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Original Article

Comprehensive analysis of transcriptome-wide expression patterns and a circRNA/lncRNA-miRNA-mRNA network in the pathogenesis of cerebral ischemia in Rattus norvegicus

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Abstract: Background: Although ischemic stroke exhibits a high prevalence in the elderly population, the involved genes and pathways are poorly understood. In this study, we proposed to identify differentially expressed genes (DEGs) and constructed a circular RAN (circRNA)/long noncoding RNA (lncRNA)/microRNA (miRNA)-mRNA network associated with the pathogenesis of ischemic stroke by using bioinformatics analysis. Methods: We constructed a rat model of middle cerebral artery occlusion (MCAO) and conducted total RNA and microRNA sequencing in brain specimens from MCAO and normal rats. Transcriptome-wide expression patterns were analyzed and DEGs were defined by applying Ballgown and a cut of log2-transformed fold-change (log2FC) ≥ 1 (or ≤ -1) with a *P* value < 0.05. We exploited Pearson correlation analysis to determine the association between the circRNA/lncRNA/mRNA network and miRNAs (*P* < 0.05 and corr ≤ -0.6), and the competing endogenous RNAs (ceRNA) interaction network was visualized with Cytoscape software and separated into subnetworks using the Molecular Complex Detection (MCODE) algorithm. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were implemented for the pathway analysis of DEGs. Results: Upregulated DEGs were significantly enhanced in positive regulation of cell migration, response to wounding, blood vessel morphogenesis, inflammatory response, and cell activation; Downregulated DEGs were associated with control of the modulation of chemical synaptic transmission, synapse organization, regulation of membrane potential, and regulation of ion transport. KEGG-pathway analysis showed that DEG-enhanced pathways were associated with the pathways of TNF signaling pathway, Fluid shear stress and atherosclerosis, NF-kappa B signaling pathway, Lipid and atherosclerosis, Human cytomegalovirus infection, Osteoclast differentiation, Chemokine signaling pathway, IL-17 signaling pathway, Viral protein interaction with cytokine and cytokine receptor, and Cytokine-cytokine receptor interaction. We uncovered several novel lncRNAs (lnc_00231, lnc_002239, lnc_004172; and a novel_circ0001704), five miRNAs (miR-200b-3p, miR-223-3p, miR-200c-3p, miR-3084a-3p, and miR-664-2-5p), and the top-10 mRNAs (upregulated mRNAs were Pdgfa, Il1b, Gdf15, Fosl1, and Cxcl2; downregulated mRNAs were Prkar2b, Olfm3, Lrrc73, Tmem38a, and Dlgap3) that were involved in ischemic stroke. Conclusions: Through bioinformatic network analysis, we identified the underlying molecular mechanisms and key central genes that may contribute to an inflammatory response after cerebral infarction.

Keywords: Ischemic stroke, differentially expressed genes, gene ontology

Introduction

Stroke is a primary cause of disability and the second leading cause of death worldwide, with the fastest growing incidence observed for the population aged 65 and older; and over 80% of stroke cases are classified as ischemic stroke (IS) [1]. Although the advancements in recanalization therapy using both pharmacologic and mechanical thrombolysis have made some progress in treating ischemic stroke, deficiencies in our understanding of the biologic mechanism(s) underlying ischemic cerebral injury still limit the amelioration of therapeutic

outcomes. Therefore, further elucidation of the pathogenesis of ischemic cerebral injury is urgently needed, uncovering new therapeutic targets to improve prognosis.

Ischemic stroke involves multiple proteins and protein-related reactions, and the mRNAs that encode the key enzymes involved are regulated by other RNAs. A microRNA (miRNA) can target multiple mRNAs, such that the mRNAs compete for binding with the miRNA, and a single gene can sponge several miRNAs as well. MiRNAs and other non-coding RNAs (ncRNAs; e. g., lncRNAs, and circ RNAs) have been implicated in cell-fate determination and various human diseases. CeRNAs can then regulate each other at the post-transcriptional level by competing for shared miRNAs. CeRNA networks link the function of protein-coding mRNAs with that of ncRNAs such as miRNAs, lncRNAs, pseudogenic RNAs, and circRNAs. Thus, a disorder in ceRNA networks could lead to the onset of human diseases.

In recent years, an increasing number of studies have shown that ncRNA transcripts and circRNAs can act as endogenous miRNAs or ceRNAs sponges, and ceRNAs communicate with and co-regulate each other by competing in their binding to shared miRNAs, thereby titrating miRNA availability [2].

NcRNA transcripts and circRNAs play regulatory roles in apoptosis, autophagy, inflammation, and hypoxic injury in a variety of diseases [2, 3]. Atherosclerosis is a chronic and multifactorial inflammatory disease that is closely associated with cardiovascular and cerebrovascular diseases, and the crosstalk between circRNAs and their competing mRNAs might be crucial in the development of atherosclerosis [4]. Some authors have ascertained that abnormal expression of lncRNAs/circRNAs may be applied as a biomarker in the diagnosis and prognosis of cerebral infarction [5], and their regulation can affect the activation of microglial cells, neuronal damage, angiogenesis, the size of cerebral infarcts, and ischemic immune inflammation after cerebral ischemia [6-10].

Therefore, the aim of this study was to seek differentially expressed genes (DEGs) associated with the progression of cerebral infarction through gene ontology (GO) bioinformatic analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway-enrichment analysis and to construct a ceRNA network to uncover their associations. A flow chart displaying the creation of a common predicted ceRNA network is shown in **Figure 1**.

Materials and methods

Acquisition of rat brain tissue

To obtain tissue samples for sequencing analysis, we constructed a permanent middle cerebral artery occlusion (pMCAO) in five male rats (purchased from Hunan Slyke Jingda Laboratory Animal Co. LTD, China) weighing 250 to 280 g, and five normal male rats of the same weight served as controls. Animals were grouped and housed at a controlled temperature (20 ± 2°C) with a 12 h light-dark cycle, and they had free access to food and water and were randomly divided into experimental group and control group. The rats were first anesthetized with 40 mg/kg sodium pentobarbital by intraperitoneal injection [11]. The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) of each rat were exposed, and the carotid bifurcation was separated. We then ligated the ECA and inserted lysine-coated nylon thread (Xi-Nong Company, China) into the ICA (18 ± 2 mm) until it blocked the origin of the middle cerebral artery (MCA); and 24 h later, the two groups of rats were deeply anesthetized and euthanized (Cervical Dislocation), and their brain tissue was removed for transcriptomic sequencing. A double-blind design was used and experienced testers evaluated the neurological deficit score to reduce bias. Neurological deficit scores were recorded as the Longa method: 0: no neurological deficit symptoms, normal activity; 1: the contralateral forelimb of the lesion could not be fully straightened; 2: turn to the opposite side when crawling; 3: walk their body to the opposite side; 4: unable to walk on their own, loss of consciousness. Rats with a score of 3 were brain-harvested for RNA sequencing.

RNA extraction, sequencing, identification and quantification of gene-expression levels

All sequencing programs were executed by Novogene Company (Beijing, China). Total RNA

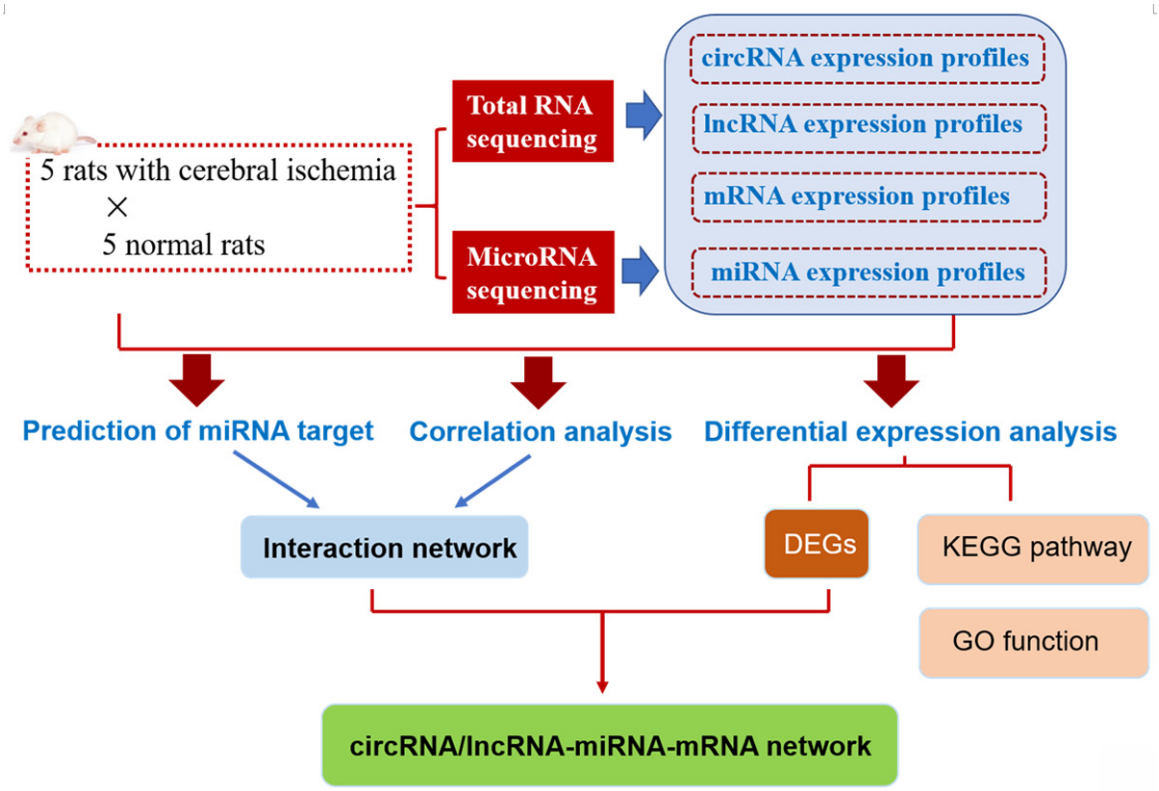


Figure 1. Flow chart of the experiment. circRNA: Circular RNA. lncRNA: Long non-coding RNA. miRNA: MicroRNA. KEGG: Kyoto Encyclopedia of Genes and Genomes. GO: Gene Ontology. DEGs: Differentially expressed genes.

from each sample was extracted using TRIzol reagent (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions, and RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was assessed using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), RNA concentration was measured using a Qubit® RNA Assay Kit in a Qubit® 2.0 Fluorometer (Life Technologies, CA, USA), and RNA integrity was determined with the RNA Nano 6000 Assay Kit using the Agilent Bioanalyzer 2100 System (Agilent Technologies, CA, USA).

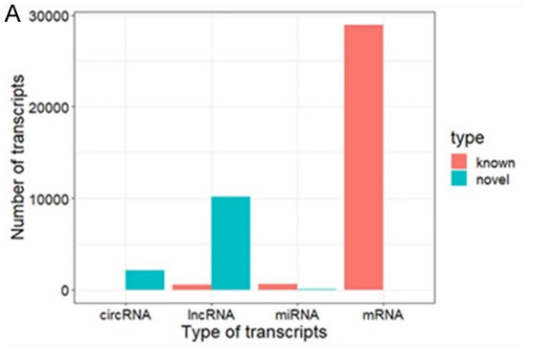
Differentially expressed transcript finder

The DESeq algorithm was applied to filter differentially expressed transcripts (DETs). DETs were defined by paired *t* test at a $P < 0.05$, (|log fold-change [logFC]| > 1 and by the Benjamini and Hochberg-corrected false discovery rate (FDR) < 0.05) based on intergroup fragment count values [12]. Our ceRNA screening criteria were (1) binding targets and (2) a negative correlation (with a $P < 0.05$ & corr ≤ -0.6) [13].

Gene ontology (GO)-enrichment and pathway analysis

GO-enrichment analysis was performed on the target gene candidates of the DE RNAs. A GOseq-based Wallenius non-central hyper-geometric distribution that adjusted for gene length bias was implemented for GO-enrichment analysis [14, 15].

KEGG is a database resource for understanding the high-level functions and utilities of biologic systems such as the cell, the organism, and the ecosystem from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/) [16]. We used KOBAS and Metascape software to analyze the statistical enrichment of the target gene candidates in the KEGG pathways [17, 18].



B

Type	Number of DEGs		
		UP	DOWN
miRNA	201	112	89
mRNA	1142	838	304
lncRNA	90	51	39
circRNA	81	72	9

Figure 2. A. Number of genes within the entire transcriptome. B. Total number of DEGs. Number of upregulated and downregulated differentially expressed circRNAs, lncRNAs, miRNAs, and mRNAs. circRNA: Circular RNA. lncRNA: Long non-coding RNA. miRNA: MicroRNA. DEGs: Differentially expressed genes.

Table 1. Top-five dysregulated mRNAs, circRNAs, lncRNAs, and miRNAs based upon *P* values are summarized

	Transcript_ID	log2FC	*P*-value	Status
mRNA	ENSRNOT00000001775	7.784898	3.60E-06	UP
	ENSRNOT00000006308	6.989001	8.85E-06	UP
	ENSRNOT00000026652	6.770399	9.68E-06	UP
	ENSRNOT00000027891	9.022332	1.26E-05	UP
	ENSRNOT00000003745	9.345564	1.95E-05	UP
	ENSRNOT00000012415	-1.232647	6.70E-05	DOWN
	ENSRNOT00000024243	-1.010741	1.05E-04	DOWN
	ENSRNOT00000061185	-1.179591	1.40E-04	DOWN
	ENSRNOT00000064060	-1.329700	1.63E-04	DOWN
	ENSRNOT00000019214	-1.580138	2.00E-04	DOWN
lncRNA	LNC_002311	23.08281	8.39E-06	UP
	LNC_002239	20.86495	0.000237	UP
	ENSRNOT00000089351	-1.431580	0.000288	DOWN
	LNC_004172	3.257991	0.000356	UP
	ENSRNOT00000076493	3.970388	0.000552	UP
miRNA	rno-miR-200b-3p	3.4234	1.69E-19	UP
	rno-miR-223-3p	3.1125	3.96E-18	UP
	rno-miR-200c-3p	3.0779	2.61E-15	UP
	rno-miR-3084a-3p	2.8743	3.44E-12	UP
	rno-miR-664-2-5p	2.5509	1.32E-11	UP
circRNA	novel_circ_0001704	1.6443	1.58E-07	UP
	novel_circ_0014701	-1.1721	3.47E-07	DOWN
	novel_circ_0014700	-1.0169	6.74E-07	DOWN
	novel_circ_0007532	1.8798	3.03E-06	UP
	novel_circ_0015562	1.8423	4.78E-06	UP

circRNA: Circular RNA. lncRNA: Long non-coding RNA. sRNA: miRNA/MicroRNA. log2FC: log2 fold change.

Statistical analysis

SPSS version 23, and R version 3.5 software were used to perform all the statistical analyses. Continuous variables were presented as mean ± SD. Differences between the two study groups were analyzed using the t test and chi-square test. The adjusted *P* value for the results was measured by the false discovery rate (FDR) method, and genes with a *P* value of < 0.01 were regarded as DEGs.

Results

Identification of DElncRNAs, DEcircRNAs, DEmiRNA, and DEmRNAs

Using RNA-Seq, we acquired large numbers of lncRNAs, circRNAs, miRNAs, and mRNAs (**Figure 2A**), and formulated statistics on the number of transcripts for subsequent analysis. According to the preset threshold, a total of 1514 DECs were identified between the MCAO and control-sample groups, including 72 upregulated and 9 downregulated circRNAs (DECs). We also uncovered 51 upregulated and 39 downregulated lncRNAs (DELs); 201 miRNAs (DEMis) containing 112 that were upregulated and 89 downregulated; and 1142 mRNAs (DEMs) that comprised 838 upregulated and 304 downregulated genes (**Figure 2B**). The top-five upregulated and downregulated DECs, DELs, DEMis, and DEMs are presented in **Table 1**. The top-five upregulated mRNAs were ENSRNOT00000001775, ENSRNOT00000006308, ENSRNOT00000026652, ENSRNOT00000027891, and ENSRNOT00000003745 (i.e, Pdgfa, Il1b, Gdf-

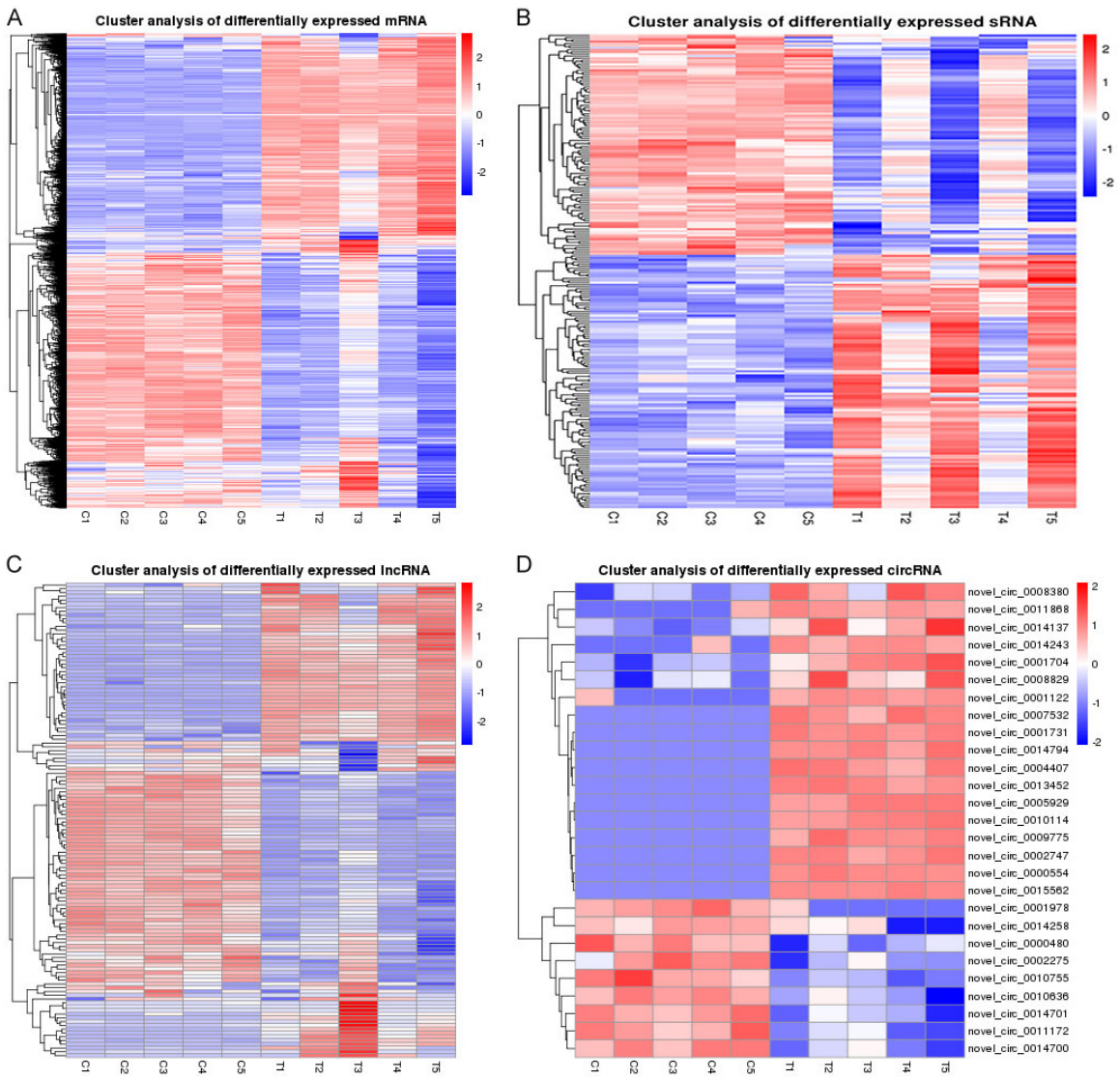


Figure 3. Heatmap analysis of DEGs in the MCAO and control groups: (A) mRNAs. (B) miRNAs. (C) lncRNAs. (D) CircRNA. Red and blue areas denote high and low relative expression, respectively. Each RNA is represented by a single row of colored boxes and each sample is designated by a single column. circRNA: Circular RNA. lncRNA: Long non-coding RNA. sRNA: miRNA/MicroRNA. C: control (n=5). T: cerebral infarction (n=5). DEGs: Differentially expressed genes. MCAO: Middle Cerebral Artery Occlusion.

15, Fosl1, and Cxcl2). The top-five low-expression mRNAs were ENSRNOT00000012415, ENSRNOT00000024243, ENSRNOT00000061185, ENSRNOT00000064060, and ENSRNOT00000019214 (i.e., Prkar2b, Olfm3, Lrrc73, Tmem38a, and Dlgap3). The clustering heatmap of differential genes is shown in (**Figure 3**).

Identification of DEcircRNAs/DElncRNAs in rats with MCAO

The Coding-Non-Coding-Index (CNCI) (v2) was set with default parameters, and we adopted the Coding Potential Calculator (CPC) (0.9-r2) to assess the extent and quality of the ORF in a transcript and to search the sequences with the known protein sequence database to clarify the coding and non-coding transcripts [19, 20]. We then applied the NCBI eukaryote protein database, setting the e-value as 1e-10 in our analysis, translated each transcript in all three possible frames, and used Pfam Scan (v1.3) to identify the occurrence of any of the known protein family domains documented in the Pfam database (release 27; we used both Pfam A and Pfam B). Any transcript with a Pfam hit was

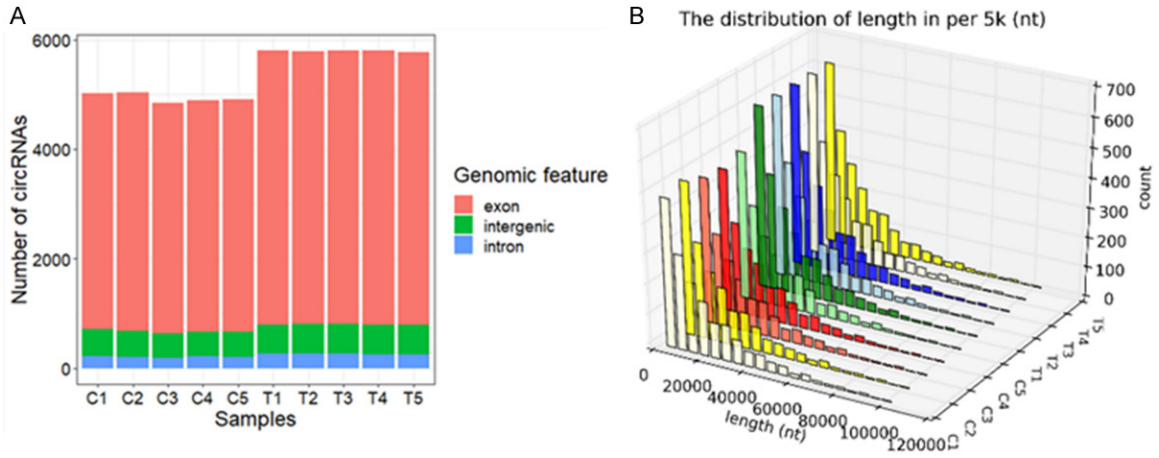


Figure 4. Sequencing of circRNA in brain tissue of the two groups of rats. A. CircRNA count. B. Length distribution of circRNA for all samples. circRNA: Circular RNA. C: control group (n=5). T: cerebral infarction group (n=5).

excluded, with fam searches reflecting default parameters of -E 0.001-domE 0.001. We then constructed multi-species genome sequence alignments and ran the phylogenetic codon substitution frequency (phyoCSF, v.20121028) with default parameters [21].

Transcripts predicted with coding potential by either/all of the four tools noted above were filtered out, and those without coding potential constituted our candidate set of lncRNAs.

The circRNAs were detected and identified using find_circ and CIRCexplorer2 [22, 23], and Circos software was used to construct the Circos figure. The raw counts were first normalized using TPM, with the normalized expression level = (readCount* 1,000,000)/libsize (libsize was the sum of the circRNA readcount) [24]. We identified 2119 previously unreported circRNAs from the two groups (**Figure 4A**) and determined a length distribution of circRNAs for all samples (**Figure 4B**).

Gene set enrichment analysis

Adopting the Metascape tool, we discerned that the differential mRNAs between the MCAO and the control groups were principally enriched in the following biologic functions: response to wounding, blood vessel morphogenesis, inflammatory response, leukocyte migration, regulation of cell adhesion, positive regulation of cell death, regulation of cytokine production, and cytokine-mediated signaling pathway for cerebral infarction (**Figure 5**). Upregulated mRNAs were primarily involved in the positive regulation of cell migration, response to wounding, blood vessel morphogenesis, inflammatory response, and cell activation (**Figure 6**). Additionally, downregulated mRNAs were primarily responsible for the modulation of chemical synaptic transmission, synapse organization, regulation of membrane potential, and regulation of ion transport (**Figure 7**). KEGG-pathway analysis indicated that MCAO DEGs were enriched in twenty pathways, and mainly enriched in the following ten pathways: TNF signaling pathway, Fluid shear stress and atherosclerosis, NF-kappa B signaling pathway, Lipid and atherosclerosis, Human cytomegalovirus infection Osteoclast differentiation, Chemokine signaling pathway, IL-17 signaling pathway, Viral protein interaction with cytokine and cytokine receptor, and Cytokine-cytokine receptor interaction (**Table 2**; **Figure 8**).

Construction of a circRNA-lncRNA-miRNA-mRNA ceRNA regulatory network in cerebral infarction

We subsequently used miRanda software to explore the ceRNA interaction between lncRNAs and miRNAs, mRNAs, and miRNAs, or circRNAs and miRNAs. The overlapping miRNAs involved in the above three ceRNA networks were also selected to construct a lncRNA/circRNA-miRNA-mRNA network, and visualized using Cytoscape software (www.cytoscape.org). We screened the ceRNA hub nodes in the network and discovered the top 20 nodes with the largest degree, betweenness, and closeness centralities (**Table 3**; **Figure 9**).

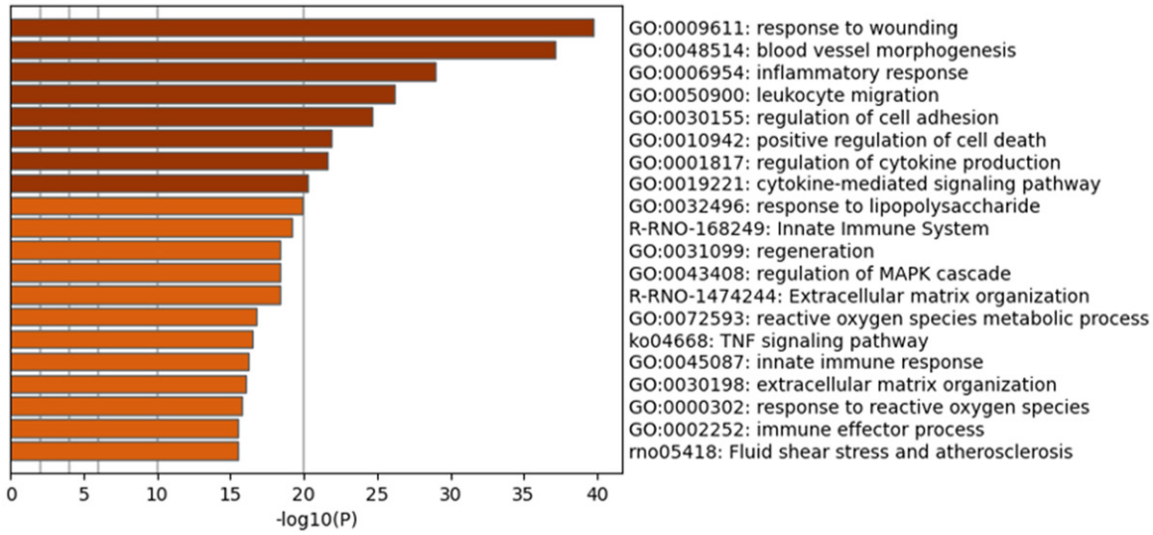


Figure 5. Bar graph of enriched terms across input gene lists, colored by *P* values. The top 20 pathways with the smallest *P*-value were taken.

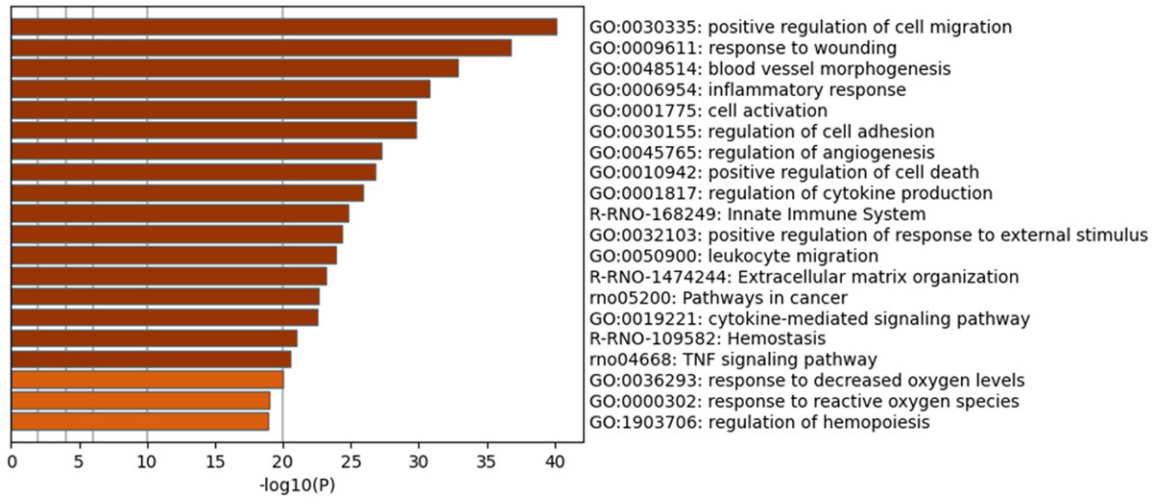


Figure 6. Bar graph of enriched terms across upregulated gene lists, colored by *P* values. The darker the color, the smaller the *P*-value.

Discussion

Great effort has been undertaken in recent years to uncover new therapeutic targets and biomarkers of ischemic stroke, including thrombolytic therapy, antiplatelet therapy, brain protection therapy, and cerebrovascular interventional therapy. However, traditional treatment methods based on antithrombotic and neuroprotective therapies are greatly limited due to their poor safety issues and treatment efficacy; this has led to high rates of mortality and disability [25]. In the cerebral system, several novel RNAs have been found to be associated with stroke, neurodegenerative disease, inflammatory diseases of the nervous system, or neurogenesis [25-27]. Although dysregulated expression of RNAs (lncRNAs, circRNAs, miRNAs, and mRNAs) and a network of their interactions have been identified and shown to influence the pathogenesis and progression of ischemic stroke [27, 28], the exact underlying mechanism(s) and the accompanying regulatory functions of ceRNAs in the stroke setting remain arcane.

In the analysis of our RNA-Seq, we detected abnormal expression of Pdgfa, Il1b, Gdf15,

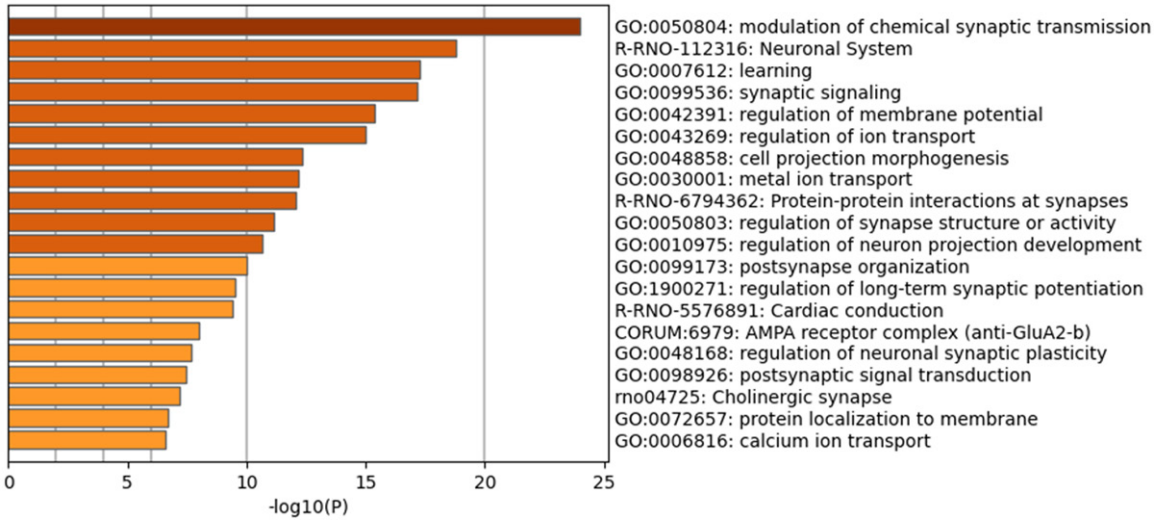


Figure 7. Bar graph of enriched terms across downregulated gene lists, colored by *P* values.

Table 2. Differential gene-enrichment pathways, with the first 20 pathways showing $P < 0.05$

KEGG_ID	Pathway_Name	Gene_Number	*P*_value
rno04668	TNF signaling pathway	36	1.9838E-16
rno05418	Fluid shear stress and atherosclerosis	39	4.2952E-14
rno04064	NF-kappa B signaling pathway	31	8.0816E-14
rno05417	Lipid and atherosclerosis	44	1.6393E-12
rno05163	Human cytomegalovirus infection	46	4.5715E-11
rno04380	Osteoclast differentiation	30	1.6988E-10
rno04062	Chemokine signaling pathway	37	3.6412E-10
rno04657	IL-17 signaling pathway	25	1.0303E-09
rno04061	Viral protein interaction with cytokine and cytokine receptor	24	1.0526E-09
rno04060	Cytokine-cytokine receptor interaction	45	2.3551E-09
rno04210	Apoptosis	30	3.2124E-09
rno04625	C-type lectin receptor signaling pathway	27	3.8754E-09
rno05323	Rheumatoid arthritis	23	7.6766E-09
rno05167	Kaposi sarcoma-associated herpesvirus infection	38	1.3186E-08
rno05169	Epstein-Barr virus infection	39	1.4123E-08
rno04010	MAPK signaling pathway	45	3.3904E-08
rno04933	AGE-RAGE signaling pathway in diabetic complications	24	3.8124E-08
rno04621	NOD-like receptor signaling pathway	33	3.9925E-08
rno05166	Human T-cell leukemia virus 1 infection	40	6.707E-08
rno05222	Small cell lung cancer	21	6.5837E-07

Gene number is the number of DEGs in each pathway. DEGs: Differentially expressed genes.

Fosl1, and Cxcl2, the top-five upregulated mRNAs. Platelet-derived growth factors (PDGFs) are robust inducers of cellular mitosis, migration, angiogenesis, and matrix modulation; and play pivotal roles in the development, homeostasis, and healing of tissues. PDGFs also act as mitogens and potent stimulators of mesenchymal cell angiogenesis; and PDGF/Rs are implicated in many pathologic processes such as atherosclerosis, fibrosis, and tumorigenesis [27]. Pdgfa belongs to the PDGF family and may occupy a critical role in ischemic diseases of the central nervous system. Abnormal expression of Pdgfa in stroke, glioblastoma,

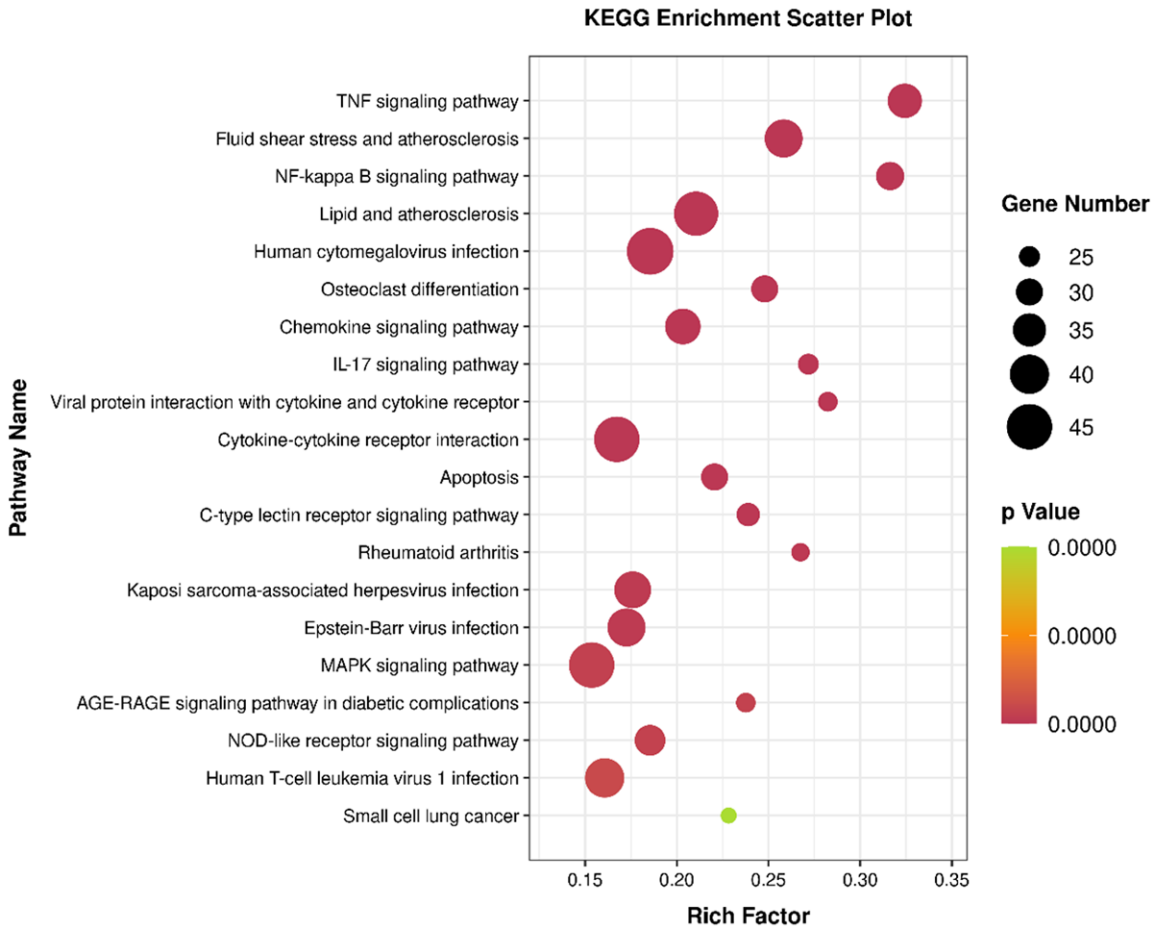


Figure 8. Gene enrichment bubble map. Genome (KEGG)-pathway enrichment of MCAO and Control DEGs. The redder the color, the more significant the statistical difference, and the larger the plot, the more genes were enriched. KEGG: Kyoto Encyclopedia of Genes and Genomes. DEGs: Differentially expressed genes. MCAO: Middle Cerebral Artery Occlusion.

and autoimmune encephalomyelitis and the pathologic processes underlying these diseases are influenced by the regulation of PDGF subunit A [28-30]. IL-β is a cytokine that initiates and regulates inflammatory processes, and it is elevated in tissues derived from animal experimentation [25]. Growth and differentiation factor 15 (GDF15) belongs to the transforming growth factor-β (TGF-β) superfamily of proteins and acts as an inflammatory marker, with a role in the pathogenesis of tumors, ischemic diseases, metabolic disorders, and neurodegenerative processes [29]. GDF15 functions are also critical for the regulation of endothelial adaptations after vascular damage [29]. FOS-related antigen 1 (FRA1) is encoded by the FOSL1 gene; belongs to the FOS protein family; principally forms an AP-1 complex with JUN family members to exert an effect; this is overexpressed in many tumors, thereby affecting various biological activities such as tumor proliferation, differentiation, invasion, and apoptosis [30]. One of the systems affected by hypoxia is the CXC chemokine system. The expression of CXCL2 is elevated after cerebral infarction, and selective inhibition of the CXCL2 pathway appears to prevent arterial plaque rupture and reduce the incidence of ischemic stroke [31]. Prkar2b, Olfm3, and Dlgap3 are downregulated mRNAs involved in ischemic stroke. The protein kinase A (PKA)-signaling cascade transduces physiologic hormone-mediated processes, and its deregulation is central to the pathogenesis of neoplastic as well as non-neoplastic diseases [32]. The PKA tetrameric holoenzyme is composed of regulatory (R) and catalytic (C) subunit dimers. The regulatory subunits are each present in alpha (α) and beta (β) isoforms

Table 3. Top 20 nodes with the largest degree of centrality and betweenness and closeness in the circRNA/lncRNA-miRNA-mRNA network

Node	Degree	Betweenness Centrality	Closeness Centrality
rno-miR-298-5p	28	0.40972379	0.47126437
rno-miR-674-5p	17	0.07540516	0.42708333
rno-miR-412-3p	16	0.08159586	0.41414141
rno-miR-149-5p	15	0.06907952	0.41
LNC_010057	13	0.09056408	0.46857143
rno-miR-6216	13	0.06010456	0.40594059
rno-miR-30c-2-3p	13	0.05013044	0.41
rno-miR-150-5p	12	0.04561329	0.40594059
rno-miR-378a-3p	12	0.02887645	0.40594059
rno-miR-15a-5p	11	0.20534168	0.34517766
rno-miR-760-3p	11	0.11060653	0.40196078
LNC_002005	11	0.06462722	0.38497653
LNC_007524	11	0.04226171	0.39613527
rno-miR-708-5p	10	0.20391827	0.3715847
LNC_001999	10	0.08416152	0.45303867
rno-miR-214-3p	10	0.08347955	0.39805825
LNC_006272	10	0.08199657	0.46327684
rno-miR-935	10	0.05867277	0.39047619
ENSRNOT00000075976	10	0.02530494	0.37104072
LNC_007522	10	0.02368285	0.37442922

(RIα, RIIα, RIβ, and RIIβ), and the PKA regulatory subunit 2B (PRKAR2B/RIIβ) is one of the four subunits of PKA-with downregulation of PRKAR2B shown to inhibit the activation of caspase-3 induced by OGD/R, thus mitigating cellular damage [32, 33]. OLFM3 was demonstrated to be differentially expressed in the cerebral cortex and serum of Alzheimer's disease (AD) patients, suggesting that OLFM3 was related to the pathogenesis of AD [34]. The postsynaptic density (PSD) is composed of numerous proteins, including a family of Discs large-associated proteins 1, 2, 3, and 4 (DLGAP1-4) that act as scaffold proteins in the PSD. The DLGAP family has been directly linked to a variety of psychological and neurological disorders [35]. Most of the molecules that up-regulated and down-regulated in our sequencing results were related to apoptosis, inflammatory response, and repellent response, suggesting that ischemic cerebral infarction may be related to the above molecules and their roles in the above pathological processes. This may be a target for future treatment of cellular damage repair or inhibition after cerebral infarction.

Importantly, miRNAs have been explored for their potential as biomarkers in the diagnosis and prognosis of brain injury in ischemic stroke, and substantial evidence suggests that miRNAs are key actors in the numerous cellular changes that follow ischemic stroke. These actions-including mitochondrial dysfunction, energy failure, cytokine-mediated cytotoxicity, oxidative stress, activation of glial cells, increased intracellular calcium levels, inflammatory responses, and the disruption of the blood-brain barrier (BBB)-target-specific miRNAs, and thus therapeutic modulation of brain injury and apoptosis can be achieved [36]. In our study, we discovered that the expression of miR-200 was augmented. The miR-200 family consists of five members that regulate the proliferation, invasion, and migration of cancer cells by inhibiting the transcription of downstream genes that include zinc finger E-box binding homeobox 1 and 2, E-cadherin, N-cadherin, transforming growth factor-β, and cancer stem cell related-proteins. Long non-coding RNAs can subsequently bind to miR-200 to regulate the proliferation and apoptosis of cancer cells [37]. Numerous studies have also indicated that members of the miR-200 family are important in glioma development, therapeutic responses, metastasis, and clinical prognosis [38]. The miR-200 family members are aberrantly expressed in several neurodegenerative diseases and participate in various cellular processes that encompass beta-amyloid (Aβ) secretion, alpha-synuclein aggregation, and DNA repair [39]. miR-223 was reported to influence several oncosuppressors; and to serve as an oncogenic driver, therapeutic target, and a biomarker of response and prognosis in most carcinomas-including breast cancer, gastroesophageal cancers, and liver cancer [40]. miR-223 is expressed differentially in a variety of tumors and may play disparate roles through pathways such as P53 [40]. miR-223 is also a hematopoietic cell-derived miRNA that is important in the regulation of monocyte-macrophage differentiation, neutrophil recruitment, and pro-inflammatory responses; can be transferred to non-myeloid cells via extracellular vesicles or

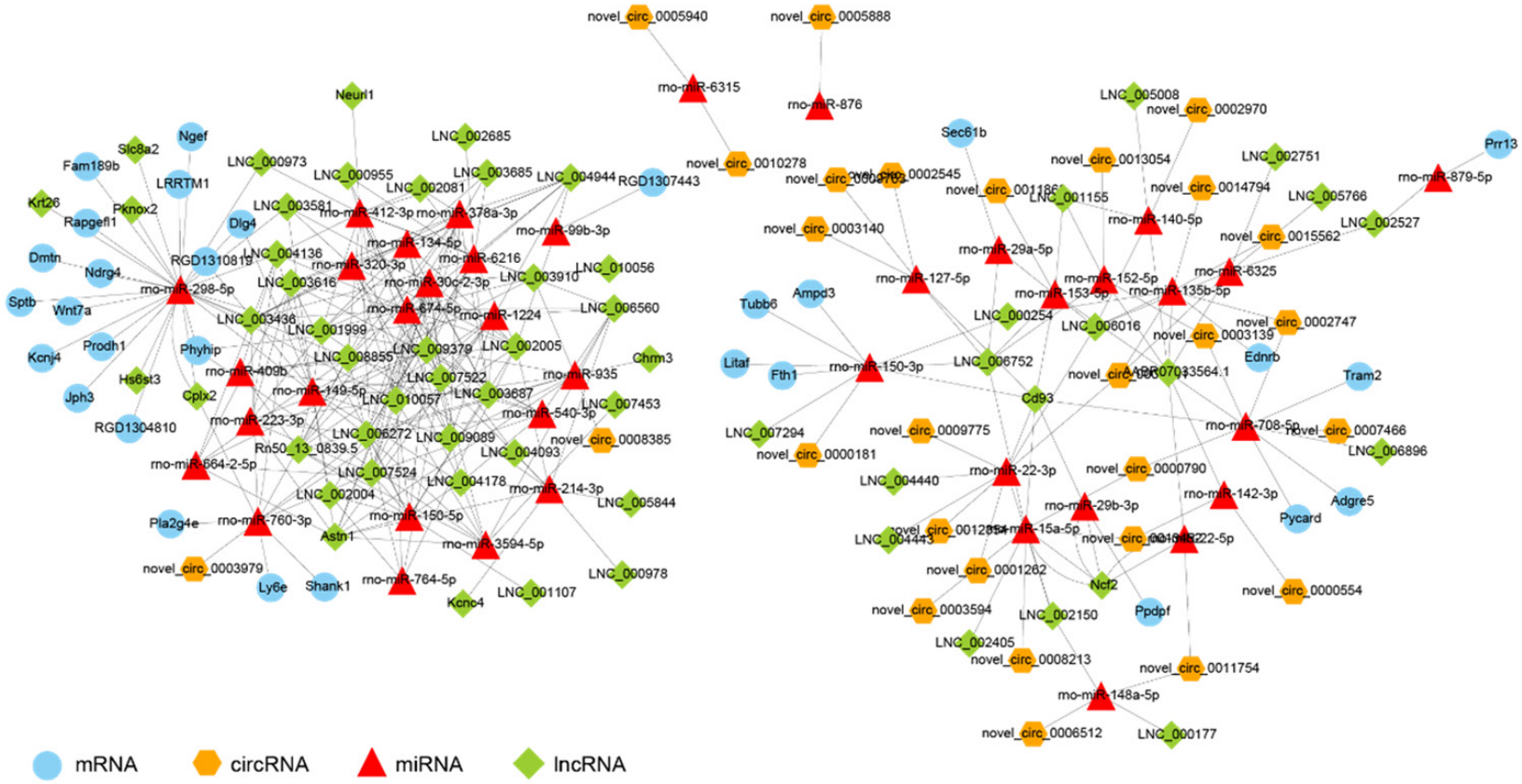


Figure 9. Differentially expressed circRNA-lncRNA-miRNA-mRNA gene-interaction network. Different color represents different RNA. circRNA: Circular RNA. lncRNA: Long non-coding RNA. miRNA: MicroRNA.

lipoproteins; is shown to regulate immune cell differentiation and inflammation [41, 42]. While we herein discerned elevated expression rates for miR-3084a-3p, miR-664-2-5p (Rn50_13_0839.5), and Cd93-202, we didn't find any literature on their functions; and they are therefore worthy of our further attention.

We performed GO-enrichment analysis on the genes in our constructed network and found that the enriched terms were relevant to ischemic stroke. Clustering of the GO terms showed that these genes were primarily enriched in cell adhesion, response to wounding, blood vessel morphogenesis, inflammatory response, leukocyte migration, positive regulation of cell death, regulation of cytokine production, and cytokine-mediated signaling pathway. Cerebral infarction is an injury caused by a series of comprehensive factors as a consequence of ischemia and hypoxia after blood-flow obstruction. Through our sequencing and bioinformatic analyses, we ascertained that a variety of biologic mechanisms were involved in cerebral ischemia. Furthermore, several miRNAs, cicRNAs, and lncRNAs have been reported to play regulatory roles during neuroprotection and angiogenesis through distinct mechanisms that involve their interactions with target-encoding genes [43].

Enrichment analysis and PPI network construction were subsequently conducted and revealed some hub genes. After predicting miRNAs targeting mRNAs, two circRNA/lncRNA-miRNA-mRNA ceRNA networks were constructed, and our results suggested that specific ceRNA axes comprise promising targets for the diagnosis of ischemic stroke.

There were several limitations to the present research study. First, our sample size was not large; thus, additional validation cohorts should be included in future studies to analyze the expression of the identified lncRNAs, circRNAs, miRNAs, and mRNAs. Second, how these novel ceRNA axes participate in the development of ischemic stroke remains unclear. Thus, further cell and animal experiments are needed to verify these findings. Future studies should also focus on identifying and verifying the novel circRNA and lncRNAs in our ceRNA network.

Conclusions

In this study, we constructed a ceRNA network with respect to MCAO in the rat and identified several associated lncRNA/circRNA-miRNA-mRNA interaction axes in the brain tissue of our rat model. This study thus provided novel insights into the genetic basis of cerebral infarction; however, further investigations are necessary to validate the underlying ceRNA mechanisms for ncRNAs and mRNAs that may be critical targets in disease treatment.

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Disclosure of conflict of interest

None.

Abbreviations

DEG, Differentially expressed gene; DEL, Differentially expressed lncRNA; DEMi, Differentially expressed miRNA; DEM, Differentially expressed mRNA; DEC, Differentially expressed circRNA; pMCAO, permanent Middle Cerebral Artery Occlusion; LncRNA, Long non-coding RNA; CircRNA, Circular RNA; miRNA, MicroRNA; MCODE, Molecular Complex Detection; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; CCA, Common Carotid Artery; ECA, External Carotid Artery; ICA, Internal Carotid Artery; ceRNAs, Competing endogenous RNAs; TCGA, The Cancer Genome Atlas; DETs, Differentially expressed transcripts; Pdgfa, Platelet-derived growth factors;

Il1β, Interleukin 1 Beta; Gdf15, Growth Differentiation Factor 15; Fosl1, FOS-related antigen 1; Cxcl2, C-X-C Motif Chemokine Ligand 2; Prkar2b, Protein kinase cAMP-dependent type II regulatory subunit beta; Olfm3, Olfactomedin 3; Lrrc73, Leucine rich repeat containing 73; Tmem38a, Transmembrane protein 38A; Dlgap3, DLG associated protein 3; CNCI, Coding-Non-Coding-Index; CPC, Coding Potential Calculator.

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