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SNHG12 inhibits oxygen-glucose deprivation-induced neuronal apoptosis via the miR-181a-5p/NEGR1 axis

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Abstract. Emerging evidence has indicated that long non-coding RNAs (lncRNAs) are closely associated with the pathogenesis of ischemic stroke. It has been reported that small nucleolar RNA host gene 12 (SNHG12) serves a critical role in ischemic stroke by acting as a competitive endogenous RNA (ceRNA). SNHG12 competes with various microRNAs (miRs) to regulate RNA transcription of specific targets. However, the effect of SNHG12 on oxygen-glucose deprivation (OGD)-induced neuronal apoptosis has rarely been reported. The present study demonstrated that SNHG12 expression was downregulated in OGD-injured SH-SY5Y cells. Furthermore, miR-181a-5p was reported as a target of SNHG12 and was negatively regulated by SNHG12. Moreover, NEGR1 was a target of miR-181a-5p, which functions as a negative regulator of NEGR1 in OGD-induced neuronal apoptosis. In summary, the results strongly confirmed the hypothesis that SNHG12 functions as a ceRNA for miR-181a-5p and regulates the expression of NEGR1 thus inhibiting OGD-induced apoptosis of SH-SY5Y cells. Neuronal apoptosis aggravates brain damage during ischemic stroke, indicating that the activation of SNHG12 and NEGR1 expression and inhibition of miR-181a-5p may be a novel strategy for the clinical treatment of ischemic stroke.

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Abbreviations: lncRNAs, long non-coding RNAs; SNHG12, small nucleolar RNA host gene 12; ceRNAs, competitive endogenous RNAs; OGD, oxygen-glucose deprivation

Key words: ischemic stroke, small nucleolar RNA host gene 12, microRNA-181a-5p, neuronal growth regulator 1, apoptosis

Introduction

Stroke, a cerebrovascular disease with high morbidity, mortality, disability rate and recurrence, is a severe threat to human health and life and remains the second leading cause of death worldwide (1,2). Ischemic strokes account for ~87% of all stroke cases, with the remainder caused mainly by primary hemorrhage (2-5). Ischemic heart disease and stroke together accounted for 15.2 million mortalities worldwide in 2015 (1), while the clinically effective treatments are limited to thrombolytic therapy and symptom management (6-8). Therefore, it is essential to elucidate the pathogenic molecular mechanism of ischemic stroke to construct a novel strategy for clinical diagnosis and treatment of ischemic cerebrovascular disease.

Non-coding RNAs (ncRNAs), including microRNAs (miRNAs or miRs), circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs), are RNAs that are transcribed from the genome; however, they are not translated into proteins (9,10). ncRNAs serve critical roles as transcriptional and post-transcriptional regulators (9). Several previous studies on the central nervous system have demonstrated that the ncRNA expression profiles are altered during a stroke, including those of various miRNAs, Piwi-interacting RNAs and lncRNAs (11,12). Previous studies on the pathophysiological changes following strokes have mainly focused on miRNAs. For instance, miR-15b has been reported to inhibit the proliferation of neural progenitor cells by downregulating Tet methylcytosine dioxygenase 3 expression (13) and miR-140 was revealed to inhibit the proliferation of astrocytes and downregulate the expression and secretion of brain-derived neurotrophic factor (14). However, compared with miRNAs, the role of lncRNAs in the pathogenesis of strokes remains unclear.

Emerging evidence has demonstrated that the dysregulation of lncRNA expression is closely associated with the pathogenesis of ischemic stroke (2,4,15). lncRNAs can act as competitive endogenous RNAs (ceRNAs) in ischemic stroke by competing with miRNAs to regulate specific RNA transcription (16). miR-181a-5p is a critical member of the miRNA family and is mainly involved in the regulation of the proliferation and apoptosis of cancer cells (17). A previous study reported that miR-181a-5p levels were significantly increased

in the serum of patients with ischemic stroke (18). However, few studies have been conducted on the role of miR-181a-5p in ischemic stroke and its regulatory mechanism (19). It has been demonstrated that a lncRNA, small nucleolar RNA host gene 12 (SNHG12), serves a critical role in ischemic stroke by acting as a ceRNA and competing with various miRNAs to regulate specific RNA transcription (20,21). SNHG12 has been revealed to downregulate the level of miR-181a-5p and enhance the sensitivity of cancer cells to cisplatin (22). Furthermore, SNHG12 was revealed to inhibit endothelial cell injury induced by oxygen-glucose deprivation and repair vascular injury following stroke (23). However, it remains unclear whether SNHG12 serves a protective role by regulating miR-181a-5p in oxygen-deprived neurons. Thus the present study aimed to investigate the regulatory function of SNHG12 and miR-181a-5p in neuronal apoptosis, which may provide novel strategies for ischemic stroke therapy.

Materials and methods

Cell culture, cell transfection and OGD treatment. The SH-SY5Y cell line was obtained from the American Type Culture Collection and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, 2 mmol/l glutamine (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with humidified air at 5% CO_2. miR-181a-5p mimics, miR-181a-5p inhibitors, small interfering RNAs (siRNAs: siControl and siNHG12), control mimics, SNHG12-expressing plasmids (pcDNA3.1-SNHG12) and corresponding negative controls (pcDNA3.1-NC) were transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The sequences were: miR-181a-5p mimics: 5'-AAC AUUCAACGCUGUCGGUGAGU-3'; mimics negative control: 5'-UUUGUACUACACAAAAGUACUG-3' siControl: 5'-GTTCTCCGAACGTGTCACGTA-3'; siNHG12: 5'-GAC CAGAAGATAATGTTCAAG-3'. At 48 h post-transfection, the cells were split for subsequent experiments

For OGD treatment *in vitro*, cells were washed three times with warm, glucose-free DMEM and placed in glucose-free DMEM, following, cells were cultured in full culture medium with normoxic oxygen at 37˚C and were maintained for 0, 3, 6, 12 and 24 h. Control cells were cultured in DMEM/high glucose at normoxia. All cells were incubated in an anaerobic chamber containing 5% CO_2 and 95% N_2 at 37˚C for the indicated time-points (24).

Brain RNA-seq database. The expression level of NEGR1 in neurons, astrocytes, oligodendrocyte precursor cells, newly formed oligodendrocytes, myelinating oligodendrocytes, microglia, and endothelial cells were obtained from a transcriptome database (http://web.stanford.edu/group/barres_lab/brain_rnaseq.html: September 2014 release) generated by RNA sequencing and a sensitive algorithm to detect alternative splicing events in each cell type.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). A total of 58 patients, n=29 ischemic stroke patients, and n=29 controls at Department of Neurology, Wenling Hospital of Traditional Chinese Medicine, between July 2013 and December 2015 were included. blood samples (10 ml) were collected from arm veins of the 29 ischemic stroke patients and 29 controls and written informed consent was obtained from all participants. The total RNA of blood samples and SH-SY5Y cell lines were extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc). Extracted RNA was reverse-transcribed into cDNA using a Takara reverse transcription kit (Takara Bio, Inc.), according to the manufacturer's protocol. RT-qPCR was performed using IQ SYBR-Green Supermix (Bio-Rad Laboratories, Inc.) and a real-time PCR detection system (Light Cycler 480; Roche Diagnostics). Thermocycling conditions were: 95˚C for 30 sec, followed by 95˚C for 10 sec and 60˚C for 30 sec for 40 cycles. The relative expression level was calculated using the $2^{-\Delta\Delta Cq}$ method (25). The sequences of the primers used were: SNHG12 forward, 5'-CGGATTTTTCCGTCTGGT CC-3' and reverse, 5'-TCTGGTCTCCCTCCTCACAAT-3'; miR-181a-5p forward, 5'-AACATTCAACGCTGTCG-3' and reverse, 5'-AACTGTGTCGTGGAG-3'; neuronal growth regulator 1 (NEGR1) forward, 5'-TGAAGCAGCGTGGGATAC AAT-3' and reverse, 5'-CCAGCGATTCCACAGACAAA-3'; GAPDH forward, 5'-GTCAACGGATTTGGTCTGTATT-3' and reverse, 5'-AGTCTTCTGGGTGGCAGTGAT-3'; and U6 forward, 5'-GCTTCGGCAGCACATATACTAA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. GAPDH was used as an internal control for mRNA and U6 small nuclear RNA was used as an internal control for lncRNA and miRNA.

Apoptosis assay. SH-SY5Y cells were treated with the indicated stimulating factors shown in Figs. 1B, 3C, 4I and 5E and harvested for staining using an Annexin V-FITC/propidium iodide apoptosis detection kit (BD Biosciences), according to the manufacturer's protocol. Briefly, harvested cells were treated with 10 μg unlabeled Annexin V for 10 min followed by Annexin V-FITC/PI staining. Cells at different apoptotic periods were detected by flow cytometry using Annexin V probe conjugated to FITC (a green fluorescent; FL1 channel) and PI (a dye for nucleic acid with red fluorescence that cannot penetrate the intact cell membrane of healthy or early apoptotic cells; FL3 channel). The proportion of all apoptotic cells, including early and late apoptotic cells, was measured and analyzed by flow cytometry using a FACS verse flow cytometer (BD Biosciences) and CELLquest software (v5.1, Becton Dickinson).

RNA immunoprecipitation (RIP) assay. RIP experiments were performed using a Magna RIP kit (EMD Millipore), according to the manufacturer's protocol. Briefly, SH-SY5Y cells were washed with cold PBS and lysed in RIP lysis buffer. Cells were then incubated with RIP buffer and magnetic beads conjugated with anti-argonaute 2 (Ago2) antibodies (EMD Millipore; cat. no. LINC00980) or NC mouse immunoglobulin G (IgG; EMD Millipore). Following this, proteins were digested with protease K buffer and immunoprecipitated RNA was isolated. Corresponding RNA was purified and detected using RT-PCR and RT-qPCR as described above.

Western blotting. SH-SY5Y cells were harvested and lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with phosphatase inhibitors and protease

inhibitors (Roche Diagnostics; cat. no. 04693132001) and centrifuged at 12,000 x g at 4˚C for 25 min. A BCA Protein Assay kit (Generay Biotech Co., Ltd.) was used to measure the concentrations of extracted protein, according to the manufacturer's protocol. A total of 30 μg of protein/lane was separated by 8-12% SDS-PAGE gel and transferred to PVDF membranes (EMD Millipore). Membranes were blocked in TBST buffer with 5% non-fat skimmed milk for 45 min at room temperature and washed three times with TBST buffer. Following this, membranes were incubated overnight with primary antibodies at 4˚C and with horseradish peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology, Inc.; cat. nos. sc-2357 and sc-516102; 1:5,000) at room temperature for 1 h. Bands were visualized using commercially ECL reagents.

The following antibodies were used: B-cell lymphoma 2 (Bcl-2; product no. 15071; 1:1,000) Bcl-2 associated X protein (Bax; cat. no. 5023; 1:1,000), cleaved caspase-3 (cat. no. 9654; 1:1,000; all from Cell Signaling Technology, Inc.), NEGR1 (cat. no. sc-137625; 1:1,000; Santa Cruz Biotechnology, Inc.) and GAPDH (cat. no. 5174; 1:2,000 Cell Signaling Technology, Inc.).

Cell Counting Kit-8 (CCK-8) assay. A total of $1x10^5$ cells were plated in 96-well plates in triplicate for 24 h and treated with the indicated stimulating factors for the indicated time-points shown in Figs. 1A, 3B, 4H and 5D, then CCK-8 reagent was added into each well and further incubated for 2 h at 37˚C, The cell viability under various conditions was measured using a CCK-8 assay (Beyotime Institute of Biotechnology), according to the manufacturer's protocol and the optical density at 450 nm (OD_{450}) value in each well was measured using a microplate reader (Quant BioTek Instruments). Cell viability rates were calculated as follows: Cell viability rate (%)=(experimental OD/control group OD) x100%.

Dual-luciferase reporter assay. Putative miR-181a-5p binding sites in SNHG12 and binding sites in the 3'-untranslated region (UTR) of NEGR1 to miR-181a-5p were predicted using StarBase (http://starbase.sysu.edu.cn/starbase2/, v2.0; September 2013 release) and TargetScan (www.targetscan.org, v7.2; March 2018 release) (26). Sequence fragments SHNG12-wild-type (WT) and SHNG12-mutant (MT) and WT and MT 3'-UTR fragments of NEGR1 containing putative miR-181a-5p binding sites were cloned into pmirGLO dual-luciferase reporter vectors (Promega Corporation) and confirmed by sequencing using Illumina MiSeq MiSeqdxDNA (Illumina, Inc.) according to the manufacturer's protocol. SH-SY5Y cells were then co-transfected with the corresponding plasmids as described above (*Cell culture, cell transfection and OGD treatment*). At 48 h post-transfection, a Dual-Luciferase Reporter Assay System (Promega Corporation) was used to measure luciferase activity. Relative luciferase activity was normalized to the *Renilla* luciferase activity.

Statistical analysis. Experiments were performed in triplicate. Data are presented as the mean ± standard error of the mean. GraphPad Prism software (version 6.01; GraphPad Software, Inc.) was used for statistical analysis of experimental data. Statistical differences were assessed using unpaired two-tailed Student's t-tests for comparison between two groups or ANOVA followed by Tukey's multiple comparisons post hoc test for comparison between >2 groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

OGD induces apoptosis in SH-SY5Y cells, reduction in SNHG12 expression and upregulation of miR-181a-5p. To create an *in vitro* model of ischemia, SH-SY5Y cells were treated with OGD (24). Viability of SH-SY5Y cells was gradually decreased in the OGD group following 0, 3, 6 12 and 24 h of treatment (Fig. 1A). Furthermore, OGD promoted apoptosis in SH-SY5Y cells compared with the control as indicated by apoptosis assays (Fig. 1B). Western blotting results for apoptosis-related proteins revealed increased expression of cleaved caspase-3 and Bax and decreased expression of Bcl-2 (Fig. 1C). To determine whether the expression levels of SNHG12 and miR-181a-5p were altered following OGD, RT-qPCR analysis was performed and the results indicated that SNHG12 expression was significantly decreased and miR-181a-5p expression was significantly increased compared with their respective controls (Fig. 1D and E). Consistent with these results, SNHG12 expression in the serum of patients with cerebral ischemia was significantly decreased and miR-181a-5p expression was significantly increased compared with healthy controls (Fig. 1D and E). These results indicated that SNHG12 and miR-181a-5p may be associated with OGD-induced apoptosis in SH-SY5Y cells.

SNHG12 targets miR-181a-5p and negatively regulates its expression. Increasing evidence has demonstrated that lncRNAs act as ceRNAs by competing with miRNAs to regulate transcription (12,16). The present study found that the expression of SNHG12 and miR-181a-5p exhibited a negative correlation in OGD-treated SH-SY5Y cells (Fig. 1D and E). Therefore, the possible association between SNHG12 and miR-181a-5p was investigated. To explore whether SNHG12 acts as a ceRNA of miR-181a-5p, the bioinformatics analysis tools StarBase (http://starbase.sysu.edu.cn/starbase2/) and TargetScan (http://www.targetscan.org) were used to predict putative SNHG12 binding sites in miR-181a-5p (27). SNHG12 contained a conserved binding site for miR-181a-5p (Fig. 2A).

To directly verify whether miR-181a-5p is a target of SNHG12, dual-luciferase reporter assays were performed in SH-SY5Y cells co-transfected with SNHG12 reporters (SNHG12-WT or SNHG12-MT) and miR-181a-5p mimics or NC-miRNA. The results demonstrated that miR-181a-5p mimics significantly reduced the activity of the SNHG12-WT reporter; however, the luciferase activity of the SNHG12-MT reporter was not affected (Fig. 2B). These results indicated that miR-181a-5p binds to SNHG12 at the predicted binding site. Furthermore, RIP assays confirmed the physical association between miR-181a-5p and SNHG12 since SNHG12 and miR-181a were enriched in Ago2 pellets compared with controls (Fig. 2C). Additionally, the exogenous overexpression of SNHG12 significantly decreased the expression of miR-181a-5p in OGD-injured SH-SY5Y cells, while siRNA-mediated downregulation of SNHG12 (siSNHG12) demonstrated the opposite effect (Fig. 2D and E).

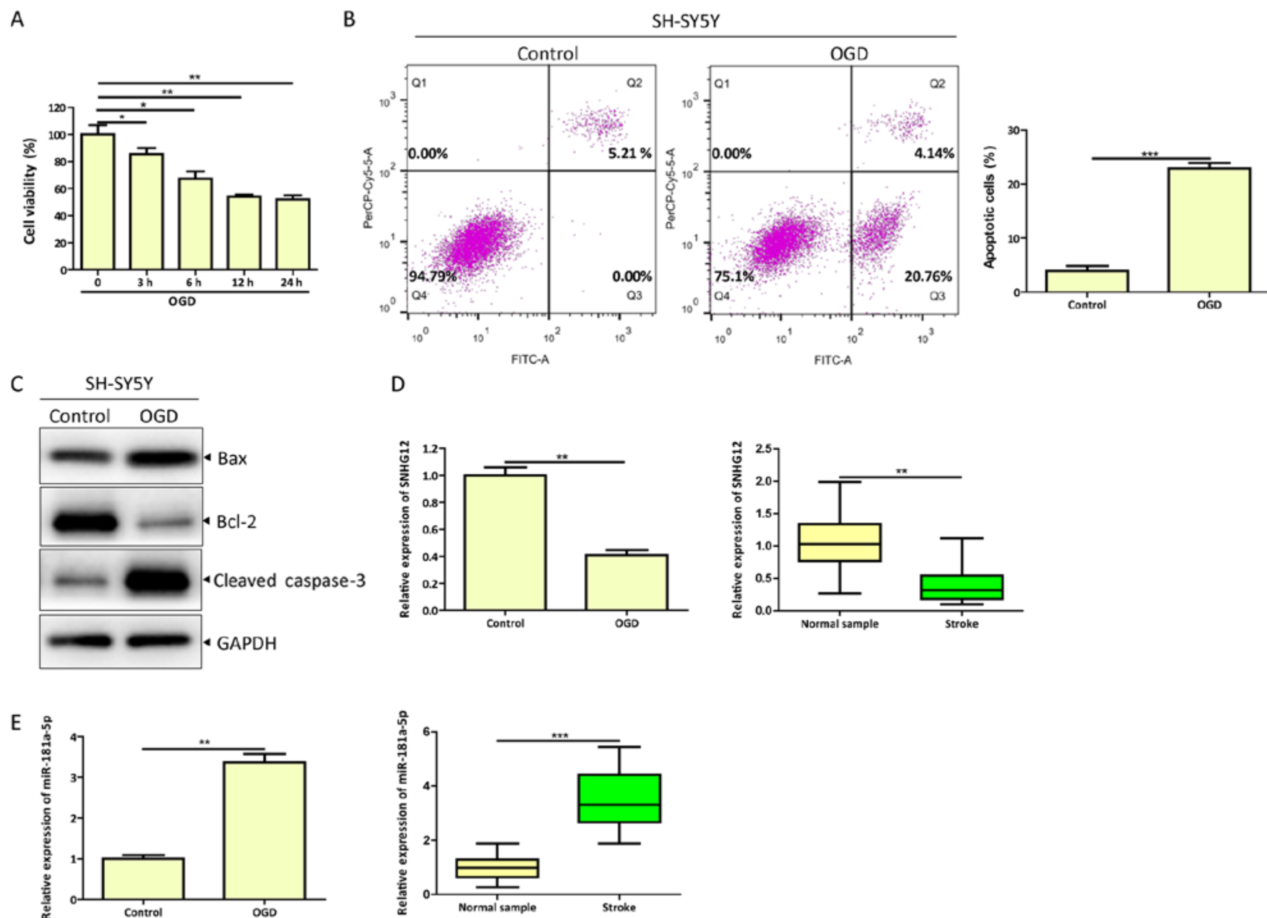

Figure 1. Induction of apoptosis in SH-SY5Y cells by OGD is accompanied by reduced SNHG12 expression and increased miR-181a-5p expression. (A) SH-SY5Y cells were seeded in a 96-well plates for 24 h, treated with OGD for the indicated time-points and cell viability was measured using a Cell Counting Kit-8. (B) Apoptosis rates of SH-SY5Y cells treated with OGD for 12 h and control was determined by flow cytometry. (C) Protein levels of Bcl-2, Bax and cleaved caspase-3 in SH-SY5Y cells treated with OGD for 12 h and controls were analyzed by western blotting. Expression levels of (D) SNHG12 and (E) miR-181a-5p in OGD-treated SH-SY5Y cells and controls or in the serum of patients with cerebral ischemia and healthy controls were measured by reverse transcription-quantitative PCR. *P<0.05, **P<0.01 and ***P<0.001. OGD, oxygen-glucose deprivation; SNHG12, small nucleolar RNA host gene 12; miR, microRNA; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X protein.

In summary, these results confirmed that SNHG12 acts as a ceRNA via direct inhibition of miR-181a-5p expression in OGD-injured SH-SY5Y cells.

SNHG12 downregulates miR-181a-5p and inhibits OGD-induced apoptosis of SH-SY5Y cells. To determine the function of SNHG12 and miR-181a-5p in the apoptosis of OGD-injured SH-SY5Y cells, miR-181a-5p mimics or NC-miRNA were transfected into SH-SY5Y cells, along with pcDNA3.1-SNHG12 or pcDNA3.1-NC. Overexpression and downregulation of miR-181a-5p was confirmed by RT-qPCR (Fig. 3A). Consistent with these results, SNHG12 overexpression reduced miR-181a-5p expression, increased cell viability and decreased apoptotic cell death compared with control group (Fig. 3A-C). Western blotting of apoptosis-related proteins Bax, Bcl-2 and cleaved caspase-3 confirmed the effects of SNHG12 overexpression on OGD-induced cell apoptosis (Fig. 3D). Furthermore, SNHG12 overexpression with co-transfected miR-181a-5p mimics restored OGD-induced SNHG12-mediated inhibition of SH-SY5Y cell apoptosis (Fig. 3C-D). These results demonstrated that SNHG12 regulated apoptosis in OGD-injured SH-SY5Y cells by downregulating miR-181a-5p expression.

miR-181a-5p inhibits NEGR1 expression and promotes apoptosis of SH-SY5Y cells. Increasing evidence has indicated that miRNAs function by binding to the 3'UTR of downstream target mRNAs to regulate target genes (27,28). Therefore, possible target genes of miR-181a-5p were predicted using the web-based miRNA database TargetScan. Bioinformatics analysis revealed that NEGR1 was a potential target gene of miR-181a-5p. Identified target sites for miR-181a-5p in the 3'UTR of NEGR1 are presented in Fig. 4A. RNA-seq results obtained from the Brain RNA-seq database (http://web.stanford.edu/group/barres_lab/brain_rnaseq.html) demonstrated that NEGR1 was highly expressed in neurons (Fig. 4B). Furthermore, NEGR1 expression was decreased in OGD-injured SH-SY5Y cells (Fig. 4C). Accordingly, dual-luciferase reporter and RIP assays confirmed that NEGR1 is a target of miR-181a-5p (Fig. 4D and E). To further confirm the regulatory effect of miR-181a-5p on NEGR1, the results for RT-qPCR and western blotting demonstrated that miR-181a-5p overexpression significantly decreased the mRNA and protein levels of NEGR1, while miR-181a-5p inhibition upregulated the mRNA and protein levels of NEGR1 (Fig. 4F and G).

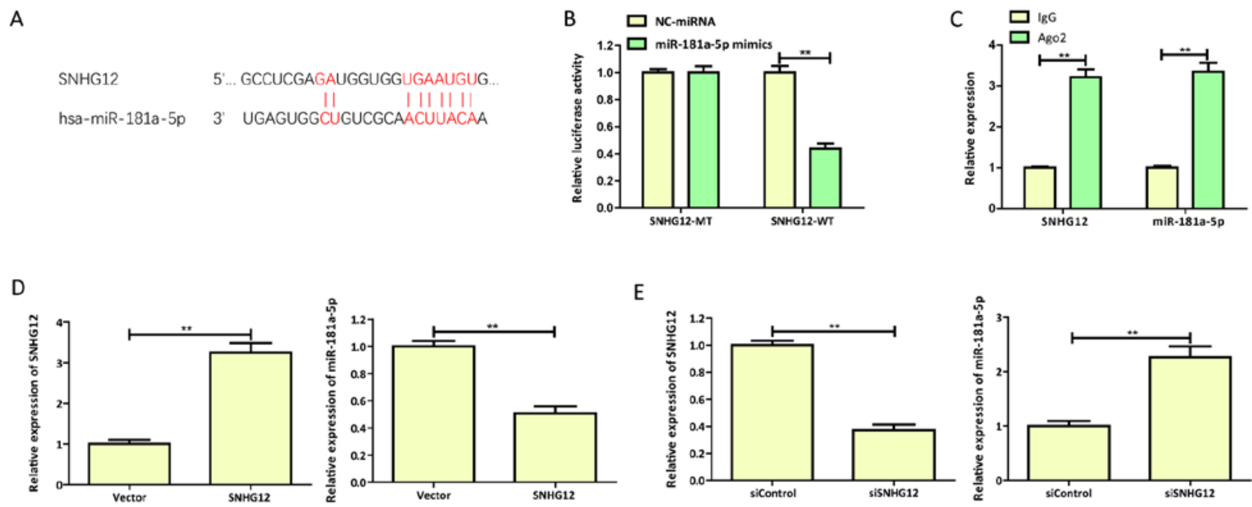

Figure 2. SNHG12 targets miR-181a-5p and negatively regulates its expression. (A) Predicted binding sequence of SNHG12 in miR-181a-5p. (B) SH-SY5Y cells were co-transfected with control or miR-181a-5p mimics and luciferase reporter vectors SNHG12-MT or SNHG12-WT, and at 48 h post-transfection relative luciferase activities was measured. (C) SH-SY5Y cells were harvested and lysed, and the lysates were used for RNA immunoprecipitation assays with Ago2 antibodies. IgG was used as the control. (D) SH-SY5Y cells were transfected with PCDNA3.1-NC or PCDNA3.1-SNHG12 expression vectors and exposed to OGD. The levels of SNHG12 and miR-181a-5p expression were measured using RT-qPCR. (E) SH-SY5Y cells were transfected with siControl or siSNHG12 and exposed to OGD and the levels of SNHG12 and miR-181a-5p were measured using RT-qPCR. **P<0.01. SNHG12, small nucleolar RNA host gene 12; miR, microRNA; Ago2, argonaute 2; IgG, immunoglobulin G; OGD, oxygen-glucose deprivation; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering.

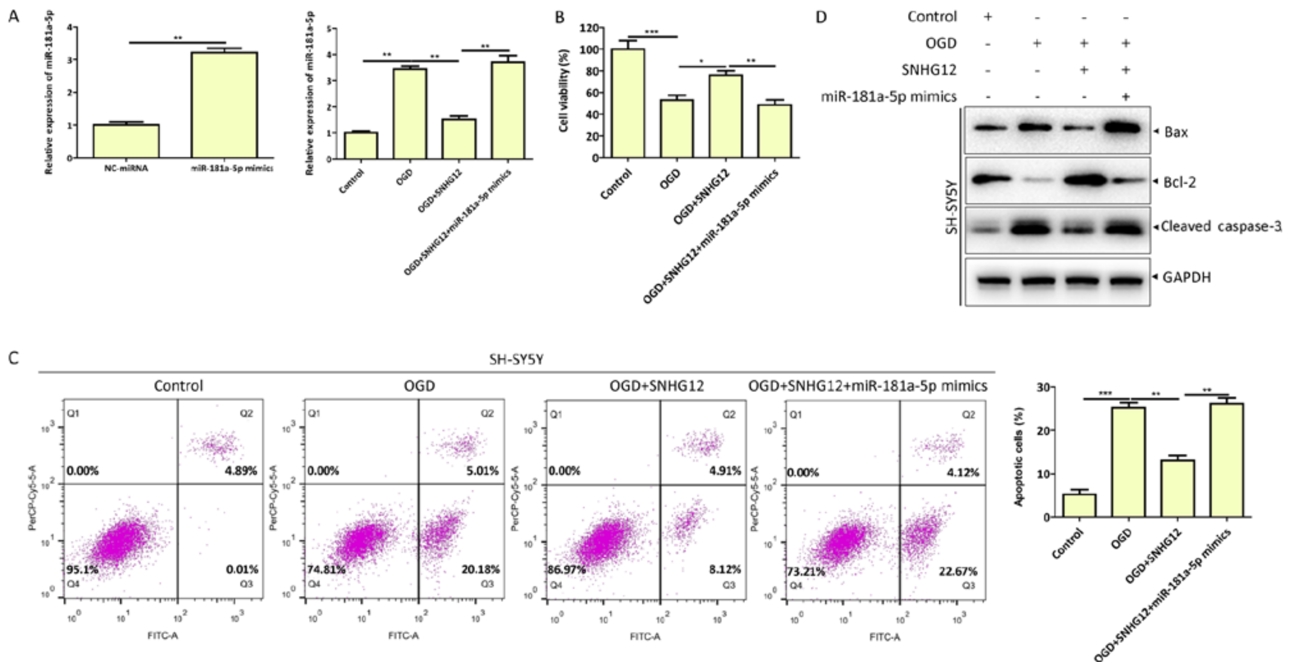

Figure 3. SNHG12 downregulates miR-181a-5p and inhibits OGD-induced apoptosis of SH-SY5Y cells. (A) At 48 post-transfection in cells overexpressing SNHG12 plasmids or controls, cells were co-transfected with NC-miRNA or miR-181a-5p mimics and SH-SY5Y cells were treated with OGD for 12 h. miR-181a-5p levels were examined by reverse transcription-quantitative PCR. (B) Cell viability was measured using Cell Counting Kit-8 assays. (C) The apoptosis rate was determined by flow cytometry. (D) Protein levels of Bcl-2, Bax and cleaved caspase-3 were analyzed by western blotting. *P<0.05, **P<0.01 and ***P<0.001. SNHG12, small nucleolar RNA host gene 12; NC, negative control; miR or miRNA, microRNA; OGD, oxygen-glucose deprivation; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X protein.

Previous studies have reported that NEGR1 is a cellular adhesion molecule involved in neurite outgrowth during neuronal development (29,30). The present study demonstrated that NEGR1 was associated with OGD-induced neuronal apoptosis. miR-181a-5p overexpression resulted in decreased cell viability (Fig. 4H) and increased apoptotic cell death (Fig. 4I) compared with the control group. NEGR1 overexpression reversed the effect of miR-181a-5p by exhibiting increased cell activity, decreased cell apoptosis, increased expression of Bcl-2 and decreased expression of cleaved caspase-3 and Bax (Fig. 4J).

In summary, these results revealed that NEGR1 is a target of miR-181a-5p and that miR-181a-5p functions as a negative

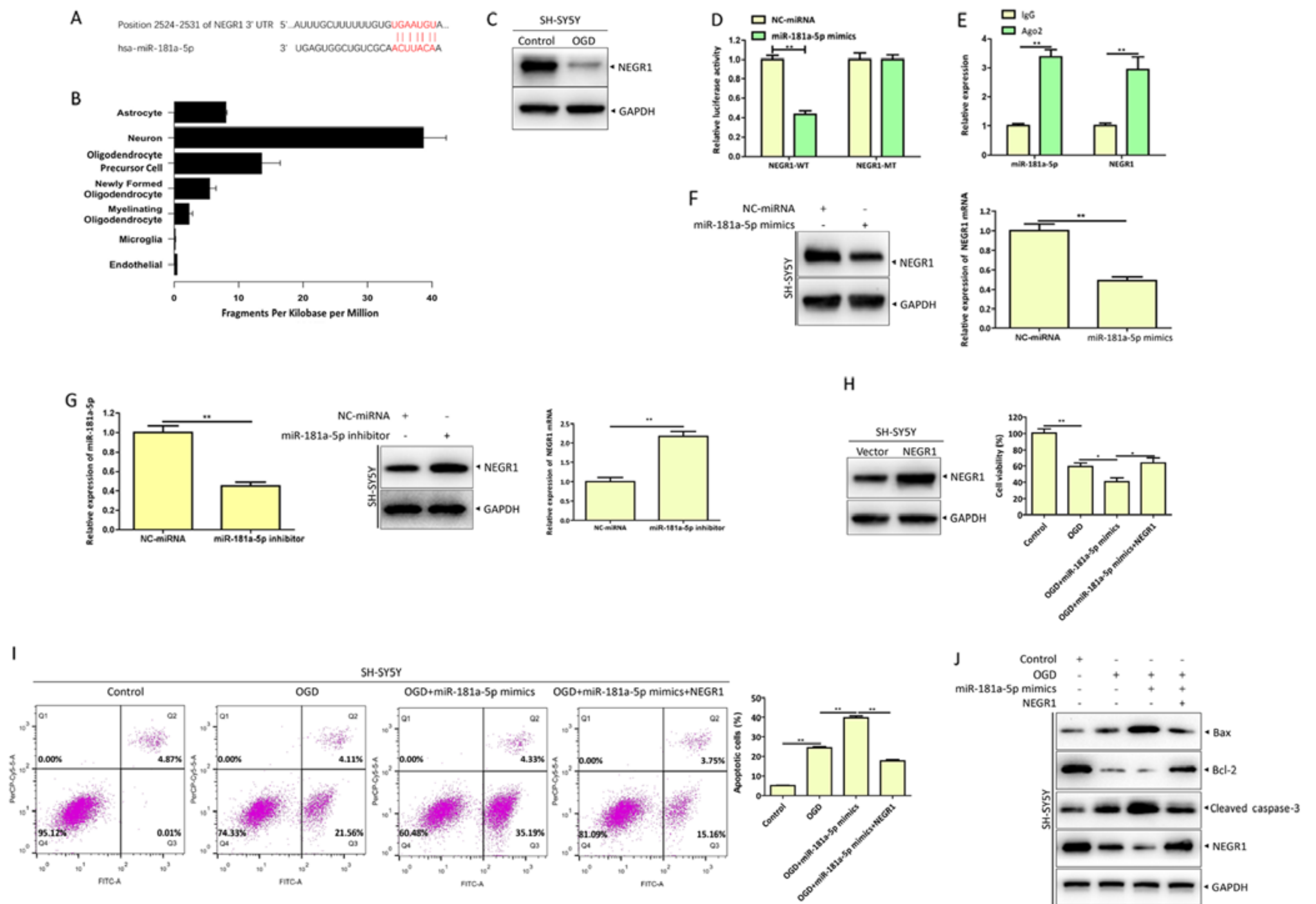

Figure 4. miR-181a-5p inhibits NEGR1 expression and promotes apoptosis of SH-SY5Y cells. (A) Binding site of NEGR1 to miR-181a-5p as predicted by TargetScan. (B) RNA-seq analysis of the expression profile of NEGR1 in various cells. (C) SH-SY5Y cells were treated with OGD for 12 h and NEGR1 expression was detected by western blotting. (D) NEGR1-WT or NEGR1-MT reporter plasmids were co-transfected with NC-miRNA or miR-181a-5p mimics, respectively, into SH-SY5Y cells. The activities of luciferase were measured using a Dual Luciferase Reporter Assay System. (E) SH-SY5Y cells were harvested and lysed, and the lysates were used for RNA immunoprecipitations assays with Ago2 antibodies. IgG was used as the control. (F) SH-SY5Y cells were transfected with NC-miRNA or miR-181a-5p mimics, and at 48 h post-transfection NEGR1 protein levels were detected by western blotting. (G) Expression levels of NEGR1 and miR-181a-5p were detected by reverse transcription-quantitative PCR. (H) NC-miRNA or miR-181a-5p mimics were transfected into SH-SY5Y cells with an overexpressed NEGR1 plasmids or controls, respectively. At 48 h post-transfection, cells were treated with OGD for 12 h and cell viability was measured via Cell Counting Kit-8 assays. (I) Apoptosis rate was determined by flow cytometry. (J) Protein levels of Bcl-2, Bax and cleaved caspase-3 were analyzed by western blotting. *P<0.05 and **P<0.01. miR or miRNA, microRNA; NEGR1, neuronal growth regulator 1; OGD, oxygen-glucose deprivation; WT, wild-type; MT, mutant; NC, negative control; Ago2, argonaute 2; IgG, immunoglobulin G; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X protein; UTR, untranslated region.

modulator of NEGR1 to regulate OGD-induced neuronal apoptosis.

SNHG12 inhibits OGD-induced apoptosis in SH-SY5Y cells through the miR-181a-5p/NEGR1 axis. To further confirm the regulation of the SNHG12/miR-181a-5p/NEGR1 axis in OGD-induced neuronal apoptosis, dual-luciferase, CCK-8 and apoptosis assays, and western blotting were performed in SH-SY5Y cells transfected with NEGR1-MT or NEGR1-WT in combination with NC-miRNA or miR-181a-5p mimics, or co-transfected with siSNHG12 or PCDNA3.1-SNHG12. SNHG12 overexpression significantly increased NEGR1-WT luciferase activity (Fig. 5A). Moreover, SNHG12 knockdown decreased the protein level of NEGR1 (Fig. 5B), while SNHG12 overexpression had the opposite effect (Fig. 5C). Additionally, SNHG12 siRNA notably abolished the effect of NEGR1 overexpression on cell viability (Fig. 5D) and apoptosis (Fig. 5E and F) of OGD-injured SH-SY5Y cells.

Collectively, these results strongly confirmed the hypothesis that SNHG12 functions as a ceRNA for miR-181a-5p to regulate the expression of NEGR1 and to inhibit the OGD-induced apoptosis of SH-SY5Y cells.

Discussion

There is ample evidence indicating that ischemic stroke is caused by intracranial thrombosis or extracranial embolism, resulting in a lack of blood flow and supply of oxygen and nutrients (4,19,31). Subsequently, multiple complex factors lead to the destruction of the blood-brain barrier following ischemic stroke, which further aggravates brain damage and a series of neurological events, including oxidative stress, toxicity of excitatory amino acids, excessively high calcium ion concentrations and increased apoptosis (4,31,32). Due to this, it is crucial to understand the molecular mechanism underlying neuronal apoptosis in ischemic stroke to establish

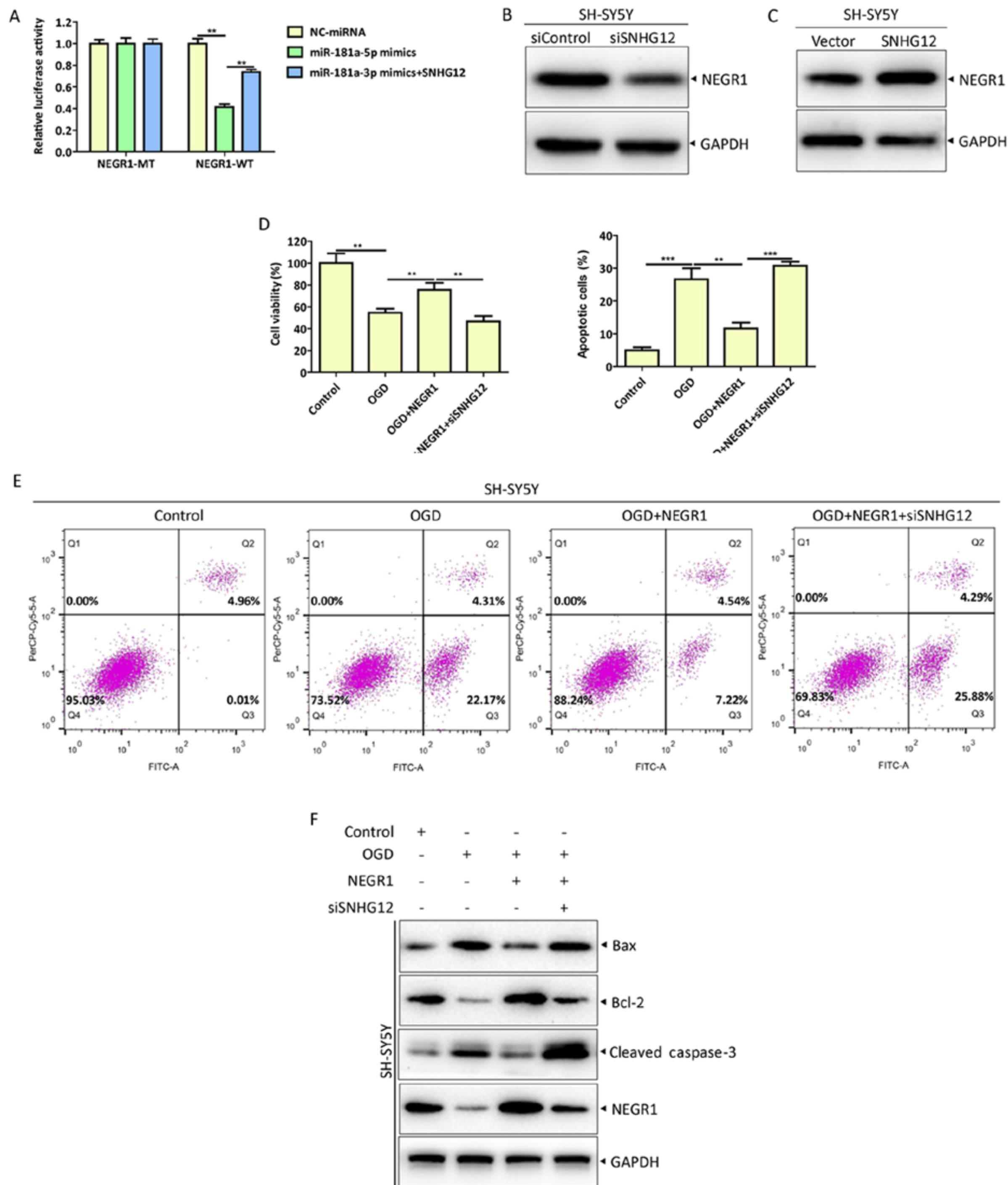

Figure 5. SNHG12 inhibits OGD-induced apoptosis in SH-SY5Y cells through the miR-181a-5p/NEGR1 axis. (A) Cells were co-transfected with the indicated plasmids or miRNAs and luciferase activity was measured using a Dual-Luciferase Reporter Assay System. Protein levels of NEGR1 were analyzed by western blotting in (B) SNHG12 knockdown or (C) overexpressed SH-SY5Y cells. NEGR1 overexpression and internal control plasmids were co-transfected with siSNHG12 or siControl into SH-SY5Y cells, respectively. At 48 h post-transfection, cells were treated with OGD for 12 h. (D) Cell viability was measured by Cell Counting Kit-8 assays. (E) Apoptosis rates were determined by flow cytometry. (F) Protein levels of Bcl-2, Bax and cleaved caspase-3 were analyzed by western blotting. **P<0.01 and ***P<0.001. SNHG12, small nucleolar RNA host gene 12; OGD, oxygen-glucose deprivation; miR or miRNA, microRNA; NEGR1, neuronal growth regulator 1; si, small interfering; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X protein; MT, mutant; WT, wild-type; NC, negative control

a novel strategy for the clinical diagnosis and treatment of ischemic cerebrovascular disease.

Emerging evidence has demonstrated that abnormal expression of ncRNA, including miRNAs, circRNAs and lncRNAs, is closely associated with the pathogenesis of ischemic stroke (6,9,20). SNHG12 is one of numerous lncRNAs involved in the progression of ischemic stroke. Long *et al* (33) reported that SNHG12 suppressed brain microvascular endothelial cell injury induced by oxygen-glucose deprivation/reoxygenation by targeting miR-199a. Yin *et al* (21) reported that SNHG12 interacted with miR-199a to attenuate apoptosis and cerebral ischemia/reperfusion injury via the

AMP-activated protein kinase (AMPK) signaling pathway under oxygen-glucose deprivation/reoxygenation conditions. Furthermore, silencing SNHG12 enhanced the effects of mesenchymal stem cells by reducing apoptosis and autophagy of brain microvascular endothelial cells by activating the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin signaling pathway (34). SNHG12 functions as a ceRNA for miR-150 to mediate VEGF expression and promote angiogenesis and alleviate ischemic stroke (23). Additionally, miR-181a-5p is reportedly involved in ischemia (35). As miR-181a-5p is a brain-enriched miRNA that exhibits elevated expression levels in the cerebrospinal fluid of patients with acute ischemic stroke (36), miR-181a-5p inhibition enhanced estradiol-mediated stroke protection in females partly by augmenting estrogen receptor α production in mouse and human astrocytes (37). Moreover, previous studies have demonstrated that miR-181a-5p inhibition has a protective effect in male rodent models of stroke and forebrain ischemia by targeting stress proteins and apoptotic regulators (18,35).

Recently, Wang *et al* (22) demonstrated a regulatory association between SNHG12 and miR-181a-5p by revealing that SNHG12 downregulated the level of miR-181a-5p and enhanced the sensitivity of cancer cells to cisplatin (22). However, the specific function of SNHG12 and miR-181a-5p in the process of OGD-induced neuronal apoptosis and their therapeutic effects remain unclear. The present study demonstrated that OGD leads to an induction of apoptosis in SH-SY5Y cells, reduction in SNHG12 expression and upregulation of miR-181a-5p. This indicated that the expression of SNHG12 and miR-181a-5p were negatively associated in OGD-treated SH-SY5Y cells. Therefore, the possible association between SNHG12 and miR-181a-5p was investigated. The results reported that miR-181a-5p was a target of SNHG12 and negatively regulated its expression. Furthermore, miR-181a-5p functioned as a negative modulator of NEGR1 to regulate OGD-induced neuronal apoptosis. Previous studies have demonstrated that NEGR1 is a cellular adhesion molecule involved in neurite outgrowth during neuronal development (29,30). The present study reported, to the best of our knowledge, for the first time that NEGR1 inhibited OGD-induced cell apoptosis. While the present study only discussed the mechanism at one time-point, it is not necessary to discuss the same mechanism at different time-points. Different time-points under OGD treatment may influence on the proportion of apoptosis and the expression level of SNHG12, miR-181a-5p and NEGR1, not the association between SNHG12, miR-181a-5p and NEGR1. RIP assays, western blotting, dual-luciferase reporter assays and other experimental results fully supported the conclusion that SNHG12 functioned as a ceRNA for miR-181a-5p and regulated the expression of NEGR1 thus inhibiting OGD-induced apoptosis in SH-SY5Y cells. Similarly, previous studies (21,38) have discussed the interaction mechanisms of SNHG12/miR-199a/SIRT1 and lncRNA CRNDE/miR-181a-5p/Wnt/β-catenin axis at a single time-point.

In summary, the present study revealed that SNHG12 inhibited OGD-induced neuronal apoptosis via the miR-181a-5p/NEGR1 axis. This indicated that SNHG12 and NEGR1 serve anti-apoptotic roles and miR-181a-5p serves a pro-apoptotic role in neurons following OGD injury. Therefore, the inhibition of miR-181a-5p and upregulation of SNHG12 and NEGR1 may be potential therapeutic strategies for the treatment of cerebral ischemic injury. Future studies are required to determine whether SNHG12 functions as a ceRNA for miR-181a-5p to regulate the expression of NEGR1 *in vivo*.

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Availability of data and materials

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

Authors' contributions

YY conducted experiments, acquired and analyzed the data, and drafted the manuscript. LC conducted experiments and acquired data. JZ analyzed and interpreted data, and revised the manuscript. LX conceptualized and designed the data, analyzed and interpreted data, and drafted and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Blood samples were collected from patients with cerebral ischemia and healthy controls, and all participants provided written informed consent. The present study was approved by the Ethics Committee of the Wenling Hospital of Traditional Chinese Medicine, Wenling, Zhejiang 317500, China (Project ID: H-I-2012-011).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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