Inflammation Related circRNAs Polymorphism and Ischemic Stroke Prognosis

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Research

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Abstract

Background CircRNAs belong to a novel class of noncoding RNAs that are generated by exons of genes by alternative mRNA splicing and involved in pathophysiological processes of ischemic stroke by regulating neuroinflammation.

Method A total of 982 patients were enrolled in our study for stroke recovery analysis. The aim of our study was to first explore the association between the inflammation related circRNA polymorphism and functional outcome 3 months after ischemic stroke by using multivariate logistic regression model. Next, we further investigated the role of circRNA polymorphism in predicting stroke recurrence by using cox proportional hazards regression model. Five circRNA polymorphisms were genotyped by using polymerase chain reaction and ligation detection reaction method.

Results We identified circ-STAT3(signal transducer and activator of transcription) rs2293152 GG genotype to be associated with poorer recovery 90 days after stroke (OR=1.452; 95%CI:1.165-4.362, p=0.016). After adjusting for confound factors, the association for rs2293152 with 3 months outcome after IS was stronger, suggesting a mechanism that rs2293152 is an independent risk factor for stroke recovery (OR=2.255; 95%CI:1.034-2.038, p=0.031). However, no other circRNA polymorphisms (rs41274714, rs10870141, rs10485505, rs4911154) was associated with functional outcome 3 months after stroke in any genetic models. Subgroup analysis revealed that the negative effect of rs2293152 GG genotype was greater in female and older patients, subjects with history of hypertension. Additionally, all the circRNA polymorphisms were not correlated with recurrent risk of ischemic stroke.

Conclusion Our results indicated that circ-STAT3 might be a novel biomarker for predicting functional outcome after stroke and an important contributor to the ischemic stroke recovery.

Introduction

Ischemic stroke ranks the major cause for mortality and disability in China [1]. Although thrombolysis is the most effective method to improve the functional outcome for the IS patients, only a small part of patients can access to this treatment due to limited window [1]. In order to develop more effective and feasible strategies for ameliorating brain injury and disability after stroke, we need to understand the pathophysiology of cerebral ischemia from the molecular perspective. Genetic factors could influence functional recovery and recurrent stroke risk, accounting for unexplained factor in stroke recovery [2]. Previous studies have reported some candidate SNPs such as apolipoprotein E and BDNF (brain derived neurotrophic factor) gene variants was associated with post-stroke recovery functional outcome after ischemic stroke [3]. However, the studies on the functional roles of circRNA in brain injury and repair after IS are just beginning. Identifying non-coding features would provide a comprehensive map and uncover potential processes of IS.

Circular RNAs (circRNAs) are new class of noncoding RNA generated by the back-splicing of introns or exons [4]. CircRNAs may act as miRNA sponges by binding to microRNA response elements and regulate

the expression of miRNA [5]. Besides, circRNAs could also exert transcriptional regulation and post-transcriptional regulation on gene expression by binding to RNA-associated proteins [6]. The generation procedure of circRNAs competed with linear spicing and influenced production of linear mRNAs. Recent study indicated that several circRNAs were aberrantly expressed in the ischemic cerebral tissue in MACO animal model as well as blood of IS patients [7]. It is suggested that circRNAs could be biomarkers for IS diagnosis and prediction of stroke outcomes. Furthermore, circRNAs was reported to contribute to pathophysiology process of stroke by mediating neuroinflammation, apoptosis, atherosclerosis and neurogenesis [8].

Recent studies have provided evidence on single nucleotide polymorphisms (SNPs) associated with circRNA expression. Ahmed et al. [9] integrated circular RNA expression from RNA-seq data of lymphoblastoid cell lines with genome sequence variation from the 1000 Genomes Project and identified thousands of cis-acting genetic variants at the circRNA influencing its expression, referred to as circRNA quantitative trait loci (circQTLs). Additionally, circQTLs existed independently of eQTLs and exerted no effect on mRNA expression. Furthermore, recent studies have also identified 196,255 circQTLs, which might influence circRNA expression by altering the canonical back-splicing sites [10]. Holdt's work revealed that the presence of specific intronic binding sites may contribute to circRNA biogenesis [11]. These results revealed that genetic factors could influence circRNA expression variation and enrich for the GWAS SNP associated with complex diseases. However, the study about genetic variants within circRNAs and ischemic stroke is in the early stage.

CircRNA DLGAP4 was located on chrome 20 and generated from the exons 8, 9 10 of DLGAP4 gene. Bai *et al*., and his colleagues [12] first found that circDLGAP4 controlled the endothelial–mesenchymal transition and be involved in blood-brain barrier (BBB) integrity. Circ-DLGAP4 promoted the maintenance of BBB integrity and improved functional outcome after stroke by sponging miR-143. Subsequently, Zhu's study indicated that circ-DLGAP4 was negatively related with the inflammation cytokines level (TNF-α, IL-6, IL-8, IL-22) in IS patients [13]. Additionally, Wang et al. identified that circ-ITCH suppressed the active of Wnt/β-catenin signaling activation by sponging miR-214 and miR-17 through increasing expression of its ITCH linear isoform [14]. The Wnt/β-catenin signaling not only played important roles in microglia activity and neuro-inflammation but also be crucial for regulating synaptic plasticity and BBB integrity and function [15]. Circ-STAT3 were derived from exons 12, 13, and 14 of STAT3 (Signal Transducer and Activator of Transcription 3) and involved in pro-inflammatory cytokines signaling [16]. Another study by Zhang et al. [17], has found that circ-TRAF2 was associated with colorectal cancer risk through regulating neuro-inflammation. Neuro-inflammation is a vital pathogenesis after stroke, which can cause secondary brain damage and unfavorable functional recovery [18]. Furthermore, microglia activation and BBB integrity are two important factors in the regulation of ischemia-induced neuroinflammatory process. Under this background, we speculated that circ-DLGAP4, circ-ITCH, circ-TRAF2 and circ-STAT3 were involved in the progression of IS recovery. The prognosis on neurological deficit can be divided into functional outcome and stroke recurrence. The aim of our study was to first explore the association between the neuro-inflammation related circRNA SNPs and functional outcome after stroke. Next, we further investigated the role of circRNA polymorphisms in predicting stroke recurrence.

Methods

Study subjects

Our study included 982 first-ever suffered from ischemic stroke and hospitalized in Department of Neurology, the First Affiliated Hospital of China Medical University between November 2016 and December 2019. Eligible cases were diagnosed ischemic stroke for the first time according to clinical manifestation and neurological examination (computed tomography and magnetic resonance imaging). The National Institute of Health stroke scale (NIHSS) score and modified Rankin Scale (mRS) score were used to assess stroke severity and functional outcome of the disease, the former was carried out on admission and the later was implemented after 3 months of the disease onset respectively. Patients with mRS score less than or equal 2 were defined as good outcome, while others were classified into poor outcome group. Patients who emerged new neurological impairments or pre-existing symptoms exacerbated after 21 days from the first-ever attacked were considered as recurrent cases. Moreover, the definition and boundary of hypertension, diabetes mellitus, dyslipidemia, smoking and drinking were same as our previous study [19]. All the patients were followed up by clinical visit or telephone interview until stroke recurrence or the latest follow up. Our study was approved by Ethics Committee of the First Hospital of China Medical University and in accordance with the principles of the Helsinki Declaration, all participants have signed the informed consents.

SNP Selection and Genotyping.

The dbSNP database (http://www.ncbi.nlm.nih.gov/SNP) and circBase (http://www.circbase.org) was used for selecting snp for circRNAs. The rule for tagSNPs selection is as following: minor allele frequency (MAF) larger than 0.05 and linkage disequilibrium (LD) patterns r^2 less than 0.8. circ-DLGAP4 rs41274714; circ-STAT3 rs2293152; circ-TRAF2 rs10870141; circ-ITCH rs10485505, rs4911154 were selected for our study. All polymorphisms were genotyped by PCR-LDR method (polymerase chain reaction and ligation detection reaction), which have been described in our previous research [19].

Statistical Analysis

We used Chi-square and t-test to calculate and compare the discrepancy of categorical and continuous variables respectively. The association between circRNA SNPs and functional outcome after stroke were accessed using multivariate logistic regression model, 95% confidence interval (95% CI) and odds ratio (OR) were calculated. The associations between circRNA SNP polymorphisms and stroke recurrence risk were accessed by cox proportional hazards regression model and by calculating hazard ratio (HR) and 95% CI. Data analysis was performed by using the SPSS 17. $P < 0.05$ was considered as statistical significance. The potential functional effect of circRNA polymorphisms on functional outcome of IS was performed by using Haploreg v4.1 webserver (http://www.broad institute.org/mammals/haploreg/haploreg/Php).

Results

Clinical Characteristic of patients group by short-term outcome

The clinical characteristics of participants were listed in Table 1. Among them, 263 cases (29.9%) were recognized as poor outcome, 615 patients (70.1%) had a favorable outcome. The age, NIHSS score, diabetes and stroke subtype were associated with functional outcome of IS. Patients with mRS of 3–6 are prone to older, diabetes status, large artery atherosclerosis-IS and higher NIHSS score.

Table 1
,Clinical characteristics of patients grouped by short-term prognosis

Variable	MRS(0–2) N = 615	MRS(3–5) N = 263	*p*
Age ≥ 60	350	186	0.000
Male	424	170	0.212
Hypertension	474	208	0.511
Diabetes	221	126	0.001
Hyperlipidemia	247	93	0.181
Smoking	253	115	0.477
Drinking	134	68	0.190
NIHSS	3.86 ± 1.784	10.34 ± 3.284	0.000
TOAST	293	190	0.000

CircRNA polymorphisms and short-term outcome of IS

Short-term prognosis of IS was assessed at 3 months after stroke by mRS and the results were shown in Table 2. Patients with GG genotype had a trend to be unfavorable outcome compared to CC genotype (p = 0.071). Furthermore, we identified circ-STAT3 rs2293152 GG genotype to be associated with poorer outcome and greater disability under recessive model 90 days post-stroke (OR = 1.452; 95% CI :1.034–2.038; p = 0.031). In other words, compared with GG, patients with the CG + CC genotype had a higher probability of good recovery. After adjusting confound factors, the GG genotype of rs2293152 is associated with a 2.255-fold higher risk of having a poorer outcome as compared to CG + CC genotype, which is statistically significant (OR = 2.255; 95% CI :1.165–4.362; p = 0.016), as shown in Table 3. However, no other circRNA polymorphisms (circ-DLGAP4 rs41274714, circ-TRAF2 rs10870141, circ-ITCH rs10485505, rs4911154) were associated with functional outcome 3 months after stroke in any genetic models.

Table 2
, circRNA polymorphism and their association with IS short-term outcome after 3 months

SNP	MRS(0-2)	MRS(3-5)	*p*	*OR*	95%CI
	N=615	N=263			
rs10485505					
CC	447	193	Reference		
CT	152	67	0.903	1.021	0.732-1.425
TT	16	3	0.177	0.434	0.125-1.508
Dominant model					
CT+TT VS CC	168/449	70/193	0.83	0.965	0.697-1.337
Recessive model					
TT VS CT+CC	16/599	3/260	0.173	0.432	0.125-1.495
rs10870141					
AA	454	200			
AG	144	56	0.486	0.883	0.622-1.254
GG	17	7	0.883	0.935	0.382-2.289
Dominant model					
GG+AG VS AA	161/454	63/200	0.489	0.888	0.635-1.242
Recessive model					
GG VS AG+AA	17/598	7/256	0.932	0.962	0.394-2.384
Rs2293152					
CC	178	70			
GC	316	124	0.99	0.998	0.706-1.410
GG	121	69	0.071	1.45	0.967-2.174
Dominant model					
GG+CG VS CC	437/178	193/70	0.483	1.123	0.812-1.553
Recessive model					
GG VS CG+CC	121/494	69/194	0.031	1.452	1.034-2.038
Rs41274714					

GG	531	217			
AG	81	46	0.101	1.39	0.936-2.062
AA	3	0	0.269	0.71	0.678-0.743
Dominant model					
AA+AG VSGG	84/531	46/217	0.143	0.143	0.905-1.984
Recessive model					
AA VS AG+GG	3/612	0/263	0.257	0.257	——
Rs49111154					
GG	411	187			
GA	179	66	0.213	0.81	0.582-1.128
AA	25	10	0.737	0.879	0.414-1.868
Dominant model					
AA+GA VS GG	204/411	76/187	0.213	0.819	0.598-1.122
Recessive model					
AA VS GA+GG	25/590	10/253	0.855	0.933	0.442-1.971

Table 3
, Stratification analysis for rs2293152 with short-term outcome according to the common factors

Variables	GG mRS (0–2)/(3–5)	CC + CG mRS (0–2)/(3–5)	*P*	OR (95% CI)
Age (years)				
< 60	56/17	209/60	0.858	1.057(0.572–1.954)
≥ 60	65/52	285/134	0.012	1.701(1.12–2.586)
Gender				
Male	85/37	339/133	0.64	1.11 (0.718–1.714)
Female	36/32	155/61	0.004	2.256(1.289–3.957)
Hypertension				
No	33/6	108/49	0.049	0.401 (0.158–1.019)
Yes	88/63	386/145	0.001	1.906 (1.309–2.775)
Diabetes				
No	74/35	320/102	0.091	1.484(0.937–2.350)
Yes	47/34	174/92	0.226	1.32 (0.823–2.275)
Hyperlipidemia				
No	66/43	302/127	0.048	1.549 (1.001–2.397)
Yes	55/26	192/67	0.272	1.355 (0.787–2.332)

Stratification analysis

To further access the effect of circRNA polymorphisms on functional outcome of post-stroke, stratified analysis was performed by subgroups of common factors using recessive model (GG vs CG + CC) (Table 4). The increased risk for rs2293152 GG genotype was more evident in sub-group of older subjects (OR = 1.701, 95% CI = 1.12–2.586, p = 0.012), females (OR = 2.256,95%CI = 1.289–3.957, p = 0.004), indicating the effect was enhanced by the potential interactions between rs2293152 and age, gender. Additionally, individuals with the GG genotype had a 1.906-fold increased risk of unfavorable outcome in hypertension group, which indicated that the negative effect was more pronounced in subjects who had history of hypertension (OR = 1,906, 95%CI:1.309–2.775, p = 0.001).

Table 4
, Short-term outcome prognosis factors of ischemic stroke in the logistic regression analysis

	p	OR	95%CI
Sex	0.448	0.762	0.378–1.536
Age	0.779	1.087	0.608–1.944
Hypertension	0.560	1.225	0.619–2.423
Diabetes	0.227	1.407	0.809–2.445
Hypercholesterolemia	0.117	0.634	0.359–1.120
Smoking	0.114	1.727	0.877-3.400
Drinking	0.707	0.873	0.429–1.776
NIHSS	0.000	2.936	2.494–3.456
Genotype GG of rs2293152	**0.016**	2.255	1.165–4.362

CircRNA polymorphisms and IS recurrence

A total of 982 the patients were enrolled in our study for stroke recurrence analysis, among them 42 patients (1.5%) were lost to follow up. The median follow-up time was 14 months. Basic characteristics of patients classified by stroke recurrence were summarized in Table 5. We found that age and stroke subtype were related to IS recurrence. In the further analysis, none of the five polymorphisms was significantly associated with stroke recurrence in any genetic models from cox regression analysis (Table 6).

Table 5
,Clinical characteristics of patients grouped by long-term prognosis

Variables	Patients	Recurrence	Log-rank p
	N=940 (%)	N=139	
Age			**0.019**
≤55	358	42	
>55	582	97	
Sex			0.78
Male	634	94	
Female	306	45	
Diabetes			0.096
No	560	76	
Yes	380	63	
Smoking			0.286
No	551	84	
Yes	589	55	
TOAST			**0.024**
LAA	527	91	
SVD	413	48	
Hypertension			0.132
No	211	26	
Yes	729	113	
Hyperlipidemia			0.918
No	574	83	
Yes	366	56	
Drinking			0.536
No	724	108	
YES	216	31	

Table 6
, Association between circRNA polymorphism and IS recurrence

Genotype of SNP	Patients	Recurrence	Log-rank *P*	HR	95 % CI
rs10485505					
CC	687	97			
CT	233	39	0.25		
TT	20	3	0.701		
Dominant model					
CT+TT VS CC	97/687	42/253	0.686	1.267	0.402-3.999
Recessive model					
TT VS CT+CC	136/920	20-Mar	0.227	1.252	0.87-1.802
rs10870141					
AA	698	105			
AG	217	32	0.535		
GG	25	2	0.394		
Dominant model					
GG+AG VS AA	105/698	34/242	0.534	0.884	0.600-1.303
Recessive model					
GG VS AG+AA	137/915	25-Feb	0.462	0.592	0.146-2.395
Rs2293152					
CC	267	37			
GC	467	70	0.726		
GG	204	32	0.679		
Dominant model					
GG+CG VS CC	37/267	102/673	0.716	1.073	0.736-1.564
Recessive model					
GG VS CG+CC	107/736	32/204	0.881	1.031	0.693-1.534
Rs41274714					
GG	797	116			
AG	140	23	0.616		

AA	3	0	0.473		
Dominant model					
AA+AG VSGG	116/797	23/143	0.76	1.072	0.684-1.681
Recessive model					
AA VS AG+GG	139/937	0/3	0.93	-	-
Rs49111154					
GG	634	89			
GA	266	45	0.349		
AA	40	5	0.781		
Dominant model					
AA+GA VS GG	89/634	50/306	0.33	1.19	0.839-1.686
Recessive model					
AA VS GA+GG	134/900	May-40	0.691	0.834	0.341-2.039

Discussion

In our study, we accessed the possibility of circRNA polymorphisms as prognostic biomarkers for IS. To the best of our knowledge, our study is the first study to examine the role of circ-RNA in the recurrence and recovery of IS. Our findings identified that circ-STAT3 rs2293152 GG genotype were significantly associated with unfavorable functional outcome of IS, and rs2293152 could be served as prognostic biomarkers for IS patients. However, the other four SNPs were not associated with functional outcome of IS after 3 months. We also failed to find the association between all the circRNA polymorphisms and IS recurrence risk. Our study will provide novel perspectives for prognosis prediction and target gene-therapy for IS.

Recent studies have suggested that circRNAs might be novel diagnostic and prognostic biomarkers for the disease. Zuo et al. [20] found that three differentially expressed circRNAs (circFUNDC1, circPDS5B, and circCDC14A)in blood of IS patients through two stage studies. Subsequently, the study of Dong et al. [7] indicated that 521 differentially expressed circRNAs (373 increased and 148 circRNAs decreased) in the IS group compared with controls. CircRNA expression profiles were altered significantly in the PBMCs of IS patient and may be participate in the pathogenesis of IS. Meantime, recent association studies revealed that circRNAs polymorphisms conferred prognostic and susceptibility biomarkers for the disease. Burd et al. [21] suggested that the rs7341786 within 9p21 contribute to atherosclerotic vascular disease susceptibility through regulation of ANRIL splicing and circular ANRIL expression. In addition, Paraboschi et al. reported that hsa-circ_0043813 from the STAT3 gene was associated with multiple sclerosis risk and the genotype CC of rs2293152 could increase circ-STAT3 expression [16]. Moreover,

Zhang and his colleges [17]. have found that rs25497 in circ-TUBB was associated with colorectal cancer risk in both Chinese and European populations by influencing the expression of ELF5 targeted to miR-4664-3p. Additionally, another study indicated that circ-FOXO3 rs12196996 at the gene flanking intron was associated with coronary artery disease risk in the Chinese Han population, which affected circ-FOXO3 expression, but not linear FOXO3 levels [22]. Furthermore, Guo et al. [23] found that circ-ITCH rs10485505 and rs4911154 were significantly associated with increased hepatocellular carcinoma risk. Until now, study about the circRNA polymorphisms and stroke prognosis is fewer.

Our study demonstrated that circ-STAT3 rs2293152 predicted functional outcome after stroke, carrying GG genotype exhibited worse outcomes 3 months post-stroke. We identified that the GG genotype was associated with increased risk of unfavorable outcome of stroke and that the CC + CG genotype was associated with a better outcome at 3 months. After adjustment for NIHSS score and other factors, the association for rs2293152 after 3 months outcome after IS was stronger, suggesting that rs2293152 is an independent risk factor for stroke recovery. However, no significant association between circ-DLGAP4 rs41274714, circ-TRAF2 rs10870141, circ-ITCH rs10485505, rs4911154 and short-term outcome of stroke was detected. Sub-group analysis revealed that the negative effect of rs2293152 GG genotype was greater in female, older patients and subjects with hypertension status. The results indicated the interaction of age, sex, blood pressure and rs2293152 enhanced the poor recovery of IS. There is a fact that risk factors for stroke recovery including hypertension, depression, atrial fibrillation were significantly more common in women [24], which may contribute the different risk among the gender. Moreover, individuals with older age are prone to have other chronic disease compared with youngers, which may influence stroke recovery. Additionally, all the circRNA polymorphisms were not correlated with a recurrent ischemic stroke risk.

The possible explanation of circ-STAT3 role in short-term prognosis might be as follows. First, the genetic variation at circ-STAT3 might influence the expression of STAT3 by functioning as miRNA sponge [25]. Second, we speculated that circ-STAT3 rs2293152 located at flanking intron may act as circ-eQTL and affect the circRNA biogenesis, which eventually influenced the expression level of circ-STAT3 [11]. This is consistent with Liu et al. study that genetic variants of circRNA within flanking intron region would influence circRNA expression [10]. Third, functional prediction revealed that rs2293152 was located at potential functional regions and might alter the binding affinity of regulatory motifs, which would influence circ-STAT3 expression. From the above evidence, we can speculate that the effects of circ-STAT3 rs2293152 on short-term prognosis of stroke seem to be mediated by regulating of circ-STAT3 expression. The increased expression of circ-STAT3 might promote the release of inflammatory cytokines and activate the inflammatory response. In addition, STAT3 may also regulated astrocyte activation through targeting of the JAK2/STAT3 pathway [26]. Astrocyte activation can aggravate inflammatory reactions and brain injury. Thus, circ-STAT3 may influence IS recovery by influence the neuro-inflammation processes after neural injury.

The highlights of this study were as follows. First, previous studies focused mainly on protein-coding genes, but our studies emphasized non-coding RNAs such as circRNAs. Our study is first and

comprehensive study to demonstrate the potential role of circRNAs polymorphisms for functional outcome after stroke. Besides, NIHSS score was an important factor for the short-term outcome after stroke, which was took into account in our study. Thus, logistic regression was applied in our study to adjust the confound factors. Finally, we also did stratification analysis to find the interactions between rs2293152 and sex, age. However, there are limitations in our study. First, the sample size of our study was not large enough, further studies in different populations with larger sample are needed to validate the association between the circRNAs polymorphisms and functional outcome after stroke. Moreover, functional mechanisms of rs2293152 on the short-term prognosis after stroke are still not clear, which is needed to be clarified in the further study.

Our study demonstrated that circ-STAT3 rs2293152 GG genotype was associated with unfavorable outcomes 3 months after stroke. Moreover, rs2293152 of circ-STAT3 can also be used as the biomarker for predicting functional outcome after stroke. Further research is needed to explore the exact biological mechanism of these genetic variations on stroke recovery. A comprehensive understanding of genetic variants effect on stroke recovery is needed for setting up personalized therapeutic interventions after stroke.

Declarations

Acknowledgments

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Conflict of interest

The authors have no conflict of interests.

Availability of data and material

The data used in our study are available from the authors on reasonable request.

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Authors' contributions

Designed the experiments: Ruixia Zhu,Xu Liu；Performed the experiments: Ruixia Zhu, Xu Liu ,Jingjing zhao；Analyzed the data: Xu Liu ,Qianwen Wang,Hongtao Chang. Wrote the paper: Ruixia Zhu ,Qianwen Wang

Compliance with ethical standards and consent to participate

This study was approved by the ethics committee of the First Affiliated Hospital of China Medical University approval, in accordance with the principles of the Helsinki Declaration（AF-SOP-07-1.0-01). Written informed consents were obtained from all the participants.

Consent for publication

Not applicable.

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