



Original Article

## Knockdown of lncRNA AU020206 could inhibit microglia apoptosis in ischemic stroke

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### Article Info

### Abstract



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The diagnostic biomarkers associated with ischemic stroke (IS) that may have clinical utility remain elucidated. Thus, the potential functional lncRNAs in IS were explored. The Gene Expression Omnibus database provided the transcriptome profile of IS for download. WGCNA analysis and integrated bioinformatics were used to find genes that were differentially expressed (DEGs). The Starbase database created the lncRNA-based ceRNA network. In order to investigate the molecular mechanism and involved pathways of DEGs in IS, functional enrichment analysis was carried out. Using qRT-PCR, lncRNAs identified as putative IS biomarkers were confirmed to be expressed in a permanent middle cerebral artery occlusion (MCAO) model. Using the annexin V/PI apoptosis test, the amount of apoptosis in oxygen-glucose deprivation (OGD) cells was measured. A total of 1600 common differentially expressed - protein-coding RNA (DE-pcRNAs) and 26 DE-lncRNAs were identified. The results of enrichment analysis indicate that the cytokine may be regulated by common DE-pcRNAs and are vital in the progress of IS. A lncRNAs-mediated ceRNA network including lncRNAs AU020206, Brip1os, F630028O10Rik and 9530082P21Rik was constructed. The expression of these lncRNAs was significantly increased in MCAO model. Knockdown of lncRNA AU020206 inhibited microglia apoptosis in OGD cell model. We constructed a lncRNAs-mediated ceRNA network and found that lncRNA AU020206 inhibited microglia apoptosis in OGD cell model. These findings provided further evidence for the diagnosis and a novel avenue for targeted therapy of IS.

**Keywords:** Ischemic stroke, Bioinformatics, lncRNAs, ceRNA network.

### 1. Introduction

One of the leading causes of mortality and a leading contributor to adult disability globally is ischemic stroke (IS), which makes up 75–80% of stroke [1, 2]. As a most common type of acute cerebrovascular disease, the pathophysiological effects of IS are driven by a multitude of complex molecules and caused by various factors [3]. With the arrival of the aging population worldwide, IS has become a common and frequently occurring disease among the middle-aged and elderly population. It has the characteristics of acute onset, severe illness, rapid change, disability, and high mortality rate [4]. However, the specific molecular mechanism of ischemic stroke is not entirely clear, and there lack of potential diagnostic markers for IS early warning. Hence, there is an urgent need to explore effective diagnostic biomarkers and the underlying mechanisms in IS.

Long non-coding RNA (lncRNA) molecules are functional RNA molecules with a length exceeding 200 nt, but it has no or lacks an open reading coding framework [5]. lncRNAs involve regulatory sequences at the transcription, reverse transcription, and epigenetic levels, and has spatiotemporal and tissue specificity in the operation of the central nervous system [6]. lncRNAs contain miRNA-responsive elements (MREs) that can bind to miRNA

through MREs, thereby inhibiting the negative regulatory effect of miRNA on mRNA and increasing mRNA expression [7]. Understanding the functional roles of lncRNAs in ischemic stroke is crucial, given their potential contribution to the disease pathology [8]. Previous studies revealed that lncRNAs have emerged as a novel class of regulatory molecules with the potential to attenuate or aggravate the pathogenic mechanisms following IS. These mechanisms include oxidative stress, neuroinflammation, cell death signaling, blood-brain barrier dysfunction, and angiogenesis [9]. Chen et al. discovered that the Notch1 pathway in IS is regulated by lncRNA GAS5 acting as a ceRNA for miR-137 [10]. According to Zhang et al., lncRNA SNHG1 controls IS cerebrovascular diseases as a ceRNA by HIF-1 $\alpha$ /VEGF signaling [11]. MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) has been extensively studied due to its involvement in the pathophysiological processes of IS, which was considered a potential therapeutic target [9]. However, the functions and roles of many other lncRNAs are unclear. To understand more detailed mechanisms of the lncRNAs in the pathogenesis of IS, further research is necessary.

Here, the aim of the study is to investigate the biomarkers and functions of lncRNAs in IS. Integrated bioinformatics analysis including the WGCNA analysis to

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construct the lncRNA-based ceRNA network by public database. In addition, four DE-lncRNAs were found to be significant in the ceRNA network and serve as potential biomarkers of IS. Their expression was verified in a model of permanent middle cerebral artery occlusion (MCAO). Finally, the function of lncRNA AU020206 was performed using an oxygen-glucose deprivation (OGD) cell model. This research offered further proof that IS exists, and it may open up a new treatment option for IS patients.

## 2. Materials and Methods

### 2.1. Data acquisition

The Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>) provided the transcriptome profile of the control tissues and IS tissues (GSE137482) [12]. This dataset includes 12 IS and 6 normal samples of C57BL/6 mice. 28 miRNAs related to IS were acquired from the study of Cai et al. [13].

### 2.2. Identification of DEGs

After the transcriptome profile of IS and the normal groups normalized, the expression of differentially expressed-protein coding RNAs (DE-pcRNAs) and DE-lncRNAs were analyzed via DESeq2 R package (threshold:  $\text{adj-p-value} < 0.05$  and  $\text{fold change} \geq 1.5$ ).

### 2.3. WGCNA

Using the WGCNA, a free-scale gene co-expression network was built [14, 15]. A co-expression network for every gene in IS and normal abdominal aorta samples was created using the "WGCNA" R package.

### 2.4. Functional enrichment analysis

The "clusterprofiler" R software package was used to perform GO and KEGG enrichment analysis in order to investigate the possible biological process and enrichment route of DEGs. The standard of statistical significance is that the adjusted P-value is less than 0.05. [16, 17].

### 2.5. Construction of lncRNA-based ceRNA network

The StarBase (<http://starbase.sysu.edu.cn/>) was used to predict miRNA-pcRNA, lncRNA-miRNA interaction information [18]. Then, using Cytoscape (version v3.7.2, <https://cytoscape.org/>), the miRNAs that controlled both mRNA and lncRNA were chosen for the creation of the ceRNA network.

### 2.6. Animals and mouse MCAO model

We bought twelve C57BL/6J female mice (8–9 weeks

old, 22–25 g) from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). After one week of adaptive feeding, the mice were used to construct the MCAO model, as described earlier [19]. In short, mice were anesthetized with a mixture of isoflurane and O<sub>2</sub>/N<sub>2</sub>. Next, carefully thread an upward-pointing 6-0 nylon monofilament suture into the internal carotid artery from the external carotid artery. After reperfusion, the mice were subjected to deep anesthesia and their brains were obtained for further analysis. The sham surgery was also performed in control mice [20]. Every animal experiment was carried out in compliance with the Second Affiliated Hospital of Nanchang University's authorized procedure.

### 2.7. QRT-PCR

Using TRIzol, RNA was isolated from cells or tissues. Using Nano drop 2000C, the concentration and purity of RNA were determined. RNA were reverse transcribed into cDNA using PCR kit. Then qRT-PCR reaction was conducted according to the following settings: 95°C 35 s, 60°C 30 s, 95°C 10 s, 65°C 5 s. After the reaction, the amplification curve and fusion curve were confirmed, and the 2<sup>-ΔΔCt</sup> value to represent the relative expression of mRNA was calculated. Primers synthesized by Fuzhou Shangya Biosynthesis (Fuzhou, China) are shown in Table 1.

### 2.8. OGD cell model

Mouse microglial line BV2 was purchased from Merck millipore (#scc103, Darmstadt, Germany) and cultured in DMEM (# a4192101, GIBCO, Rockville, MD, USA) containing 10% fetal bovine serum (#16000044, GIBCO, Rockville, MD, USA), 100 U/ml penicillin and 100 g/ml streptomycin. The microglia were treated with 1 × PBS for rinsing twice, and then the cells were cultured with Earle's balanced salt solution in a 0.5% O<sub>2</sub>, 94.5% N<sub>2</sub>, 5% CO<sub>2</sub> incubator at 37°C for 2 h. Subsequently, the cell culture medium of OGD group and normal group was replaced with normal culture medium, and cultured at 37°C for 12 h.

### 2.9. Cell transfection

Corresponding lentiviruses expressing sequence siRNAs specific to lncRNA AU020206 were designed and synthesized by Fuzhou Shangya Biosynthesis (Fuzhou, China) to knockdown lncRNA AU020206 in BV2 cells. Si-NC was used as the negative control. Transfections were conducted by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

**Table 1.** Specific RNAs primers for quantitative qRT-PCR analysis.

Gene name	Sequence (5'-3')
β-actin	F: CTCATCCTGGCCTCGCTGT
	R: GCTGTCACCTTCACCGTTCC
AU020206	F: GGATGATAGACTCTGGCCAGG
	R: CCCACTAAACAAAATGCCTCT
Brip1os	F: ACCAGCCACGCAACCTAAC
	R: CCCTCCAAGTCCCATCCAG
F630028O10Rik	F: AAGACAAAGGCACCACTTCAATG
	R: CCACCAGCAGTTTTCTAAAGGATG
9530082P21Rik	F: CCTGGCCTTTGCTTGGGATA
	R: TAGCAGGACTCAGGAGCCAT

### 2.10. Statistical analysis

All experimental data were obtained through three independent experiments using Statistic Package for Social Science (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA). The measurement data are expressed in terms of mean±SD, and the significance between two groups was calculated by t-test. P<0.05 indicates statistical significance.

## 3. Results

### 3.1. Identification of the DE-pcRNAs and DE-lncRNAs in IS

To screen the difference in molecular expression between IS tissue and normal tissue, DE-pcRNAs and DE-lncRNAs were identified. A total of 2200 DE-pcRNAs were obtained, which includes 1703 up-regulated and 497 down-regulated DE-pcRNAs (Figure 1A). Besides, we also identified 41 DE-lncRNAs including 29 up-regulated and 12 down-regulated genes (Figure 1B). Furthermore, to understand the expression distribution of DEGs in-between the IS group and the normal group, hierarchical cluster algorithm analysis on DE-pcRNAs and DE-lncRNAs was performed and shown in the heat maps, respectively (Figure 1C and D).

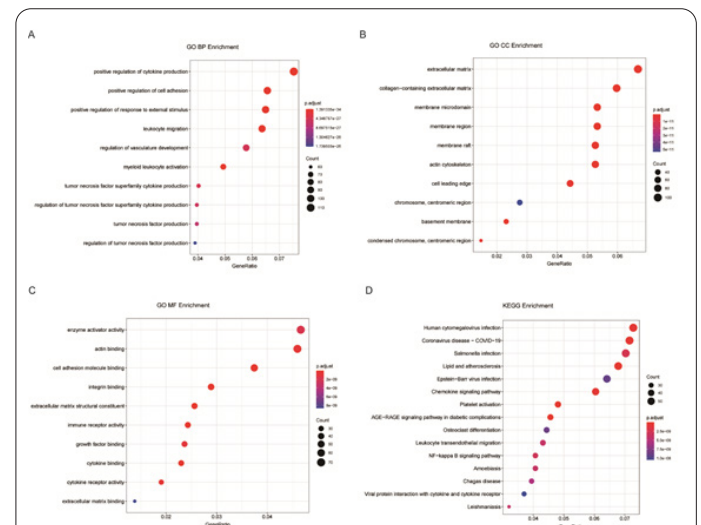
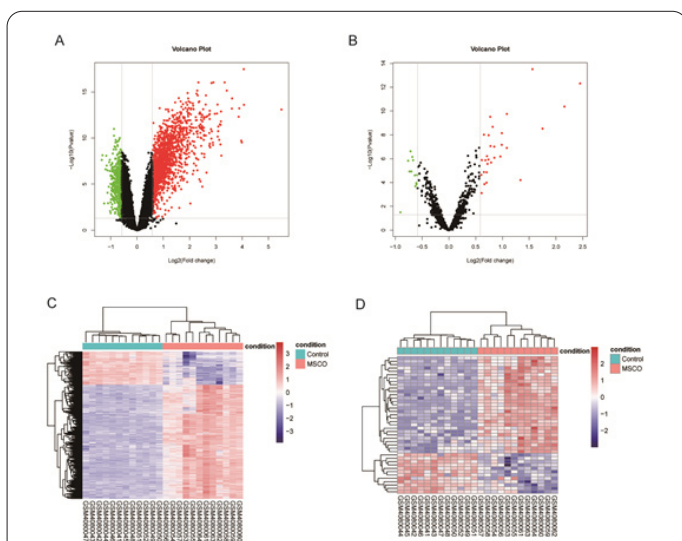
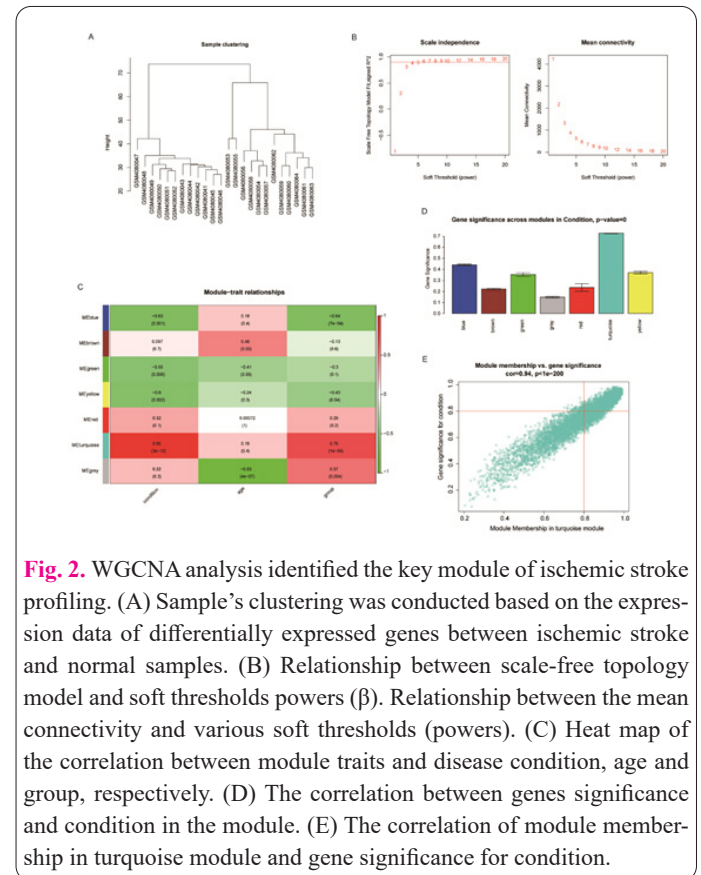
### 3.2. WGCNA analysis identified the key module of IS profiling

To investigate the co-expression network most highly related to IS progression, WGCNA analysis was conducted according to 24 samples in GSE137482 datasets (Figure 2A). A power of  $\beta = 4$  (scale-free  $R^2 = 0.9$ ) was chosen for the soft-thresholding parameter to ensure the network (Figure 2B), and seven modules in the condition were detected. Thereafter, the turquoise module including 5841 genes was found to have the highest correlation with IS condition (turquoise,  $r=0.95$ ,  $p=3e^{-12}$ ) (Figure 2C, D). The turquoise module was thus selected as the important module for further examination. Then, by comparing the

correlation between modules and IS condition, 2118 hub genes with strong correlation between modules and stroke occurrence were screened (module membership value > 0.8 and gene significance value > 0.8 as the screening criteria) (Figure 2E).

### 3.3. Functional enrichment analysis of common DEGs

Then, we conducted Venn analysis to acquire more credible DE-pcRNAs and DE-lncRNAs. As shown in Figure S1, 1600 common DE-pcRNAs and 26 DE-lncRNAs were identified. GO analysis contains biological processes (BP) (Figure 3A), cell components (CC) (Figure 3B), and molecular functions (MF) (Figure 3C). The top 10 significantly



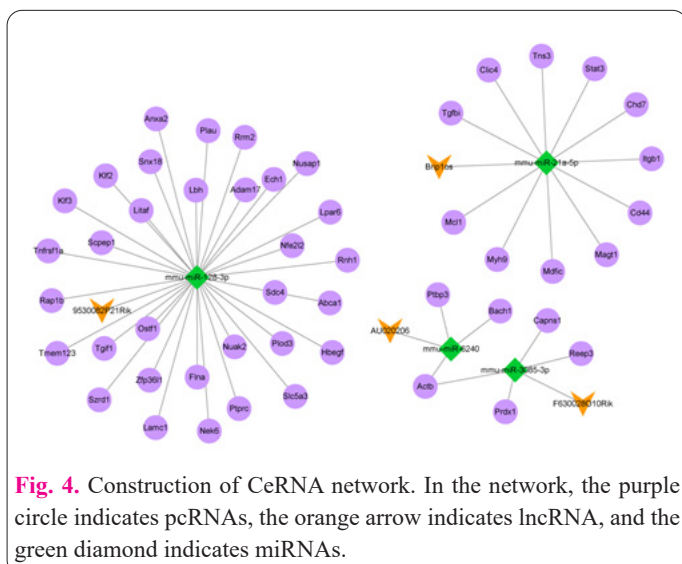
enriched terms including the generation of cytokines and cell adhesion are positively regulated, and regulation of vasculature development was remarkably enriched in BP (Figure 3A), cytokine receptor activity, cytokine binding and immune receptor activity were enriched in MF (Figure 3C). Besides, we also carried out KEGG pathways analysis of DE-pcRNAs. The results showed that lipid and atherosclerosis, chemokine signaling pathway, platelet activation and so on pathways were remarkably enriched (Figure 3D). These results indicate that the cytokine may regulated by common DE- pcRNAs and are significant in the progress of IS [21].

### 3.4. Identification and validation of lncRNAs in ceRNA network

Using Starbase, a ceRNA network containing pcRNAs, lncRNAs, and miRNAs was created to study the interactions between DEGs [18]. A total of 1600 common DE-pcRNAs and 26 common DE-lncRNAs were acquired from previous results. In addition, 28 miRNAs related to IS were obtained from research of Cai et al [13]. As shown in Figure 4, the ceRNA network included 49 DE-pcRNAs, 4 DE-miRNAs, and 4 DE-lncRNAs, in which we found that DE-lncRNAs AU020206, Brip1os, F630028O10Rik and 9530082P21Rik can bind to miRNA in the network. Nevertheless, the effect of these four lncRNAs on the IS has not been reported yet. Thereafter, we performed qRT-PCR for further validation in MCAO. As shown in Figure 5, the relative expression of lncRNAs AU020206, Brip1os, F630028O10Rik and 9530082P21Rik were significantly increased in MCAO model groups compared to control groups. Furthermore, we carried out the GO enrichment analysis based on the pcRNAs involved in ceRNA network to investigate the function of the four lncRNAs. The GO terms including endothelium development/ differentiation, response to wounding, T cell activation, etc. in BP were significantly enriched (Figure S2A). Additionally, there was a substantial enrichment in signaling pathways related to lipid and atherosclerosis, fluid shear stress and atherosclerosis, and focal adhesion (Figure S2B).

### 3.5. Knockdown of lncRNA AU020206 inhibited microglia apoptosis in OGD model

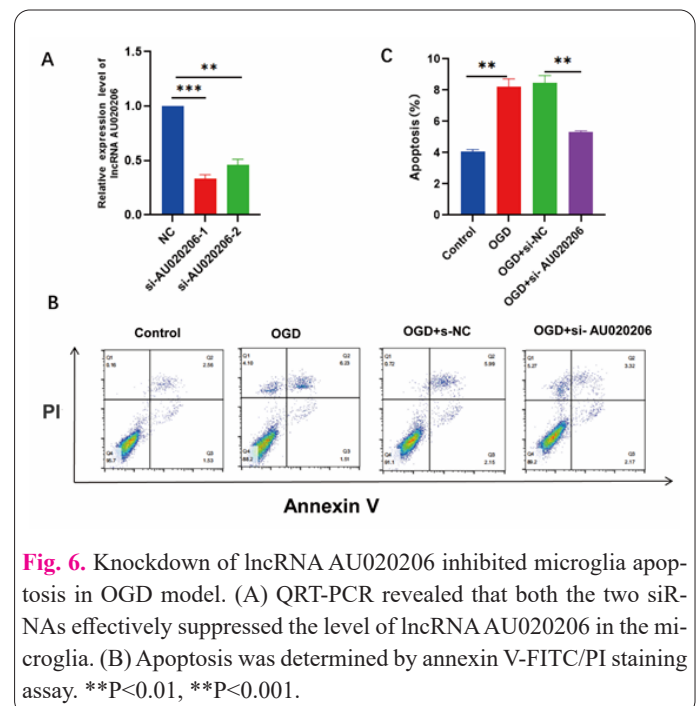
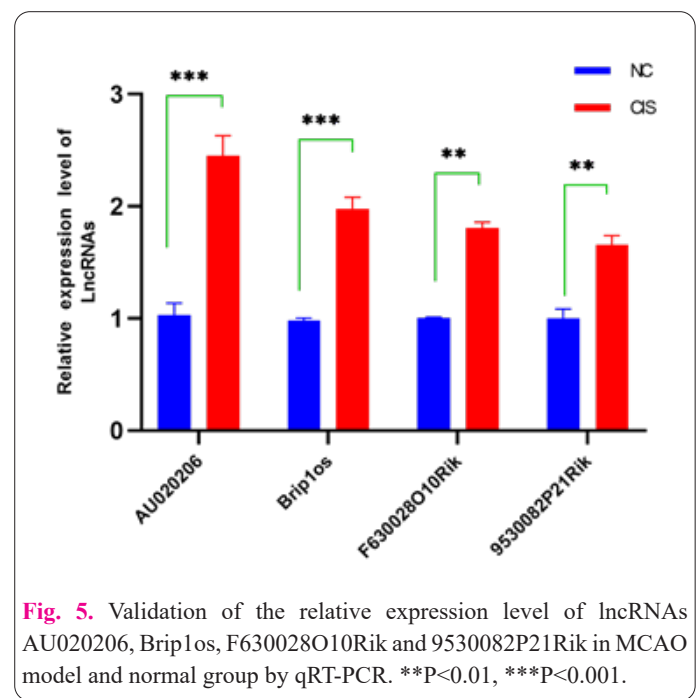
Among the four DE-lncRNAs above, lncRNA AU020206 showed the most significant differences. Then,



we knocked down the expression of lncRNA AU020206 using siRNA1 and siRNA2 in microglia. The results of qRT-PCR revealed that both the two siRNAs effectively suppressed the level of lncRNA AU020206 in the microglia (Figure 6A). The result of annexin V/PI analysis demonstrated that OGD caused microglia apoptosis, and the apoptosis rate was higher than normal group (Figure 6B and C). Knockdown of lncRNA AU020206 significantly inhibited microglia apoptosis in OGD cells (Figure 6B and C). These data suggested that knockdown of AU020206 protects microglia from apoptosis.

## 4. Discussion

IS is an acute brain injury that accounts for about 80% of all strokes and is the leading cause of morbidity and mortality worldwide. [2, 22, 23]. Notably, IS has become an increasing social and public health burden in the world. Increasing evidence indicates that the pathophysiologi-



cal processes in IS have been the primary concern [24]. Nevertheless, the diagnostic biomarkers associated with IS that may have clinical utility remain elucidated. In this present study, we carried out the integrated bioinformatics analysis to screen out the DEGs in IS based on the RNA-seq dataset downloaded from GEO database. The WGCNA was used to identify gene modules under both IS and normal circumstances and to build a free-scale gene co-expression network, and related module was used to identify candidate biomarkers in IS. A Total of 1600 common DE-pcRNAs and 26 DE-lncRNAs were screened.

Using novel technologies like RNA-seq, deep sequencing, and microarrays, the majority of aberrantly expressed lncRNAs have been found in IS patients or animals that have been injured by ischemia [24-26]. More and more evidences have suggested that lncRNAs that acting as ceRNAs function as crucial mediators to regulate the pathogenesis of IS [23]. For example, the lncRNA Malat1 acts as a ceRNA to modulate PTEN expression and sponge miR-205-3p to control apoptosis in IS and to contribute to bebeerine-mediated suppression of HMGB1 in post-stroke inflammation [27, 28]. According to Gai et al., the miR-126/SOX6 signaling pathway is how lncRNA CHRF controls the development of IS [29]. In this case, a lncRNA-mediated ceRNA network was built for more research using the common DE-pcRNAs, DE-lncRNAs, and DE-miRNAs.

The cytokines in experimental stroke and are therefore potential targets in future stroke therapy [21]. The activation of the immune system contributes significantly to the development of IS pathology [30]. Chemokine could induce the directional migration of cell; they control the chemotaxis of immune cells. GO analysis indicated the significantly enriched terms including positive regulation of cytokine production was significantly enriched in biological process, cytokine receptor activity, cytokine binding and immune receptor activity were significantly enriched in molecular function. Besides, chemokine pathway was also enriched, indicating that the cytokine may regulated by common DE-pcRNAs and are significant in the progress of IS [21].

For the first time, we identified these four lncRNAs as potential biomarkers for IS, including lncRNA AU020206, Brip1os, F630028O10Rik and 9530082P21Rik, which have not been reported in IS yet. Zhang et al. indicated that Brip1os, AU020206, are potential therapeutic targets for atherosclerosis [31], which is consistent with our KEGG results that the pathways like fluid shear stress and atherosclerosis, lipid and atherosclerosis were significantly enriched. Qin et al. revealed that F630028O10Rik (abbreviated as F63) is a novel lncRNA that was significantly up-regulated in tumors isolated from mice [32]. Besides, lncRNA-F630028O10Rik was indicated to be promoted by TLR4 after spinal cord injury and promoted microglial pyroptosis [33]. As for 9530082P21Rik, is a novel lncRNA that have never been reported before. The expression of these lncRNAs showed enhanced expression in MCAO model. These lncRNAs were suggested to be associated with endothelium development/ differentiation, response to wounding, T cell activation in biological process by GO enrichment analysis. These findings contribute to a better understanding of the roles and processes of lncRNAs in IS.

## 5. Conclusion

In conclusion, we constructed an lncRNA-mediated ceRNA network and verified the expression of lncRNAs AU020206, Brip1os, F630028O10Rik and 9530082P21Rik. What's more, we preliminarily demonstrated the function of lncRNA AU020206 in an OGD cell model. Further investigating the functions and mechanisms of the lncRNA AU020206 in biological systems may lead to opportunities for providing novel therapeutic targets of IS.

## Conflict of interests

The author has no conflicts with any step of the article preparation.

## Consent for publications

The author read and approved the final manuscript for publication.

## Ethics approval and consent to participate

No human or animals were used in the present research.

## Informed consent

The authors declare not used any patients in this research.

## Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

## Author contributions

Sihui Song, Zhihua Chen and Jianming Zhu designed the research. Sihui Song, Zhihua Chen and Qiang Liu performed the experiments. Sihong Shu and Jianwei Lei visualized the results. Sihui Song and Jianming Zhu wrote manuscript. Jianming Zhu provided experimental resource. All authors reviewed and approved the final manuscript.

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