

Downregulated miRNA-324-5p aggravates neuronal injury induced by oxygen-glucose deprivation via modulating RAN

JUNQUAN GU*, SHUHUA GUI*, LINLIN HU, LIANG KONG, MEIQI DI and YINMING WANG

Department of Neurology, The Third People's Hospital of Wuxi, Wuxi, Jiangsu 214041, P.R. China

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Abstract. Differentially expressed miRNAs in the GEO profile of ischemic stroke were analyzed to clarify the specific role of microRNA-324-5p (miRNA-324-5p) in ischemic stroke and the potential mechanism. After screening out miRNA-324-5p, its level in peripheral blood of stroke patients and *in vitro* oxygen-glucose deprivation (OGD)-induced primary rat neurons was determined by quantitative real-time polymerase chain reaction (qRT-PCR). Regulatory effects of miRNA-324-5p on viability, and apoptosis of OGD-induced neurons were evaluated by CCK-8 and Annexin V fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining, respectively. Glucose uptake and caspase-3 activity in OGD-induced neurons transfected with miRNA-324-5p mimics or inhibitor were also examined. The binding of miRNA-324-5p to its target gene RAN was analyzed by dual-luciferase reporter gene assay and western blot analysis. By analyzing the data of GSE46266 profile, miRNA-324-5p expression was shown markedly lower in MCAO rats relative to controls. Identically, we also observed the downregulated miRNA-324-5p in peripheral blood of stroke patients and *in vitro* OGD-induced primary neurons. Overexpression of miRNA-324-5p accelerated viability, induced apoptosis and strengthened glucose uptake ability of OGD-induced neurons. Knockdown of miRNA-324-5p, conversely, obtained the opposite results. Furthermore, we confirmed the binding of miRNA-324-5p to RAN, the target gene that was negatively regulated by miRNA-324-5p. Importantly, RAN overexpression partially reversed the regulatory effect of miRNA-324-5p on viability and glucose uptake of OGD-induced neurons. miRNA-324-5p is downregulated after ischemic stroke, which aggravates the disease condition by inhibiting neuronal proliferation and glucose uptake via upregulating RAN.

Introduction

Ischemic stroke is a cerebrovascular disease that seriously threatens human health. High rates of incidence, mortality, disability and recurrence, as well as many serious complications are the characteristics of ischemic stroke. With the improved life quality and fast-paced lifestyle, the incidence of ischemic stroke has markedly increased and the disease onset is becoming younger. So far, thrombolysis is the most effective treatment for ischemic stroke (1,2). However, thrombolysis has a strict time window limitation. Only 5% of ischemic stroke patients are eligible for thrombolysis, and unfortunately, they suffer from thrombolysis-related bleeding risk (3). Therapeutic efficacy of thrombolysis is not satisfactory. Only 50% ischemic stroke patients are able to achieve recanalization after thrombolysis, and they may also experience revascularization in the future (4). Therefore, it is urgent to search for novel targets for clinical treatment of ischemic stroke.

In recent years, many studies have reported that microRNAs (miRNAs) are able to regulate pathological processes of cerebral ischemia in different stages (5). miRNAs are a class of non-coding small RNAs with 19-23 nt in length that are highly conserved in sequence (6). miRNAs degrade or inhibit translation of mRNAs at post-transcriptional level and are completely or incompletely complementary to them. They participate in various biological progressions, including development, differentiation, innate immune response and adaptive immune response (7). Currently, there are hundreds of miRNAs discovered in human brain, which are involved in the development and pathophysiology of the nervous system (8).

miRNA-324-5p is located on chromosome 17p13.1. Deficiency of miRNA-324-5p in medulloblastoma is proved to be associated with del (17p) (9). In addition, studies have confirmed that miRNA-324-5p regulates metastasis, invasion, stemness and drug-resistance of hepatocellular carcinoma (10,11). The role of miRNA-324-5p in ischemic stroke, however, remains unclear.

Patients and methods

Sample collection. A total of 80 cases of acute ischemic stroke patients admitted to the Third People's Hospital of Wuxi (Wuxi, China) from July 2015 to March 2017 were enrolled. Moreover, 80 cases of healthy controls undergoing physical examination at the same period were enrolled as controls. Written informed

Correspondence to: Dr Yinming Wang, Department of Neurology, The Third People's Hospital of Wuxi, 585 Xinyuanbei Road, Liangxi, Wuxi, Jiangsu 214041, P.R. China
E-mail: rfrwc072@163.com

*Contributed equally

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consent was obtained before the study, which was approved by the Ethic Committee of the Third People's Hospital of Wuxi.

Analysis of differentially expressed miRNAs. Analysis of differentially expressed miRNAs was performed using the GEO2R online analysis tool (<http://www.ncbi.nlm.nih.gov/geo/geo2r/>) of the GEO database. GEO2R identifies the differentially expressed genes by variance analysis and t-test using the R project for statistical computing. Genes with a fold difference ≥ 1.5 and $P < 0.05$ were considered to be differentially expressed.

Primary neuron extraction. After anesthetizing and disinfecting the pregnant rats, gestational sac was harvested and placed in sterile Hank's Balanced Salt Solution (HBSS). The epidermis and skull were cut with ophthalmology, and the cortical brain tissue was harvested using an elbow microscopic sputum. After peeling off meninges and blood vessels, cortical tissues were cut, digested in 0.125% trypsin at 37°C for 15 min and cultured. Primary neurons were seeded in a 6-well plate and NB27 medium was applied 2 h later. At 7 days of culture, primary neurons were collected for subsequent experiments.

Establishment of oxygen-glucose deprivation (OGD) model in primary neurons. Primary neurons were cultured in glucose-free Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) and maintained in a three-gas incubator for 2 h. Neurons were then incubated in glucose-containing DMEM and maintained at 37°C, 5% CO₂ incubator for 12, 24 and 48 h, respectively.

Glucose uptake determination. Primary neurons were seeded in the 24-well plate and glucose uptake percentage was determined using the glucose oxidase-peroxidase method. Neurons were stimulated with synthetic insulin (100 nmol/l) for 6 h. The supernatant of each well was collected, centrifuged at 1,500 x g at 4°C for 5 min and subjected to absorbance determination. Percentage of glucose uptake was finally calculated.

Caspase-3 activation determination. Primary neurons were lysed in 100 μ l of lysate solution and the supernatant transferred into the pre-cold Eppendorf (EP) tube, then incubated with 10 μ l of Ac-DEVD-pNA (2 mmol/l) for 60-120 min. After color change, absorbance at 405 nm was determined.

Cell transfection. Transfection was performed when the confluence was up to 80-90% following the instructions of Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The final dose of miRNA-324-5p mimics and inhibitor was adjusted to 50 nmol/l.

Cell apoptosis determination. Primary neurons were incubated with 10 μ l of Annexin V fluorescein isothiocyanate (FITC) and 5 μ l of propidium iodide (PI) in the dark. Finally, cells were suspended in 1X binding buffer for 20 min in dark, followed by flow cytometry detection (Partec AG).

Western blot analysis. Total protein was extracted from cell lysis, quantified and electrophoresed. After transferring on

polyvinylidene fluoride (PVDF) membranes (EMD Millipore), they were incubated with primary antibodies at 4°C. The following day, membranes were incubated with the corresponding secondary antibody for 2 h. Bands were exposed with enhanced chemiluminescence, and integral optical density was analyzed by gel imaging analysis system (NIH).

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR). We used TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) to extract total RNA. Cell lysis was mixed with chloroform, centrifuged at 12,000 x g at 4°C for 10 min and the precipitate was incubated with isopropanol. After centrifugation, the precipitate was washed with 75% ethanol, air dried and diluted in diethyl pyrocarbonate (DEPC) water (Beyotime). The extracted RNA was subjected to reverse transcription using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) and amplified by SYBR[®]-Green Master Mix (Takara). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference. Primer sequences were as follows: miRNA-324-5p, forward, GCTATCACAGAGCATTTTCTCAT and reverse, TGCACCAAACACGACTTTTAACC; RAN, forward, GGTGGTACTGGAAAAACGACC and reverse, CCAAGGTGGCTACATACTTCT.

Dual-luciferase reporter gene assay. RAN 3'-untranslated region (3'UTR) containing the wild-type or mutant-type sequences of the miRNA-324-5p target binding sites was cloned into the luciferase reporter vector, respectively. It was co-transfected with miRNA-324-5p mimics/NC in neurons for 48 h, followed by luciferase activity determination.

Cell counting kit-8 (CCK-8) assay. Primary neurons were seeded in a 96-well plate with 5x10³ cells per well. At the appointed time-points, 10 μ l of CCK-8 (Dojindo) solution was added to each well and incubated at 37°C for 2 h. The wavelength at 450 nm was detected by a microplate reader.

Statistical analysis. Data were analyzed by Statistical Product and Service Solutions (SPSS) 20.0 statistical software (IBM, Corp.). Quantitative data were represented as mean \pm standard deviation (mean \pm SD) and analyzed by the t-test. $P < 0.05$ was considered as statistically significant.

Results

Downregulated miRNA-324-5p in ischemic stroke. We downloaded the miRNA profile GSE46266 from the GEO database and analyzed the differentially expressed miRNAs in ischemic stroke rats (Fig. 1A). It is indicated that miRNA-324-5p was markedly downregulated in MCAO rats relative to controls (Fig. 1B). Peripheral blood samples of ischemic stroke patients and normal samples were collected in the Third People's Hospital of Wuxi. Identically, miRNA-324-5p level remained lower in blood samples of ischemic stroke patients (Fig. 1C). Subsequently, we constructed the *in vitro* model of ischemic stroke by OGD induction in primary rat neurons. As qRT-PCR data revealed, miRNA-324-5p level was downregulated by OGD induction, and gradually decreased with the prolongation of reperfusion (Fig. 1D).

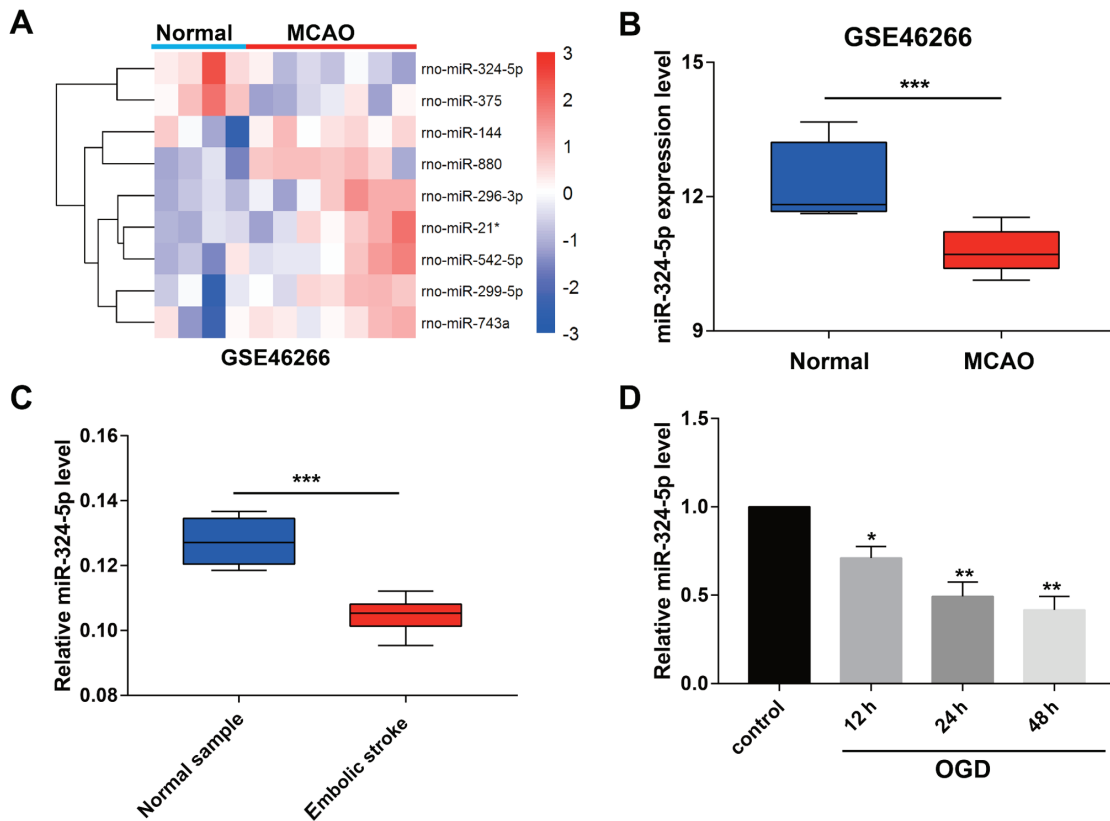


Figure 1. Downregulated miR-324-5p in ischemic stroke. (A) The miRNA profile GSE46266 from the GEO database. (B) GSE46266 dataset showed that miR-324-5p was markedly downregulated in MCAO rats relative to controls (compared with normal, ***P<0.001). (C) miR-324-5p was downregulated in blood samples of ischemic stroke patients (compared with normal, ***P<0.001). (D) miR-324-5p level was downregulated by OGD induction in primary neurons, and gradually decreased with the prolongation of reperfusion (compared with control, *P<0.05, **P<0.01).

miRNA-324-5p participates in OGD-induced cerebral ischemic injury. To elucidate the biological function of miRNA-324-5p, we first transfected miRNA-324-5p mimics or inhibitor in OGD-induced primary neurons to test their transfection efficacy (Fig. 2A). Viability was remarkably elevated in OGD-induced primary neurons overexpressing miRNA-324-5p. Conversely, knockdown of miRNA-324-5p achieved the opposite trend (Fig. 2B). Glucose uptake was accelerated by miRNA-324-5p overexpression (Fig. 2D). However, we observed inhibited neuronal apoptosis after miRNA-324-5p overexpression as the decreased caspase-3 activity and apoptotic rate revealed (Fig. 2C and E).

miRNA-324-5p inhibits RAN expression. miRNA is capable of inhibiting the transcription and translation of target mRNAs by binding to them. Here, we predicted the binding between miRNA-324-5p and RAN by bioinformatics method (Fig. 3A). Luciferase activity was remarkably reduced in cells co-transfected with RAN-WT and miRNA-324-5p mimics, whereas it did not change in those transfected with RAN-WT, indicating the binding of RAN to miRNA-324-5p (Fig. 3B). Both mRNA and protein levels of RAN were negatively regulated by miRNA-324-5p (Fig. 3C and D).

RAN overexpression accelerates OGD-induced cerebral ischemic injury. Contrary to the expression pattern of miRNA-324-5p, RAN was gradually upregulated by OGD induction at both mRNA and protein levels (Fig. 4A-C).

Transfection of pcDNA-RAN sufficiently upregulated RAN level in OGD-induced primary neurons (Fig. 4D and E). It was found that RAN overexpression decreased viability and glucose uptake, but enhanced apoptotic rate of primary neurons (Fig. 4F-H).

We speculate the involvement of RAN in miRNA-324-5p-mediated ischemic stroke. OGD-induced primary neurons were transfected with miRNA-324-5p mimics or miRNA-324-5p mimics + pcDNA-RAN. Increased viability and glucose uptake due to miRNA-324-5p overexpression were partially reversed by RAN overexpression (Fig. 5A and B). The data demonstrated that miRNA-324-5p alleviated ischemic stroke by downregulating RAN.

Discussion

Ischemic brain injury involves complex pathological processes. miRNAs, as novel biological hallmarks, have been identified to be crucial in different stages of cerebral ischemic injury (12-15). *In vitro* and *in vivo* experiments have demonstrated that overexpression of miRNA-134 exacerbates cell death and apoptosis. miR-134 deficiency in OGD-induced N2A cells and ischemic brain tissues upregulate the protein level of HSPA128. Moreover, miR-134 knockdown could markedly reduce brain infarct size, alleviate nerve cell damage and elevate neurological function score in mice (16). Sun *et al* (17) reported that miR-124 overexpression remarkably enlarges cerebral infarction area and

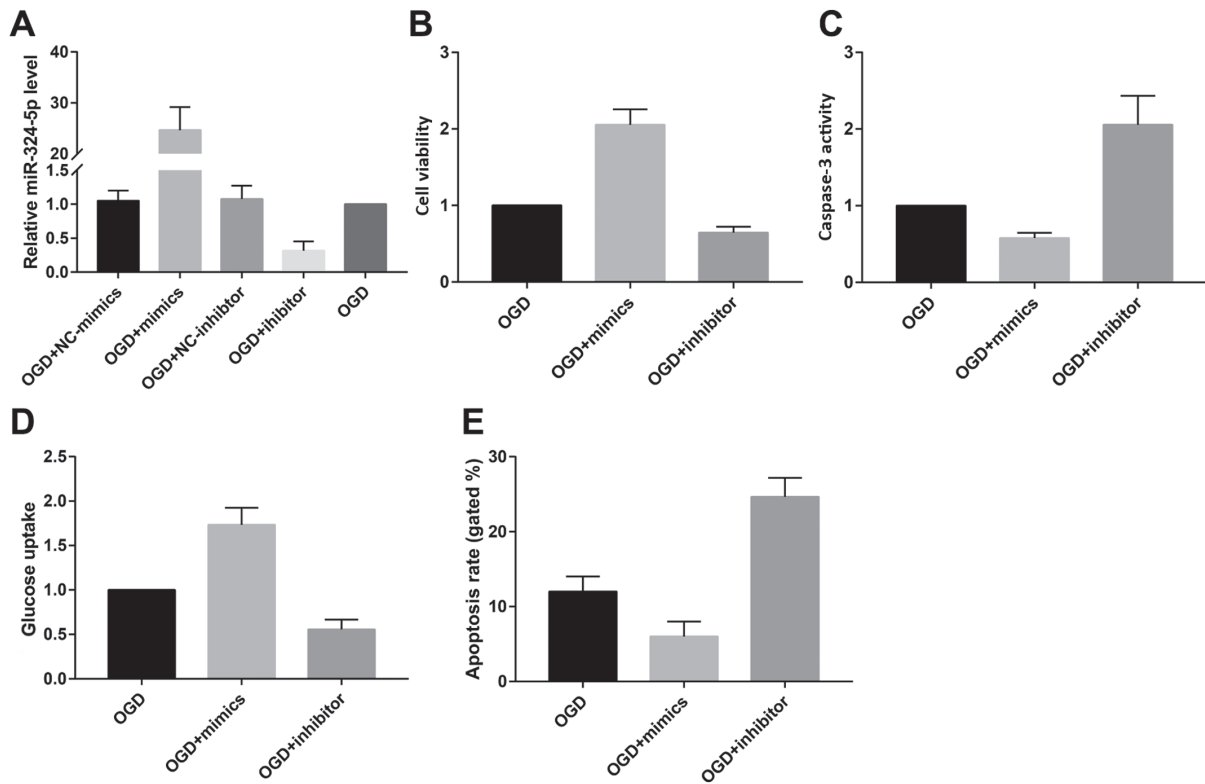


Figure 2. miR-324-5p participates in OGD-induced cerebral ischemic injury. (A) Transfection efficacy of miR-324-5p mimics or inhibitor in OGD-induced primary neurons. (B) Cell viability was remarkably elevated in OGD-induced primary neurons transfected with miR-324-5p mimics, and inhibited by transfection of miR-324-5p inhibitor. (C) Caspase-3 activity was remarkably inhibited in OGD-induced primary neurons transfected with miR-324-5p mimics, and elevated by transfection of miR-324-5p inhibitor. (D) Glucose uptake was remarkably elevated in OGD-induced primary neurons transfected with miR-324-5p mimics, and inhibited by transfection of miR-324-5p inhibitor. (E) Apoptotic rate was remarkably inhibited in OGD-induced primary neurons transfected with miR-324-5p mimics, and elevated by transfection of miR-324-5p inhibitor.

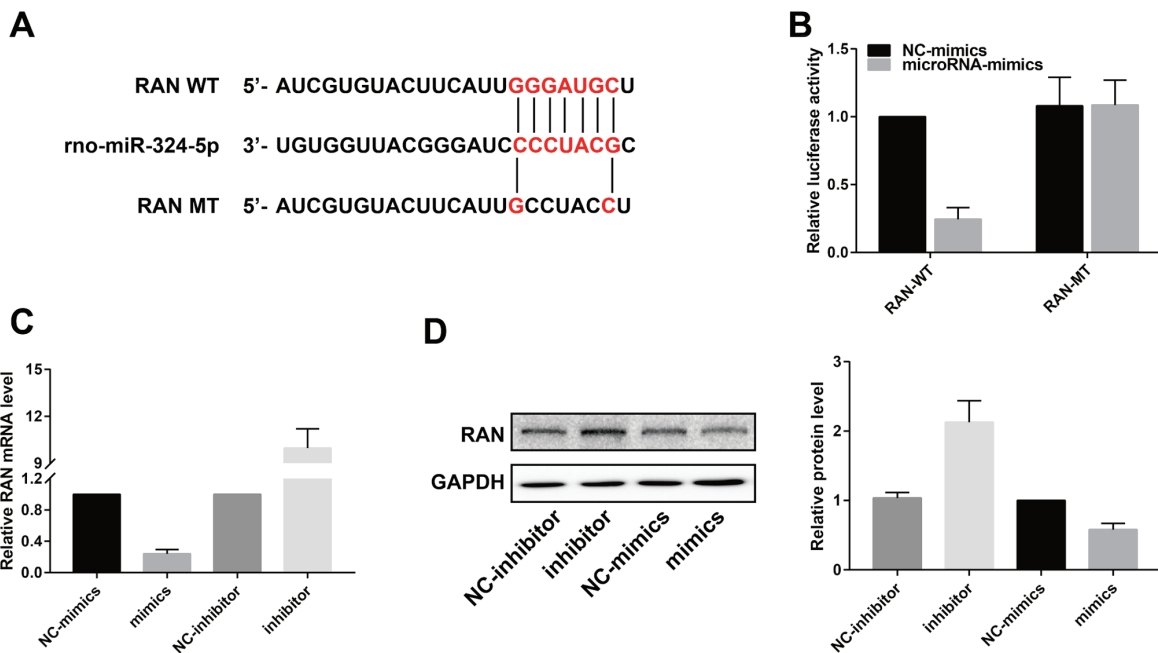


Figure 3. miR-324-5p inhibits RAN expression. (A) Binding sequence between miR-324-5p and RAN predicted by bioinformatics method. (B) Luciferase activity was remarkably reduced in cells co-transfected with RAN-WT and miR-324-5p mimics, whereas it did not change in those transfected with RAN-MT. (C and D) The mRNA (C) and protein levels (D) of RAN were negatively regulated by miR-324-5p.

downregulated miR-124 exerts a protective role in ischemic stroke by inhibiting neuronal apoptosis. Overexpression of

miR-424 alleviates ischemic brain damage by inhibiting G1/S phase transition and microglial activation (18).

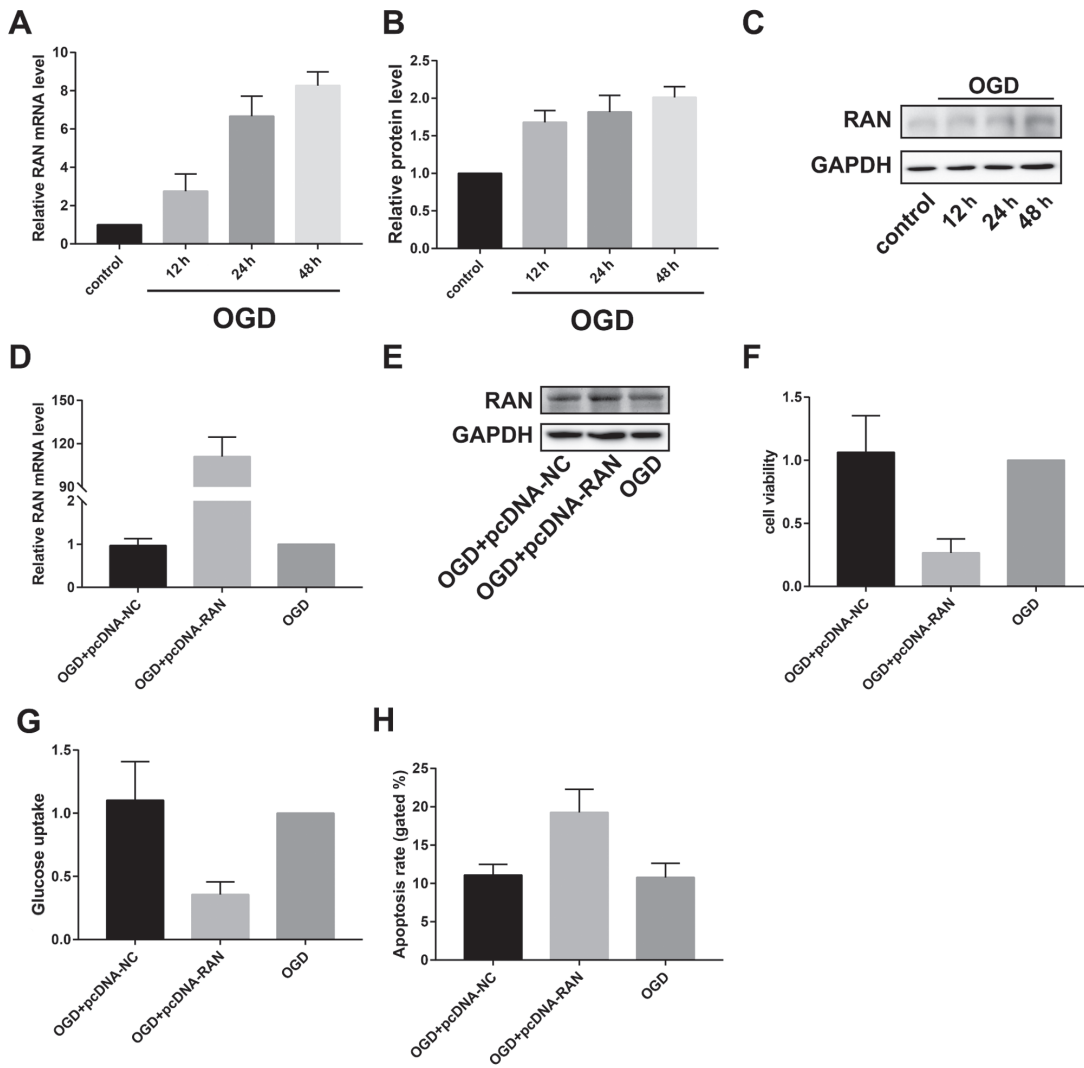


Figure 4. RAN overexpression accelerates OGD-induced cerebral ischemic injury. (A-C) The mRNA (A) and protein levels (B and C) of RAN are gradually upregulated by OGD induction. (D and E) Transfection efficacy of pcDNA-RAN in OGD-induced primary neurons at mRNA (D) and protein levels (E). (F) Cell viability decreased by RAN overexpression. (G) Glucose uptake decreased by RAN overexpression. (H) Apoptotic rate increased by RAN overexpression.

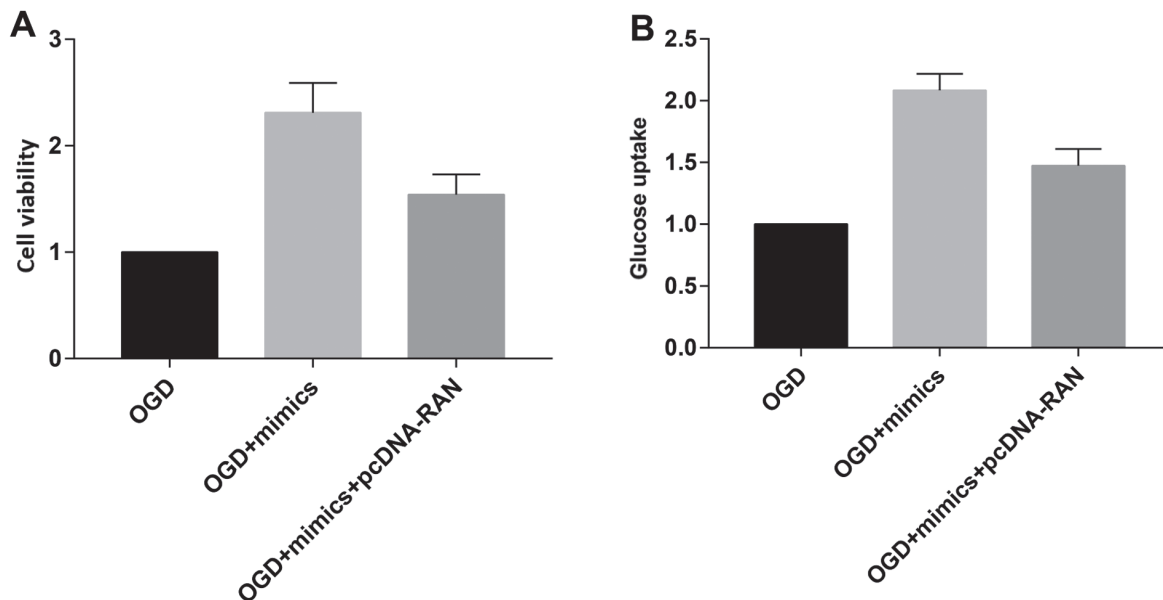


Figure 5. RAN overexpression reverses the role of miR-324-5p. OGD-induced primary neurons were transfected with miR-324-5p mimics or miR-324-5p mimics+pcDNA-RAN. (A) Cell viability in treated neurons. (B) Glucose uptake in treated neurons.

A relevant study screened out the target gene of miRNA-181, GRP78, a classic marker of endoplasmic reticulum stress. miRNA-181 is downregulated in the cerebral ischemic penumbra, which accelerates the progression of cerebral injury by inducing neuronal apoptosis through upregulating GRP78 (19). Differentially expressed miRNAs in circulating cerebral ischemia may serve as diagnostic and prognostic hallmarks (20–22).

In this study, we found that miRNA-324-5p was closely related to cerebral ischemic injury. miRNA-324-5p was identified to be downregulated in the selected GEO profile, peripheral blood of stroke patients and OGD-induced primary neurons. Overexpression of miRNA-324-5p accelerated viability, induced apoptosis and strengthened glucose uptake ability of OGD-induced neurons. Subsequently, RAN was predicted to be the target gene of miRNA-324-5p, which was negatively regulated by it.

RAN is a 25 kDa GTPase, distributed in nucleus by binding to GTP or cytoplasm by binding to GDP. RAN exerts biological functions in eukaryotic cells, including nuclear transport, mitosis and formation of nuclear membrane and nuclear pore complexes (23). Recent studies have shown that RAN is associated with cell fates, such as cell death, proliferation, differentiation, immortalization and tumorigenesis. RAN dysfunction will lead to unlimited cell proliferation. By analyzing serous epithelial ovarian cancer, RAN expression was shown negatively correlated to disease prognosis (24). Moreover, transfection of siRNA RAN reduces viability of human-derived tumor cell lines (H1299, DLD-1), suggesting that RAN is an effective antitumor target (25). An *in vitro* experiment demonstrated that RAN knockdown inhibits HepG2 cells to proliferate, exerting remarkable antitumor function (26). The specific role of RAN in ischemic stroke remains unclear. Our results show that RAN overexpression inhibited viability, glucose uptake and induced apoptosis of OGD-induced primary neurons. Importantly, RAN overexpression partially reversed the regulatory effect of miRNA-324-5p on ischemic stroke.

Due to the limited experimental conditions, we only examined the biological effects of miRNA-324-5p *in vitro*. Its protective effect on cerebral ischemia-reperfusion injury and post-ischemic adaptation require validation in an *in vivo* model.

In conclusion, miRNA-324-5p is downregulated at post-stroke, which aggravates the progression of stroke by inhibiting neuronal proliferation and glucose uptake via upregulating RAN.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SG, JG and YW designed the study and performed the experiments, SG, LH and LK collected the data, JG and MD analyzed the data, SG, JG and YW prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Third People's Hospital of Wuxi (Wuxi, China). Signed informed consents were obtained from the patients or the guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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