Reports of Biochemistry & Molecular Biology
Vol.12, No.3, Oct 2023

Original article

www.RBMB.net

Association of Circulating Circular RNAs (hg38_circ_0008980, and CircDLGAP4) in Diagnosis, Diseases Severity, and Prognosis of Ischemic Stroke

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Abstract

Background: Fast diagnosing ischemic stroke (IS) is a critical issue in clinical studies, as it allows more effective therapy and stops the progression of IS. The blood level of circular RNAs (CircRNAs) after stroke may be a rapid diagnostic marker.

Methods: In this study, the blood level of circRNAs was evaluated using a real-time polymerase chain reaction (PCR). We used logistic and linear regression analysis to assess the potential of circRNAs levels with the risk of IS.

Results: circRNA DLG associated protein 4 (CircDLGAP4) was decreased in patients compared with controls, and logistic regression showed its expression negatively associated with IS risk. The expression level of human genome version 38_Circular_0008980 (hg38_circ_0008980) was reduced significantly in patients with small vessel disease (SVD), and the linear regression analysis showed a negative relationship between hg38_circ_0008980 expressions with SVD subtype. hg38_circ_0008980 expression relative to controls showed a significant association with IS risk.

Conclusions: Taken together, we found a significant decrease in the level of hg38_circ_0008980 after IS; it may act as a novel circRNA in IS pathophysiology with a positive correlation with stroke severity.

Keywords: Biomarkers, Circular RNAs, Ischemic strokes.

Introduction

Stroke is the most common cause of morbidity, mortality, and long-term disability in developed and developing countries (1, 2). There are several mimics and camouflages for ischemic stroke (IS) (3-5). Thus, finding a rapid diagnostic test to confirm the clinical diagnosis and more effective therapy for ischemic stroke is necessary (6).

One of the endogenous single-stranded RNAs with a closed covalent-circle structure is circRNAs (7). They are produced during post-transcriptional modification by back-splicing of mRNAs or Long noncoding RNAs (lncRNAs) and, because of their covalent structure as well as resistance to exonuclease are much more stable than linear RNA, thus can serve as a diagnostic marker in the peripheral blood of patients (8). CircRNAs can regulate the expression of specific target genes (9). Several circRNAs are involved in the regulation of ischemic-reperfusion injury (IRI) (10, 11) and other cerebral diseases (12, 13). CircRNAs may also act as a diagnostic biomarker for discriminating neurological disorders (14), while they mainly have a sponge function for the regulation of microRNA (miRNA) (15). Animal studies have revealed a close association of cerebral circRNA expression with IS (10, 16, 17).

The circDLGAP4 is a sponge for miR-143,

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Received: 10 Jul, 2023; Accepted: 26 Sep, 2023

[DOI: 10.61186/rbmb.12.3.476]

and in animal models has been shown that the overexpression of circDLGAP4 attenuated the neurological deficits, the infarct areas, and blood-brain damage (11). Bai et al. have shown that circDLGAP4 levels were significantly decreased in acute IS patients. They demonstrated that circDLGAP4 could inhibit miR-143 activity (11). Silencing of miR-143 exerted a protective effect against cerebrovascular damage (18). The circDLGAP4-miR-143 network exerts specific critical regulatory impact on the permeability of the blood-brain barrier (BBB), attenuates IS outcome (19, 20).

Another circRNA showing an important regulatory effect on BBB integrity is It is a novel circRNAs could sponge hsa-miR-660-5p and exert regulatory effects of meningitic E. coli penetration on BBB (21). hg38_circ_0008980 mentioned above, is likely to have a regulatory effect on BBB permeability in IS. There is no evidence about the hg38_circ_0008980 expression after IS. Thus, we hypothesized that the expression of hg38_circ_0008980 may alter after IS and serve as a significant diagnostic marker for IS. Therefore, we evaluated for the first time the expression levels of novel circRNA, hg38_circ_0008980, and also circDLGAP4 level in peripheral blood of 75 IS patients and 75 controls by real-time PCR to determine their expression levels within the first 24 hours after IS to correlate these levels with the stroke severity, clinical parameters as well as their possible application for diagnosis and prognosis of IS.

Materials and Methods

Study subjects

This is a case-control study conducted at Namazi Hospital in Shiraz. All patients underwent brain diffusion-weighted magnetic resonance imaging (MRI) or non-contrast computed tomography (CT) to differentiate IS from intracerebral hemorrhage. According to the Guidelines, stroke is an acute neurologic disorder lasting more than 24 hours (or < 24 hours with neuroimaging evidence) (22). In this study, inclusion criteria were as follows: patients must be admitted within 24 h from the onset of stroke without a history of the previous stroke, older than 18 years, and we could follow them regularly. Seventy-five controls from Shiraz's population were randomly included in our study and were matched for sex and age with cases. The ages of the patients and controls ranged from 30-95 years. Controls with a history of stroke, transient ischemic attack (TIA), or severe brain disorders were excluded. This study, hypertension and diabetes were diagnosed according to these criteria: for hypertension: receiving antihypertensive therapy or blood pressure ≥ 140/90 mmHg on two occasions at least 24 h apart (23). Diabetes mellitus: receiving treatment with hypoglycemic drugs or two fasting glucose >126 mg/dl (7.0 mmol/L) and 2-h post-load glucose >200 mg/dl (11.1 mmol/L) or (24). To indicate the severity of stroke, we used the National Institutes of Health Stroke Scale (NIHSS) score on admission; higher, scores represent greater severity (25). Six months after admission, the functional outcomes were obtained according to the modified Rankin scale (mRS) blinded to the level of CircRNA (26). Different types of IS were diagnosed according to the trial of org 101072 in acute stroke treatment (TOAST) classification (27). Ethics approval for this study was obtained by the local ethics committee of the Arsanjan Branch, Islamic Azad University, Iran (IR.IAU.A.REC.1399.029). Peripheral venous blood samples were collected after taking written informed consent from all subjects (or their proxy respondents). The blood samples were collected from 75 patients admitted in 0-24 h after stroke symptoms.

Measurement of the circRNAs levels

TRIzol Reagent (GeneAll, Seoul, South Korea) was used to isolate total RNA from blood samples according to the thiocyanate-phenol-chloroform method. Using complementary DNA (cDNA) synthesis Kit (Yektatajhiz, Iran) for cDNA synthesis from RNA samples with the A260/A230 and

[DOI: 10.61186/rbmb.12.3.476]

A260/A280 ratios greater than 1.7 (28). We used quantitative real-time PCR for circDLGAP4 and hg38_circ_0008980 levels measurement using RealQ Plus 2x Master Mix Green Low ROX™ (Ampliqon, Denmark). *TATA box-binding protein* (TBP) was used as an internal reference for circRNAs detection. The Quantstudio 3 Real-Time PCR System (Applied Biosystems, Foster City, USA) was used while the thermal-cycling settings were one repeat (10 min at 95 °C) accompanied by 40 cycles (15 s at 95 °C, 30 s at 62 °C, 45 s at 72 °C) and one cycle (5 min 72 °C). The melting phase was (15 s at 95 °C, 30 s at 72 °C, 15 s at 95 °C). Variation in expression levels was expressed using the cycle threshold (Ct) values. ΔCt represents the difference of Ct between *TBP* and the target gene. The relative circRNAs expression levels for every individual were defined using $2^{-\Delta Ct}$. The results were calculated with the $2^{-\Delta Ct}$ method (29). The primer sequences were used as follows (4, 11, 21): (Direction; 5′-3′)

CircDLGAP4;
Forward: ACGGCTACTGGTTCCTAAAGC
Reverse: GGGGTCTTCTTATACGCCACT

hg38_circ_0008980;
Forward: CTCCACCAGATGTCAGTT-3′,
Reverse: TACAGGCAGAGGGTATTTG

TBP;
Forward: CCCGAAACGCCGAATATAATC
Reverse: TCTGGACTGTTCTTCACTCTTG

Statistical analysis
The circRNAs expression levels were shown as mean ± standard error (SE). The following tests were used for different analyses in our study, the student's t-test for comparing the mean expression of the circ-RNAs between case and control groups. One-Way Analysis of Variance (ANOVA) for comparing the circRNA expression levels between different types of strokes. A comparison between categorical data was carried out using a chi-square test. The expression level of circRNAs was compared between cases and controls using an independent two-sample t-test. We used logistic regression analyses to evaluate the association of circRNA expression with the risk of IS. The relationship between circRNA levels with clinical parameters was analyzed using linear regression. By receiver operating characteristic curve (ROC) curve analysis, the diagnostic and prognostic values were estimated, and the findings were presented as the area under the curve (AUC). Correlations were analyzed using the Spearman correlation. The analyses used the statistical package for the social sciences (SPSS) software (version 19.0) and GraphPad Prism 5.0. The *p*-value of < 0.05 was regarded as statistically significant.

Results

Demographic and clinical characteristics of all participants (IS patients and controls)
In total, 75 first-ever stroke patients with symptom onset within 24 hours were recruited and assessed in this study (27 females (36%) and 48 males (64%) aged from 32 to 90 years old (the mean age and standard deviation were 64.79 ± 1.78)). The control group was similar in age, sex, and body mass index (BMI) to the case group (Table 1). The mean age of the control group was 64.55±1.68. Risk factors such as diabetes, hypertension, and smoking were demonstrated to be more prevalent in cases compared to controls. In laboratory findings, there were no significant differences in the levels of triglyceride and total cholesterol between the IS cases and the controls; however, low-density lipoprotein was significantly higher, and high-density lipoprotein levels were significantly lower in IS cases compared to controls (Table 1).

[DOI: 10.61186/rbmb.12.3.476]

Table 1. Demographic and clinical characteristics of the participants.

Characteristics		Cases (n = 75)	Controls (n = 75)	*p*
Male, n (%)		48 (64%)	48 (64%)	0.999[a]
Female, n (%)		27 (36%)	27 (36%)	
Age (years)		64.79±1.78	64.55±1.68	0.922[b]
BMI (kg/m^2)		25.64±0.53	25.99±0.45	0.619[b]
Hypertension, n (%)	Yes	46.66%	13.33%	0.641
	No	53.33%	60 %	
Diabetes, n (%)	Yes	24%	26.66%	0.452
	No	76%	73.33%	
Smoking, n (%)	Yes	18.66%	4%	0.005[a**]
	No	81.33%	96%	
Drinking, n (%)	Yes	4%	0%	0.080[a]
	No	96%	100%	
Hyperlipidemia	Yes	14.16%	10.66%	0.461[a]
	No	85.33%	83.33%	
Laboratory findings				
Triglyceride (mmol/L)		117.83±5.30	122.73±7.58	0.597[b]
Total Cholesterol (mmol/L)		144.35±5.29	114.89±4.37	0.999[b]
Low Density Lipoprotein (mmol/L)		91.25±3.209	81.24±3.243	0.030[b*]
High Density Lipoprotein (mmol/L)		36.07±0.847	41.41±1.265	0.001[b***]
Large artery atherosclerosis, n (%)		30 (40%)		
Small-vessel occlusion, n (%)		26 (34.66%)		
Cardio embolism, n (%)		7 (9.33%)		
Undetermined, n (%)		12 (16%)		
NIHSS at admission				
≤6		40%		
≥7		60%		
mRS at 3 months at admission				
0-2		30.66%		
3-6		96.33%		
mRS at 6 months				
0-2		48.43%		
3-6		51.56%		

Data were shown as mean ± SD or as n (%). [a]Chi-square Test; [b]Independent two-sample T-test.
BMI, body mass index; NIHSS, National Institutes of Health Stroke Scale; mRS, modified Rankin Scale.
*$p < 0.05$. **$p < 0.01$. ***$p = 0.001$.

[DOI: 10.61186/rbmb.12.3.476]

Expression of hg38_circ_0008980 and circDLGAP4 in IS patients were lower than controls.

The blood levels of circDLGAP4 and hg38_circ_0008980 in IS patients were significantly lower than the controls at 0-24 h after stroke respectively [(1.90 ± 0.11 *vs.* 2.23 ± 0.10) and (1.71 ± 0.13 *vs.* 2.37 ± 0.24)] ($p < 0.05$, Fig. 1A). Furthermore, One-Way ANOVA analysis showed the level of hg38_circ_0008980 and circDLGAP4 in SVD cases was significantly lower than those in the controls ($p < 0.05$) (Figs. 1B and C) [hg38_circ_0008980; F value (4, 145) = 2.259, $p = 0.0656$] and [circDLGAP4; F value (4, 145) = 3.506, $p = 0.0092$]. However, a significant decrease in circDLGAP4 expression was reported in patients with SVD relative to UD (Fig. 1C).

logistic regression analysis also revealed the significant negative association of hg38_circ_0008980 and circDLGAP4 expression with the risk of IS (adjusted OR = -0.412; 95% CI: 0.49-0.88, $p < 0.01$) and (adjusted OR = -0.404; 95% CI: 0.45-0.98, $p < 0.05$) respectively (Data not shown).

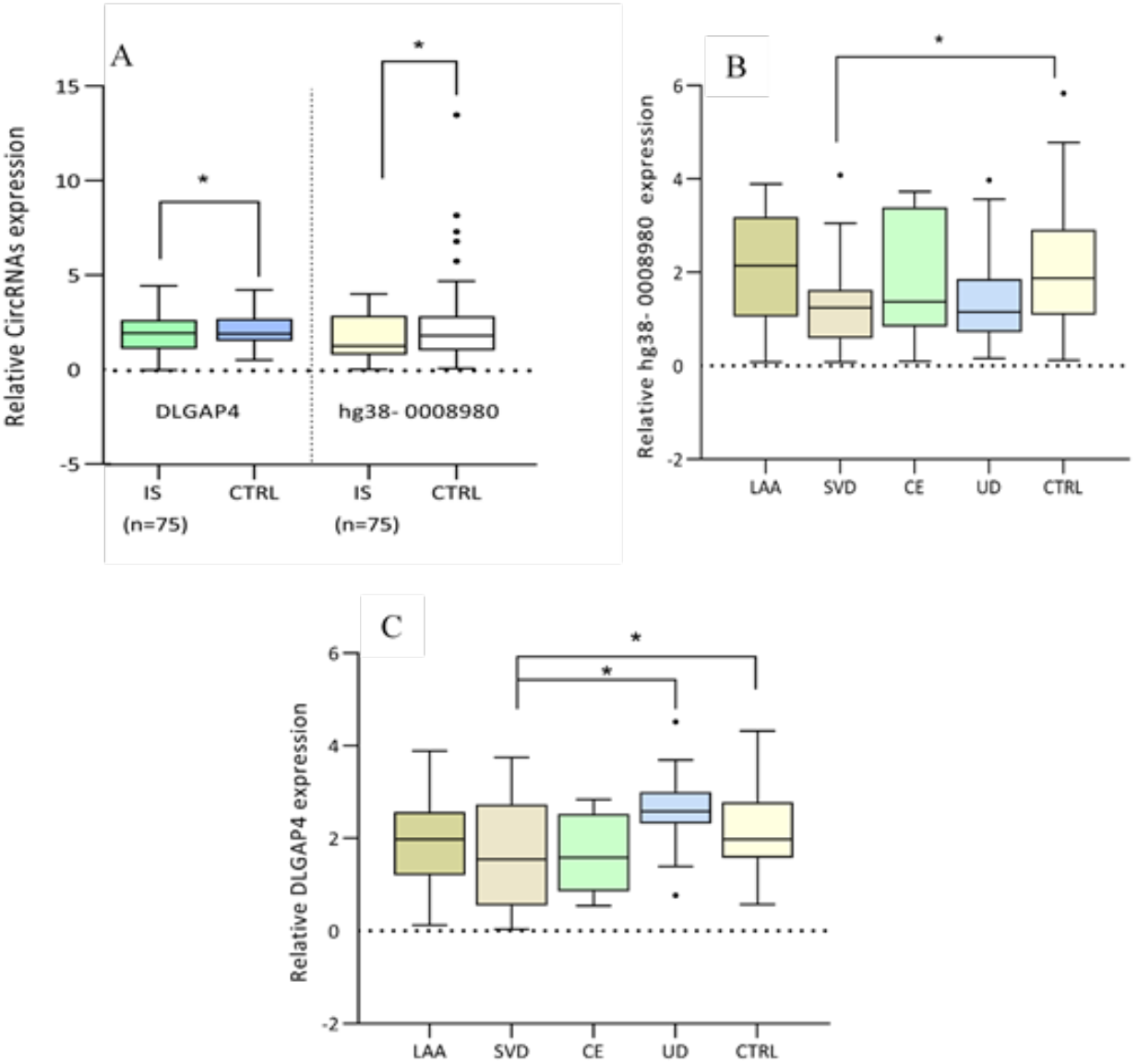

Fig. 1. The expression levels of hg38_circ_0008980 and circ circDLGAP4 in patients, controls, and different subtypes of ischemic stroke. (A) Independent Student's t-test revealed that hg38_circ_0008980 and circDLGAP4 levels were lower than controls. (B, and C) The comparison of hg38_circ_0008980 and circDLGAP4 levels in different subtypes of IS by One-Way ANOVA analysis. Results were expressed as mean ± SEM. *$p < 0.05$. ***$p < 0.001$. Abbreviations: IS, ischemic stroke; CTRL, control; LAA, large-artery atherosclerosis; SVD, small-vessel disease; CE, cardioembolism; UD, undetermined.

[DOI: 10.61186/rbmb.12.3.476]

Association between clinical variables with circRNAs (hg38_circ_0008980 and circDLGAP4) expression

The association between clinical variables and circRNAs expression was defined by subgroup analyses. The level of hg38_circ_0008980 expression was significantly higher in patients with NIHSS scores > 7.

Furthermore, we also used linear regression analysis to detect the association between the circRNA levels with clinical parameters and types of stroke. We found significant negative relationships between hg38_circ_0008980 expression and SVD subtype ($p = 0.008$, Beta = -0.036) and also between circDLGAP4 level and BMI in IS patients ($p = 0.02$, Beta = - 0.29) (Table 2).

Table 2. Linear regression analysis for the association between clinical parameters with circDLGAP4 and hg38_circ_0008980 in IS patients.

Variables	Beta circDLGAP4	95 % CI		p-value	Beta hg38_circ_0008980	95 % CI		p-value
SEX	0.076	-0.327	0.644	0.516	-0.155	-0.952	0.201	0.198
Age	-0.125	-0.024	0.008	0.309	0.080	-0.013	0.025	0.527
BMI	-0.292	-0.119	-0.009	**0.022***	-0.066	-0.081	0.048	0.609
NIHSS	0.038	-0.032	0.044	0.757	0.097	-0.028	0.063	0.440
MRS	-0.148	-0.281	0.064	0.214	-0.122	-0.308	0.102	0.319
HTN	0.104	-0.256	0.677	0.371	0.090	-0.345	0.765	0.453
DM	-0.033	-0.666	0.509	0.790	0.117	-0.381	1.01	0.367
SVD	-0.150	-0.870	0.236	0.257	-0.367	-1.55	-0.240	**0.008****
CE	-0.109	-1.21	0.462	0.373	-0.095	-1.37	0.618	0.451
UD	0.215	-0.079	1.258	0.083	-0.197	-1.42	0.169	0.121

BMI, body mass index; NIHSS, National Institutes of Health Stroke Scale; mRS, modified Rankin Scale DM, diabetes mellitus; IHD, Ischemic heart disease, HTN, Hypertension: HLP, Hyperlipidemia; SVD, Small-vessel disease; CE, cardioembolism; UD, undetermined, *$p < 0.05$. **$p < 0.01$.

Moreover, the Spearman correlation also revealed that the hg38_circ_0008980 level was significantly correlated with NIHSS scores (Fig. 2A). We did not find a significant correlation between circDLGAP4 expression levels with NIHSS scores. Spearman test showed a significant negative relationship between circDLGAP4 level and BMI in IS patients (Fig. 2B). Our results revealed a positive correlation between the level of circDLGAP4 and high-density lipoprotein (HDL) in IS patients.

Diagnostic and prognostic value of circulating circRNAs (hg38_circ_0008980 and circDLGAP4) in IS

The expression level of circDLGAP4 and hg38_circ_0008980 in 0-24 h after stroke also showed a nonsignificant diagnostic value for discriminating IS patients from the controls (Figs. 3B and C). The sensitivity and specificity were 78.67% and 38.67 % for the diagnostic value of circDLGAP4 level and 56 %, and 60 %, for hg38_circ_0008980 ($p > 0.05$).

In our study, we considered the mRS score of 3-6 in 6 months after stroke as an unfavorable functional outcome and the mRS score of 0-2 as a favorable outcome. The circDLGAP4 and hg38_circ_0008980 levels showed no significant predictive prognosis for a 6-month adverse outcome relative to a good outcome with an AUC of 0.58 and 0.55, respectively. These results demonstrated the expression levels of two circular RNA could not be identified as a functional outcome prediction marker for IS. The sensitivity and specificity for circDLGAP4 and hg38_circ_0008980 were as follows (80.77% and 39.13%) and (71.15% and 47.83%) respectively (Data not shown).

[DOI: 10.61186/rbmb.12.3.476]

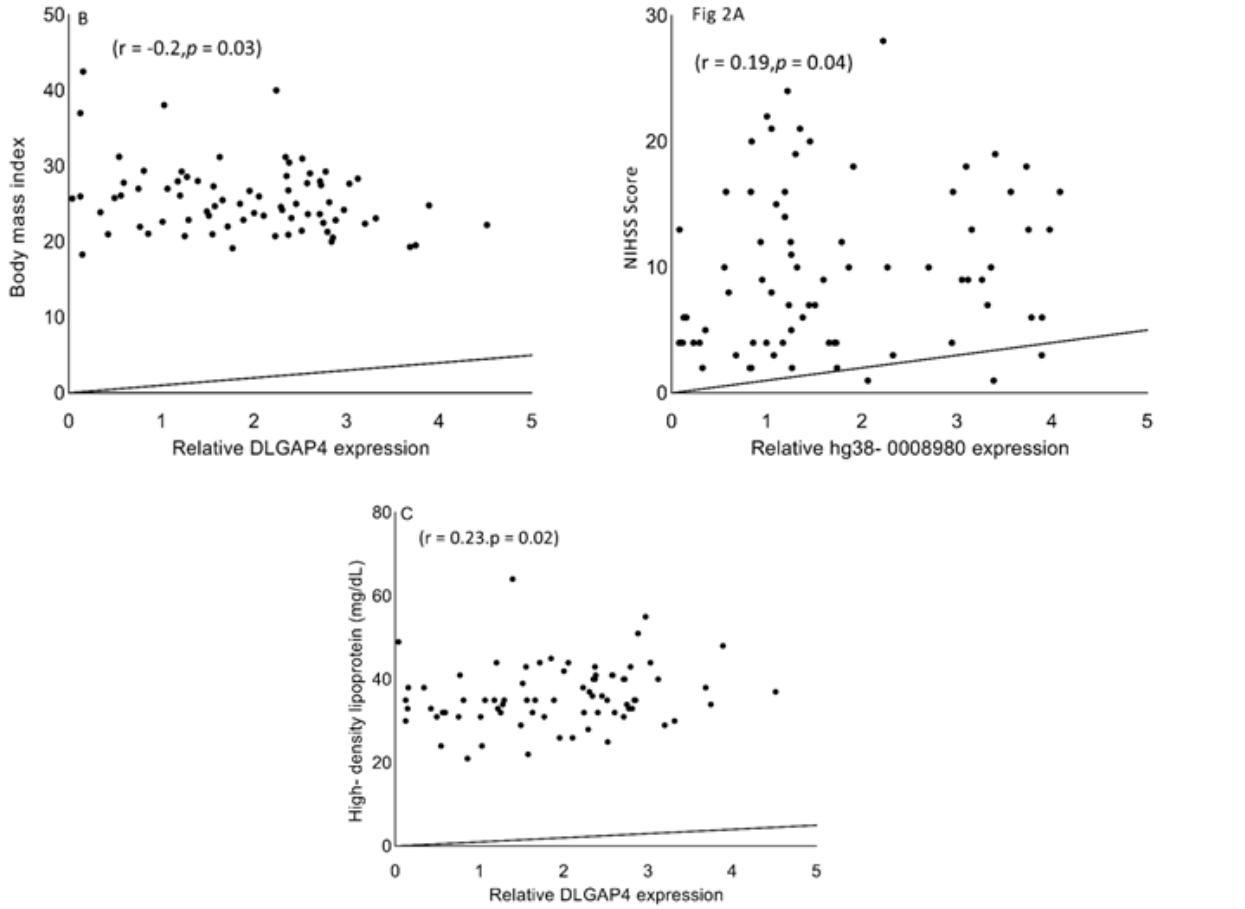

Fig. 2. The Spearman Correlation between expression levels of circRNAs with NIHSS and BMI in patients. (A) The Spearman Correlation between expression of hg38_circ_0008980 and NIHSS in patients (B) Correlation between expression of circDLGAP4 and BMI (C) Correlation between expression of circDLGAP4 and HDL. NIHSS, National Institutes of Health Stroke Scale; BMI, body mass index; HDL, high-density lipoprotein.

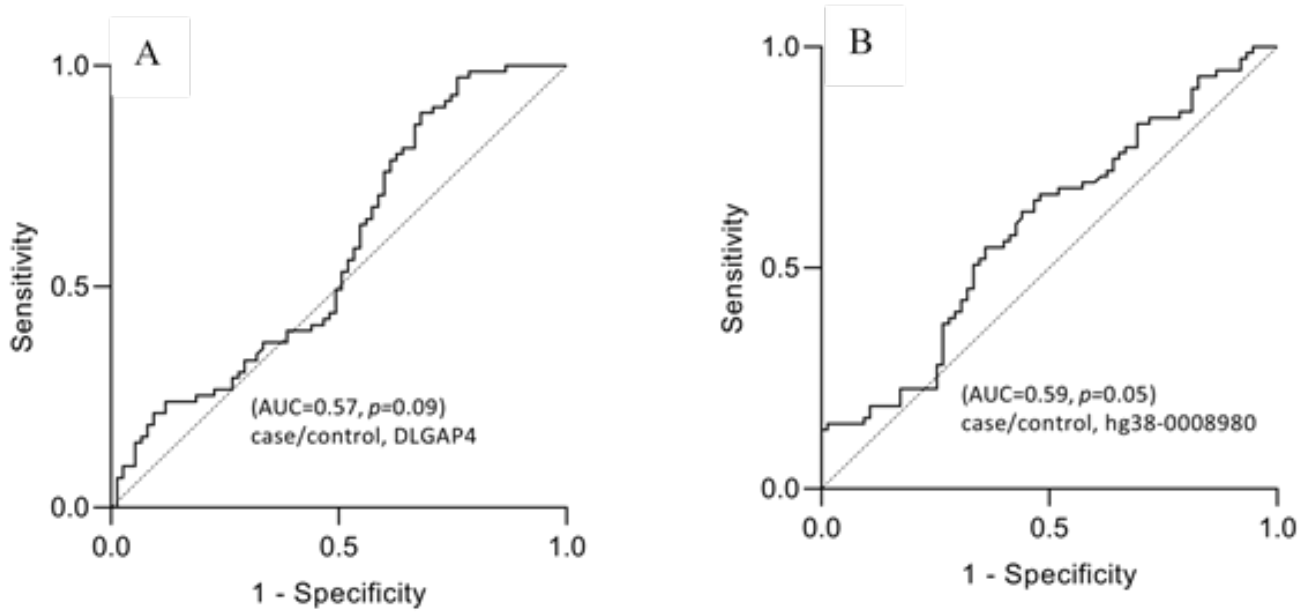

Fig. 3. ROC curves. (A, B) ROC curve analyses of circDLGAP4 and hg38_circ_0008980 for discriminating IS patients from the controls. ROC, receiver operating characteristic; AUC, area under the curve.

[DOI: 10.61186/rbmb.12.3.476]

Discussion

We found a significant decrease in the level of hg38_circ_0008980 after IS; it may act as a novel circRNA in IS pathophysiology with a positive correlation with stroke severity. Our research was the first study that assessed hg38_circ_0008980 expression 0-24 hours after IS. It showed that hg38_circ_0008980 was decreased in patients compared with controls, and logistic regression showed a significant association of its expression with IS risk. CircDLGAP4 expression was negatively associated with IS risk. CircDLGAP4 had a negative correlation with BMI (r = -0.2, p = 0.03) and a positive correlation with HDL (r = 0.23, p = 0.02).

In our study, a significant decrease in the expression level of circDLGAP4 was seen 0-24 h after IS. This result is consistent with previous studies that demonstrated the downregulation of circDLGAP4 and its protective effect in IS patients (11, 30). We could not find the negative correlation between circDLGAP4 expression and NIHSS score, but logistic regression analysis showed the negative association between circDLGAP4 levels with risk of IS. This result was confirmed by the significant negative and positive correlation between BMI and HDL with circDLGAP4 levels, respectively. Because the decrease in BMI and increase in HDL level decrease the risk of IS (31, 32). Our results showed patients with lower BMI and higher HDL relative to others had elevated circDLGAP4 expression with a lower risk of IS.

In the Zho study, the mean NIHSS score was 7.9 ± 3.4, while the mean NIHSS score in our patients was 9.8 ± 0.74. Therefore, the value of circDLGAP4 might not be well correlated with NIHSS in patients with severe IS. The expression of circDLGAP4 showed a significant decrease relative to controls in our patients with SVD type. Cerebral small vessel disease has a significant relationship with type 2 diabetes mellitus (33, 34). A previous study showed that circDLGAP4 promotes diabetic kidney disease injury by sponging miR-143 (35). According to those mentioned above, there is a possibility of a correlation between circDLGAP4-miR-143 and diabetes and SVD.

hg38_circ_0008980 expression showed significant downregulation 0-24 h after stroke, while logistic regression analysis represented a negative association with risk of IS. Subgroup analysis and Spearman test showed a significant positive correlation between the hg38_circ_0008980 level and NIHSS score. Moreover, linear regression has also demonstrated the negative relationship between hg38_circ_0008980 level and SVD stroke that was confirmed by the significant decrease in hg38_circ_0008980 expression level close to controls. We also found the lower mean NIHSS score in SVD patients relative to the others (7.96 ± 1.09 *vs* 9.8 ± 0.74), respectively. Therefore, it seems that hg38_circ_0008980 level as a novel circRNA may have a positively correlate with stroke severity, especially in patients with SVD.

Our study had some limitations. It was a single-center study with a relatively sample size. A small number of IS patients with cardioembolism (CE) stroke had enrolled in our study; therefore, in this study, the value of circRNAs in patients with CE might not be well evaluated. In addition, we did not assess the association of inflammatory biomarkers such as highly sensitive C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrotic factor-alfa (TNF-α) with CircRNAs.

As extensive studies showed better prognosis but still a high recurrence rate of IS (36), if the association of CircRNAs with stroke and its predisposing factors becomes well-proven in translational studies, their measurements can be used as indicators of efficacy of the preventive and therapeutic policies for stroke. This was the first study revealing a significant decrease in hg38_circ_0008980 expression in IS patients. Our results showed a significant positive correlation of hg38_circ_0008980 with NIHSS expression after stroke. A larger sample size with more information needs to reach the appropriate statistical power. A small number

[DOI: 10.61186/rbmb.12.3.476]

of IS patients with CE stroke had enrolled in our study; therefore, in this study, the value of circRNAs in patients with CE might not be well evaluated. To clarify the role of circulating circular RNAs (hg38_circ_0008980 and circDLGAP4) in IS. Additional research with a larger sample size is needed to study the diagnostic and prognostic value and the downstream effects of these circRNAs. Further investigations are necessary to find the time-dependent changes in expression levels of these circRNAs after IS and their correlation with stroke severity. Taken together, circulating hg38_circ_0008980 level showed a positive correlation with stroke severity. We also found the downregulation of circDLGAP4 after stroke, while its expression showed a negative association with the risk of IS.

Acknowledgments

The present study was derived from a thesis by Mastaneh Zeraatiannejad. This work was supported by Islamic Azad University, Zarghan Branch, Zarghan, Iran.

Funding

No financial assistance was received in support of the study.

Conflicts of Interest

The authors have no relevant financial or nonfinancial interests to disclose. Authors declared that the publishing of this article does not constitute a conflict of interest with respect to the research, authorship, and/or publication of this article.

Ethical statements

Ethics approval for this study was obtained by the local ethics committee of the Arsanjan Branch, Islamic Azad University, Iran (IR.IAU.A.REC.1399.029). Peripheral venous blood samples were collected after taking written informed consent from all subjects (or their proxy respondents).

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[DOI: 10.61186/rbmb.12.3.476]

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