754558 G allele was previously reported to lead to conformational change and increase the stability of the 3′UTR structure of *NLRP3* mRNA, resulting in a higher expression level (Hitomi et al. 2009), our results also demonstrated this trend in Chinese Han population. NLRP3 was known to serve as a key mediator of the innate immune response, and inadequate or

Table 4 Stratification analysis of rs10754558 with Hypertension status in IS cases

Genetic model	Characteristics	Genotypic/allelic numbers		OR (95 % C.I.)[a]	*P*
		N	N		
Suzhou population					
	Hypertension status	Yes	No		
Co-dominant model	CC	75	77	Reference	
	CG	190	116	1.68 (1.14–2.48)	**0.009**
	GG	106	33	3.27 (1.98–5.27)	**<0.0001**
Dominant model	CC	75	77	Reference	
	CG+GG	296	149	2.03 (1.41–2.79)	**0.0001**
Recessive model	CC+CG	265	193	reference	
	GG	106	33	2.31(1.52-3.69)	**0.0009**
Additive model	C allele	340	270	reference	
	G allele	402	182	1.76 (1.31–2.24)	**<0.0001**
Beijing population					
	Hypertension status	Yes	No		
Co-dominant model	CC	62	66	Reference	
	CG	150	101	1.59 (1.03–2.46)	**0.04**
	GG	95	31	3.27 (1.93–5.52)	**<0.0001**
Dominant model	CC	62	66	Reference	
	CG+GG	245	132	1.99 (1.31–2.99)	**0.0002**
Recessive model	CC+CG	212	167	Reference	
	GG	95	31	2.49 (1.51–3.77)	**0.0001**
Additive model	C allele	274	233	Reference	
	G allele	340	163	1.76 (1.31–2.24)	**<0.0001**
Pooled population					
	Hypertension status	Yes	No		
Co-dominant model	CC	137	143	Reference	
	CG	340	217	1.65 (1.27–2.15)	**0.0008**
	GG	201	64	3.25 (2.43–4.62)	**<0.0001**
Dominant model	CC	137	143	Reference	
	CG+GG	541	281	2.09 (1.51–2.65)	**<0.0001**
Recessive model	CC+CG	477	360	Reference	
	GG	201	64	2.38 (1.71–3.39)	**<0.0001**
Additive model	C allele	614	503	Reference	
Additive model	G allele	742	345	1.79 (1.41–2.11)	**<0.0001**

[a] Adjusted for proper confounders except for additive model. Bold characters indicate significant difference between two groups (95 % C.I.s do not cut 1)

excessive reaction will cause damage to the human body. Enhanced NLRP3 expression may activate the inflammasome through the recognition of several cell stress molecules (Gram et al. 2012). Inflammasome has been found play a key role in the innate immune response reduced inflammation, innate immunity plays a pivotal role in inflammation-related neuronal injury, which is associated with IS.

An interesting find in present study was the interaction of *NLRP3* rs10754558 and hypertension status in IS cases. At least one report supported the correlation between *NLRP3* polymorphism and hypertension (Suárez and Buelvas 2015), but the possible association *NLRP3* rs10754558 and hypertension risk was not validated to our knowledge. Known as a stroke prediction marker, hypertension was also one of general prevention strategies (Esenwa and Gutierrez 2015), if the possible association *NLRP3* rs10754558 and hypertension risk was identified, *NLRP3* rs10754558 would be a promising marker to guide both stroke and hypertension therapy and prevention.

We are aware that our study had several limitations, specifically the replication study in other populations, more primary data from in vivo analysis and further functional analysis. Considering location of the locus and previous

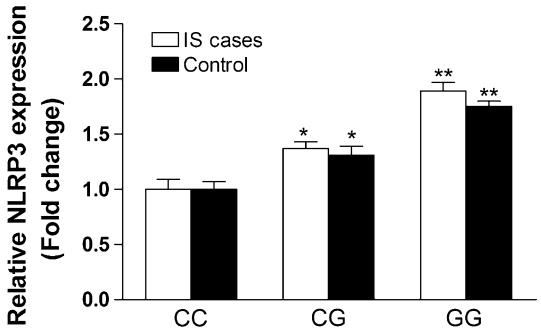

Fig. 1 *NLRP3* transcription level analysis in different genotyping groups of PBMCs from IS patients. *$P < 0.01$ versus CC group, **$P < 0.001$ versus CC group

report (Zhao et al. 2015), we speculated that G allele may disrupt the primary binding site, which in turn influences the potential miRNA-MicroRNA-223 recruitment and binding, thus *NLRP3* transcription was upregulated.

Taken together, our data indicated that genetic polymorphisms in *NLRP3* may influence IS risk in Chinese Han population. However, this IS-associated polymorphism rs10754558 showed none direct demonstration that rs150376137 is causative due to a genetic conception that another genetic variation may link to rs10754558 is the true mutation driving the association. Finally, the replication of our studies in other populations and further in vivo analysis are required for complete elucidation of the roles of *NLRP3* polymorphisms in predisposition for IS.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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