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Molecular epidemiological study of exosomes *circZNF609*, *circPUM1*, *IGF2* with ischemic stroke

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Abstract

Background Ischemic stroke (IS) is a common cardiovascular disease (CVD). Insulin-like growth factor 2 (*IGF2*), *circZNF609*, and *circPUM1* are involved in metabolic regulation, vascular health, neuroprotection, and inflammation modulation and are relevant to IS mechanisms. This study investigated the effects of plasma exosomal expression of *circZNF609*, *circPUM1*, and *IGF2* on IS.

Methods The expression of *circZNF609*, *circPUM1*, and *IGF2* mRNA in exosomes was detected in 145 patients with IS and 290 controls using real-time qPCR in a cross-sectional study. *Q1–Q4* represents the quartile groups based on the target gene expression levels.

Results There was no significant difference in the expression levels of *circZNF609* and *circPUM1* in the plasma exosomes between the IS and control groups ($P > 0.05$). However, a nonlinear relationship between the expression levels of *circZNF609* in the IS group ($P < 0.05$). Exosomal *IGF2* mRNA expression in the IS group was significantly lower than that in the control group ($P = 0.043$).

The multifactorial adjusted results showed that in the case–control study of IS, *circZNF609* in plasma exosomes was associated with a reduced risk of disease in group *Q2* (adjusted *OR*: 0.565; $P = 0.035$) compared to that in group *Q1*, the low-expression group. In plasma exosomes, *circZNF609* expression in group *Q4* was associated with a reduced risk of disease in group *Q1* (adjusted *OR*: 0.654; $P = 0.004$) compared to that in group *Q1* (low expression). Plasma exosomes with *IGF2* showed a reduced risk in the *Q4* group with high *IGF2* expression compared to that in the *Q1* group with low *IGF2* expression (adjusted *OR*: 0.543; $P = 0.042$).

Conclusions This study suggests that the low expression of *circZNF609*, *circPUM1*, and *IGF2* in peripheral blood plasma exosomes could pose a potential risk for IS and serve as biomarkers for clinical treatment.

Keywords Exosomes, Hypertension, Ischemic stroke, Circular RNA

Introduction

Globally, 41 million people lose their lives to noncommunicable diseases each year, with cardiovascular disease being the primary cause of 17.9 million deaths annually [1]. Stroke is the second leading cause of mortality worldwide,with an increasing incidence. The impact of stroke on disability-adjusted life years (DALY) is increasing owing to population growth and an intensifying aging

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trend [2, 3]. The pathogenesis of ischemic stroke is multifaceted and influenced by vascular risk factors, which in turn are coregulated by genetic background and conditions through epigenetic mechanisms [4]. Stroke is characterized by acute disruption of the cerebral circulation resulting from various factors. Vascular diseases that impede cerebral blood flow can lead to brain tissue damage or dysfunction, potentially resulting in fatality if sustained for more than 24 h [5]. Ischemic stroke (IS) is the most prevalent type of stroke and is associated with high rates of disability and mortality [6]. The primary pathogenesis of IS involves cerebral thrombosis or vascular occlusion, leading to local ischemia and hypoxia. This, in turn, disrupts energy metabolism within brain cells, triggering a cascade of pathological processes, including calcium overload, oxidative stress, and inflammatory reactions. These interconnected pathological mechanisms ultimately culminate in the widespread death of brain cells in affected regions [7, 8].

CircRNAs are endogenous noncoding RNA that form a covalent closed-loop structure by linking the 3′and 5′ends, making them resistant to exonucleases. CircRNAs are more stable than their linear counterparts. CircRNAs can regulate the expression of target genes by regulating the transcription of parental mRNAs, are extensively present in human tissues, and serve as crucial regulators in various diseases and pathophysiological processes, including cancer, neurological diseases, and cardiovascular diseases [9]. Studies have indicated that circRNAs contribute to stroke development, are implicated in processes such as atherosclerosis, neuroinflammation, neurogenesis, apoptosis, cell migration, and intimal regeneration [10, 11], and have been established to participate in gene expression regulation, as well as the onset and progression of hypertension and stroke [12–14].

Exosomes, which range in size from 30 to 200 nm, are extracellular vesicles that contain a diverse array of biomolecules, including proteins, lipids, DNA, and RNA. These vesicles play crucial roles in regulating organ injury, modulating the extracellular matrix, and facilitating intercellular communication by transmitting signals and molecules to neighboring cells. Research indicates that Exosomes are key mediators of inflammatory responses, cell proliferation, regulation of the extracellular microenvironment, and the induction of immune responses. Exosomes are currently recognized as valuable biomarkers and prognostic indicators for a wide range of diseases affecting various areas such as development, immunity, tissue homeostasis, cancer, and neurodegenerative conditions. They also show promise for gene and drug delivery, which significantly influences their clinical application [15, 16]. Notably, most small molecules, including nucleic acids, struggle to penetrate the blood–brain barrier. However, exosomes can traverse this barrier, facilitating the transport of nucleic acid molecules into the brain or peripheral blood to exert their biological effects. These unique characteristics make exosomes potential carriers for therapeutic interventions for neurological disorders [17, 18]. Recent studies have indicated that circRNAs can be transferred to exosomes, where they are more abundant than at intracellular levels [19–21]. However, the expression profile, biological function, and form of circRNAs in exosomes in stroke research remains largely unexplored. Some studies have suggested that exosome-derived circRNAs could serve as valuable diagnostic and evaluative tools for acute cerebral infarction and potentially become biomarkers for this condition [22]. These findings imply that exosome-derived circRNAs offer insights into the mechanisms of acute cerebral infarction and present a novel approach for diagnosing and treating this disease.

CircZNF609 is a covalently closed-loop RNA [23]. The expression of *circZNF609* in peripheral blood leukocytes is downregulated in patients with coronary artery disease compared to the normal population, suggesting that *circZNF609* is an independent protective factor against coronary artery disease [24]. Based on bioinformatics predictions and reports in the literature, researchers hypothesized that *circZNF609* may play a protective role in coronary artery disease by interacting with miRNAs to block the inflammatory response through the regulation of the *miR-138-5p/AKT1* or *miR-150-5p/Smad7* pathways [25]. Reports have shown that Downregulation of *circZNF609* during myocardial ischemia/reperfusion (I/R) remodeling can exert a protective effect and attenuate left ventricular dysfunction after acute I/R injury. In addition, in vitro studies have found that *circZNF609* regulates cardiomyocyte survival and proliferation by modulating the crosstalk between the Hippo-YAP and *Akt* signaling pathways [26]. Many studies have found an association between *circZNF609* and cardiovascular disease [27, 28], but no study has yet clearly demonstrated the correlation between *circZNF609* and IS in plasma exosomes.*CircPUM1* has become a hotspot in cancer research since 2018. C*ircPUM1* expression is closely associated with disease progression in multiple cancer types. Despite the differences in its function in different diseases, *circPUM1*'s role in processes such as cell proliferation, apoptosis, and migration makes it a molecule of interest. Although the specific role of *circPUM1* in IS has not yet been clarified, its extensive involvement in other diseases suggests that it plays an important role in IS pathology. *CircPUM1* in exosomes may affect neuronal survival and function by regulating intracellular signaling pathways, which in turn affects the onset and progression

of IS [29]. However, findings on *circPUM1* function and regulation have been inconsistent, implying that further exploration is required.

IGF2, a mediator of growth hormone-induced metabolic and anabolic effects, also plays a role in the regulation of cell growth, differentiation, and apoptosis and exerts beneficial effects on acute myocardial ischemia. *IGF2* plays a crucial role in regulating neuronal growth, survival, and differentiation [30]. *IGF2* also affects angiogenesis, particularly by upregulating vascular endothelial growth factor (*VEGF*), which plays a role in the formation and maintenance of the cerebral vascular system [31]. Animal experiments have shown a progressive increase in endogenous *IGF2* levels after hemorrhagic stroke in rats, revealing the critical role of this factor in recovery after brain injury, as well as its role as a growth and anti-inflammatory factor.

The relationship between *circZNF609*, *circPUM1,* and *IGF2* levels and IS in plasma exosomes has not been reported. Carrying out research on the etiologic mechanisms of IS for early intervention is important for the prevention and control of cardiovascular disease in the population and may provide clues for the targets of clinical intervention drugs.

Materials and methods

Study population

In this IS case–control study, 145 patients with IS were recruited from two medical institutions: Wuhu General Hospital and the Second People's Hospital in Wuhu City. In addition, 290 control participants without a history of stroke, matched for age (± 3 years) and sex, were selected from the same area. The inclusion criteria for IS cases were that a CT or MRI of the brain was performed, and the patient's signs or symptoms lasted for more than 24 h. Patients with hemorrhagic stroke, transient ischemic attack with symptoms lasting less than 24 h, acute coronary syndromes, tumors, autoimmune diseases, and severe renal failure. Patients who had received or were receiving specific treatments (e.g., thrombolytic therapy) could have affected the results of the study, as well as patients with other neurological disorders, such as Alzheimer's disease and Parkinson's disease, which could have affected the results. The control inclusion criterion was to select a population in the same or neighboring area as the cases for epidemiologic investigation and to serve as a control group matched for sex and age. Considering that hypertension is a major risk factor for stroke, a control group of patients with hypertension was selected to exclude the confounding effects of hypertension on the results. The exclusion criteria were malignant tumors, other serious heart, brain, or kidney diseases, and stroke.

Questionnaire survey and physical examination

Demographic characteristics, smoking and drinking habits, disease history, and medication history were assessed using the questionnaires (Supplementary Document 1). All members of the research team underwent rigorous standardization training and certification. Participants who smoked at least one cigarette pack per day during their lifetime were categorized as smokers. Individuals were classified as drinkers if they consumed alcohol at least three times per week. Physical assessments were performed at least thrice, including measurements of systolic blood pressure (SBP, in mmHg) and diastolic blood pressure (DBP, in mmHg). Hypertension was defined as either a self-reported history of elevated blood pressure, measured blood pressure levels of SBP ≥ 140 mmHg or DBP ≥ 90 mmHg, or recent use of antihypertensive drugs. Diabetes was indicated by fasting blood glucose levels (GLU) ≥ 7.0 mmol/L, self-reported history of diabetes, or current use of blood sugar-lowering medications. Dyslipidemia was identified based on abnormal lipid profile levels [total cholesterol (TC) ≥ 6.22 mmol/L, triglycerides (TG) ≥ 2.26 mmol/L, low-density lipoprotein cholesterol (LDL-C) ≥ 4.13 mmol/L, high-density lipoprotein cholesterol (HDL-C) < 1.04 mmol/L, self-reported dyslipidemia diagnosis, or the ongoing use of lipid-lowering drugs.

Blood collection and biochemical index detection

Blood samples collected from participants were stored in EDTA anticoagulation tubes for preservation. Biochemical parameters, such as TC, TG, HDL-C, LDL-C, and GLU levels, were collected from the study subjects. The following assays were used: glucose oxidase, cholesterol oxidase–peroxidase, glycerol phosphate oxidase–peroxidase, catalase removal, and sulfate precipitation.

Exosomal RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction

Plasma exosomal RNA was extracted using an exosomal RNA extraction kit(ExoRNeasy Midi Kit 77,144; QIAGEN, Germany). High-quality RNA was efficiently extracted from the samples, and the isolated RNA was reverse-transcribed into cDNA using the PrimeScriptTM RT kit (Takara, Japan, RR047A). The expression of exosomal *circZNF609*, *circPUM1*, *and IGF2* mRNA was detected using SYBR Green real-time quantitative polymerase chain reaction.Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The Primer-BLAST tool was used to design the candidate primer sequences. According to the primer design principles, the gene sequences were keyed in, and the primer design parameters, such as length, melting temperature (Tm), and GC content, were adjusted to

ensure that the primers had good specificity and amplification efficiency. After the design was completed, primer sequences were synthesized by Shanghai Sangong Biotechnology Co. The primer sequences of formal experimental genes (Table 1). After the primer design was completed, the primers were compared and analyzed using the BLAST tool to ensure that the primers were specifically bound to the target gene without other nonspecific binding sites. At the same time, the gradient dilution experiment was carried out to test the amplification efficiency of the primers, and the results showed that the amplification efficiency of the primers ranged from 90 to 110%, which was in line with the experimental requirements. The qPCR reaction system included Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen,11,733–046, USA), RNase-free dH2O, forward primer, reverse primer, and cDNA. Samples were incubated at 95∘C for 5 min, followed by 40 cycles of 95∘C for 10 s, 57∘C for 20 s, and 72∘C for 20 s on a QuantStudio™ 7 Flex Real-Time PCR System platform (Applied Biosystems, 4,485,700, CA). The extraction process was performed in a sterile environment, and laboratory personnel wore sterile gloves and masks and used RNase-free consumables and reagents to avoid exogenous RNase contamination. The integrity and concentration of the extracted RNA were examined using gel electrophoresis and a NanoDrop spectrophotometer to ensure that the quality of the RNA met the requirements of subsequent experiments.

Statistical analysis

The demographic and clinical characteristics of the subjects, as well as the expression levels of genes, were analyzed using SPSS software (version 26.0). Restricted Cubic Spline (RCS) analysis was conducted using R 4.3.1, and statistical results were presented as two-sided $P<0.05$ to indicate statistically significant differences.

Categorical information was described by the composition ratio (%), and differences between groups were compared using χ^2; measured data obeying normal distribution were expressed as $\bar{x} \pm S$, and comparisons between groups were made using the independent samples t-test; measured data not conforming to normal distribution were expressed as M ($P_{25} \sim P_{75}$), and comparisons between two groups were made using the Mann–Whitney U test.

Logistic regression models were used to analyze the association between *circZNF609*, *circPUM1*, and *IGF2* mRNA expression and the risk of morbidity associated with IS, and the (*OR*) and 95% confidence intervals (95% *CI*) were calculated. Nonlinear associations between *circZNF609*, *circPUM1*, and *IGF2* mRNA levels and the risk of developing IS were analyzed using Restricted Cubic Splines (RCS).

Result

Exosome identification results

Exosome transmission electron microscopy (TEM) findings

By analyzing the photographs taken by transmission electron microscopy (TEM), it can be clearly seen that the bilateral vesicle membrane of the exosomes shown in the figure is in the form of a vesicle-like structure, which has the typical biological characteristics of an exosome (Fig. 1).

Experimental results of exosome NTA particle size analysis

NTA particle size analysis showed that the average particle size was 107.1 nm (reference standard: 30 ~ 200 nm), and the concentration was 7.2×10^{11} (Particles/mL). The particle size and concentration are shown in Fig. 2.

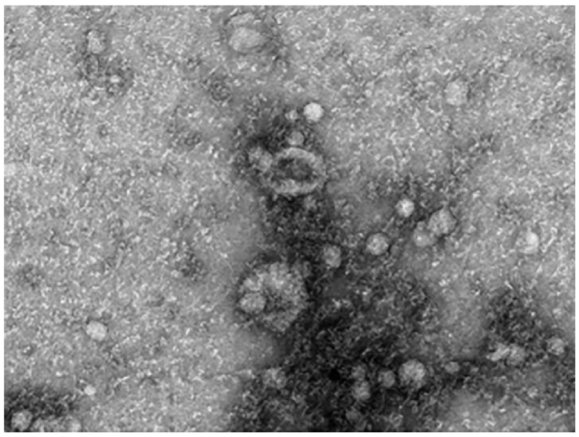

Fig. 1 Morphology of plasma exosomes under transmission electron microscopy

Table 1 Specific primer sequences

Gene	Forward primer (5′−3′)	Reverse primer (3′−5′)	Length (bp)
circZNF609	CAGCGCTCAATCCTTTGGGA	GACCTGCCACATTGGTCAGTA	131
circPUM1	GCAGAACATCAGGTGCGTTC	GGTCCATCTTTGCTGGATTCATC	137
IGF2	TCCAGTTCGTCTGTGGGGACC	CAGCACTCCTCAACGATGCC	91
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG	197

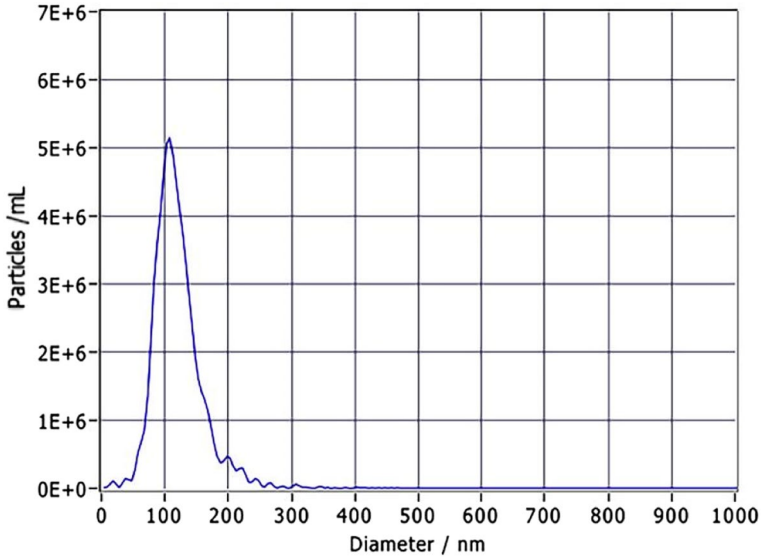

Fig. 2 Schematic diagram of particle size and concentration

Characteristics of the participants

A total of 435 subjects, including 145 patients with IS and 290 controls, were included in this IS case–control study. The differences between cases and controls were not statistically significant in terms of sex or age ($P>0.05$). The systolic blood pressure in the IS case group (148.72 mmHg) was higher than that in the control group (139.12 mmHg), the diastolic blood pressure in the case group(83.80 mmHg) was higher than that in the control group (78.29 mmHg), and the differences were statistically significant (all $P<0.001$), the LDL-C level in the hypertensive case group was higher (2.79 mmol/L) than that in the control group (2.44 mmol/L). In addition, the history of hypertension was higher in the IS group (62.8%) than in the control group (50.0%) ($Z=6.340$, $P=0.012$), history of diabetes mellitus was higher in the case group (22.8%) than in the control group (14.8%) ($Z=4.217$, $P=0.040$), and the rate of alcohol consumption was higher in the case group (33.8%) than in the control group (21.0%) ($Z=8.329$, $P=0.004$). The differences in TC, TG, HDL-C, and GLU levels and the presence of dyslipidemia were not statistically significant ($P>0.05$), as shown in Table 2.

Analysis of exosome circZNF609 expression levels

In IS controls, the difference in plasma exosomal *circZNF609* expression levels was not statistically significant ($P>0.05$). According to subgroup analysis, in the < 65 years age population, the plasma exosome *circZNF609* expression level of IS cases was [0.547(0.149,0.946)], which was lower than that of the control group [0.763(0.391,2.827)] ($Z=1.969$, $P=0.049$); in the population not suffering from hypertension, the IS case plasma exosome *circZNF609* expression level was [0.752 (0.368,1.555)], which was lower than the control level [0.989 (0.565,1.775)] ($Z=2.006$, $P=0.045$), as shown in Table 3. In RCS analysis, a nonlinear correlation was found between plasma exosomal *circZNF609* expression levels and the risk of ischemic stroke development ($P_{overall}=0.040$, $P_{non\text{-}linear}=0.025$), as shown in Fig. 3.

Analysis of exosome circPUM1 expression levels

In the IS controls, the difference in plasma exosomal *circPUM1* expression levels was not statistically significant ($P>0.05$). In the dyslipidemia subgroup, the plasma exosome *circPUM1* expression level of IS patients was [0.579 (0.354,1.491)], which was lower than that of the controls [0.908 (0.549,1.616)] ($Z=2.213$, $P=0.034$), as shown in Table 4. In RCS analysis, no nonlinear correlation was found between plasma exosomal *circPUM1* expression levels and the risk of IS development ($P_{overall}=0.033$, $P_{non\text{-}linear}=0.293$), as shown in Fig. 4.

Analysis of exosome IGF2 expression levels

The plasma exosomal *IGF2* expression level in patients with IS was [0.895 (0.420,1.416)] lower than that in the control group [0.991 (0.592,1.672)] ($Z=2.022$, $P=0.043$).

In the < 65 years subgroup, the plasma exosomal *IGF2* expression level in the IS case group was [0.586 (0.300,0.919)], which was lower than that in the control group [0.993 (0.562,1.665)] ($Z=3.091$, $P=0.002$). In the nonsmoking subgroup, the plasma exosomal *IGF2* expression level in the IS case group was [0.867

Table 2 Demographic and clinical characteristics of the subjects

Characteristics	Group	Subjects in the genetic case–control study		*t/Z/χ2*	*P*
		Control (*n* = 290)	IS (*n* = 145)		
Sex [n(%)]	Male	182(62.8)	91(62.8)	0.000	1.000
	Female	108(37.2)	54(37.2)		
Age (year)		68.51 ± 9.82	68.85 ± 9.99	0.333	0.739
SBP (mmHg)		139.12 ± 19.38	148.72 ± 21.80	4.672	**< 0.001**
DBP (mmHg)		78.29 ± 10.41	83.80 ± 14.06	4.181	**< 0.001**
TC (mmol/L)		4.53 ± 0.96	4.44 ± 0.99	0.902	0.368
TG (mmol/L)		1.19(0.82,1.76)	1.28(0.99,1.80)	1.635	0.102
HDL-C (mmol/L)		1.29 ± 0.35	1.30 ± 0.32	0.284	0.776
LDL-C (mmol/L)		2.44 ± 0.70	2.79 ± 0.99	3.826	**< 0.001**
GLU (mmol/L)		5.46(5.02,6.04)	5.51(4.83,6.86)	0.819	0.413
Smoking [n(%)]	No	237(81.7)	112(77.2)	1.225	0.268
	Yes	53(18.3)	33(22.8)		
Drinking [n(%)]	No	229(79.0)	96(66.2)	8.329	**0.004**
	Yes	61(21.0)	49(33.8)		
Dyslipidemia [n(%)]	No	184(63.4)	97(66.9)	0.503	0.478
	Yes	106(36.6)	48(33.1)		
Hypertension [n(%)]	No	145(50.0)	54(37.2)	6.340	**0.012**
	Yes	145(50.0)	91(62.8)		
Diabetes [n(%)]	No	247(85.2)	112(77.2)	4.217	**0.040**
	Yes	43(14.8)	33(22.8)		

Table 3 Stratification analyses for *circZNF609* gene mRNA expression($2^{-\Delta\Delta CT}$)

Factor	Stratum	Control (*n* = 290)	IS (*n* = 145)	*Z*	*P*
mRNA expression		0.994(0.548,1.934)	0.867 (0.410,1.967)	1.374	0.170
Sex	Male	1.036 (0.583,2.064)	0.867 (0.411,1.862)	1.380	0.168
	Female	0.906 (0.511,1.717)	0.869 (0.363,2.186)	0.501	0.616
Age	≤ 65 years	0.763 (0.391,2.827)	0.547 (0.149,0.946)	1.969	**0.049**
	> 65 years	1.031 (0.576,1.729)	0.941 (0.457,2.217)	0.182	0.855
Smoking	No	0.996 (0.530,1.743)	0.848 (0.370,2.016)	1.399	0.162
	Yes	0.960 (0.559,2.152)	1.076 (0.465,1.973)	0.378	0.706
Drinking	No	0.942 (0.521,1.831)	0.842 (0.368,2.142)	1.282	0.200
	Yes	1.155 (0.730,2.061)	0.923 (0.453,1.913)	0.740	0.459
Dyslipidemia	No	1.057 (0.566,2.133)	0.896 (0.411,2.186)	0.978	0.328
	Yes	0.873 (0.519,1.73)	0.799 (0.39,1.687)	1.115	0.265
Hypertension	No	0.989 (0.565,1.775)	0.752 (0.368,1.555)	2.006	**0.045**
	Yes	0.996 (0.529,1.964)	0.902 (0.442,2.244)	0.126	0.899
Diabetes	No	0.996 (0.558,2.006)	0.902 (0.419,2.108)	0.775	0.438
	Yes	0.960 (0.505,1.728)	0.710 (0.357,1.344)	1.254	0.210

(0.394,1.402)], which was lower than that in the control group [1.012(0.597,1.665)] ($Z = 2.448$, $P = 0.013$) (Table 5).

In the RCS analysis, no nonlinear correlation was found between plasma exosomal *IGF2* expression

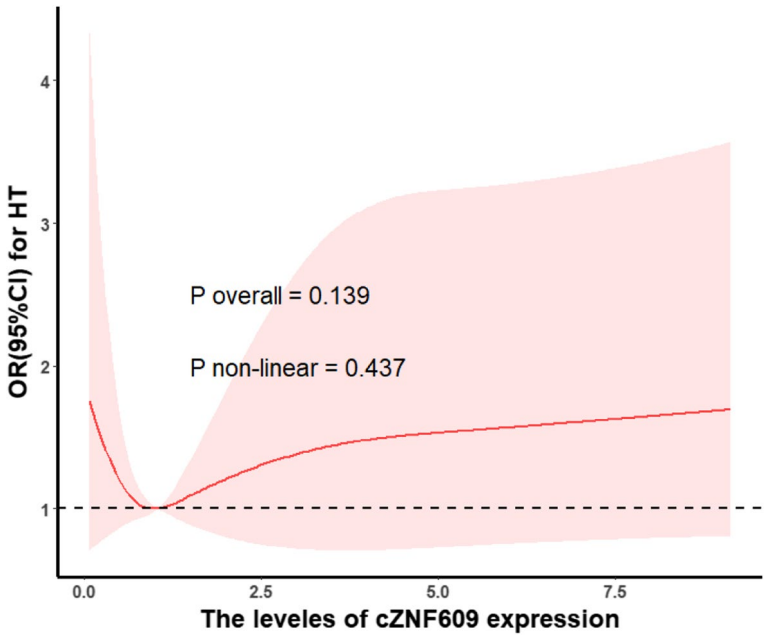

Fig. 3 ORs and 95% CIs of exosome *circZNF609* expression levels in relation to the risk of ischemic stroke development

Table 4 Stratification analyses for *circPUM1* gene mRNA expression($2^{-\Delta\Delta CT}$)

Factor	Stratum	Control ($n=290$)	IS ($n=145$)	*Z*	*P*
mRNA expression		0.986 (0.567,1.708)	0.939 (0.496,1.567)	1.355	0.175
Sex	Male	0.975 (0.553,1.733)	0.926 (0.499,1.37)	1.735	0.083
	Female	1.010 (0.611,1.709)	1.015 (0.494,2.165)	0.244	0.807
Age	≤65 years	0.981 (0.526,2.353)	0.845 (0.496,1.316)	1.231	0.218
	>65 years	0.992 (0.599,1.645)	0.981 (0.494,1.723)	0.809	0.418
Smoking	No	0.973 (0.567,1.733)	0.941 (0.519,1.733)	0.684	0.494
	Yes	1.031 (0.575,1.583)	0.878 (0.427,1.376)	1.525	0.127
Drinking	No	0.973 (0.565,1.733)	0.972 (0.479,1.661)	0.916	0.360
	Yes	1.052 (0.645,1.64)	0.855 (0.551,1.456)	1.224	0.221
Dyslipidemia	No	1.068 (0.604,1.804)	1.005 (0.628,1.71)	0.332	0.740
	Yes	0.908 (0.549,1.616)	0.579 (0.354,1.491)	2.213	**0.034**
Hypertension	No	1.086 (0.565,1.842)	0.940 (0.521,1.564)	0.843	0.400
	Yes	0.959(0.591,1.612)	0.915(0.444,1.661)	0.986	0.324
Diabetes	No	0.981(0.591,1.678)	0.939(0.504,1.688)	0.993	0.321
	Yes	1.119(0.487,1.927)	0.933(0.489,1.383)	1.001	0.317

levels and the risk of ischemic stroke development ($P_{overall}=0.024$, $P_{non\text{-}linear}=0.051$) (Fig. 5).

Regression analysis of the association between gene expression levels and the risk of developing IS

The results of multifactorial adjustment showed that *circZNF609* in plasma exosomes was associated with a reduced risk of disease in the low expression groups *Q1*, *Q2,* and *Q4* ($P<0.05$). The risk of *IGF2* in plasma exosomes was reduced in group *Q1* with low expression and in group *Q4* with high expression ($P<0.05$), as shown in Table 6.

Discussion

circRNAs are formed by reverse splicing of mRNA precursors of host genes [32]. In recent years, with the continuous development of high-throughput sequencing technology and bioinformatics algorithms, researchers have identified many circRNAs in human tissues and bodily fluids [33]. These circRNAs exhibit specific

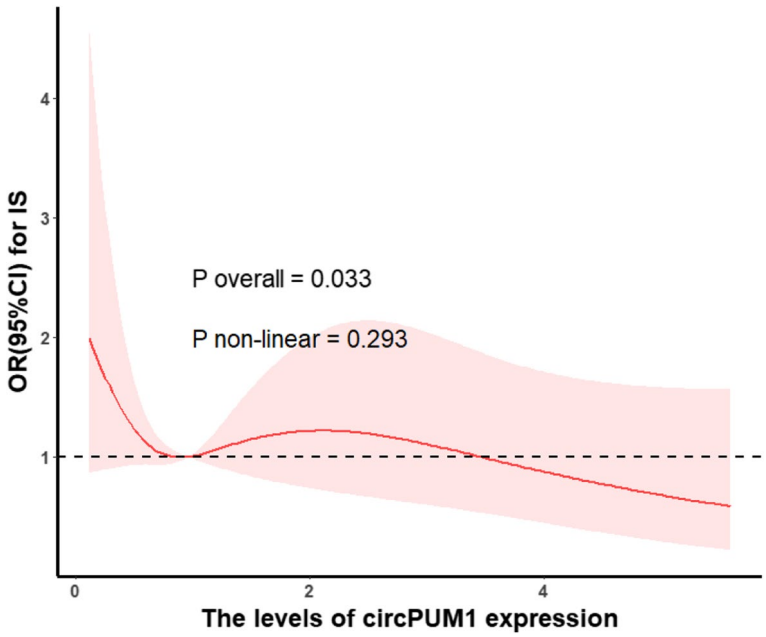

Fig. 4 ORs and 95% CIs of exosome *circPUM1* expression levels in relation to the risk of ischemic stroke development

Table 5 Stratification analyses for *IGF2* gene mRNA expression($2^{-\Delta\Delta CT}$)

Factor	Stratum	Control ($n=290$)	IS ($n=145$)	*Z*	*P*
mRNA expression		0.991 (0.592,1.672)	0.895 (0.420,1.416)	2.022	**0.043**
Sex	Male	1.085 (0.614,1.763)	0.928 (0.426,1.611)	1.499	0.134
	Female	0.889 (0.539,1.479)	0.734 (0.413,1.242)	1.396	0.163
Age	≤65 years	0.993 (0.562,1.665)	0.586 (0.300,0.919)	3.091	**0.002**
	>65 years	0.991 (0.595,1.687)	0.954 (0.460,1.769)	0.500	0.617
Smoking	No	1.012 (0.597,1.665)	0.867 (0.394,1.402)	2.488	**0.013**
	Yes	0.852 (0.507,1.729)	0.987 (0.590,1.461)	0.450	0.653
Drinking	No	0.989 (0.598,1.661)	0.870 (0.390,1.441)	1.909	0.056
	Yes	0.999 (0.558,1.716)	0.919 (0.471,1.336)	0.822	0.411
Dyslipidemia	No	1.07 (0.603,1.692)	0.916 (0.468,1.617)	1.250	0.211
	Yes	0.948 (0.543,1.63)	0.822 (0.388,1.34)	1.912	0.056
Hypertension	No	0.976 (0.529,1.695)	0.867 (0.442,1.341)	1.534	0.125
	Yes	1.005 (0.641,1.668)	0.916 (0.39,1.735)	1.360	0.174
Diabetes	No	1.005 (0.594,1.668)	0.899 (0.441,1.344)	1.949	0.051
	Yes	0.856 (0.531,1.697)	0.775 (0.294,2.058)	0.371	0.711

expression patterns in different cell types or tissues, with high stability [34]. The distribution of circRNAs in plasma exosomes reflects the expression of host genes. Cell adhesion molecule receptors and calcium adhesion proteins have been found to play a regulatory role in the inflammatory response based on the functional annotation of host genes, which is consistent with the role of the inflammatory response in the injury process after stroke [35]. *Circ_0043837* and *circ_0001801* in exosomes have been found to be important predictors of large-artery atherosclerotic (LAA) stroke through large-sample validation. Moreover, compared to plasma circRNAs, exosomal circRNAs showed better results in diagnosing LAA stroke [36].

In this study, we found that plasma exosomal *circZNF609* expression levels were lower in the IS case group than in the control group in the <65-year-old population, and in the HT-unaffected subgroup, exosomal *circZNF609* expression levels were lower in the IS case group than in the control group. The results of multifactorial

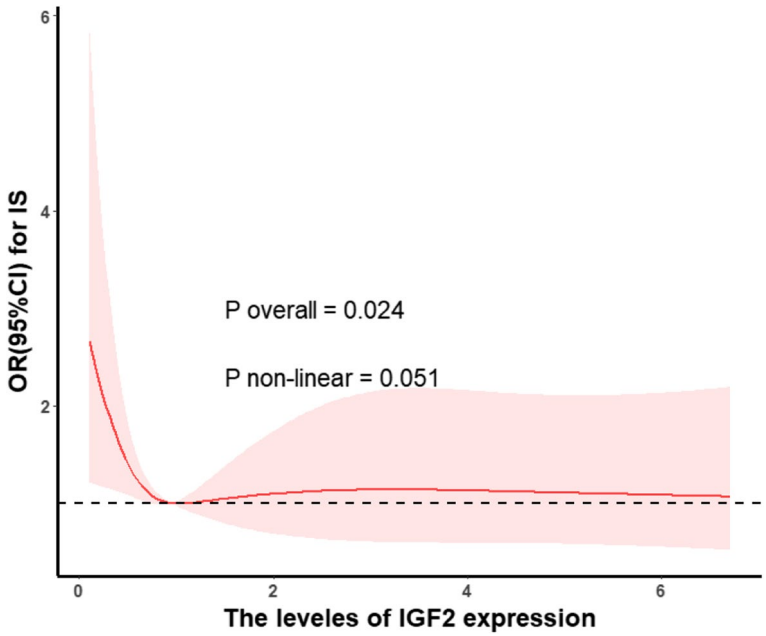

Fig. 5 ORs and 95% CIs of exosome *IGF2* expression levels in relation to the risk of ischemic stroke development

Table 6 Logistic regression analysis of the association between gene expression levels and risk of developing IS

Gene	Group	*OR (95%CI)*	*P*	*OR (95%CI)* [a]	*P*[a]
circZNF609	*Q1*	reference		reference	
	Q2	0.565 (0.318,1.001)	**0.050**	0.565 (0.315,1.013)	**0.035**
	Q3	0.437 (0.242,0.788)	**0.006**	0.416 (0.228,0.761)	0.055
	Q4	0.675 (0.384,1.185)	0.171	0.654 (0.368,1.164)	**0.004**
circPUM1	*Q1*	reference		reference	
	Q2	0.843 (0.475,1.496)	0.559	0.833 (0.462,1.501)	0.543
	Q3	0.703 (0.392,1.26)	0.237	0.667 (0.366,1.215)	0.185
	Q4	0.782 (0.438,1.395)	0.405	0.773 (0.425,1.404)	0.397
IGF2	*Q1*	reference		reference	
	Q2	0.736 (0.422,1.285)	0.281	0.709 (0.400,1.256)	0.238
	Q3	0.565 (0.319,1.001)	**0.050**	0.563 (0.313,1.014)	0.056
	Q4	0.565 (0.319,1.001)	**0.050**	0.543 (0.301,0.978)	**0.042**

[a] Adjusted for age, sex, smoking, alcohol consumption, hypertension, diabetes mellitus, and dyslipidemia; Q1 first quartile(0%−25%), Q2 second quartile(25%−50%), Q3 third quartile(50%−75%), and Q4 fourth quartile(75%−100%)

adjustment showed that *circZNF609* in plasma exosomes was associated with a reduced risk of disease relative to the low expression group, group *Q1*, and the high expression groups, groups *Q2* and *Q4*. In recent years, patients with stroke have become younger [37]. Endothelial dysfunction and injury play important roles in the pathogenesis of cardiovascular diseases such as coronary artery disease and HT. *circZNF609* is abundantly expressed in endothelial cells, and its dysregulated expression is associated with impaired vascular function. Under normal conditions, inhibition of *circZNF609* expression increased endothelial cell viability and proliferation and accelerated endothelial cell migration, suggesting a potential role for *circZNF609* in promoting angiogenesis. It has been shown that *circZNF609* in the circulatory system is associated with left ventricular dysfunction in patients with myocardial infarction [38]. Notably, the trend of *circZNF609* in cardiac tissues does not coincide with that of *circZNF609* in the blood of patients [39, 40]. RNA levels in the circulatory system are complexly regulated, and *circZNF609* in peripheral blood may be associated with extracellular vesicular transport [41]. In addition, reduced accumulation and secretion of *circZNF609* in cardiac tissues may also lead to the opposite observation. The nonsignificant association of *circZNF609* with IS risk in the overall population may be due to the presence of comorbidities and other factors between subgroups. Differences in the distribution of comorbidities, such as hypertension and diabetes mellitus, in different subgroups may have interfered with the

direct association between circZNF609 and IS. Follow-up studies will further analyze the effects of comorbidities on *circZNF609* expression and IS risk to clarify its mechanism of action in the overall population.

CircPUM1 plays an active role in promoting the proliferation, migration, and invasion of trophoblasts and inhibiting apoptosis and inflammatory responses [42, 43]. Cerebral ischemia–reperfusion injury occurs as a result of blood flow recovery after ischemic stroke and may result in severe damage to the brain tissue. Previous studies have revealed that cerebral ischemia–reperfusion injury involves a variety of physiological and pathological processes, such as inflammatory responses, neuronal damage, and oxidative stress, which ultimately lead to apoptosis and necrosis. Animal experiments have shown that *circPUM1* levels decrease following cerebral ischemia/reperfusion injury in mice. Further experimental results showed that inhibition of *circPUM1* exacerbated neuronal apoptosis, inflammation, and cytotoxicity. M*iR-340-5p* and *DDX5*, which are downstream molecular pathways of *circPUM1*, were found to have reduced expression levels of *circPUM1* and DEAD-box deconjugate 5 in cellular experiments in middle cerebral artery occlusion mice, whereas *miR-340-5p* expression levels increased. Specifically, *circPUM1* increases the expression level of *DDX5* by binding to *miR-340-5p*, which regulates neuronal apoptosis and inflammatory processes [44]. It has been reported that *circPUM1* is dysregulated in patients with lung and ovarian cancers and is involved in cancer progression. *circPUM1* sponges *miR-760* in polycystic ovary syndrome (PCOS) cells. Subsequently, researchers evaluated the effects of *circPUM1* on PCOS cells and found that overexpression of *circPUM1* promoted the proliferative capacity of cells and inhibited the apoptotic rate [45, 46].

The results of this study showed that the plasma exosomal *IGF2* expression levels in ischemic stroke patients were significantly lower than the levels in the control group, and the difference was statistically significant. This finding suggests that *IGF2* plays an important role in the pathophysiological response to ischemic stroke.*IGF2*, as the most abundant insulin-like growth factor in the brain, has attracted increasing attention for its role in central nervous system disorders.*IGF2* is involved in the formation of myelin sheaths, which are associated with nerve conduction.*IGF2* is involved in the formation of neuronal cells and is related to neural conduction through the activation of the *IGF1R* and subsequent triggering of the *PI3K/Akt* and *MAPK /ERK* signaling pathways, which play a key role in neuronal cell formation, maturation, differentiation, and survival [47]. After stroke, the *IGF2R*-mediated antioxidant and neuroprotective effects of *IGF2* are particularly important for alleviating oxidative stress and secondary brain damage caused by mitochondrial dysfunction [48]. Atherosclerosis is a major risk factor for stroke, and some studies have reported an association between *IGF2* and atherosclerosis [49].

IGF2 regulates endothelial cell function by promoting cell migration, tube formation, and production of the vasodilator nitric oxide. In addition, *IGF2* is involved in the modulation of calcium homeostasis and enhancement of acetylcholinesterase.*IGF2* affects the inflammatory phenotype of macrophages by regulating proton channels in the cytoplasm. Low doses of *IGF2* bind to *IGF2R* and induce *IGF2R* internalization, which promotes the facilitation of cytoplasmic H + entry into the mitochondria, enhances oxidative phosphorylation, and exhibits an antiinflammatory phenotype [50]. *IGF2* is overexpressed in many cancers and is associated with poor prognosis. Exosomal *miR-543* inhibits ovarian cancer cell proliferation by targeting *IGF2* [50]. Exosomes released by tumor cells activate normal fibroblasts into cancer-associated fibroblasts by translocating *IGF2* to recipient cells and activating *PI3K/Akt* signaling, suggesting that *IGF2* plays a key role in regulating cell proliferation and migration [51].

In this study, we found that *IGF2* in plasma exosomes reduced the risk of disease in the high-expression *Q4* group relative to the low-expression group *Q1*. Studies in animal models have found that *IGF2* can effectively improve myocardial function after coronary artery occlusion and that this improvement is closely related to the maintenance of myocardial structural integrity [52]. By intracoronary or direct intracardiac injection of *IGF2*, the area of myocardial infarction induced by coronary artery occlusion in pigs can be effectively reduced by intracoronary or cardiac function improved [53]. Gabi et al. showed that overexpression of *IGF2* can further improve the left ventricular ejection fraction and reduce the myocardial infarction area [53].

Bai et al. showed that *circFUNDC1* expression is elevated in serum-derived exosomes from patients with IS. *circFUNDC1* inhibits homologous phosphatase-tensin protein activity by enriching miR-375. Further studies have shown that the knockdown of *circFUNDC1* effectively attenuates human cerebral microvascular endothelial cell injury induced by oxygen and glucose deprivation (OGD) model [54].

MicroRNAs (miRNAs) are small noncoding RNAs that play crucial roles in the post-transcriptional regulation of gene expression. They are involved in various pathological processes, including ischemic stroke (IS), by modulating inflammation, apoptosis, and angiogenesis. For instance, the levels of circulatory *microRNA-1* and *microRNA-221* are closely correlated with changes in other cardiac markers that are altered in

cardiovascular disease. The potential of *microRNA-1* and *microRNA-221* as noninvasive biomarkers is important for monitoring myocardial infarction (MI) risk and predicting the likelihood of arterial narrowing or blockage [55]. Mansouri et al.. found that the levels of circulating *miR-19a* in patients with acute myocardial infarction (AMI) were significantly higher than those in controls. M*iR-19a* may have prognostic value and could serve as a promising molecular target for the early diagnosis and prognosis of AMI [56].

Our study has several limitations, including the need for larger sample sizes and multicenter studies to validate our results further. In addition, we were unable to assess the prognostic value of *circZNF609*, *circPUM1*, and *IGF2* in major cardiovascular events due to the lack of patient follow-up information. Moreover, the roles of *circZNF609*, *circPUM1*, and *IGF2* in the causal pathway between hypertension and ischemic stroke have not yet been fully elucidated. This study had a cross-sectional design. Although associations were found between *circZNF609, circPUM1*, and *IGF2* expression and ischemic stroke risk, a clear causal relationship could not be established. It was not possible to determine whether changes in gene expression led to the development of ischemic stroke or whether changes in gene expression were caused by ischemic stroke. Follow-up studies need to use longitudinal studies or intervention study designs to resolve causality better. Therefore, the prognostic value of *circZNF609*, *circPUM1*, and *IGF2* and their role in pathogenesis needs to be further evaluated in subsequent studies.In this study, we selected GAPDH as the internal reference gene primarily because of its stable expression across various tissues and cell types, and its widespread use in circRNA and mRNA expression analyses. However, we also acknowledge that the use of a single reference gene may have certain limitations.In future studies, we will consider introducing additional reference genes (such as 18S rRNA) for validation to ensure the reliability of the results.

In the future, we will carry out animal experiments to detect changes in the expression of *circZNF609*, *circPUM1,* and *IGF2* in the plasma exosomes of animal models by constructing an animal model of ischemic stroke and observing the effects of intervening in the expression of these molecules on neurological function, cerebral infarction volume, and other indices of the animal model to validate its therapeutic potential. Interventional trials, such as the administration of exogenous *IGF2* or drugs regulating the expression of *circZNF609* and *circPUM1*, should be considered to observe the therapeutic effects in patients with IS.

Conclusions

The association between *circZNF609, circPUM1*, and *IGF2* expression in plasma exosomes and the risk of ischemic stroke identified in this study is expected to provide new diagnostic markers for clinical use. In the future, more accurate diagnostic methods could be developed based on these markers, such as detecting the expression levels of these genes in the plasma exosomes of patients, assisting in the early diagnosis of ischemic stroke, achieving timely intervention and treatment, and improving patient prognosis. The exploration of potential therapeutic targets also provides a direction for the development of new therapeutic strategies, such as intervention in the IGF2-related signaling pathway, which may become a new way to treat ischemic stroke. Further research is required to validate these results.

Supplementary Information

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Supplementary Material 1.

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Authors' contributions

Yan Chen and Yingshui Yao conceptualized and designed the study and edited and proofed the manuscript. Suhai Fei conducted experiments, participated in data organization,conducted data analysis, drafted the manuscript, and interpreted the research results. Miao Xu conducted data analysis, contributing to the robustness of the results and proofed the manuscript. Zhenfeng Liu, Haining Xie, Yue Yu, Yinghu Chu collected the samples and collected the data. Yuelong Jin, Lijun Zhu, and Zhengmei Fang proofed the manuscript. All authors consent to take responsibility for the content of the work.

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Data availability

The data that support the findings of this study are available from the Institute of Chronic Disease Prevention and Control, Wannan Medical College, Anhui Province, but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. The Data and full trial protocol are however available from Yan Chen author upon reasonable request and with permission of the Institute of Chronic Disease Prevention and Control, Wannan Medical College, Anhui Province. The contact information is the email address of the author Yan Chen.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the First Affiliated Yijishan Hospital of Wannan Medical College (Approval No.32 (2018)). The trial adheres to the principles of the Helsinki declaration. Written informed consent has been obtained from all subjects or their caregivers.

Consent for publication
Consent for publication was obtained from the participants.

Competing interests
The authors declare no competing interests.

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