Upregulated LINC00319 aggravates neuronal injury induced by oxygen-glucose deprivation via modulating miR-200a-3p

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Abstract. Ischemic stroke is one of the main causes of physical disability and mortality worldwide. Long non-coding RNAs (lncRNAs) are reported to be dysregulated in various biological progressions and serve important roles in pathological processes of cerebral ischemia. However, their biological actions and potential mechanisms in the progression of ischemic stroke remain unknown. The present study aimed to investigate the functions of LINC00319 on ischemic brain injury. It was identified that LINC00319 was significantly upregulated in the Gene Expression Omnibus profile of ischemic stroke. Furthermore, LINC00319 overexpression elevated caspase-3 activity and increased the apoptotic rate of neuronal cells, as well as decreased cell viability and glucose uptake. It was also demonstrated that LINC00319 participated in oxygen-glucose deprivation (OGD)-induced cerebral ischemic injury. LINC00319 could competitively bind with microRNA (miR)-200a-3p and decrease its expression. Moreover, miR-200a-3p could partly offset the negative effects of LINC00319 overexpression on neuronal injury caused by OGD. Collectively, the present results suggested that LINC00319 promoted apoptosis and aggravated neuronal injury induced by OGD by regulating miR-200a-3p, which may be important for ischemic stroke treatment.

Introduction

Stroke is one of the leading causes of mortality worldwide, and ischemic stroke accounts for 80-85% of all strokes (1). Ischemic stroke is associated with multiple physiological and pathological processes, including neuronal apoptosis, inflammation, oxidative stress and excitotoxicity, ultimately leading to neuronal death (2,3). However, only 20% of patients with stroke receive effective treatment due to its increased risk of symptomatic intracerebral hemorrhage (4). Increasing evidence has revealed that ischemia typically involves a range of neural activities, including hypoxia, oxidative stress and inflammation (5), and these processes lead to necrosis, autophagy and apoptosis in the ischemic brain (6). Ischemic stroke severely compromises the quality of life of the patient, but there is currently no effective medication to accelerate rehabilitation (7). Therefore, the development of effective therapies is urgently required.

Long non-coding RNA (lncRNAs) are considered as pseudogenes that compete for microRNA (miRNA/miR) binding, as well as serving a key role in gene regulation and cell development (8,9). LncRNAs and miRNAs also present competing endogenous RNA (ceRNA) activity (10). It was reported that lncRNAs are involved in numerous complicated biological processes, including ischemic stroke. For example, lncRNA-1810034E14Rik exerts anti-inflammatory effects in ischemic stroke (11). Moreover, metastasis-associate lung adenocarcinoma transcript 1 (MALAT1) promotes cerebral ischemia-reperfusion injury by competitively binding miR-145 by affecting aquaporin 4 expression (12).

It has been noted that lncRNA 319 (LINC00319) has a role in a variety of tumors. For instance, LINC00319 promotes the proliferation and invasion of lung cancer cells (13,14). Furthermore, LINC00319 exerts oncogenic roles by repressing miR-3127 in bladder cancer progression (15,16), but its detailed role in ischemic brain injury is yet to be elucidated.

The aim of the present study was to investigate the role of LINC00319 in ischemic stroke. The current data suggested that LINC00319 expression was significantly upregulated in ischemic stroke. Furthermore, LINC00319 promoted the progression of ischemic stroke by modulating miR-200a-3p. Thus, the present study provides a novel insight into the underlying molecular mechanism of ischemic stroke.

Materials and methods

Human samples. Patients with acute ischemic stroke (n=50) admitted to The First Affiliated Hospital of Jiamusi University (Jiamusi, China) between January 2018 and January 2020

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were selected for the study. Blood samples were collected from patients with acute ischemic stroke (males, n=27; females, n=23; age range, 31-72 years; median age, 51.34 years). The etiology of stroke was classified according to the TOAST classification criteria (17). Patients with neurological diseases, cardiac embolism, transient ischemic attack, hemorrhagic infarction, occult cerebrovascular malformation or traumatic cerebrovascular disease were excluded. The present study was approved by the Ethics Committee of Jiamusi University. All procedures were in agreement with the Declaration of Helsinki (18). Written informed consent was obtained from all patients. The study was approved by the Institutional Review Board of The First Affiliated Hospital of Jiamusi University (approval no. JUIRBR-2019-301).

Analysis of differentially expressed lncRNAs. The microarray data for the current study was obtained from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) using the accession nos. GSE46266, GSE58294 and GSE22255 (19). Normalized analysis of differentially expressed genes was performed using RStudio 3.5.1 software and related R software packages (Bioconductor, RStudio, Inc.; https://www.rstudio.com/). LncRNAs with a fold-change ≥2 and P<0.01 were identified as differentially expressed. Gene Ontology (GO) analysis was used to evaluate LINC00319 positive-associated genes in GEO datasets using the Database for Annotation, Visualization and Integrated Discovery v6.8 (https://david.ncifcrf.gov/).

Primary neuron culture. Neonatal clean grade Sprague-Dawley rats (n=50; age, <24 h) were obtained from the Experimental Animal Center of Jiamusi University. Primary culture of cortical neurons in rats was performed using a previously described method (20). The rats were placed in an anesthesia induction box and anesthetized with isoflurane (induced concentration, 3-4%) for 2-3 min and anesthesia was maintained using 1-1.5% isoflurane. The rats were decapitated, and the heads were soaked with 75% alcohol for 1 min under aseptic conditions, and the whole brain was dissected. Ophthalmic scissors were used to repeatedly cut the cortex into pieces. Subsequently, the tissues were incubated with 2 ml 0.25% trypsin (cat. no. C0201; Beyotime Institute of Biotechnology) at 37˚C for 30 min. Next, the tissue mass was prepared into a suspension via digestion. Finally, $1x10^7$ cells were inoculated in a six-well culture plate. After 24 h, the complete culture medium [DMEM/F12 (cat. no. 670087; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 2% B27 (cat. no. 27010; Engreen Biosystem Co., Ltd.)] was changed and cells were cultured for 3 days. The Directive 2010/63/EU on the protection of animals used for scientific purposes stipulates that a competent person shall perform killing using a method that is appropriate for respective species. All applicable international, national and/or institutional guidelines or the care and use of animals were followed. The animal study was approved by Institutional Review Board of The First Affiliated Hospital of Jiamusi University (approval no. JUIRBR-2019-017).

To inhibit the proliferation of glial cells and hybrid cells, primary neurons were cultured in cytosine arabidopsis culture medium (final concentration, 2.5 μg/ml, half solution; cat. no. C0525; Tokyo Chemical Industry Co., Ltd.) for 3 days. The complete culture medium was changed once every 3 days, and half volume of the medium was changed each time.

Establishment of an oxygen-glucose deprivation (OGD) model. An OGD model can simulate *in vivo* cerebral ischemia and is also used as an *in vitro* ischemic model (21). Neurons were cultured in DMEM with low glucose (cat. no. 11885076; Gibco; Thermo Fisher Scientific, Inc.) in a wet incubator at 37˚C, 95% N_2 and 5% O_2 for 6 h. During reperfusion, the neurons were cultured in DMEM with high glucose (cat. no. 10313021; Gibco; Thermo Fisher Scientific, Inc.) replenished with 2% B-27 Plus Supplement (cat. no. A3582801; Gibco; Thermo Fisher Scientific, Inc.) and incubated in a constant oxygen incubator for 12, 24 or 48 h. Cells kept in normal medium and normal conditions were used as controls.

Glucose uptake assay. To measure changes in glucose metabolism, neurons were inoculated in a 96-well plate. Neurons were then treated with or without 100 nM/l insulin (cat. no. P3376; Beyotime Institute of Biotechnology) for 20 min. Subsequently, 0.01 mM 2-deoxyglucose (2DG; cat. no. ST1024; Beyotime Institute of Biotechnology) was added to cells and incubated for 20 min at 37˚C. Cells were washed with PBS three times, harvested and 2DG uptake was assessed using the Glucose Uptake Assay kit (Colorimetric) (cat. no. ab136955; Abcam) following the manufacturer's instructions.

Caspase-3 activity detection. Neurons were seeded in six-well plates. The cell supernatant was obtained and centrifuged at 500 x g at room temperature for 5 min. Caspase-3 activity was detected with a Caspase-3 Assay kit (cat. no. ab39383; Abcam), according to the manufacturer's instructions. The activity of caspase-3 was measured at a wavelength of 405 nm with a Tecan microplate reader (Infinite F50; Tecan Group, Ltd.).

Cell transfection. Neurons were transfected with 20 μM of LINC00319 overexpression plasmid (OE-LINC00319), small interfering (si) RNA LINC00319 (si-LINC00319), miR-200a-3p mimic, miR-inhibitor and corresponding negative controls (NC) with scramble sequence (si-NC or miR-NC/miR-inhibitor NC) or empty vector (OE-NC) at room temperature, which were synthesized by Shanghai GenePharma Co., Ltd. Lipofectamine® 2000 (cat. no. 11668030; Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect neurons according to manufacturer's protocol. Cells were incubated at 37˚C for 6 h. The culture medium was then changed with fresh DMEM containing 10% FBS. The plasmid sequences are listed in Table I. After 48 h of transfection, the cells were harvested for subsequent experiments.

RNA extraction and reverse transcription-quantitative (RT-q)PCR. After transfection, neuron cells were collected. Total RNA was extracted and lysed using TRIzol® reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.), and then reverse transcribed into cDNA utilizing a PrimeScript RT-PCR kit (cat. no. RR014A; Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. qPCR was performed using a SYBR-Green PCR Master Mix

Table I. Plasmid sequences.

Plasmid	Sequence (5'-3')
siRNA-LINC00319	F: GCTGTAATGTGCTGTGACT R: AGTCACAGCACATTACAGC
OE-LINC00319	F: GACUAAACAAGGUCUUAAUTT R: AUUAAGACCUUGUUUAGUCTT
si-NC	F: UUCUCCGAACGUGUCACGU R: ACGUGACACGUUCGGAGAA
miR-200a-3p mimic	F: UAACACUGUCUGGUAACGAU R: GCGGGUCACCUUUGAACAUC
miR-200a-3p inhibitor	F: AUUGUGACAGACCAUUGCU R: UGGGGUCGUCGGUCUAGGG
miR-200a-3p mimic NC	F: UUCUCCGAACGUGUCACGU R: ACGUGACACGUUCGGAGAA
miR-200a-3p inhibitor NC	F: GAACAGGUAGUCUGAACACUG R: CAGUACUUUUGUGUAGUACAA

F, forward; R, reverse; siRNA, si, small interfering; NC, negative control; miR, microRNA.

(cat. no. 4309155; Invitrogen; Thermo Fisher Scientific, Inc.) on a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec, and 60˚C for 60 sec. GAPDH and U6 were used as controls. The primer sequences used were as follows: LINC00319 forward, 5'-GAACCTCAGTTCCTGGCCTC-3' and reverse, 5'-GAGGCCAGGAACTGAGGTTC-3'; GAPDH forward, 5'-AGCCACATCGCTCAGACAC-3' and reverse, 5'-GCCCAATACGACCAAATCC-3'; miR-200a-3p forward, 5'-TAACACTGTCTGGTAACGATGT-3' and reverse, 5'-CAG GTCCAGTTTTTTTTTTTTTT-3'; and U6 forward, 5'-CTC GCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCA CGAATTTGCGT-3'. Relative gene expression levels were calculated by comparing to the expression level of the internal standard using the $2^{-\Delta\Delta Cq}$ method (22).

Luciferase reporter assay. The interaction between LINC00319 and miR-200a-3p was predicted using TargetScan (http://www.targetscan.org/vert_72/) and starBase v2.0 (http://starbase.sysu.edu.cn/). Among all the statistically relevant miRNAs, miR-335-5p, miR-141-3p and miR-200a-3p were obtained from two databases and selected for further experiments.

A luciferase reporter assay was used to evaluate potential binding between LINC00319 and miR-200a-3p. The psiCHECK2 vector (GeneChem, Inc.) was conducted to construct a LINC00319 3'-untranslated region (UTR)-containing reporter. The 3'-UTRs of LINC00319, as well as its wild-type (WT) and mutant (MUT) binding sites with miR-200a-3p, were amplified and cloned into luciferase reporter vectors. miR-200a-3p mimics and plasmids (20 μM) were co-transfected for 48 h using Lipofectamine® 2000. The luciferase activity was analyze using a Dual-Luciferase Reporter Assay System (cat. no. E1910; Promega Corporation) according to the manufacturer's instructions. *Renilla* luciferase intensity was utilized as an internal control.

RNA immunoprecipitation (RIP) assay. RIP was performed with EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation kit (cat. no. 17-701; EMD Millipore) according to the manufacturer's protocols. RNA fractions extracted from RIP were analyzed via qPCR.

Cell viability and cell apoptosis. Cell proliferation was measured using a Cell Counting Kit-8 (CCK-8; cat. no. C0037; Beyotime Institute of Biotechnology) assay. Neuron cells were collected 24 h post-transfection and were seeded into 96-well plates at the density of $2x10^3$ cells/well. Subsequently, CCK-8 solution was added and incubated for 2 h at 37˚C at days 1, 2 and 3 post seeding. The absorbance was measured at a wavelength of 450 nm with a Tecan microplate reader (Infinite F50; Tecan Group, Ltd.).

Apoptosis was analyzed using a Hoechst 33258 detection assay kit (cat. no. C1017; Beyotime Institute of Biotechnology) in accordance with the manufacturer's instructions. Neurons ($1x10^5$) were collected and seeded in cell slides for 24 h, which were precoated with 0.1% poly-L-lysine at 4˚C for 12 h. Subsequently, the glass slides were fixed with 4% paraformaldehyde at room temperature for 15 min. The nuclei were stained with Hoechst 33258 (10 μg/ml) for 20 min and observed under a fluorescence microscope (Olympus Corporation) at x200 magnification.

Western blotting. Total protein was extracted from neuronal cells with RIPA lysis buffer (cat. no. C05-01001; BIOSS). Protein concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology). Proteins (30 μg/lane) were separated via 10% SDS-PAGE gel and transferred onto PVDF membranes (cat. no. 3010040001; EMD Millipore).

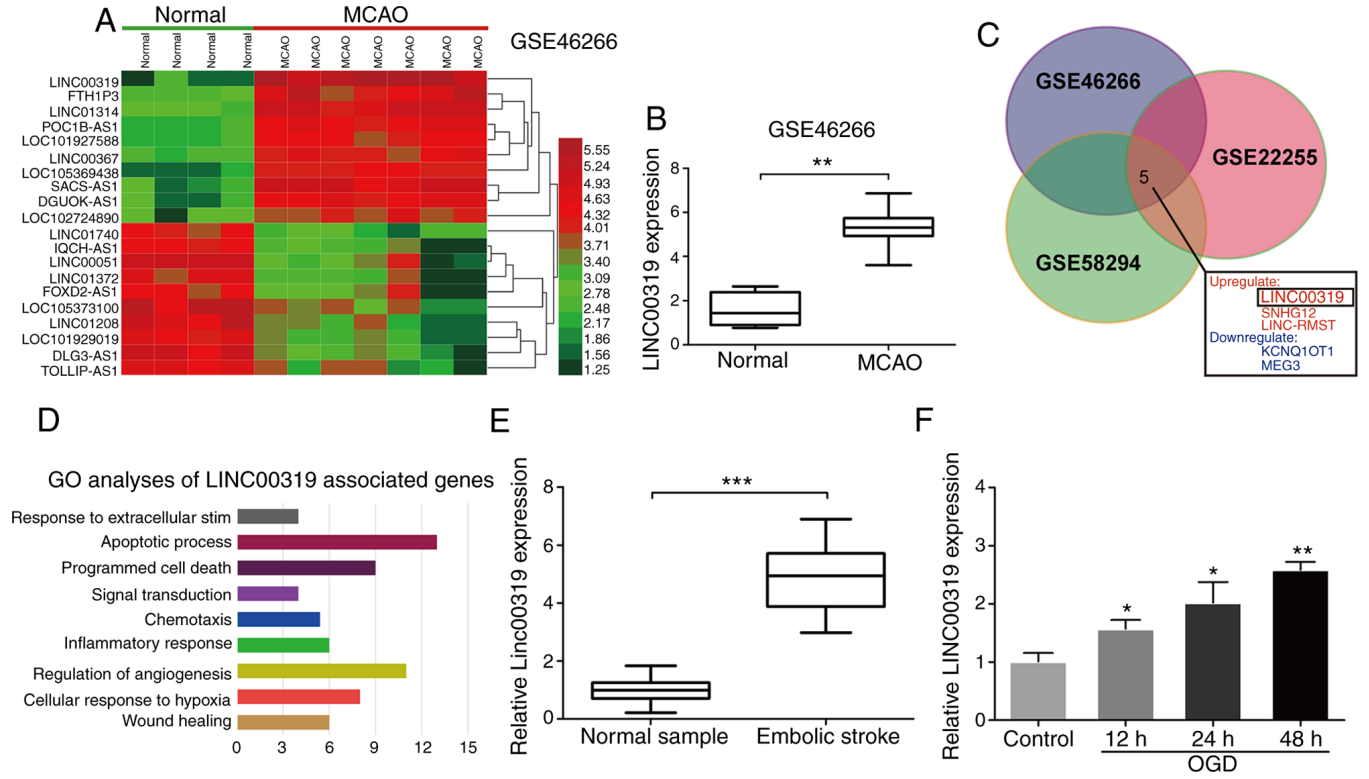

Figure 1. Upregulated LINC00319 in ischemic stroke. (A) Heatmaps were constructed using the differential lncRNA between normal and MCAO rat model detected, as by significance analysis in GSE46266 database. (B) GSE46266 dataset demonstrated that LINC00319 was markedly upregulated in MCAO rats compared with normal group. (C) A total of five significantly differential-expressed lncRNAs were identified among GSE46266, GSE22255 and GSE58294 datasets. (D) GO analysis was performed using the LINC00319 positive-associated genes in GEO datasets. (E) Reverse transcription-quantitative PCR analysis indicated that LINC00319 was upregulated in blood samples of patients with ischemic stroke (n=50), as compared with healthy group. (F) LINC00319 expression was upregulated by OGD induction in primary neurons, and was gradually increased with the prolongation of reperfusion (compared with control). *P<0.05, **P<0.01, ***P<0.001. Data are presented as the mean ± SD of three independent experiments. GO, Gene Ontology; GEO, Gene Expression Omnibus; MCAO, middle cerebral artery occlusion; lncRNA, long non-coding RNA; OGD, oxygen-glucose deprivation.

After blocking in 5% BSA (cat. no. ST025; Beyotime Institute of Biotechnology) for 1 h at room temperature, membranes were incubated with the following primary antibodies at 4˚C overnight: Rabbit anti-caspase-3 (rabbit polyclonal antibody; cat. no. 66470-2-lg; 1:500; Wuhan Sanying Biotechnology) and mouse anti-GAPDH (mouse monoclonal antibody; 1:1,000; cat. no. SC-47724; Santa Cruz Biotechnology, Inc.). Following primary antibody incubation, membranes were incubated with horseradish peroxidase-conjugated anti-mouse (cat. no. ab6728) or anti-rabbit (cat. no. ab6721; both at 1:2,000; both from Abcam) secondary antibodies for 1 h at room temperature. Protein bands were visualized via ECL detection (cat. no. P0018S; Beyotime Institute of Biotechnology) with a ChemiDoc™ MP Imaging detection System (Bio-Rad Laboratories, Inc.) and analyzed by Image Lab software (version 3.0; Bio-Rad Laboratories, Inc.).

Statistical analysis. Statistical analyses were conducted using SPSS version 21.0 (IBM Corp.) and graphs were plotted using GraphPad Prism 6.0 (GraphPad Software, Inc.). Data are presented as the mean ± SD. Comparisons between groups were determined using a two-tailed Student's t-test or one-way ANOVA with Tukey's post hoc test. Aberrantly expressed lncRNAs were examined based on the Benjamini-Hochberg method (23). The association between the expression of LINC00319 and the expression of miR-200a-3p was analyzed using Pearson's correlation. Experiments were independently performed three times. P<0.05 was considered to indicate a statistically significant difference.

Results

LINC00319 is significantly upregulated in ischemic stroke. To identify vital lncRNAs involved in ischemic stroke, differentially expressed lncRNAs were analyzed via bioinformatics analysis in a middle cerebral artery occlusion (MCAO) rat model in the GSE46266 dataset (Fig. 1A). The top 20 aberrantly expressed lncRNAs were selected with cut-off values of $\log_2$ fold-change level (FC) >2 and false discovery rate (FDR) <0.01. It was observed that LINC00319 was significantly overexpressed in MCAO rat models compared with normal models (Fig. 1B). For further analysis, a Venn diagram was conducted using FunRich to visualize the overlapping lncRNAs among GSE46266, GSE58294 and GSE22255 datasets. In total, five intersecting lncRNAs were identified and investigated according to their logFC level (Fig. 1C). LncRNA-LINC00319 was present on the list of significantly upregulated lncRNAs (Fig. 1C). Gene Ontology (GO) analysis demonstrated that LINC00319 was closely associated with 'Apoptotic process' (Fig. 1D).

Peripheral blood samples were collected from patients with ischemic stroke and physical examination volunteers from the

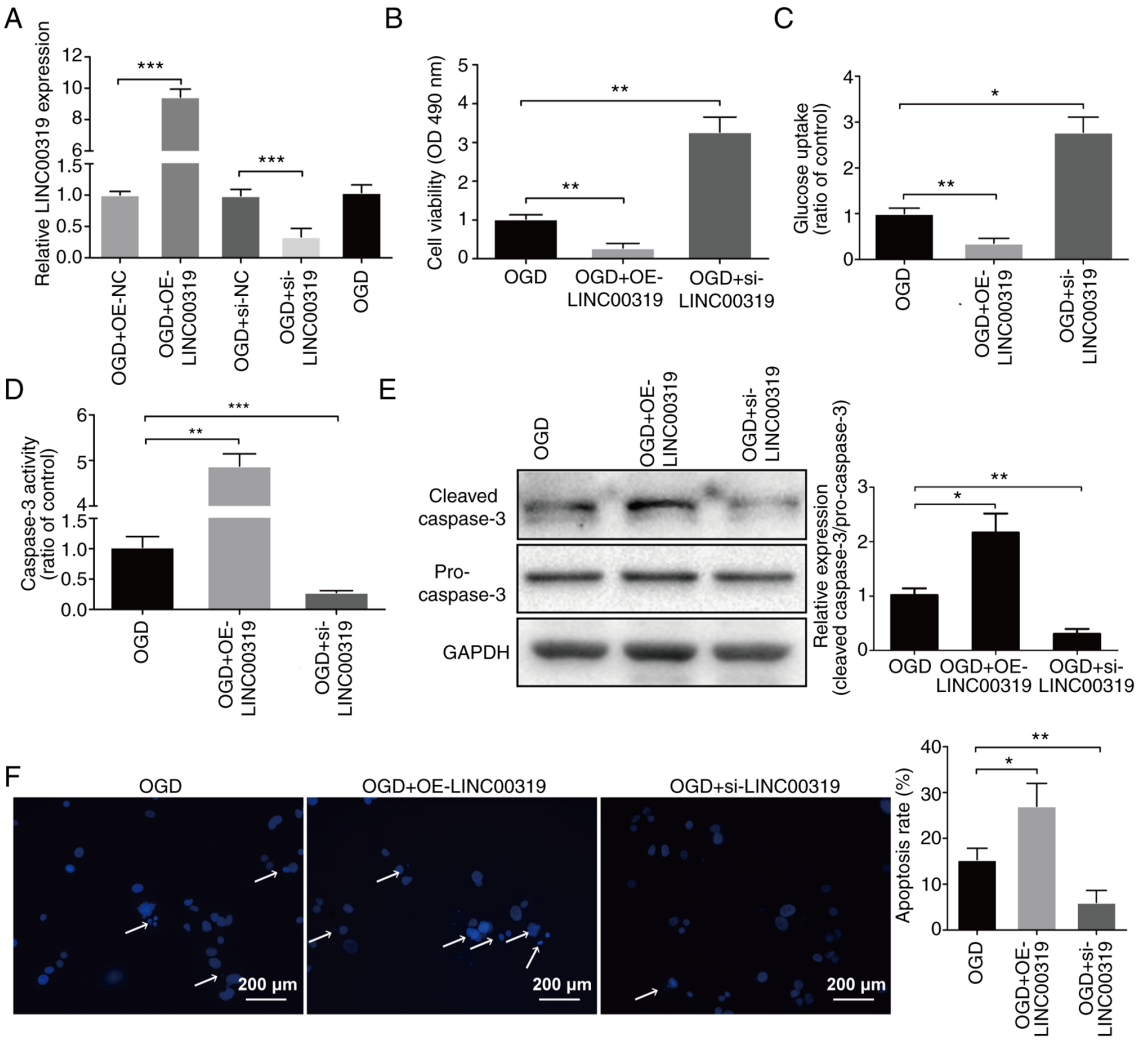

Figure 2. LINC00319 participates in OGD-induced cerebral ischemic injury. (A) Transfection efficacy of pcDNA3.1 (OE-LINC00319) or siRNA (si-LINC00319) in OGD-induced primary neurons as assessed using a reverse transcription-quantitative PCR assay. (B) Cell viability was detected in OGD-induced primary neurons transfected with OE or si-LINC00319 with a Cell Counting Kit-8 assay. (C) Caspase-3 activity was detected in OGD-induced primary neurons transfected with OE or si-LINC00319 with a Caspase-3 Fluorometric Assay. (D) Glucose uptake was measured in OGD-induced primary neurons transfected with OE or si-LINC00319 via the glucose oxidase-peroxidase method. (E) The expression of Caspase-3 was detected in OGD induced primary neurons transfected with OE or si-LINC00319 by western blotting assay (F). Apoptotic rate was measured in OGD-induced primary neurons transfected with OE or si-LINC00319 via Hoechst-33258 assay. *P<0.05, **P<0.01, ***P<0.001. Data are presented as the mean ± SD of three independent experimentsOGD, oxygen-glucose deprivation; siRNA, small interfering RNA; OE, overexpression.

First Affiliated Hospital of Jiamusi University. Consistently. LINC00319 expression was significantly upregulated in patients with ischemic stroke compared with control samples (Fig. 1E). Next, ischemic stroke models were established via OGD in primary neurons *in vitro*. The results demonstrated that LINC00319 was upregulated under ischemia-reperfusion induction and increased gradually with the extension of reperfusion time (Fig. 1F).

LINC00319 participates in OGD-induced ischemic stroke. To investigate the biological function of LINC00319 in ischemic stroke, LINC00319 was overexpressed with pcDNA3.1 (OE-LINC00319) and silenced with siRNA (si-LINC00319) in primary neurons (Fig. 2A). LINC00319 overexpression in ischemic stroke significantly decreased cell viability, while LINC00319 knockdown significantly increased cell viability (Fig. 2B). Furthermore, LINC00319 knockdown significantly increased glucose uptake (Fig. 2C). Caspase-3 activity and protein level measurement results suggested that LINC00319 knockdown significantly inhibited neuronal apoptosis (Fig. 2D and E). The apoptosis assay also demonstrated similar results (Fig. 2F). Thus, the results indicated that LINC00319 participated in OGD-induced ischemic injury.

Correlation between LINC00319 and miR-200a-3p. To detect the subcellular localization of LINC00319, the nuclear and cytoplasmic fractions of neurons were collected. LINC00319 expression was measured in different subcellular fractions, and it was found that LINC00319 expression was higher in cytoplasmic fractions compared with that in the nucleus (Fig. 3A). These results indicated that LINC00319 may exert both transcriptional and post-transcriptional regulatory roles in neuron cells.

Using TargetScan and starBase v2.0 database blast prediction, it was demonstrated that LINC00319 contained a putative targeting site for miR-335-5p, miR-141-3p

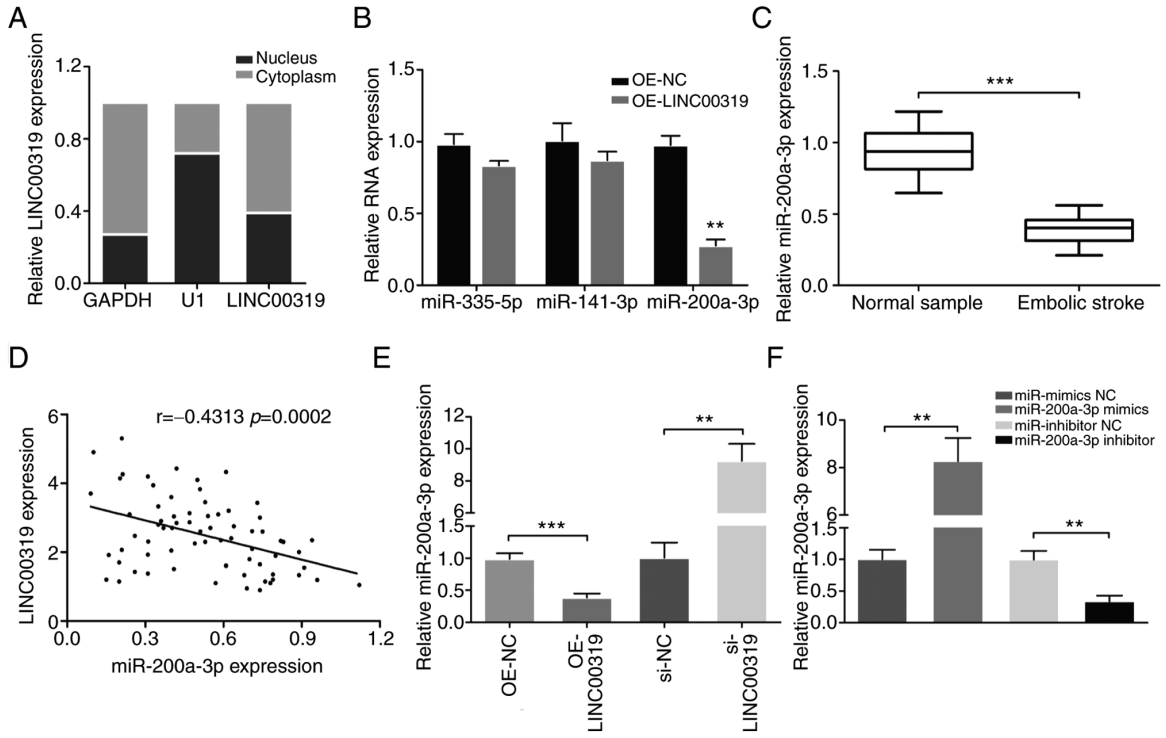

Figure 3. Correlation between LINC00319 and miR-200a-3p. (A) LINC00319 expression in different subcellular fractions cells was measured via RT-qPCR in primary neurons. Black range indicates nuclear fraction, Gray range indicates cytoplasmic fraction. (B) RT-qPCR analysis of miR-335-5p, miR-141-3p and miR-200a-3p in primary neurons cells transfected with OE-LINC00319. (C) RT-qPCR analysis indicated that miR-200a-3p was downregulated in blood samples of patients with ischemic stroke (compared with healthy group). (D) Pearson's correlation analysis identified a negative relationship between LINC00319 expression and miR-200a-3p expression in 50 patients with ischemic stroke. (E) mRNA expression of miR-200a-3p was negatively regulated by LINC00319. (F) Transfection efficacy of miR-200a-3p mimics or inhibitor in OGD-induced primary neurons assessed via RT-qPCR assay. **P<0.01, ***P<0.001. Data are presented as the mean ± SD of three independent experimentsRT-qPCR, reverse transcription-quantitative PCR; OGD, oxygen-glucose deprivation; OE, overexpression; miR, microRNA.

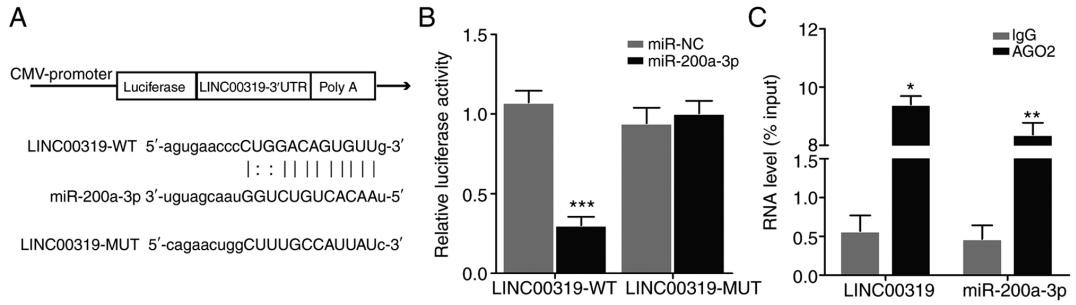

Figure 4. LINC00319 binds directly to miR-200a-3p. (A) Predicted miR-200a-3p binding sites on LINC00319, and schematic of WT and mutant psiCHECK2-LINC00319 constructs. (B) Luciferase activity was significantly decreased in cells co-transfected with LINC00319-WT and miR-200a-3p mimics, whereas it did not change in those transfected with LINC00319-WT. (C) Association between LINC00319 and miR-200a-3p with Ago2. *P<0.05, **P<0.01, ***P<0.001. Data are presented as the mean ± SD of three independent experiments. miR, microRNA; WT, wild-type.

and miR-200a-3p. Furthermore, the expression levels of miR-335-5p, miR-141-3p and miR-200a-3p were measured in primary neurons overexpressing LINC00319. The results demonstrated that LINC00319 overexpression significantly decreased the expression of miR-200a-3p (Fig. 3B). Moreover, miR-200a-3p expression was significantly decreased in the blood samples of patients with ischemic stroke compared with the healthy control group (Fig. 3C). The mRNA expression of miR-200A-3p was negatively correlated with LINC00319 in patients with ischemic stroke (r=-0.4313, P<0.001; Fig. 3D).

Subsequently, the effects of LINC00319 on miR-200a-3p *in vitro* were examined. The results demonstrated that LINC00319 knockdown promoted miR-200a-3p expression (Fig. 3E). In the follow-up experiments, miR-200a-3p mimics and inhibitor were successfully transfected into OGD-induced primary neurons (Fig. 3F). Collectively, a negative interaction was identified between LINC00319 and miR-200a-3p.

LINC00319 directly binds to miR-200a-3p. To elucidate the functional relationship between LINC00319 and miR-200a-3p, bioinformatics analyses were performed. Using starBase v2.0 (http://starbase.sysu.edu.cn/mirMrna.php) and TargetScan (http://www.targetscan.org), the binding pattern between LINC00319 and miR-200a-3p was investigated. In addition,

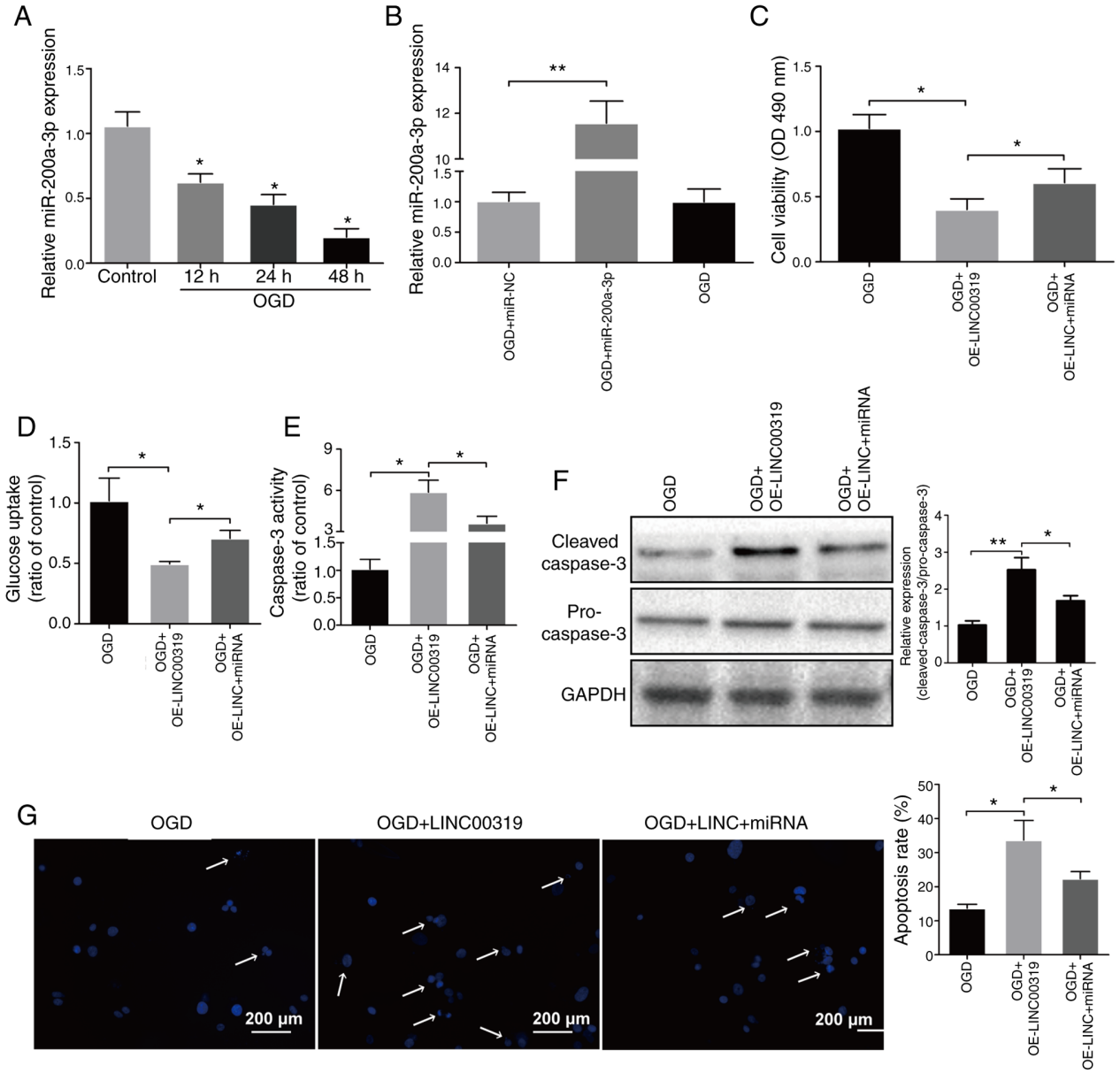

Figure 5. miR-200a-3p overexpression reverses the role of LINC00319. (A) miR-200a-3p expression was downregulated by OGD induction in primary neurons, and gradually increased with the prolongation of reperfusion (compared with control). (B) Transfection efficacy of miR-200a-3p mimics and miR-NC in OGD-induced primary neurons, as measured via reverse transcription-quantitative PCR assay. (C-F) Cell viability, caspase-3 activity, glucose uptake and apoptotic rate were detected in OGD-induced primary neurons transfected with OE-LINC00319 or OE-LINC00319 + miR-200a-3p. (G) Apoptotic rate was measured in OGD induced primary neurons transfected with OE-LINC00319 or OE-LINC00319 + miR-200a-3p via Hoechst-33258 assay. *P<0.05, **P<0.01. Data are presented as the mean ± SD of three independent experiments. OGD, oxygen-glucose deprivation; OE, overexpression; miR, microRNA.

a structural mutation was induced in LINC00319 (Fig. 4A). The luciferase reporter gene was then detected. The results demonstrated that miR-200a-3p inhibited the activity of LINC00319-WT reporter (Fig. 4B), indicating that LINC00319 and miR-200a-3p interacted directly at this binding site. Furthermore, the RIP assay results suggested that LINC00319 could directly bind to miR-200a-3p (Fig. 4C).

miR-200a-3p overexpression facilitates OGD-induced cerebral ischemic injury. To investigate the effects of miR-200a-3p in ischemic stroke, its expression levels were detected in neurons. miR-200a-3p expression significantly declined with the prolongation of reperfusion (Fig. 5A). Subsequently, OGD-induced neurons were transfected with miR-200a-3p (Fig. 5B). It was found that the declining cell viability and glucose uptake due to LINC00319 overexpression were partially reversed by miR-200a-3p (Fig. 5C and D). In addition, caspase-3 activity, protein levels and apoptosis rates were increased after LINC00319 overexpression, but were decreased when co-transfected with miR-200a-3p (Fig. 5E-G). Therefore, the results indicated that miR-200a-3p could reverse the effects of LINC00319 in OGD-induced cerebral ischemic injury.

Discussion

Ischemic stroke is a major public health issue resulting in mortality and disability and is more difficult to treat compared

with other cerebral diseases (24). Ischemic stroke is considered one of the most serious diseases worldwide, threatening the health and life of aging populations (25). It is well known that lncRNAs are present in large numbers in the central nervous system (26). Moreover, lncRNAs serve a crucial role in cerebral function and in nervous system diseases, particularly in central lesions (27-29). The dysregulation of lncRNAs is associated with dysplasia and pathological process of the brain (29). Previous studies have reported that lncRNAs can interact with miRNAs, DNA and proteins to adjust and control gene expression (30). LncRNAs can also promote apoptosis and angiogenesis, as well as cause inflammation and cell death in ischemic stroke (31). It has been revealed that lncRNAs MALAT1, H19, taurine-upregulated gene 1, maternally expressed gene 3, small nucleolar RNA host gene 14, C2dat1, ANRIL and FosDT are upregulated in cerebral ischemia (32).

miRNAs, which are short non-coding RNAs (18-21 nucleotides), can inhibit the function of protein-coding transcripts, leading to changes in cell structure and function (33,34). Previous studies have observed that miRNAs regulate responses involved in curative effects in the conditions of stroke and neuroinflammation. Furthermore, miRNAs are key regulators of inflammation in ischemic stroke. A previous study proposed that miR-669c-3p exerts a protective role in ischemic stroke via the inhibition of MyD88 signaling (35). Moreover, miR-665-3p protects microglia from OGD-induced apoptosis and inflammation (36). miR-98 decreases the infiltration of proinflammatory factors and ameliorates the neurological outcome of ischemic/reperfusion stroke in mice (37), while intracerebral injection of miR-494 agomir decreases neuronal apoptosis and cerebral infarct volume in the acute phase of MCAO (38).

Recent studies have shown that lncRNA host gene 15 (SNHG15) was abnormally expressed in ischemic stroke and inhibited miR-18a expression and activated the ERK/MEK pathway to promote ischemic stroke progression (39). The researchers found that lncRNA HULC was associated with high risk, severe illness, and poor prognosis in patients with acute ischemic stroke (40). LncRNA MACC1-AS1 alleviated microvascular endothelial cell injury, promoted angiogenesis by regulating mir-6867-5p/TWIST1, and played a protective role in ischemic stroke (41). In addition, the researchers found that lncRNA RMRP had a protective effect on ischemic stroke by activating the PI3K/Akt signaling pathway through valproate (42). It was found that lncRNA Nespas played an anti-inflammatory and anti-apoptotic role in ischemic stroke by inhibiting TAK1 (transforming growth factor-β-activated kinase 1) (43). Another study showed that the knockdown of lncRNA KCNQ1OT1 increased cell activity and inhibited the autophagy induced by ischemic stroke through the miR-200a/FOXO3/ATG7 pathway (25).

In the present study, LINC00319 was screened via bioinformatics analysis. The expression of LINC00319 was significantly increased in ischemic stroke. To the best of our knowledge, there has been no previous research regarding the role of LINC00319 in ischemic stroke. The present bioinformatics analysis suggested that LINC00319 may be involved in apoptosis and the regulation of angiogenesis. Furthermore, it was demonstrated that LINC00319 could aggravate OGD-induced cerebral ischemic injury by competitively binding miR-200a-3p.

lncRNAs and miRNAs serve crucial roles in oxidative stress-induced brain injury (44). For instance, it has been revealed that lncRNA-KCNQ1OT1 is significantly elevated and promotes autophagy via the miR-200a/FOXO3/autophagy related 7 axis in ischemic stroke (25). In addition, lncRNA-Meg3 acts as a ceRNA by targeting the miR-21/programmed cell death 4 signaling pathway to accommodate ischemic neuron death (45). Another study reported that downregulated expression of MALAT1 weakened neuronal death by regulating miR-30a expression to suppress autophagy in cerebral ischemic stroke (46). Collectively, these aforementioned studies revealed that lncRNAs could serve vital roles as a ceRNA for miRNAs in cerebral ischemic stroke.

In conclusion, the present study demonstrated that LINC00319 contributed to brain damage and promoted apoptosis in cerebral ischemic stroke. Furthermore, LINC00319 exacerbated the progression of ischemic stroke by binding miR-200a-3p to inhibit neuronal proliferation and decrease glucose uptake. The present data suggested that the LINC00319/miR-200a-3p axis may facilitate the development of novel therapeutic strategies to intervene in ischemic stroke.

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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

HY and HW designed the study and performed the experiments. HY, HL and XZ collected the data. XZ, HY, HL and YY performed the data processing, statistical analysis and bioinformatics investigations. HY and HL prepared the manuscript. HY and HW confirm the authenticity of the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Institutional Review Board of Jiamusi University (Jiamusi, China; approval no. JUIRBR-2019-301) and all procedures were performed in accordance with national (D.L.n.26, March 4th, 2014) and international laws and policies (directive 2010/63/EU). Written informed consent was provided by all patients prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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