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RESEARCH ARTICLE

Increased plasma levels of circPTP4A2 and circTLK2 are associated with stroke injury

Xingzhi Wang[1,2,3,*], Shenyang Zhang[1,3,4,*], Zuohui Zhang[1,3], Jie Zu[1,3], Hongjuan Shi[1,3], Lu Yu[1,3], Bingchen Lv[1,3], Likun Cui[1,3], Wenqi Mao[1,3], Di Wu[1,3] & Guiyun Cui[1,3]

[1]Department of Neurology, The Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu, China
[2]Jiangsu Key Laboratory of Brain Disease and Bioinformation, Research Center for Biochemistry and Molecular Biology, Xuzhou Medical University, Xuzhou, Jiangsu, China
[3]Institute of Stroke Research, Xuzhou Medical University, Xuzhou, Jiangsu, China
[4]School of Medicine, Southeast University, Nanjing, Jiangsu, China

Correspondence
Guiyun Cui, Department of Neurology, The Affiliated Hospital of Xuzhou Medical University, No. 99 Huaihai West Road, Quanshan District, Xuzhou, Jiangsu, China. Tel: +86 13852159170; Fax: +86 051685802273; E-mail: gyc171722014@163.com

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*These authors contributed equally to this work.

Abstract

Objective: Accumulating studies have shown that circulating circular RNAs (circRNAs) represent novel biomarkers for many human diseases. We investigated whether plasma circPTP4A2 and circTLK2 levels are associated with stroke severity, infarct volume, stroke etiology, and functional outcome in acute ischemic stroke (AIS) patients. **Methods**: We applied quantitative real-time PCR (qPCR) to measure plasma circPTP4A2 and circTLK2 levels of 236 AIS patients within 72 h of symptoms onset and 136 healthy controls. We further assessed the National Institutes of Health Stroke Scale (NIHSS), infarct size, the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification and the 90-day modified Rankin scale (mRS) for each patient. **Results**: At admission, plasma circPTP4A2 and circTLK2 levels in patients with moderate to severe stroke were significantly higher compared to those with mild stroke. Logistic regression and receiver-operating characteristic (ROC) curve analyses indicated that they might function as predictive biomarkers for moderate to severe stroke. We also observed a medium positive correlation between these two circRNAs and NIHSS. Plasma circPTP4A2 and circTLK2 levels were slight positively correlated with cerebral infarct volume only in anterior circulation infarction (ACI) patients. Levels of both circPTP4A2 and circTLK2 were closely related with large artery atherosclerosis (LAA) stroke. Moreover, changes within 7 days after admission in circPTP4A2 and circTLK2 were able to predict unfavorable clinical outcome 90 days after AIS. **Interpretation**: These results demonstrate that plasma circPTP4A2 and circTLK2 strongly correlated with severity, subtypes and prognosis of AIS, and they could serve as promising biomarkers.

Introduction

Stroke is the second most common cause of mortality and third most frequent cause of disability worldwide.[1] According to the Global Burden of Disease Study, 3.94 million patients experience stroke in China in 2019, of which 2.87 million are acute ischemic stroke (AIS).[2] The assessment of stroke severity, infarct core, and etiology categorization are crucial for guiding selection of therapeutic options. In addition, prediction of functional outcome after AIS is also necessary for optimizing clinical care and allocation of medical resources. Most of the reported studies for stroke markers focused mainly on proteins, such as matrix metalloproteinase-9 (MMP-9),[3] neurofilament light chain (NfL),[4] myeloperoxidase (MPO),[5] and sphingosine-1-phosphate (S1P).[6] However, existing blood biomarkers for ischemic stroke are of limited clinical value due to their poor specificity and diagnostic delay.[7] As such, there is an urgent need for accurate biomarkers of AIS to guide the disease management and individualized therapy.

Circular RNAs (circRNAs) are a novel class of noncoding RNAs with a continuous loop structure, produced by a back-splicing of the 5′ and the 3′ ends covalently join.

Differential expression of circRNAs have been associated with multiple human diseases, including cancer, neurological disorders, immune system diseases and cardiovascular diseases.[8] Accumulating evidence reveals that circRNAs are highly enriched in peripheral blood and can be accurately detected in human plasma.[9,10] Therefore, circulating circRNAs have great potential as ideal biomarkers. Our previous studies indicated that plasma levels of circPTP4A2 and circTLK2 significantly upregulated in AIS patients.[11] However, the associations of these two circRNAs with stroke severity and outcome were not clearly characterized, which has limited their clinical applicability as biomarkers.

Thus, in the present study we sought to analyze association of circPTP4A2 and circTLK2 with stroke severity as assessed by NIH stroke scale (NIHSS) score and infarct volume. Additionally, we further evaluate their association with stroke etiology and the role of these two circRNAs in predicting functional outcome.

Methods

Study design and participants

This was a prospective, observational, single-center study, which is a continuation of our previous study.[11] All eligible patients admitted to our stroke unit of the Affiliated Hospital of Xuzhou Medical University were enrolled between May 2021 and December 2022. The study protocol was approved by the Ethics Committee of the Affiliated Hospital of Xuzhou Medical University (XYFY2021-KL107-01) and registered at the US National Institutes of Health Clinical Trials Registry (NCT05098340). All patients or their proxies provided informed consent before enrollment.

The inclusion criteria for the AIS patients were as follows: (a) Age between 18 and 80 years; (b) Admission for first-ever AIS within 72 h after onset. The exclusion criteria were the following: (a) Patients with transient ischemic attack (TIA), intracerebral hemorrhage (ICH), subarachnoid hemorrhage (SAH), cerebral traumata or brain tumors. (b) Patients with severe liver and kidney dysfunction, severe heart failure, severe infectious, or systematic inflammatory diseases or malignant tumors. (c) Pregnant or breast-feeding women. (d) Patients with a history of surgery within the past 90 days. All AIS patients received acute treatment and nursing care in the stroke unit in compliance with Chinese guidelines. In addition, 136 age-matched healthy control subjects were recruited from the physical examination center in the Affiliated Hospital of Xuzhou Medical University.

CircRNA measurement

The plasma RNA isolation and quantitative real-time PCR (qPCR) were performed as our previously published method.[11] In brief, blood samples from all AIS patients were taken immediately after admission to the hospital and before therapy was administered. Afterward, the plasma was separated and centrifuged at 2000 *g* for 10 min at 4°C. Total RNA was isolated using TRIzol LS reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA concentration was checked using NanoPhotometer® spectrophotometer (IMPLEN, USA). For qPCR assay, cDNA was synthesized using the HiScript® Q RT SuperMix for qPCR Kit (R123-01, Vazyme, China) and then reactions were performed in the Bio-Rad CFX96 real-time system using UltraSYBR Mixture (CWBIO, China). Cycling conditions for qPCR were carried out using manufacturer's recommended protocol and qPCR assays were performed in triplicates. The relative expression of circRNAs analyzed by $2^{-\Delta\Delta Ct}$ method normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Primer sequences were synthesized by RiBoBio (Guangzhou, China) with the following sequences: circPTP4A2 (hsa_circ_0007364)-fw: 5′-GGAATCCACGTTCTAGTTTTTCG-3′; circPTP4A2 (hsa_circ_0007364)-rev: 5′-TCTCGGTGTCCAGGAGTCTTC-3′; circTLK2 (hsa_circ_0045128)-fw: 5′-GCTTTTCAGAATCTTATCAACGACG-3′; circTLK2 (hsa_circ_0045128)-rev: 5′-CATGAGGGTTGGCAGAGCAG-3′; GAPDH-fw: 5′-GAACGGGAAGCTCACTGG-3′; GAPDH-rev: 5′-GCCTGCTTCACCACCTTCT-3′.

Data collection and assessment

After admission, complete baseline data on demographic characteristics, clinical features, medical history, biochemical indexes, and routine laboratory determinations were collected. Neurological severity was assessed using the NIHSS[12] at baseline by neurologists trained in it. In accordance with previous research,[13] we defined minor stroke as <6 and moderate to severe stroke as ≥6 on the NIHSS score. The etiologic subtypes of ischemic stroke was classified using the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria.[14] All patients underwent MRI within 72 h of stroke onset with a 1.5 Tesla or 3.0 Tesla units. The acute infarct areas were semi-automatically labeled using the open-source segmentation tool ITK-SNAP (version 3.8.0).[15] The stroke volume was calculated in the labeled regions by two blinded advanced neurologists using ITK-SNAP software. Functional outcome was evaluated by modified Rankin scale (mRS) at 90 days after occurrence of stroke. Favorable and unfavorable functional outcomes were defined as mRS scores of 0–2 and 3–6 points, respectively.[16]

Statistical analysis

Normality of data distribution was assured using Kolmogorov–Smirnov tests. Normal continuous variables were presented as mean ± standard deviation and were analyzed using Student's t-test or one-way ANOVA. Nonnormal continuous variables were presented as median (interquartile range [IQR]) and were analyzed using Mann–Whitney U test or Kruskal–Wallis ANOVA. Categorical variables were described as frequency (percentage) and compared using chi-square or Fisher's exact test. For paired comparisons, the Wilcoxon matched-pairs test was used.

The association of circRNAs expression levels with NIHSS scores and infarct volumes were assessed using the Spearman's correlation coefficient. Both unadjusted and adjusted logistic regression models were constructed to determine the relationship between circRNAs expression levels and stroke severity (moderate-to-severe stroke). Covariates with a univariate association with moderate to severe stroke at p-value <0.2 were included in the multivariate model. Odds ratios (OR) with 95% confidence intervals (CI) were calculated for each candidate variable. Receiver operating characteristic (ROC) curve analysis was used to evaluate the predictive value of circRNAs expression in predicting stroke severity or outcome. Youden index was used to identify the optimal cutoff threshold for determining sensitivity and specificity. Furthermore, combined ROC analysis based on binary logistic regression were performed to assess the predictive value for combined circRNAs.

All statistical analyses were performed using SPSS 26.0 (IBM corporation, CA, USA), and statistical significance was determined at $p < 0.05$ (two-sided).

Results

Patient characteristics

Between May 2021 and December 2022, a total of 275 eligible patients with AIS consented to participate in the study. Of these, 39 patients were excluded because of insufficient volume of the hematology sample (17 patients), incomplete laboratory data (18 patients), and missing NIHSS scores on admission (4 patients). Therefore, 236 AIS patients and 136 age-matched HCs were included in the final analysis. As shown in Table 1, patients with AIS had higher rates of hypertension, diabetes mellitus and atrial fibrillation, and showed higher blood glucose, higher low-density lipoprotein cholesterol (LDL-C), and lower high-density lipoprotein cholesterol (HDL-C) levels in comparison to HCs. Then the AIS patients were stratified into two groups according to NIHSS score on admission: minor stroke group (n = 91, NIHSS score <6) and moderate-to-severe stroke group (n = 145, NIHSS score ≥6). When compared to patients with minor stroke, patients with moderate to severe stroke had higher baseline NIHSS scores, larger infarct volumes and a longer time from onset to hospitalization. Moreover, the stroke etiology also showed different constituent ratio between the two groups according to the TOAST criteria (p = 0.002). The etiology of minor stroke was mainly small artery occlusion (SAO) while the etiology of moderate to severe stroke was large artery atherothrombotic (LAA). Table 1 displays baseline characteristics of all participants.

CircRNA levels and stroke severity

Consistent with our previous publication,[11] plasma levels of circPTP4A2 (median = 1.92, IQR = 1.28; $p < 0.001$; Fig. 1A) and circTLK2 (median = 1.58, IQR = 1.08; $p < 0.001$; Fig. 1B) were significantly upregulated in 236 AIS patients when compared to 136 matched healthy controls. Notably, the relative expression level of circPTP4A2 (median = 2.13, IQR = 1.36; $p < 0.001$; Fig. 1A) and circTLK2 (median = 1.82, IQR = 1.18; $p < 0.001$; Fig. 1B) were increased in patients with moderate to severe stroke when compared to patients with minor stroke. Immediately afterward, we limited our analysis to HCs (n = 136) and examined the difference in the expression of circRNAs among different subgroups based on stroke risk factors. These risk factors included age, sex, hyperlipidemia, diabetes mellitus, dyslipidemia, atrial fibrillation, atrial fibrillation, smoking status, and drinking alcohol. However, we did not observe any significant change in circPTP4A2 and circTLK2 expression between various subgroups of HCs with different stroke risk factors (Table S1).

Next, ROC curve was constructed to evaluate the predictive ability of individual circRNA and the combined circRNAs for stroke severity. The area under the curves (AUCs) of circPTP4A2 and circTLK2 for prediction of moderate to severe stroke as opposed to minor stroke were 0.716 (95% CI: 0.649–0.783, $p < 0.001$) and 0.670 (95% CI: 0.599–0.741, $p < 0.001$), respectively. Furthermore, the combination of these two circRNAs showed greater predictive ability, with an AUC of 0.746 (95% CI: 0.681–0.810, $p < 0.001$), a sensitivity of 0.779 and specificity of 0.615 (Fig. 1C).

To evaluate the predictive power of circPTP4A2 and circTLK2 for stroke severity, the binary logistic regression analyses were performed in AIS patients. The univariate analysis suggested that the increased circPTP4A2 and circTLK2 were closely associated with moderate-to-severe

Table 1. Baseline characteristics of the study population.

	HCs	All stroke patients	*p* value[a]	Minor stroke	Moderate-to-severe stroke	*p* value[b]
Demographic characteristics						
Total, *n*	136	236		91	145	
Age, years, median (IQR)	66 (16)	66 (16)	0.435[c]	64 (16)	66 (17)	0.266[c]
Female, *n* (%)	59 (43.4)	82 (34.7)	0.098[d]	27 (29.7)	55 (37.9)	0.195[d]
Prior vascular risk factors, *n* (%)						
Hypertension	59 (43.4)	155 (65.7)	<0.001[d]	58 (63.7)	97 (66.9)	0.619[d]
Diabetes mellitus	21 (15.4)	67 (28.4)	0.005[d]	28 (30.8)	39 (26.9)	0.521[d]
Dyslipidemia	32 (23.5)	58 (24.6)	0.820[d]	25 (27.5)	33 (22.8)	0.413[d]
Atrial fibrillation	2 (1.5)	38 (16.1)	<0.001[d]	11 (12.1)	27 (18.6)	0.184[d]
Coronary artery disease	15 (11.0)	35 (14.8)	0.301[d]	12 (13.2)	23 (15.9)	0.574[d]
Current smoking	18 (13.2)	49 (20.8)	0.069[d]	18 (19.8)	31 (21.4)	0.768[d]
Alcohol abuse	4 (2.9)	15 (6.4)	0.150[d]	6 (6.6)	9 (6.2)	0.906[d]
NIHSS at admission, median (IQR)	NA	6 (4)		3 (2)	8 (5)	<0.001[c]
Infarct volume, mL, median (IQR)	NA	10.5 (10.5)		6.4 (6.9)	13.7 (15.4)	<0.001[c]
Stroke subtype, *n* (%)						0.002[e]
LAA	NA	113 (47.9)		37 (40.7)	76 (52.4)	
CE	NA	40 (16.9)		10 (11.0)	30 (20.7)	
SAO	NA	77 (32.6)		39 (42.9)	38 (26.2)	
Other determined	NA	6 (2.5)		5 (5.5)	1 (0.7)	
Undetermined	NA	0 (0)		0 (0)	0 (0)	
Biochemical indexes on admission, median (IQR)						
Serum glucose, mmol/L	5.29 (1.08)	5.94 (2.19)	<0.001[c]	5.80 (2.41)	6.00 (2.05)	0.290[c]
TG, mmol/L	1.19 (0.70)	1.26 (0.65)	0.376[c]	1.29 (0.74)	1.26 (0.64)	0.152[c]
TC, mmol/L	4.16 (1.35)	4.28 (1.32)	0.126[c]	4.34 (1.32)	4.28 (1.46)	0.535[c]
HDL-C, mmol/L	1.10 (0.46)	1.04 (0.28)	0.036[c]	1.04 (0.27)	1.04 (0.30)	0.763[c]
LDL-C, mmol/L	2.42 (1.09)	2.66 (1.15)	0.006[c]	2.75 (0.99)	2.66 (1.19)	0.497[c]
Patients received alteplase or endovascular therapy, *n* (%)	NA	21 (17.8)		5 (5.5)	16 (11.0)	0.146[d]
Time from onset to hospitalization, h, median (IQR)	NA	15 (15.5)		12.0 (18.0)	16.0 (14.0)	0.023[c]

CE, cardioembolic; HCs, healthy control subjects; HDL-C, high-density lipoprotein cholesterol; IQR, interquartile range; LAA, large artery atherothrombotic; LDL-C, low-density lipoprotein cholesterol; NA, not available; SAO, small artery occlusion; TC, total cholesterol; TG, triglyceride.
[a]All stroke patients versus HCs.
[b]Minor stroke versus moderate-to-severe stroke.
[c]Mann–Whitney *U*-test.
[d]Chi-squared test.
[e]Fisher's exact test.

stroke. In the multivariate logistic regression model, high level of expression of circPTP4A2 and circTLK2 remained significant predictors of moderate-to-severe stroke after adjustment for confounders such as gender, atrial fibrillation, stroke subtype, receiving alteplase or endovascular therapy and time from onset to hospitalization. The ORs and 95% CIs of circPTP4A2 and circTLK2 for moderate to severe stroke are detailed in Table 2.

Association of circRNA levels with NIHSS score and infarct volume

Spearman rank correlation indicated that plasma levels of circPTP4A2 ($\rho = 0.374$, $p < 0.001$) and circTLK2 ($\rho = 0.315$, $p < 0.001$) in AIS patients have medium positive correlations with initial NIHSS score (Fig. 2A). Among 236 AIS patients, 24 patients were further excluded because of insufficient MRI image quality. Therefore, we analyzed the correlation between circRNA levels and cerebral infarct volume in the remaining 212 AIS patients. Results from the Spearman rank correlation revealed no significant correlation between circRNA levels and cerebral infarct volume (Fig. 2B). Moreover, plasma levels of circPTP4A2 ($\rho = 0.186$, $p = 0.015$) and circTLK2 ($\rho = 0.178$, $p = 0.021$) had a slight positive association with cerebral infarct volume in anterior circulation infarction (ACI) patients, but not in posterior circulation infarction (PCI) patients (Fig. 2C,D).

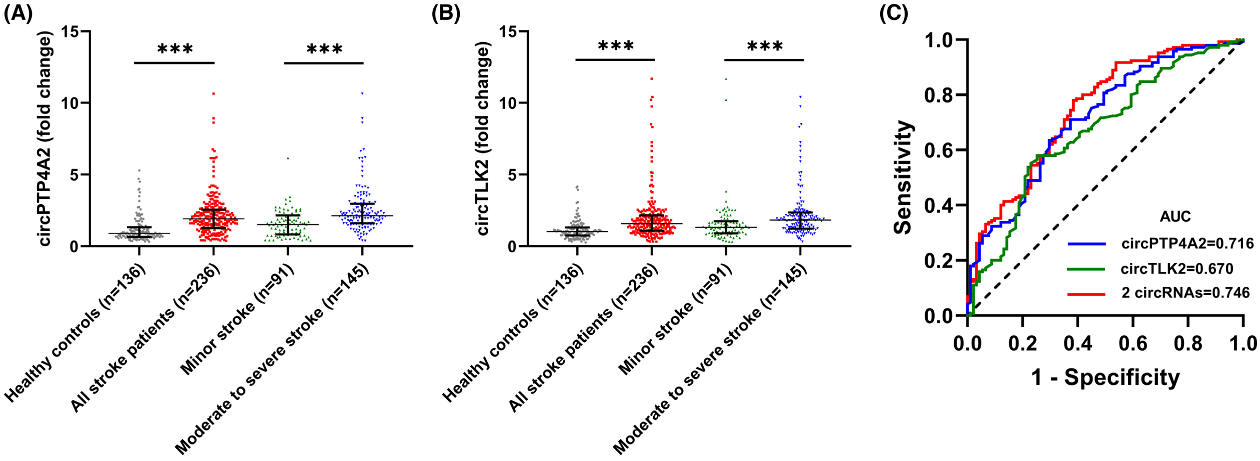

Figure 1. Correlations of circRNA levels with neurological deficit severity of AIS. Relative levels of (A) circPTP4A2 and (B) circTLK2 via qPCR in healthy controls, all stroke patients, minor stroke, and moderate-to-severe stroke groups. Median ± interquartile range, Mann–Whitney *U*-test. ***$p < 0.001$. (C) ROC curve for circPTP4A2 and circTLK2 on predicting moderate-to-severe stroke. AUC, area under the receiver operating curve.

Table 2. Univariate and multivariate logistic regression analysis for stroke severity.

	Univariate analysis			Multivariate analysis		
Variable	OR	95% CI	*p* value	OR	95% CI	*p* value
Age	1.008	0.984–1.032	0.505			
Gender	1.449	0.827–2.539	0.196	1.770	0.937–3.343	0.079
Hypertension	0.870	0.502–1.507	0.619			
Diabetes mellitus	1.208	0.678–2.151	0.521			
Dyslipidemia	1.286	0.704–2.347	0.414			
Atrial fibrillation	0.601	0.282–1.280	0.187	0.385	0.165–0.903	0.028
Coronary artery disease	0.806	0.379–1.711	0.574			
Current smoking	0.907	0.473–1.738	0.768			
Alcohol abuse	1.067	0.367–3.103	0.906			
Stroke subtype	0.653	0.493–0.865	0.003	0.819	0.593–1.130	0.224
Serum glucose, mmol/L	0.999	0.917–1.089	0.988			
TG, mmol/L	0.901	0.664–1.223	0.504			
TC, mmol/L	0.957	0.745–1.230	0.733			
HDL-C, mmol/L	1.040	0.563–1.928	0.900			
LDL-C, mmol/L	0.953	0.698–1.300	0.761			
Patients received alteplase or endovascular therapy	0.469	0.166–1.327	0.154	0.935	0.270–3.234	0.915
Time from onset to hospitalization	1.010	0.995–1.027	0.199	1.012	0.993–1.030	0.228
circPTP4A2	2.273	1.622–3.187	<0.001	2.468	1.689–3.604	<0.001
circTLK2	1.371	1.073–1.751	0.012	1.357	1.035–1.779	0.027

CI, confidence interval; OR, odds ratio.

CircRNA levels and stroke subtype

In this study, stroke etiologies in the 236 AIS patients included LAA ($n = 113$), cardioembolic (CE, $n = 40$), SAO ($n = 77$), other determined etiology ($n = 6$), and undetermined etiology ($n = 0$). Due to the small number of AIS patients in subtypes of other determined etiology and undetermined etiology, stroke types were categorized as LAA, CE, and SAO. As shown in Fig. 3A, plasma levels of circPTP4A2 was significantly increased in LAA, CE, and SAO groups (LAA vs. HCs: $p < 0.001$; CE vs. HCs: $p = 0.004$; SAO vs. HCs: $p < 0.001$). Although there was a trend toward elevated circPTP4A2 levels in the LAA group, this did not meet statistical significance when comparing circPTP4A2 levels in the LAA group with levels in the CE and SAO groups (LAA vs. CE: $p = 0.025$; LAA vs. SAO: $p = 0.048$). Similarly, plasma levels of circTLK2 was increased in LAA, and SAO groups (LAA

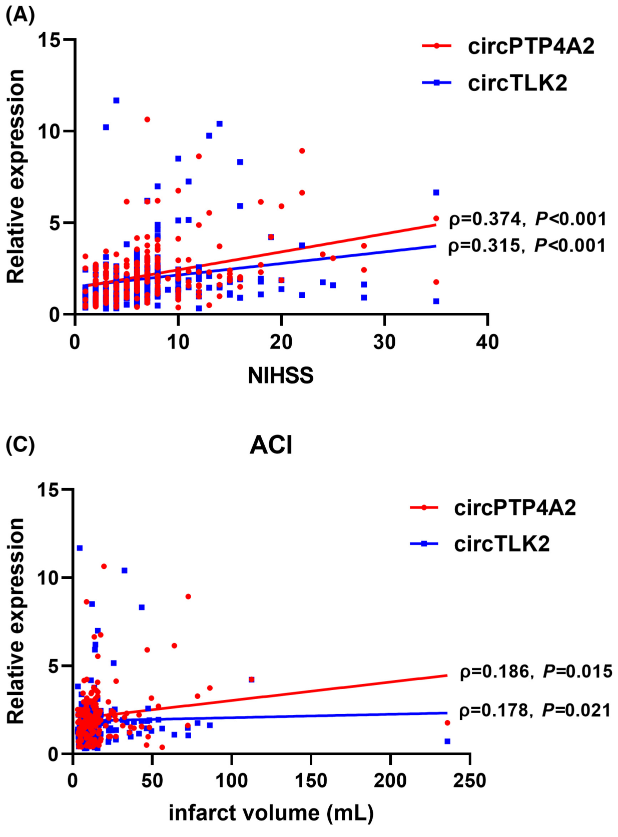

Figure 2. Relationship of circRNA expression levels with NIHSS score and infarct volume. (A) Spearman rank correlations between circRNA expression levels and NIHSS score in AIS patients. *N* = 236. (B–D) Spearman rank correlations between circRNA expression levels and cerebral infarct volume in AIS patients (*N* = 212), ACI patients (*N* = 169) or PCI patients (*N* = 43). ACI, anterior circulation infarction; PCI, posterior circulation infarction.

vs. HC: $p < 0.001$; CE vs. HC: $p = 0.045$; SAO vs. HC: $p < 0.001$; Fig. 3B), and the increase in LAA group was more evident (LAA vs. CE: $p = 0.005$; LAA vs. SAO: $p = 0.025$; Fig. 3B).

CircRNA levels and stroke outcome

To explore the significance of circRNA levels in predicting stroke outcome, we followed up with AIS patients at 3 months after stroke. All 236 AIS patients completed the follow-up, of which 176 patients had a favorable outcome (mRS $\leq$ 2), and 60 had a unfavorable outcome (mRS > 2). When compared with patients with favorable outcomes, those with unfavorable outcomes usually had higher NIHSS scores, larger infarct volumes, a longer time from onset to hospitalization and a greater proportion of LAA and CE (Table 3). However, the baseline levels of circPTP4A2 and circTLK2 failed to distinguish patients with favorable and unfavorable prognosis (Fig. 4A–C). To further investigate the association between the changes of circRNA levels and stroke outcome, we examined plasma levels of circPTP4A2 and circTLK2 from 48 AIS patients on Days 1 and 7 after stroke. As shown in Fig. 5A–F, the levels of circPTP4A2 and circTLK2 declined from Day 1 to Day 7 in favorable prognosis patients. In contrast, the levels of circPTP4A2 and circTLK2 were upregulated from Day 1 to Day 7 in unfavorable prognosis patients. Next, ROC curve analyses were performed according to the changes of circRNA levels within 7 days after hospital admission to predict unfavorable prognosis in stroke (ΔcircRNA = levels of circRNA on Day 7—levels of circRNA on Day 1). The AUCs of ΔcircPTP4A2 and ΔcircTLK2 for differentiating between favorable and unfavorable outcome were 0.809 (95%CI: 0.670–0.947, $p < 0.001$) and 0.796 (95%CI: 0.665–0.927, $p = 0.001$), respectively. In addition, the combination of these two ΔcircRNAs showed greater discriminatory ability (AUC = 0.838, 95%CI: 0.719–0.956) than the individual circRNA, with a sensitivity of 75.0%, specificity of 85.7% (Fig. 5G).

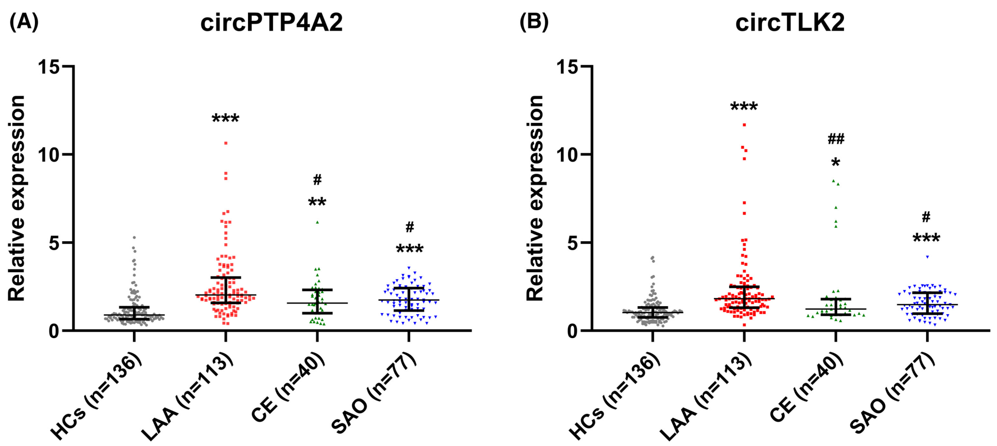

Figure 3. Relative levels of (A) circPTP4A2 and (B) circTLK2 in different subtypes of stroke according to TOAST classification. Median ± interquartile range, Kruskal–Wallis ANOVA test followed by Bonferroni correction pairwise comparisons.*$p < 0.05$ versus HCs; **$p < 0.01$ versus HCs; ***$p < 0.001$ versus HCs; $^{\#}p < 0.05$ versus LAA; $^{\#\#}p < 0.01$ versus LAA.

Discussion

In this study, we confirmed that increased plasma levels of circPTP4A2 and circTLK2 were associated with the stroke severity reflected by the NIHSS score. Furthermore, both circPTP4A2 and circTLK2 have also been shown to correlate with TOAST subtypes of AIS. Besides, the changes of these two circRNAs within 7 days of admission were able to predict unfavorable clinical outcome. Our results highlight the superior applicability of circPTP4A2 and circTLK2 as potential biomarkers for AIS.

Until now, several published studies have reported expression patterns of circRNAs in AIS patients, which raised the possibility of using them as novel biomarkers.[17–19] For example, Zuo et al.[19] found that circFUNDC1, circCDC14A and circPDS5B were significantly upregulated in the plasma of AIS patients. Liu et al.[18] demonstrated plasma circOGDH expression level elevated in AIS patients when compared with noncerebrovascular disease controls. The aforementioned studies, however, focused solely on the diagnostic value of circRNAs in AIS patients, with no assessment used for stroke severity. By contrast, this study highlights the accuracy of circRNAs in predicting stroke severity. Currently, the NIHSS is the standard assessment of stroke severity, which is widely used clinically.[20] The data of this study indicated that increased plasma levels of circPTP4A2 and circTLK2 were directly related to more severe stroke as assessed by NIHSS. Correlation analysis showed that there were medium positive correlations between levels of these two circRNAs and NIHSS score. Thus, expression levels of circPTP4A2 and circTLK2 might provide additional predictive information of illness severity for AIS patients, especially for those patients who were unable to cooperate with neurologic examination. Nevertheless, the application values of circRNA in stroke severity assessment still required a large sample study before potential application in the clinic.

Another important indicator of stroke severity is cerebral infarct volume. In this study, the levels of circPTP4A2 and circTLK2 were only weakly associated with cerebral infarct volume in ACI patients, did not appear to be significantly associated with cerebral infarct volume PCI patients. That is to say, we were unable to demonstrate the relevance of both circPTP4A2 and circTLK2 for cerebral infarct volume within 72 h of stroke onset. Indeed, our preceding study has revealed that plasma levels of circPTP4A2 and circTLK2 peaked within 24 h poststroke onset.[11] Consequently, the time interval from stroke onset to blood sample collection in the present study might be too long to describe appropriately the relationship between peak of expression of these two circRNAs and cerebral infarct volume. As generally known, cerebral infarct volume assessment is extremely important to determine the strategy of recanalization treatment in the hyperacute phase of AIS. Modern neuroimaging equipment, such as MRI and RAPID automated software are expensive and relatively time consuming.[21,22] Therefore, the value of circPTP4A2 and circTLK2 in the assessment of cerebral infarct volume needs further study at the hyperacute stage of AIS.

At present, there are relatively few studies available on expression of circRNAs to correlate stroke etiology. Xiao et al.[23] identified circulating exosomal circ_0043837 and circ_0001801 as predictive biomarkers for LAA stroke. Li et al.[24] found that hsa_circRNA_0003574 might serve as a potential therapeutic target and a novel biomarker for ischemic stroke caused by intracranial atherosclerotic

Table 3. Baseline characteristics of AIS patients with different outcomes.

	Favorable	Unfavorable	p value[a]
Demographic characteristics			
Total, *n*	176	60	
Age, years, median (IQR)	64 (17)	69 (13)	0.009[b]
Female, *n* (%)	59 (33.5)	23 (38.3)	0.499[c]
Prior vascular risk factors, *n* (%)			
Hypertension	119 (67.6)	36 (60)	0.283[c]
Diabetes mellitus	50 (28.4)	17 (28.3)	0.991[c]
Dyslipidemia	45 (25.6)	13 (21.7)	0.544[c]
Atrial fibrillation	27 (15.3)	11 (18.3)	0.586[c]
Coronary artery disease	26 (14.8)	9 (15.0)	0.966[c]
Current smoking	34 (19.3)	15 (25.0)	0.349[c]
Alcohol abuse	12 (6.8)	3 (5.0)	0.618[c]
NIHSS at admission, median (IQR)	6 (4)	12 (8)	<0.001[b]
Infarct volume, mL, median (IQR)	8.6 (9.05)	22.7 (34.70)	<0.001[b]
Stroke subtype, *n* (%)			0.001[d]
LAA	74 (42.0)	39 (65.0)	
CE	28 (15.9)	12 (20.0)	
SAO	68 (38.6)	9 (15.0)	
Other determined	6 (3.4)	0 (0)	
Undetermined	0 (0)	0 (0)	
Biochemical indexes on admission, median (IQR)			
Serum glucose, mmol/L	5.84 (1.89)	6.31 (2.10)	0.311[b]
TG, mmol/L	1.29 (0.66)	1.20 (0.62)	0.419[b]
TC, mmol/L	4.28 (1.24)	4.28 (1.52)	0.978[b]
HDL-C, mmol/L	1.04 (0.32)	1.05 (0.28)	0.894[b]
LDL-C, mmol/L	2.66 (1.15)	2.66 (0.97)	0.977[b]
Patients received alteplase or endovascular therapy, *n* (%)	13 (7.4)	8 (13.3)	0.162[c]
Time from onset to hospitalization, h, median (IQR)	13.0 (16.0)	24 (32.3)	<0.001[b]

CE, cardioembolic; HCs, healthy control subjects; HDL-C, high-density lipoprotein cholesterol; IQR, interquartile range; LAA, large artery atherothrombotic; LDL-C, low-density lipoprotein cholesterol; NA, not available; SAO, small artery occlusion; TC, total cholesterol; TG, triglyceride.
[a]Unfavorable stroke versus Favorable stroke.
[b]Mann–Whitney *U*-test.
[c]Chi-squared test.
[d]Fisher's exact test.

stenosis. In this study, we also found that the levels of circPTP4A2 and circTLK2 were elevated in LAA, CE, and SAO groups, with more pronounced in LAA group. Rupture of an atherosclerotic plaque is a frequent cause for ischemic stroke. Immune-inflammatory activation contribute directly to atherogenesis, and also to formation of vulnerable plaques.[25] In a previous study, we identified circPTP4A2 and circTLK2 as highly associated with the immune-inflammatory mechanism of ischemic stroke by using bioinformatics analysis.[11] Thus, both circPTP4A2 and circTLK2 might be involved in formation and development of atherosclerotic plaque. However, further studies are warranted to understand the role of circPTP4A2 and circTLK2 in the pathogenesis of LAA stroke.

Accurate prediction of outcome is beneficial for informed decision-making about clinical management and rehabilitation program after AIS. Although baseline levels of circPTP4A2 and circTLK2 did not predict stroke outcome in our study, the changes of these two circRNAs within 7 days of admission provided useful prognostic information in AIS patients. Recently, several high-quality clinical studies have indicated that some blood-based biomarkers were correlated poor outcome after stroke.[3,26–28] However, most identified biomarkers were not routinely applied in clinical practice. This study indicated that the levels of circPTP4A2 and circTLK2 showed a significant downward trend from Day 1 to Day 7 in favorable prognosis patients, while they showed a significant upward trend from Day 1 to Day 7 in unfavorable prognosis patients. Thus, dynamic monitoring of circPTP4A2 and circTLK2 may have contributed to improve discrimination of stroke prognosis.

Although the sources and biological mechanisms of increased circPTP4A2 and circTLK2 remain to be established, our data provided several important clues. Specifically, increased levels of circPTP4A2 and circTLK2 were

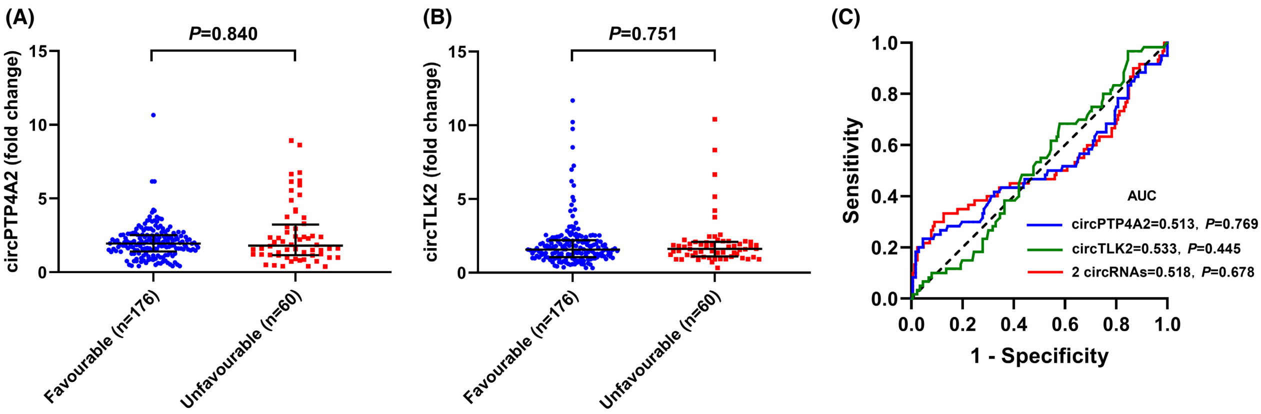

Figure 4. Correlations of circRNA levels with stroke outcome. Relative levels of (A) circPTP4A2 and (B) circTLK2 via qPCR in favorable outcome (mRS ≤ 2) and unfavorable outcome (mRS > 2) group. Median ± interquartile range, Mann–Whitney *U*-test. (C) ROC curve for circPTP4A2 and circTLK2 on predicting unfavorable outcome at 3 months after stroke. AUC, area under the receiver operating curve.

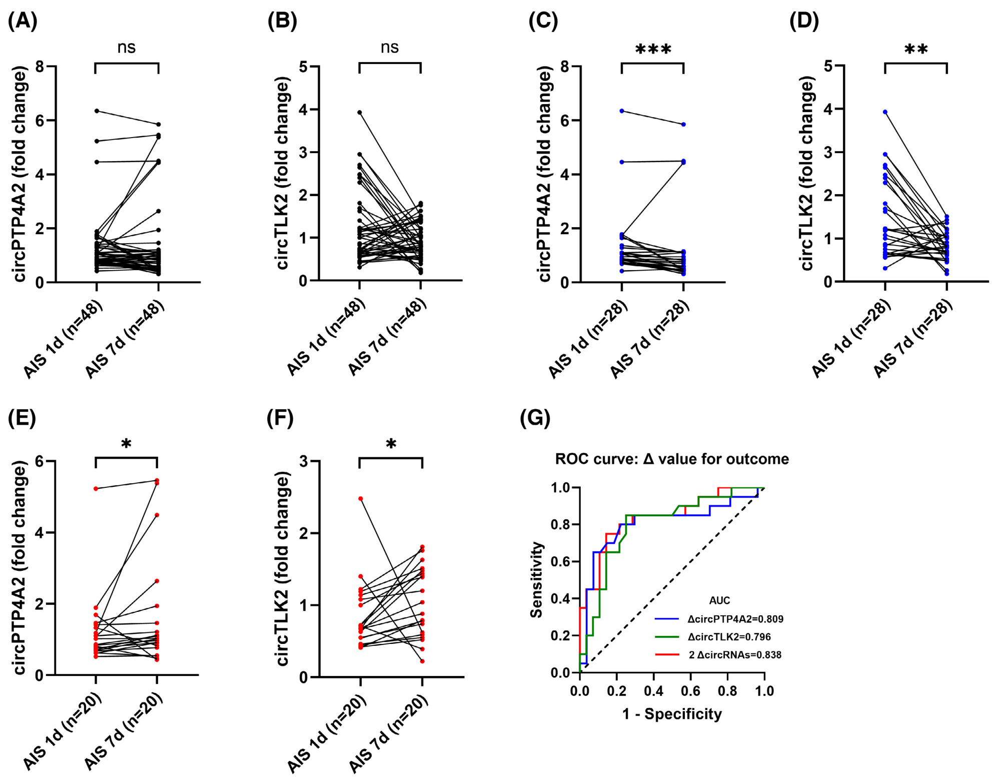

Figure 5. Relative levels of circRNA in AIS patients on Day 1 and Day 7, and their values in predicting outcome. (A, B) For all patients; (C, D) for patients with favorable outcome (mRS ≤ 2); (E, F) for patients with unfavorable outcome (mRS > 2). Wilcoxon matched-pairs test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. (G) ROC curve was constructed according to Δvalue (relative levels of circRNA on Day 7 minus relative levels on Day 1). AUC, area under the receiver operating curve.

more common in patients with moderate to severe stroke and they were associated with the severity of stroke. Moreover, in various central nervous system diseases, including Alzheimer's disease, trauma stroke and brain tumors, free or exosome-encapsulated circRNAs can be released into peripheral circulation after blood–brain barrier disruption.[29] A recent study revealed that neuron-derived circRNA was released from ischemic brain tissue to circulating peripheral blood by exosome.[18] Hence, it is rational to speculate that elevated circPTP4A2 and circTLK2 could be a correlated response to aggravated ischemic injury and they could be derived from neurons or other neural cells in ischemic zone. However, such a hypothesis requires further experimental confirmation.

Several recent studies have demonstrated that circRNAs were involved in the pathogenesis of stroke-related neuroinflammation.[30–32] In a mouse stroke model, knockdown of neutrophil-derived circCDC14A inhibited astrocyte activation and alleviates neurological damage after cerebral ischemia.[31] Intriguingly, another research also reported similar findings demonstrating that specific blockage of circHECTD1 ameliorated astrocyte activation in transient middle cerebral artery occlusion (tMCAO) mouse.[30] In addition, overexpression of circ_0000831 attenuated neuroinflammation by competitively binding to miR-16-5p in microglia following cerebral ischemia.[32] Moreover, our previous research also suggested that circPTP4A2 and circTLK2 might be closely linked to immuno-inflammation following AIS.[11] The above studies indicated the implications of circRNAs in neuroinflammatory mechanism and management of ischemic stroke. Small interfering RNA (siRNA) have been engineered for various central nervous system diseases,[33] and targeted silencing of circPTP4A2 and circTLK2 as a novel stroke therapeutic strategy might be achieved in the near future.

Remarkably, this study has not only clinical value, but also important public health implication. Our data suggest that plasma circPTP4A2 and circTLK2 might contributes to risk stratification, and facilitate the selection of more effective surveillance and therapy for stroke in clinical practice, especially for patients with more severe stroke and poor prognosis. However, our research also has several limitations. First, this was a single-center study, and therefore, our results may not apply to other clinical center. External validation using large multicenter studies will be required in the future. Second, only 48 samples were collected on Days 1 and 7 after stroke. Due to small sample size, exact logistic regression model for predicting stroke prognosis cannot be constructed. Thus, more AIS patients need to be recruited in order to evaluate of the prognostic significance of dynamic changes in plasma circPTP4A2 and circTLK2. Third, association of stroke severity and plasma levels of circPTP4A2 and circTLK2 may be influenced by several factors. Even though we adjusted for numerous potential confounders, residual and unmeasured confounding could not be ruled out.

Conclusions

In conclusion, our findings suggest that elevated plasma circPTP4A2 and circTLK2 levels at admission independently associated with increased severity of stroke, and they provide additional important information regarding relationship between these two circRNAs and etiologic stroke classification. Moreover, our study raised the exciting possibility that monitoring changes in circPTP4A2 and circTLK2 might be useful for stroke outcome prediction. Further studies are necessary to unravel underlying mechanisms and clinical implications of circPTP4A2 and circTLK2 in ischemic stroke.

Author Contributions

Xingzhi Wang, Shenyang Zhang, and Guiyun Cui conceived and designed the study. Xingzhi Wang, Zuohui Zhang, Jie Zu, Hongjuan Shi, and Lu Yu collected and analyzed the clinical data. Xingzhi Wang, Shenyang Zhang, Bingchen Lv, Likun Cui, Wenqi Mao, and Di Wu carried out experiments, and prepared figures and tables. Xingzhi Wang wrote the manuscript draft.

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Conflict of Interest Statement

The authors claim no conflicts of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Comparison of circRNAs expression between various subgroups of HCs with different stroke risk factors.

