The construction of neurogenesis-related ceRNA network of ischemic stroke treated by oxymatrine

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Background **Known as a disease associated with high mortality, disability and a significant financial burden, ischemic stroke ranks as one of the three diseases threatening human health. Recent advances in omics technology created opportunities to uncover the mechanism in ischemic stroke occurrence and treatment. In this study, we aimed to construct the competitive endogenous RNA (ceRNA) networks of ischemic stroke treated by oxymatrine intervention.**

Method **The middle cerebral artery occlusion (MCAO) mouse model of ischemic stroke was constructed, and oxymatrine was administered. Then RNA-Sequencing was performed and integrated analysis of mRNAs, lncRNAs and circRNAs was conducted to reveal the pharmacology of oxymatrine. Functional enrichment analysis was performed to explore the underlying mechanism of differentially expressed (DE) mRNAs. The protein-protein interaction (PPI) network of neurogenesis-related genes and long noncoding RNAs (lncRNAs)/circular RNAs (circRNAs) based ceRNA networks were constructed.**

Results **First, this study revealed the DE-mRNAs, DE-lncRNAs and DE-circRNAs between Oxymatrine treated group and the MCAO group. Then, the common 1231 DE-mRNAs, 32 DE-lncRNAs and 31 DE-circRNAs with opposite trends were identified. The Kyoto Encyclopedia of Genes and Genomes to identify the functional enrichment of 1231 DE-mRNAs were enriched in neurogenesis-related biological processes. Based on neurogenesis-related DE-mRNAs, the PPI network was constructed, and hub genes were identified based on centrality. Finally, both the lncRNA-based and circRNAs-based ceRNA networks were constructed.**

Conclusion **In summary, this study identified novel coding and noncoding ischemic stroke targets of oxymatrine-treated MCAO. Most importantly, we identified lncRNAs and circRNAs candidates as potential oxymatrine targets and constructed the neurogenesis-related ceRNA networks. *NeuroReport* 33: 641–648 Copyright © 2022 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Introduction

A stroke is a group of diseases including ischemic and hemorrhagic strokes, caused by a sudden rupture of a blood vessel in the brain or a blockage. Due to high morbidity, mortality, recurrence rate and complications, it ranks as one of the top three diseases threatening human health [1]. The 2017 updated statistics of the American Heart Association reported that ischemic stroke ranked as number 2nd top cause of cardiovascular diseases [2]. Indeed, ischemic stroke has led to long-term disability and death around the world and millions of people die from stroke-related causes each year [3,4]. Currently, tissue plasminogen activator is the only US Food and Drug Administration-approved drug for acute ischemic stroke. Therefore, it is of great significance to search for effective and low-toxicity compounds for the treatment of stroke.

Natural products have shown promising performance for alleviating the progression of ischemic stroke. Oxymatrine is a bioactive component extracted from *sophora flavescens*. Oxymatrine has been reported for its application in stroke treatment, for the function of anti-inflammatory, antiapoptosis, antioxidant and neuroprotective effects [5–8]. Liu *et al.* [9] reported that oxymatrine reduced infarct volume by downregulating the nuclear factor-kappa B (NF-κB) expression [9]. However, due to the limitations of traditional biological research techniques, the molecular mechanism of how oxymatrine works on stroke is still poorly understood.

The development of omics technology provides us with a comprehensive grasp of stroke progression and the

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pharmacology of drug intervention. In this study, for the first time, we integrated the transcriptome to comprehensively conduct bioinformatics analysis to explore the mechanism of stroke and revealed the mechanism of oxymatrine by means of functional enrichment analysis. It is hoped to provide a theoretical basis for the improvement of clinical treatment of stroke and the discovery of ideal targets in the future.

Materials and methods

Animals and groups

Eight- to ten-week-old male C57BL/6 mice were purchased from Charles River Laboratories (Beijing, China) and used for the construction of a middle carotid artery occlusion model. All animal experiments were approved by the Ethics Committee of Hebei General Hospital (approval no. 201921). Animals were housed at a controlled temperature (20 ± 2 °C) with a 12 h light-dark cycle, and they had free access to food and water. The nine mice were randomized into the sham group ($n = 3$), middle cerebral artery occlusion (MCAO) group ($n = 3$) and MCAO-treated with Oxymatrine (300 mg/kg) group ($n = 3$).

Transient middle cerebral artery occlusion

An MCAO mouse model was constructed in our study, as described in a previous study [10,11]. Transient focal cerebral ischemia was induced by the right MCAO. Mice were anesthetized with 10% chloral hydrate (350 mg/kg). Body temperature was monitored and maintained at 36.5–37.5 °C. Briefly, after making an incision in the midline skin. The right common carotid artery, right internal carotid artery (ICA) and right external carotid artery (ECA) were exposed through a midline incision of the neck. A monofilament coated with silicone was inserted via a small nick in the right ECA and slowly pushed through the right ICA until it reached the base of the middle cerebral artery to block the blood flow into the middle cerebral artery brain territory. Cerebral ischemia through the intraluminal suture was maintained for 60 min, and it was followed by removal of the monofilament and reperfusion [11–13].

Sample preparation

Oxymatrine was purchased from Sigma (St. Louis, Missouri, USA), which was dissolved in normal saline at 4 °C. Oxymatrine (60 mg/ kg) was injected intraperitoneally 15 min after the operation, and then the cerebral cortex was harvested 24 h later. The cerebral cortices of each group were obtained after each operation and RNA extraction was performed immediately. The rest of the samples were stored at –80 °C in liquid nitrogen [11–13].

Transcriptome RNA-sequencing

RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted total RNAs were sequenced on the Illumina Novaseq 6000 platform using the standard paired-end protocol. Approximately 40 million 75-bp paired reads were acquired for each sample. Raw sequences were processed for quality control, trimming (Trimmomatic version 0.36) and alignment (STAR/2.5.1b) [14]. Downstream analyses were performed using R (version 3.1.1), with edgeR and the limma package with the voom method [15]. A false discovery rate (q value) of <0.05 and a nominal P value of <0.05 were considered statistically significant.

Bioinformatics analysis

P value < 0.05 and fold change (FC) > 2 were selected as the criteria for identifying significantly differentially expressed genes. To obtain an overview of the characteristics of differentially expressed genes (DEGs), the R package was used to generate a Venn diagram and heatmap. Gene enrichment analysis was performed based on gene ontology, and a P value <0.05 was set as the cutoff for significantly enriched functional GO term. Protein-protein interaction (PPI) was assessed by the String database (https://string-db.org/) and visualized by cytoscape.

Results

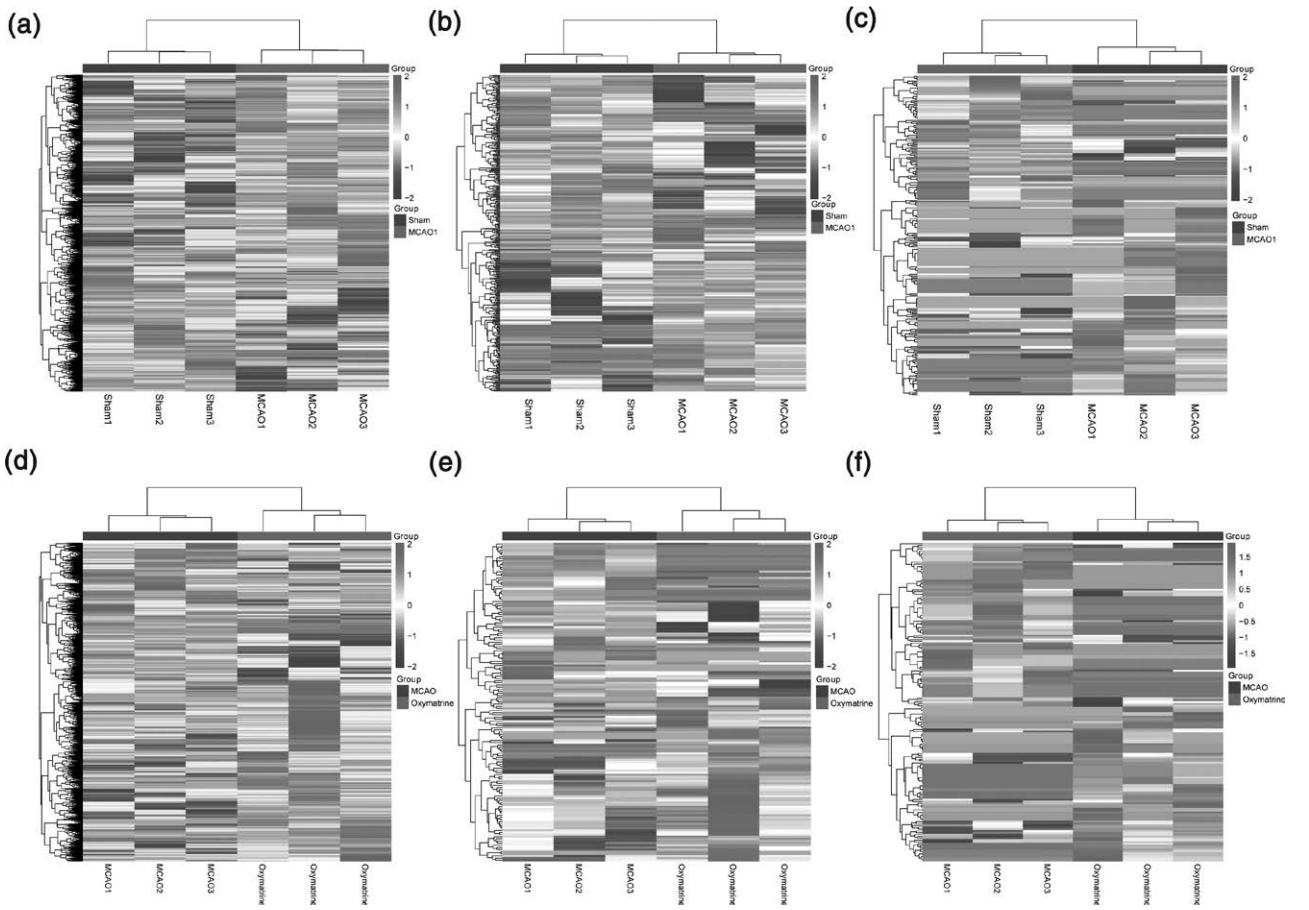
Transcriptome analysis of gene expression changes induced by oxymatrine

First, after conducting the RNA-Sequencing, we compared the transcriptome between MCAO (M) with Sham (S) and Oxymatrine (O), respectively. The DEGs (including DE-mRNAs, DE-lncRNAs, DE-circRNAs, FC >2; $P < 0.05$) were obtained. The heatmaps of DEGs, including DE-mRNAs (Fig. 1a), DE-lncRNAs (Fig. 1b) and DE-circRNAs (Fig. 1c) in MCAO (M) vs. Sham(S) were shown. Heatmaps of DEGs in two groups of Oxymatrine (O) vs. MCAO (M) were shown in Fig. 1d,e. The target genes of oxymatrine were identified. Next, the Venn analysis showed that there were 1959 common DE-mRNAs in which 1231 had the opposite trend and serve as reverse expressed genes (REGs) [upregulated in one (MCAO vs. sham) and downregulated in another one (oxymatrine vs. MCAO), or opposite] (Fig. 2a,b). Similarly, we screened out a total of 32 common DE-lncRNAs (Fig. 2c,d) and 31 common DE-circRNAs (Fig. 2e,f) with the opposite trend. These DEGs were the foundation for the construction of ceRNA networks.

Oxymatrine alleviates stroke pathology through neurogenesis pathway

To investigate the pathogenesis mechanism of ischemic stroke, the functional enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes based on common DE-mRNAs was carried out. As shown in Fig. 3a, the 1231 REGs were significantly enriched in neurogenesis-related biological processes. The expression level of neurogenesis-related REGs in MCAO (M), Sham (S) and Oxymatrine (O) groups was shown in the heatmap (Fig. 3b). Based on neurogenesis-related DE-mRNAs, the PPI network of the neurogenesis-related REGs was

Fig. 1



Transcriptome analysis of gene expression changes induced by oxymatrine. (a–c) The heatmaps of differently expressed mRNA (DE-mRNAs) (a), DE-lncRNAs (b), DE-circRNAs (c) between MCAO (M) and Sham(S) groups were shown. (d,e) The heat maps of differently expressed mRNA (DE-mRNAs) (d), DE-lncRNAs (e), DE-circRNAs (f) between oxymatrine (O) and MCAO (M) groups were shown. MCAO, middle cerebral artery occlusion.

constructed, and hub genes was identified based on centrality, as shown in Fig. 3c.

The construction of neurogenesis-related ceRNA network

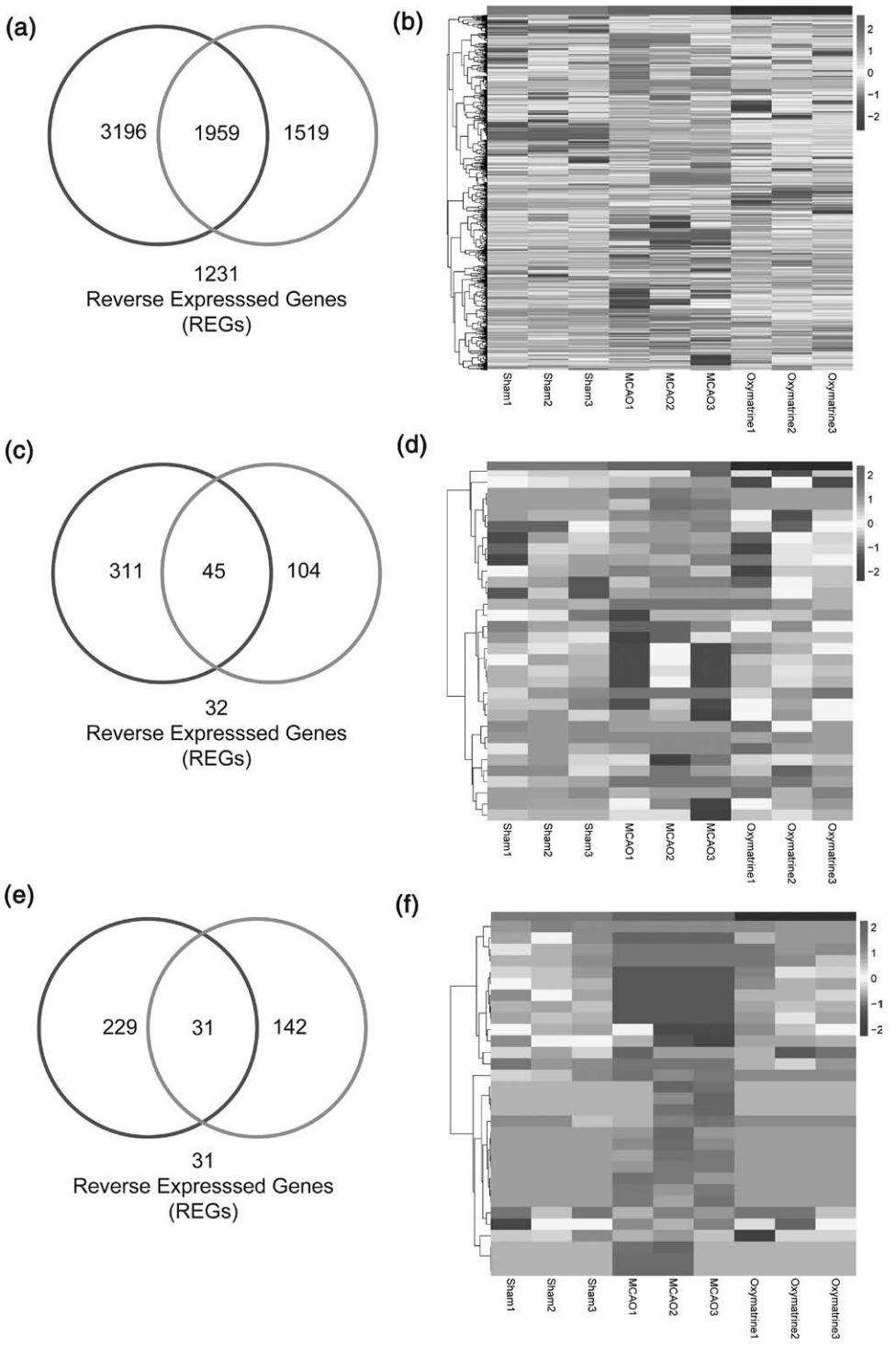
Additionally, the role of noncoding genes, including lncRNAs and circRNAs, in ischemic stroke was also investigated. LncRNA is known to regulate gene expression at multiple levels including transcription and post-transcription by regulating nearby located coding genes and distantly located genes [16]. To explore the target miRNAs of REGs, Targetscan (https://www.targetscan.org/vert_80/) [17] was used for target prediction. Hence, we constructed a lncRNA-mRNA-miRNA neurogenesis-related network (Fig. 4a), which contained a total of four DE-lncRNAs, five predicted-miRNAs and 68 DE-mRNA. By analyzing the correlation coefficient between lncRNA and mRNA expression, four lncRNAs with significant positive correlation were found to regulate 68 genes in neurogenesis-related pathways in MCAO.

CircRNAs was reported to regulate the expression of host genes [18–21]. Hence, we also constructed a neurogenesis-related circRNA-mRNA-miRNA network (Fig. 4b), which contained a total of 5 DE-circRNAs, 68 predicted-miRNAs and 86 DE-mRNAs. However, 86 genes showed significant differences in mRNA, including Apc and Foxp1, the key signaling modulators of the Wnt pathway and NFκB pathway. The mRNA expression trend of 40 genes was consistent with that of circRNAs, and the mRNA expression trend of 20 genes was inconsistent with that of circRNAs. However, whether these lncRNAs and circRNAs have functions and their mechanism needs to be further verified.

Discussion

The pathogenesis of stroke involves complex and diverse molecular network changes. In our study, we identified

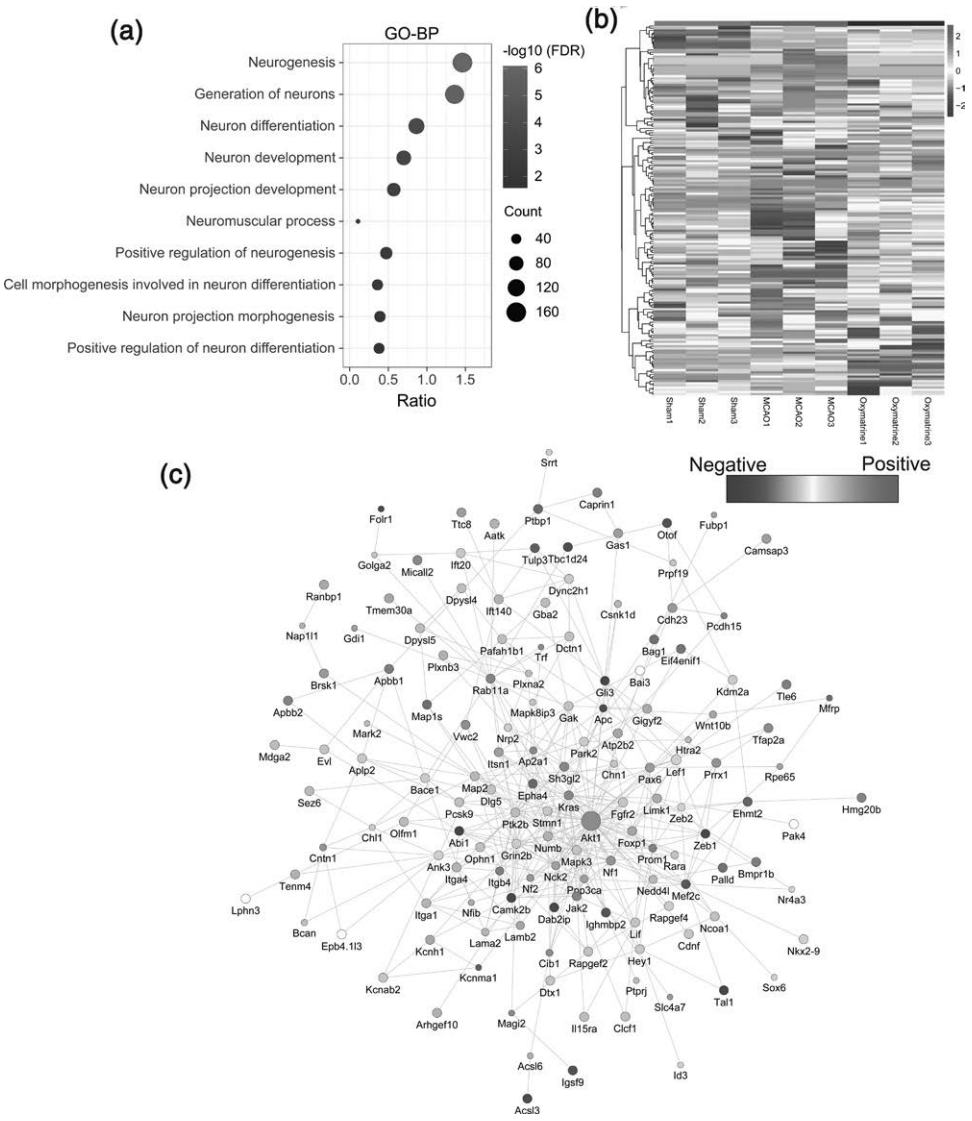
Fig. 2



The identification of reverse expressed genes (REGs). (a-b) The Venn graph (a) showing the 1959 common DE-mRNAs between M vs. S and O vs. M groups, in which the 1231 REGs were presented in heat map (b). (c,d) The Venn graph (c) showing the 45 common DE-mRNAs between M vs. S and O vs. M groups, in which the 32 REGs were presented in heat map (d). (e,f) The Venn graph (e) showing the 31 common DE-mRNAs between M vs. S and O vs. M groups, in which the 31 REGs were presented in heat map (f).

the differentially expressed circRNAs, lncRNAs, mRNAs and screened out the reverse expressed genes. Notably, we found that the common DE-mRNAs were significantly enriched in the neurogenesis signaling pathways.

Fig. 3

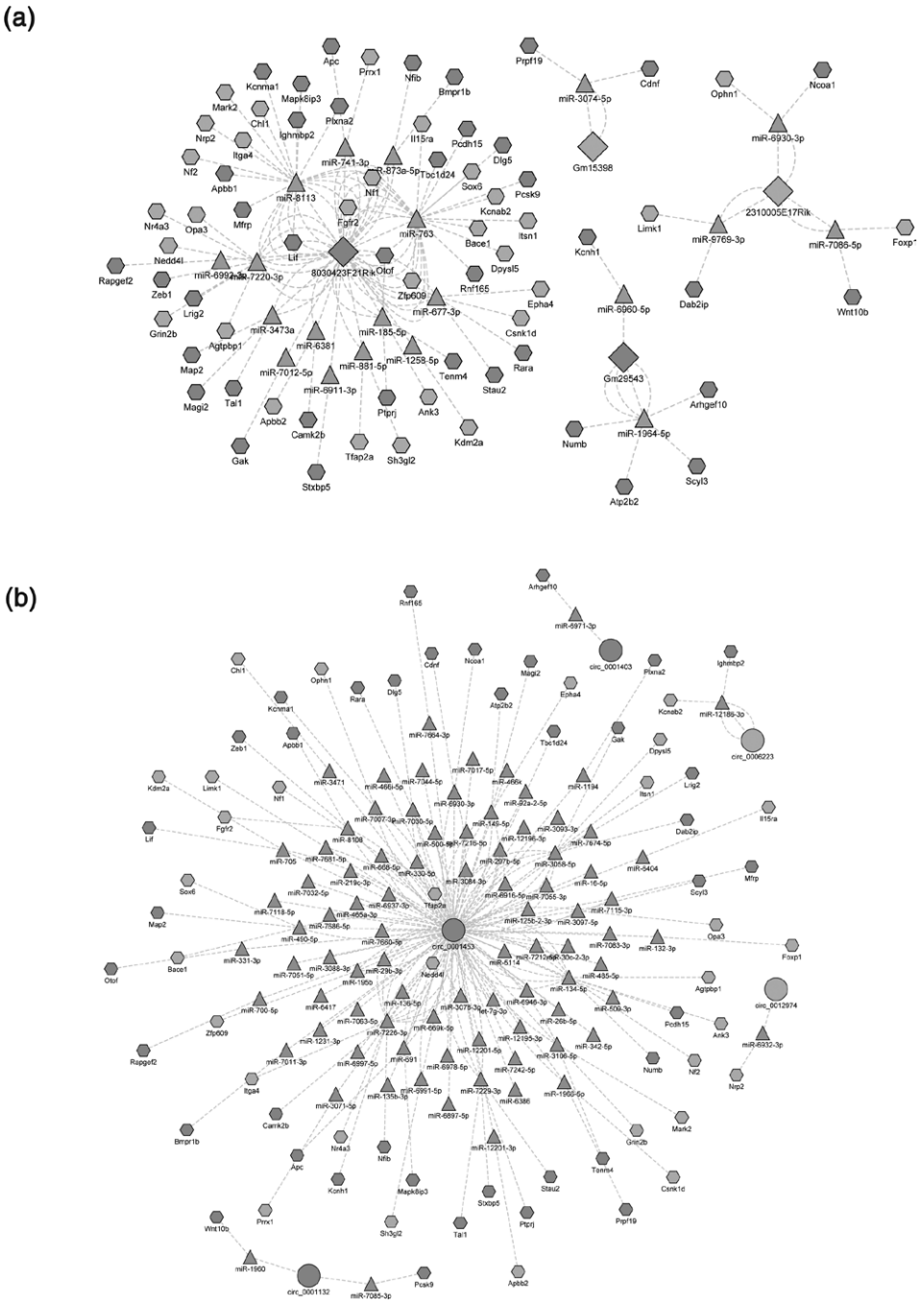


Oxymatrine alleviates stroke through the Neurogenesis pathway. (a) The KEGG enrichment analysis indicated that the 1231 reverse expressed genes were significantly enrichment in the neurogenesis pathway. (b) The heat map showing the expression of neurogenesis-related genes in sham/ MCAO/ Oxymatrine treatment groups. (c) The protein-protein interaction (PPI) network of neurogenesis-related DE-mRNAs. KEGG, Kyoto Encyclopedia of Genes and Genomes; MCAO, middle cerebral artery occlusion.

Understanding the mechanisms of neurogenesis could improve the therapeutic strategies for brain repair [22]. In some studies, approaches to enhance neurogenesis after ischemic stroke have been explored [23]. Nevertheless, these therapies have not led to successful clinical outcomes.

In our study, for the first time, the combination of transcriptome and bioinformatics was used to construct the ceRNA networks of Oxymatrine treated MCAO. Post-translational mechanisms such as alternative RNA splicing, miRNAs-mediated protein translational inhibition [24] and post-translational modifications were reported to play an important role in stroke pathogenesis [25]. The ceRNA network has been construed in some studies for ischemic stroke investigation. For example, Li *et al.* [26] constructed a lncRNA-mediated ceRNA network for investigating the immune pathogenesis of ischemic stroke. Oxymatrine has been reported to attenuate cognitive deficits and exert neuroprotection by regulating autophagy [8] and NF-kB expression [9]. In 2009, Fan et al. [27] reported that oxymatrine downregulated TLR4 expression and protection against focal ischemia. Noncoding RNAs, including lncRNAs and circRNAs, are emerging as important functional modulators and biomarkers in stroke [28–31], but their role in oxymatrine treatment of stroke has not been revealed. This study, for

Fig. 4



The construction of neurogenesis-related ceRNA network. (a) The neurogenesis-related lncRNA-mediated ceRNA network of ischemic stroke treated by oxymatrine. (b) The neurogenesis-related circRNA mediated ceRNA network of ischemic stroke treated by oxymatrine.

the first time, uncovered a list of candidate lncRNAs and circRNAs in oxymatrine intervention in MCAO.

It is worth noting that Apc and Foxp1 showed the same trend of differential expression in circRNA and lncRNAs mediated ceRNA network. Plasma APC level may be a marker or risk factor for ischemic stroke [32]. It was also reported that APC attenuates ischemic brain injury in MCAO-treated mice [33]. Besides, APC coordinates neurogenesis and cortical size during development [34]. Foxp1 levels subsequently decline during the transition to superficial-layer neurogenesis and have broad functions in cortical neurogenesis [35]. Foxp1 was also involved in neurogenesis in autism spectrum disorders

[36]. As Foxp1 has been reported to play a role in neurogenesis and regulating the NF-κB pathway [37], circRNAs and lncRNAs may regulate their host genes expression in several ways [38], we speculated that the NF-κB pathway is involved in the stroke progression and oxymatrine treatment, possibly in an indirect way.

Past reports have pointed toward secondary inflammation in the penumbra region as the cause of injury and cell death [39]. It is well known that the injury caused by a stroke can induce neurogenesis in the penumbra of the infarcted area [23]. Furthermore, microglia are a self-sustained population of immune/myeloid cells present throughout the central nervous system, and activation of microglia, the resident immune cells in the brain, would be expected after a stroke [40]. In our results, some neurogenesis-related DE-mRNA were also associated with the microglia [e.g. protein kinase B (Akt1), Epha4 and Bag1]. Xiao *et al.* [41] revealed that tetrahydrocurcumin upregulated Bag1 expression in Aβ-treated BV-2 cells. Alexaki *et al.* [42] revealed that dehydroepiandrosterone inhibits acute microglia-mediated inflammation through activation of the TrkA-Akt1/2-cyclic AMP response-element binding protein (CREB)-Jmjd3 pathway. Wei *et al.* [43] indicated that neuronal EphA4 regulates OGD/R-induced apoptosis by promoting alternative activation of microglia. This evidence suggests that this neurogenesis-related DE-mRNAs might participate in the activation of the microglia in the penumbra of the infarcted area.

In conclusion, this study revealed novel coding and noncoding targets of oxymatrine-treated mice after stroke. Furthermore, we constructed the neurogenesis-related ceRNA networks. Additionally, we identified lncRNAs and circRNAs candidates as potential oxymatrine targets. In summary, this study identified novel coding and noncoding ischemic stroke targets of oxymatrine.

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The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

X.Z. and H.W. conceived and designed the study. W.Y., W.Z. and Y.S. analyzed the data. Z.W., W.H. and Y.J performed the experiments. Y.G., X.N. and L.L. performed visualization. X.Z. and H.W. wrote the article. H.W. reviewed and edited the manuscript. All authors read and approved the final article.

Conflicts of interest

There are no conflicts of interest.

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