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Original Article

Suppression of lncRNA SNHG15 protects against cerebral ischemia-reperfusion injury by targeting miR-183-5p/FOXO1 axis

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Abstract: Background: Cerebral ischemia/reperfusion (I/R) injury is a severe complication during the treatment of patients with stroke. It has been shown that the expression of SNHG15 was increased in patients with ischemic stroke (IS). However, the function and regulatory mechanism of SNHG15 in IS remains unclear. Methods: An oxygen glucose deprivation/reoxygenation (OGD/R) cell model was use to establish an *in vitro* model of I/R injury. RT-qPCR assay was used to detect the level of SNHG15 in OGD/R-treated SH-SY5Y cells. Meanwhile, middle cerebral artery occlusion (MCAO) was used to establish an *in vivo* model of cerebral I/R injury. Results: The expression of SNHG15 was upregulated in OGD/R-treated SH-SY5Y cells. Downregulation of SNHG15 during reperfusion reduced cell death in OGD/R-treated SH-SY5Y cells. In addition, SNHG15 knockdown suppressed OGD/R-induced apoptosis in SY-SY5Y cells by attenuating intracellular ROS generation and reducing mitochondrial membrane potential (MMP) lost. In addition, SNHG15 knockdown promoted cell cycle transition in SY-SY5Y cells after OGD/R insult accompany with PI3K/Akt signaling activation. Meanwhile, mechanism investigations suggested SNHG15 knockdown downregulated the expression of FOXO1 through acting as a competitive 'sponge' of miR-183-5p. Most importantly, knockdown of SNHG15 expression *in vivo* inhibited neuronal apoptosis and decreased infarct area in MCAO rats. Conclusion: Thus, the present study indicated that SNHG15 knockdown protected against cerebral I/R injury via targeting miR-183-5p/FOXO1 axis, which may represent a potential therapeutic option for the treatment of cerebral IS.

Keywords: Stroke, lncRNA SNHG15, microRNA-183-5p, FOXO1, apoptosis

Introduction

Stroke is a clinically common cerebrovascular disease, which is classified into two types, hemorrhagic and ischemic [1]. The minority (10-20%) of stroke cases is hemorrhagic, while IS accounts for 80-90% of all stroke cases [2]. Thus, IS is a serious threat to human health [3]. Thrombolytic therapy is known as an effective therapeutic approach for IS [3-5]. However, thrombolytic therapy unavoidably results in the reperfusion injury, commonly known as cerebral I/R injury [3-5]. In addition, various pathological processes are participated in the progression of I/R injury, such as necrosis, apoptosis or neuronal apoptosis induced by oxidative stress [6, 7]. The pathogenesis of stroke is complex [8]. Therefore, identification of novel therapeutic targets is critical for improving the treatment of IS.

Long non-coding RNAs (LncRNAs) are a set of ncRNAs, with lengths longer than 200 bp [9]. It has been shown that lncRNAs could mediate gene expression at the transcriptional or post-translational levels [10]. Previous studies found that several lncRNAs, such as 1810-034E14Rik, MALAT1, SETD5-AS1, MEG3, could regulate neuron death in IS [9-12]. Deng et al found that the level of lncRNA SNHG15 was increased in patients with IS suggesting SNHG15 might be a potential diagnose biomarker [13]. However, the role of SNHG15 in IS remain largely unclear.

MiRNAs are small non-coding RNA molecules, approximately 22 nucleotides in length. MiRNAs

can bind to the 3'-UTRs of target genes and induce mRNA degradation [4]. In addition, miRNAs have been found to be involved in various processes, such as neural cell differentiation, apoptosis and survival [14]. Recently, miRNAs have emerged as biomarkers for diagnosis and treatment of a variety of human diseases, including IS [15]. In addition, lncRNAs could act as a competitive endogenous RNA (ceRNA) to compete against miRNAs, thus regulate gene expression [16]. Yan et al found that knockdown of lncRNA MEG3 could protect against I/R injury through targeting miR-21/PDCD4 signaling [12]. In this study, we first demonstrated the functional interaction among SNHG15, miR-183-5p, and FOXO1 in IS, which may provide a new therapeutic option for cerebral I/R injury.

Materials and methods

Cell culture

SH-SY5Y cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). SH-SY5Y ells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

OGD/R administration

SH-SY5Y cells were cultured for 4 h in a glucose-free medium in the hypoxic atmosphere (5% CO_2, 95% N_2). After OGD treatment, the medium was replaced with fresh DMEM medium containing 4.5 g/L glucose and 10% FBS, and incubated for 24, 48 and 72 h at 37°C under an atmosphere of 95% air and 5% CO_2.

Cell transfection

SNHG15 siRNAs were purchased from Ribobio (Guangzhou, China). The target sequences of siRNAs for SNHG15 were as follows: SNHG15 siRNA1: TATTTAGAGTCACCTGGCTAATGAA; SNHG15 siRNA2: CCCGTATGGTAAGTCAGGTGATTGA; SNHG15 siRNA3: GAATCCCAGAAAGGTTCATTGTCTT. Meanwhile, the miR-183-5p agomir, miR-183-5p antagomir, and negative control (NC) were obtained from Ribobio. After OGD administration, SH-SY5Y cells were transfected with siRNA-control, SNHG15 siRNA1, SNHG15 siRNA2, SNHG15 siRNA3, miR-183-5p agomir or miR-183-5p antagomir using Lipofectamine 2000 reagent (Thermo Fisher Scientific), and incubated in glucose- and serum-containing DMEM medium in an atmosphere of 95% air and 5% CO_2.

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

Total cellular RNA was extracted using the TRIzol reagent (Thermo Fisher Scientific). EntiLink™ 1st strand cDNA synthesis kit (ELK Biotechnology, Hubei, China) was used to synthesize complementary DNA (cDNA). Then, real-time PCR was performed using the EnTurbo™ SYBR Green PCR SuperMix (ELK Biotechnology) on an applied biosystems StepOnePlus machine (Applied Biosystems, CA, USA). The specific primers for U6 were, 5'-CTCGCTTCGGCAGCACAT-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse); for miR-183-5p were 5'-GGGCACTGGTAGAATTCACTCTC-3' (forward) and 5'-CTCAACTGGTGTCGTGGAGTC-3' (reverse); for actin were 5'-GTCCACCGCAAATGCTTCTA-3' (forward) and, 5'-TGCTGTCACCTTCACCGTTC-3' (reverse); for SNHG15 were 5'-CGGTCTTCGGCAGTCTAGTCA-3' (forward) and 5'-GGGGTGTTCAGCAACTATTCCT-3' (reverse). MiRNA expression was normalized against U6 and mRNA expression was normalized against actin using the $2^{-\Delta\Delta CT}$ method.

Cell counting kit-8 (CCK-8) assay

Cell viability was determined using the CCK-8 reagent (Beyotime, Shanghai, China). SH-SY5Y cells were plated onto 96-well plates at a density of 5000 cells/well overnight, and then subjected to OGD/R. After that, CCK-8 reagent (10 μL) were added into each well, and the cultures plates were incubated for another 2 h. The optical density (OD) value of each well was assessed by a microtiter plate reader (Bio-Rad, California, CA, USA) at the wavelength of 450 nm.

Flow cytometry assay

For the apoptosis assay, SH-SY5Y cells were washed twice with pre-cooling PBS and resuspended in binding buffer (Thermo Fisher Scientific). Then, cells were stained with 5 μL of annexin V-FITC and 5 μL of propidium iodide (PI, Thermo Fisher Scientific) in darkness for 15 min. Later on, the percentage of the apoptotic

cells was assessed with flow cytometry (BD Biosciences, Franklin Lake, NJ, USA).

For the cell cycle assay, SH-SY5Y cells were washed twice with pre-cooling PBS and fixed in 75% ethanol at 4°C overnight. After that, cells were incubated with 1 mg/mL of PI/RNase Staining Buffer (Thermo Fisher Scientific) in darkness for 30 min. Later on, cell cycle distribution was assessed with flow cytometry (BD Biosciences).

Immunofluorescence assay

SH-SY5Y cells were washed twice with PBS, and then fixed with 4% paraformaldehyde for 15 min. After that, cells were permeabilised with 0.3% Triton X-100 (Thermo Fisher Scientific) for 10 min at room temperature. Later on, cells were blocked with 1% BSA (Thermo Fisher Scientific) for 30 min, and then incubated with the Ki67 antibody (Abcam Cambridge, MA, USA) at 4°C overnight. Subsequently, cells were incubated with a goat anti-rabbit secondary antibody (1:3000, Abcam) at 37°C for 1 h. Finally, a microscope (Leica, Buffalo Grove, IL, USA) was applied to image the fluorescence signals. Nuclei was counterstained with DAPI.

Luciferase reporter assay

The 3'-UTR of the wild-type (WT) SNHG15 and mutant (MT) SNHG15 were synthesized and inserted into pmirGLO luciferase reporter vectors (Promega, Madison, WI, USA). Then, WT-SNHG15 or MT-SNHG15 plasmid was co-transfected with miR-183-5p agomir and NC respectively in SH-SY5Y cells for 48 h using lipofectamine 2000. In addition, the 3'-UTR of the WT-FOXO1 and MT-FOXO1 were synthesized and inserted into pmirGLO luciferase reporter vectors (Promega, Madison, WI, USA). After that, WT-FOXO1 or MT-FOXO1 plasmid was co-transfected with miR-183-5p agomir and NC respectively in SH-SY5Y cells for 48 h using lipofectamine 2000. Later on, luciferase activity in SH-SY5Y cells were assessed using the dual luciferase reporter assay system (Promega) and renilla luciferase activity was used as an endogenous control.

RNA pulldown assay

SNHG15 were labeled with desthiobiotinylation using the Pierce RNA 3' End Desthiobiotinylation Kit (Thermo Fisher Scientific). After that, RNA pull-down assay was conducted using the Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific) according to the manufacturer's protocols.

Reactive oxygen species (ROS) detection

SH-SY5Y cells were stained with 10 mM 2, 7-dichlorofluorescin diacetate (DCFH-DA, KeyGen Biotech Co. Ltd., Jiangsu, China) at 37°C for 30 min in darkness. After that, the cells were washed twice with PBS. Subsequently, a flow cytometer (BD Biosciences) was used to assess the fluorescent signals.

ELISA

Samples of the supernatants were collected from SH-SY5Y cells, and commercial ELISA kits were used to measure GSH and SOD contents (#A005-1, #A001-3; Jiancheng, Bioengineering Institute, Nanjing, China) in the supernatants of SH-SY5Y cells.

JC-1 staining

SH-SY5Y cells were incubated with JC-1 staining reagent (2 mL; Beyotime) for 20 min in darkness at 37°C. Following washing twice with PBS, the JC-1 fluorescence was determined using a flow cytometer (BD Biosciences).

Western blot

Equivalent amounts of proteins were subjected to 10% SDS-PAGE, and then transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). After that, the membrane was blocked with 5% non-fat milk in TBST for 1 h at room temperature, and then the membrane was incubated overnight at 4°C with the following primary antibodies against: Bcl-2 (1:1000), active caspase 9 (1:1000), active caspase 3 (1:1000), FOXO1 (1:1000), $p27^{kip1}$ (1:1000), CDK2 (1:1000), Cyclin E1 (1:1000), p-Akt (1:1000), Akt (1:1000) and β-actin (1:1000). Subsequently, the membrane was incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (1:3000) at room temperature for 1 h. Then, the bands were visualized using an enhanced chemiluminescent substrate (ECL) kit (Thermo Fisher Scientific). All antibodies were purchased from Abcam.

Animal study

8-week-old Sprague-Dawley rats were obtained from Shanghai SLAC Animal Center. The animal used and experimental protocols were approved by the Animal care Committee of the Second Hospital of Hebei Medical University, and all studies were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were anaesthetized with 10% chloral hydrate (350 mg/kg, intraperitoneal injection), followed by exposing the right common carotid artery, and external carotid artery (ECA), and internal carotid artery (ICA). After that, a 6/0 surgical nylon monofilament with a rounded tip was inserted into the ICA through right ECA, and gently advanced to occlude the middle cerebral artery (MCA). After 2 h of middle cerebral artery occlusion (MCAO), reperfusion was allowed by retracting the surgical nylon monofilament. The rats were allowed to recover for 7 days. These rats were randomly divided into the 3 groups (n = 6): Sham group; I/R group; I/R + SNHG15 siRNA3 group. SNHG15 siRNA3 was injected into the mouse cortex of mice before MCAO operation. The sham-operated animals were manipulated in the same manner but without the surgical nylon monofilament insertion.

The rats were euthanized at 7 days and the brains were sliced into 3-mm thick serial coronal sections. After that, the sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C. The infarct area was estimated by IPP program. The percentage of infarct volume was calculated as described previously [17].

Determination of cerebral water content

The wet weight of ischemic hemispheres was weighed. After that, the brain tissues were incubated in an oven at 105°C until no further decrease in weight. Later on, the dry weight of ischemic hemispheres was weighed. Subsequently, brain water content was calculated according to the formula: (wet weight - dry weight)/wet weight × 100%.

TUNEL assay

TUNEL Colorimetric IHC Detection Kit (Thermo Fisher Scientific) was applied to assess cell apoptosis in brain tissues. The images of TUNEL-positive cells were observed using a microscope (Leica).

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (version 7.0, La Jolla, CA, USA). The results of RT-qPCR, western blot, CCK-8, immunofluorescence, and flow cytometry assays were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's tests. Data are presented as the mean ± standard deviation (S.D.). All data were repeated in triplicate. The results were considered significant at *P<0.05.

Results

The expression of SNHG15 is upregulated in OGD/R-treated SH-SY5Y cells

To evaluate the effect of SNHG15 on neuronal cells with I/R injury *in vitro*, an OGD/R cell model (SH-SY5Y) was established to mimic cerebral I/R injury. As shown in **Figure 1A**, 4 h OGD and 72 h reperfusion decreased the cell viability to about 50%. In addition, OGD 4 h/reperfusion 72 h upregulated the percentage of apoptotic cells (~40%) compared with controls (~3%) (**Figure 1B**). These results indicated that OGD/R could induce neuronal death. Importantly, OGD 4 h/reperfusion 72 h notably upregulated the level of SNHG15 in SH-SY5Y cells (**Figure 1C**). Thus, we chose OGD 4 h/reperfusion 72 h as the optimum time point for the following experiments. These data suggested that the expression of SNHG15 is upregulated in OGD/R treated SH-SY5Y cells.

Downregulation of SNHG15 protected OGD/R-induced neuronal cell death

To explore the role of SNHG15 knockdown on SH-SY5Y cells subjected to OGD/R, we used three different siRNAs (SNHG15 siRNA1, SNHG15 siRNA2, SNHG15 siRNA3) to downregulate SNHG15 in cells. As indicated in **Figure 2A**, SNHG15 level was notably lower in SH-SY5Y cells transfected with SNHG15 siRNA2 or SNHG15 siRNA3 plasmids than in those transfected with siRNA-control plasmids. In addition, the results of CCK-8 and immunofluorescence assays indicated that SNHG15 knockdown during reperfusion in SH-SY5Y cells reversed OGD/R-triggered cytotoxicity; as

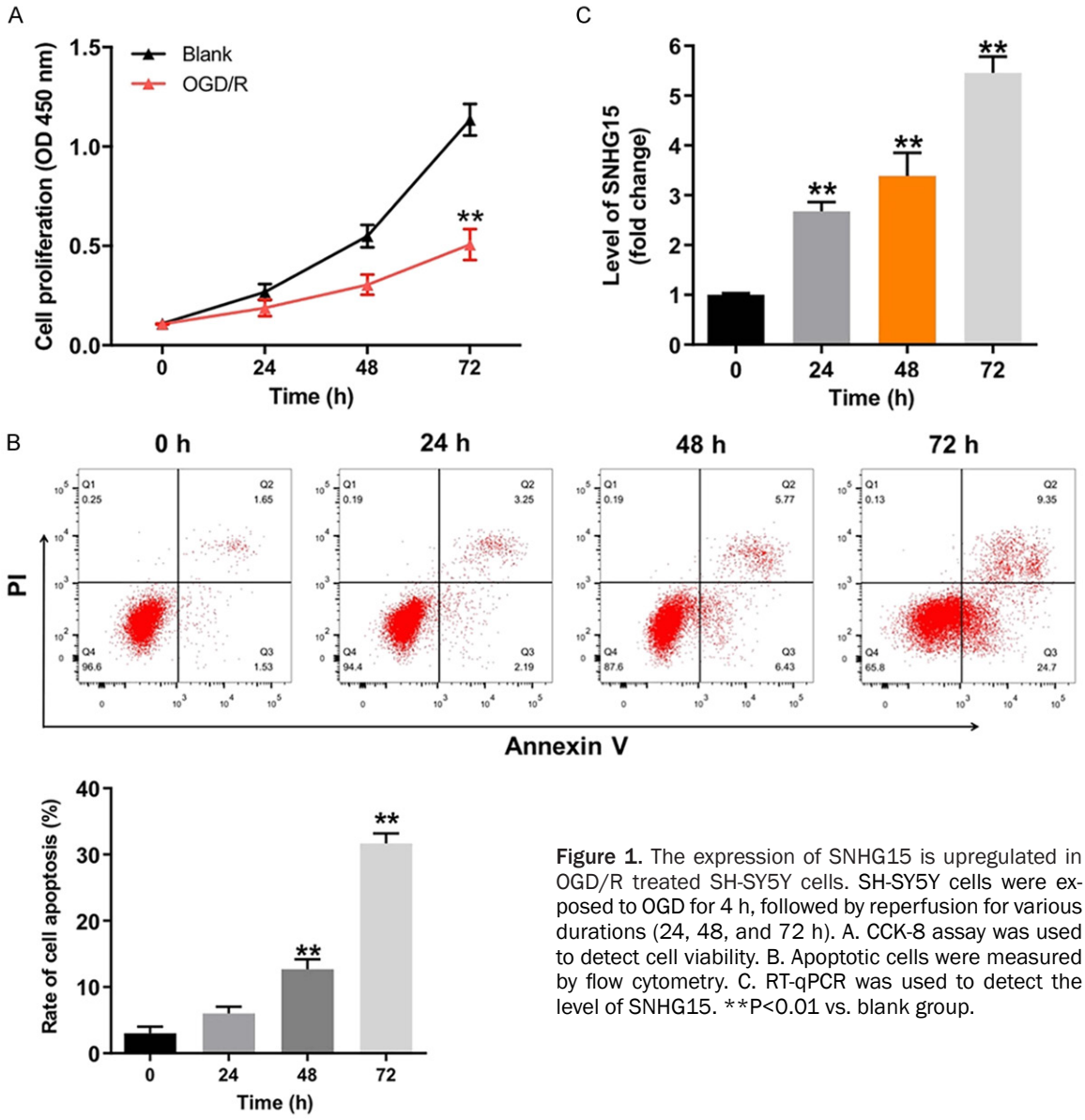


Figure 1. The expression of SNHG15 is upregulated in OGD/R treated SH-SY5Y cells. SH-SY5Y cells were exposed to OGD for 4 h, followed by reperfusion for various durations (24, 48, and 72 h). A. CCK-8 assay was used to detect cell viability. B. Apoptotic cells were measured by flow cytometry. C. RT-qPCR was used to detect the level of SNHG15. **P<0.01 vs. blank group.

shown not only by a marked increase in cell viability but also by a marked increase in the number of Ki67-positive cells (**Figure 2B-D**). These data revealed that downregulation of SNHG15 could protect OGD/R-induced neuronal cell death.

SNHG15 functions as a ceRNA of miR-183-5p in neuronal cells

Starbase (http://starbase.sysu.edu.cn) was used to identify the miRNAs interacting with SNHG15. The potential binding sites for miR-183-5p in SNHG15 were predicted, indicating that SNHG15 might be a potential target of miR-183-5p (**Figure 3A**). In addition, the level of miR-183-5p was significantly increased in SH-SY5Y cells after transfection with miR-183-5p agomir, while miR-183-5p antagomir markedly reduced the level of miR-183-5p in SH-SY5Y cells (**Figure 3B**). Moreover, the results of dual luciferase reporter assay indicated that overexpression of miR-183-5p significantly decreased the luciferase activity in SH-SY5Y cells co-transfected with SNHG15-WT; however, overexpression of miR-183-5p caused on change in luciferase activity in SH-SY5Y cells co-transfected with SNHG15-MT (**Figure 3C**). Meanwhile, the results of RNA pull-down and RT-qPCR manifested that SNHG15

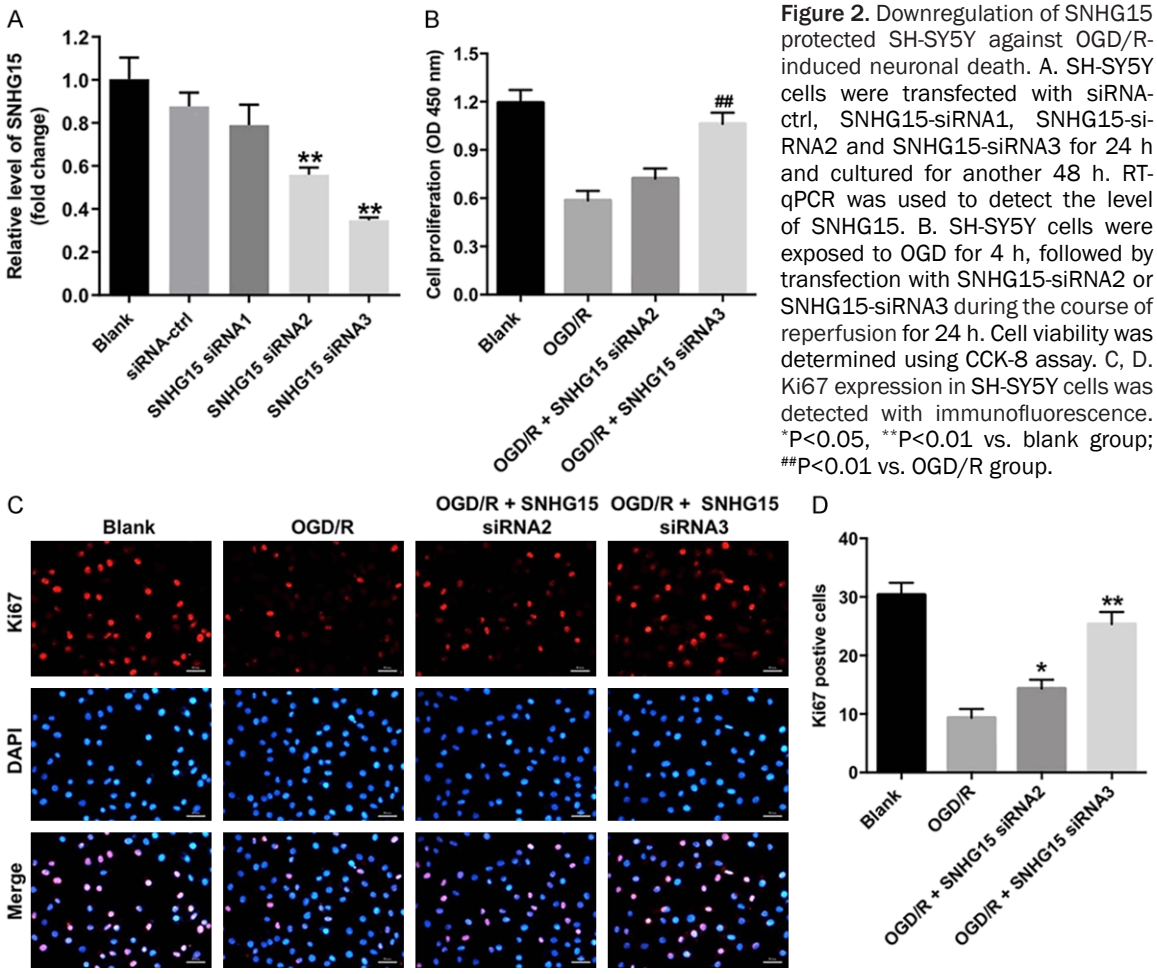


Figure 2. Downregulation of SNHG15 protected SH-SY5Y against OGD/R-induced neuronal death. A. SH-SY5Y cells were transfected with siRNA-ctrl, SNHG15-siRNA1, SNHG15-siRNA2 and SNHG15-siRNA3 for 24 h and cultured for another 48 h. RT-qPCR was used to detect the level of SNHG15. B. SH-SY5Y cells were exposed to OGD for 4 h, followed by transfection with SNHG15-siRNA2 or SNHG15-siRNA3 during the course of reperfusion for 24 h. Cell viability was determined using CCK-8 assay. C, D. Ki67 expression in SH-SY5Y cells was detected with immunofluorescence. $^{*}P<0.05$, $^{**}P<0.01$ vs. blank group; $^{\#\#}P<0.01$ vs. OGD/R group.

was pulled down by biotin-labeled miR-183-5p, indicating miR-183-5p directly interacted with SNHG15 (**Figure 3D** and **3E**). These data illustrated that SNHG15 directly binding to miR-183-5p in neuronal cells.

Knockdown of SNHG15 attenuated OGD/R-induced oxidative stress in neuronal cells via miR-183-5p

To determine whether SNHG15 knockdown could regulate oxidative stress in OGD/R-treated SH-SY5Y cells, intracellular ROS production and the levels of GSH and SOD were detected. As indicated in **Figure 4A-C**, OGD/R remarkably increased ROS production, but obviously reduced the levels of GSH and SOD in cells, indicating OGD/R administration could induce the oxidative stress in SH-SY5Y cells. Conversely, SNHG15 knockdown notably reversed OGD/R-induced oxidative stress in SH-SY5Y cells (**Figure 4A-C**). Importantly, inhibitory effect of SNHG15 knockdown on oxidative stress caused by OGD/R insult was reversed by the treatment with miR-183-5p antagomir (**Figure 4A-C**). These results suggested that downregulation of SNHG15 could attenuate OGD/R-triggered oxidative stress in neuronal cells via miR-183-5p.

Downregulation of SNHG15 abolished OGD/R-triggered apoptosis in neuronal cells via miR-183-5p

To assess whether SNHG15 downregulation could affect mitochondrial function in neuronal cells during OGD/R insult, MMP was detected using JC-1 staining assay. As indicated in **Figure 5A**, OGD/R injury markedly reduced MMP in cells, whereas downregulation of SNHG15 prevented the loss of MMP in OGD/R-treated SH-SY5Y cells. When SH-SY5Y cells under OGD/R were treated with SNHG15 siRNA3 together with miR-183-5p antagomir,

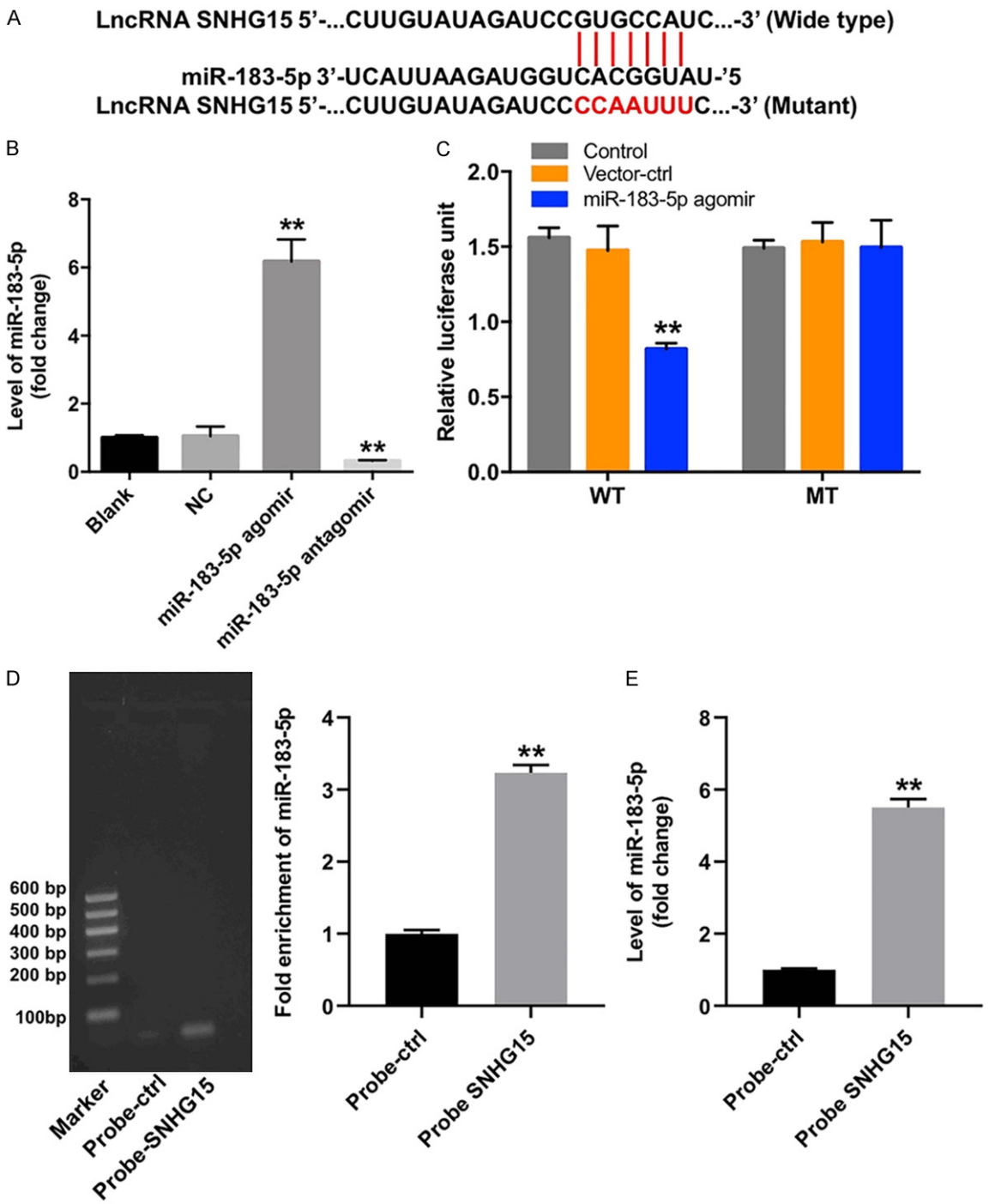


Figure 3. SNHG15 functions as a ceRNA of miR-183-5p in SH-SY5Y cells. A. The putative binding sites of miR-183-5p on SNHG15. B. SH-SY5Y cells were transfected with NC, miR-183-5p agomir and miR-183-5p antagomir for 72 h. The level of miR-183-5p in cells was detect using RT-qPCR assay. **P<0.01 vs. NC group. C. Luciferase assay of cells transfected with SNHG15-WT or SNHG15-MT reporter together with miR-183-5p or NC. **P<0.01 vs. vector-ctrl group. D. RNA pull-down analysis determined the interaction of miR-183-5p and SNHG15. E. The level of miR-183-5p in SH-SY5Y cells was detect using RT-qPCR assay. **P<0.01 vs. probe-ctrl group.

the effect of SNHG15 on MMP was reversed by miR-183-5p knockdown (**Figure 5A**). In addition, downregulation of SNHG15 notably attenuated OGD/R-induced apoptosis in SH-SY5Y

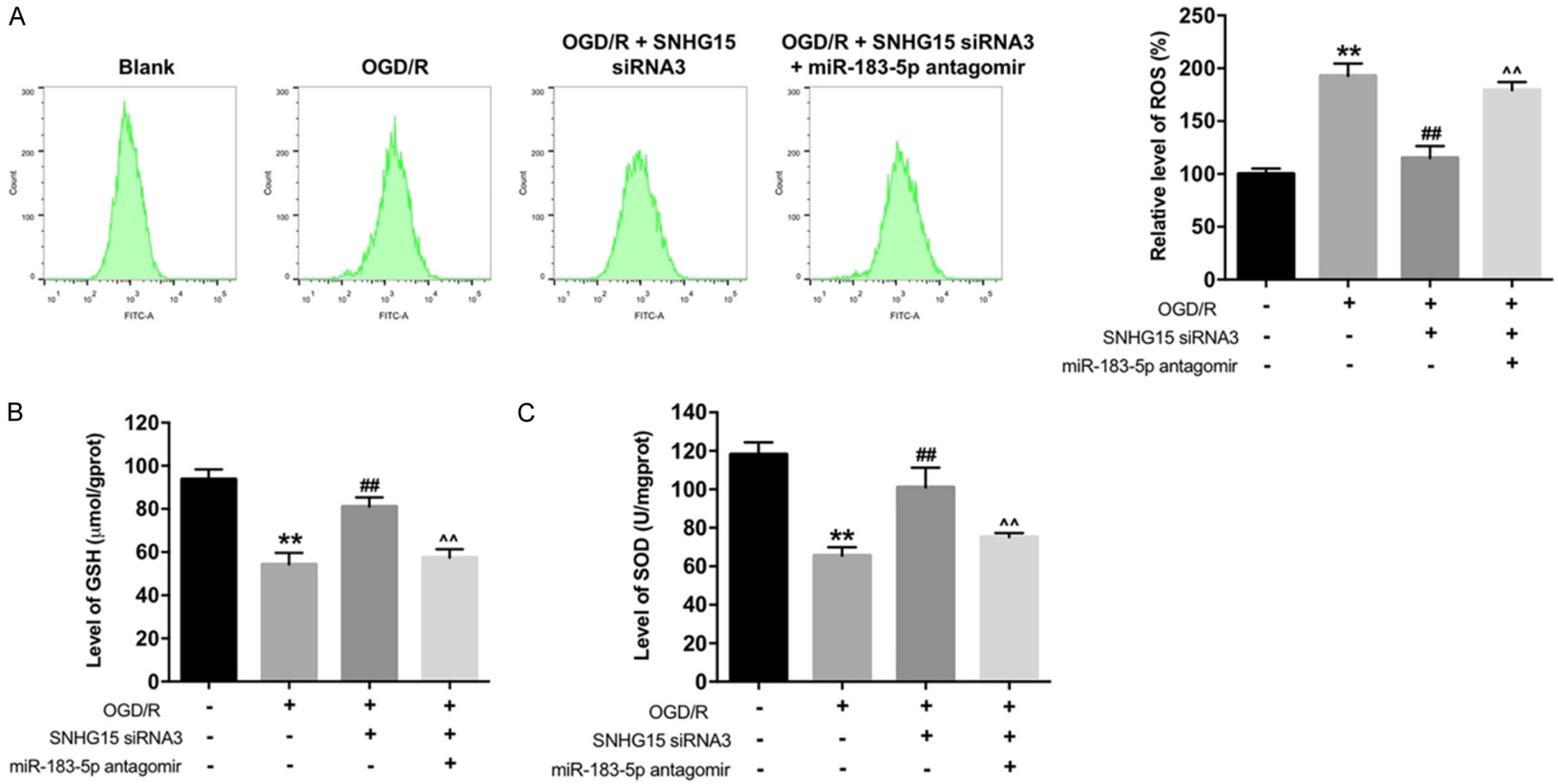


Figure 4. Downregulation of SNHG15 attenuated OGD/R-triggered oxidative stress in neuronal cells via miR-183-5p. SH-SY5Y cells were exposed to OGD for 4 h, followed by transfection with SNHG15-siRNA3 for 24 h in the presence or absence of the miR-183-5p antagomir during the course of reperfusion. A. ROS production was assessed by flow cytometry. B, C. ELISA was used to detect the levels of GSH and SOD in the supernatants of SH-SY5Y cells. $^{**}P<0.01$ vs. blank group; $^{\#\#}P<0.01$ vs. OGD/R group; $^{\wedge\wedge}P<0.01$ vs. OGD/R + SNHG15 siRNA3 group.

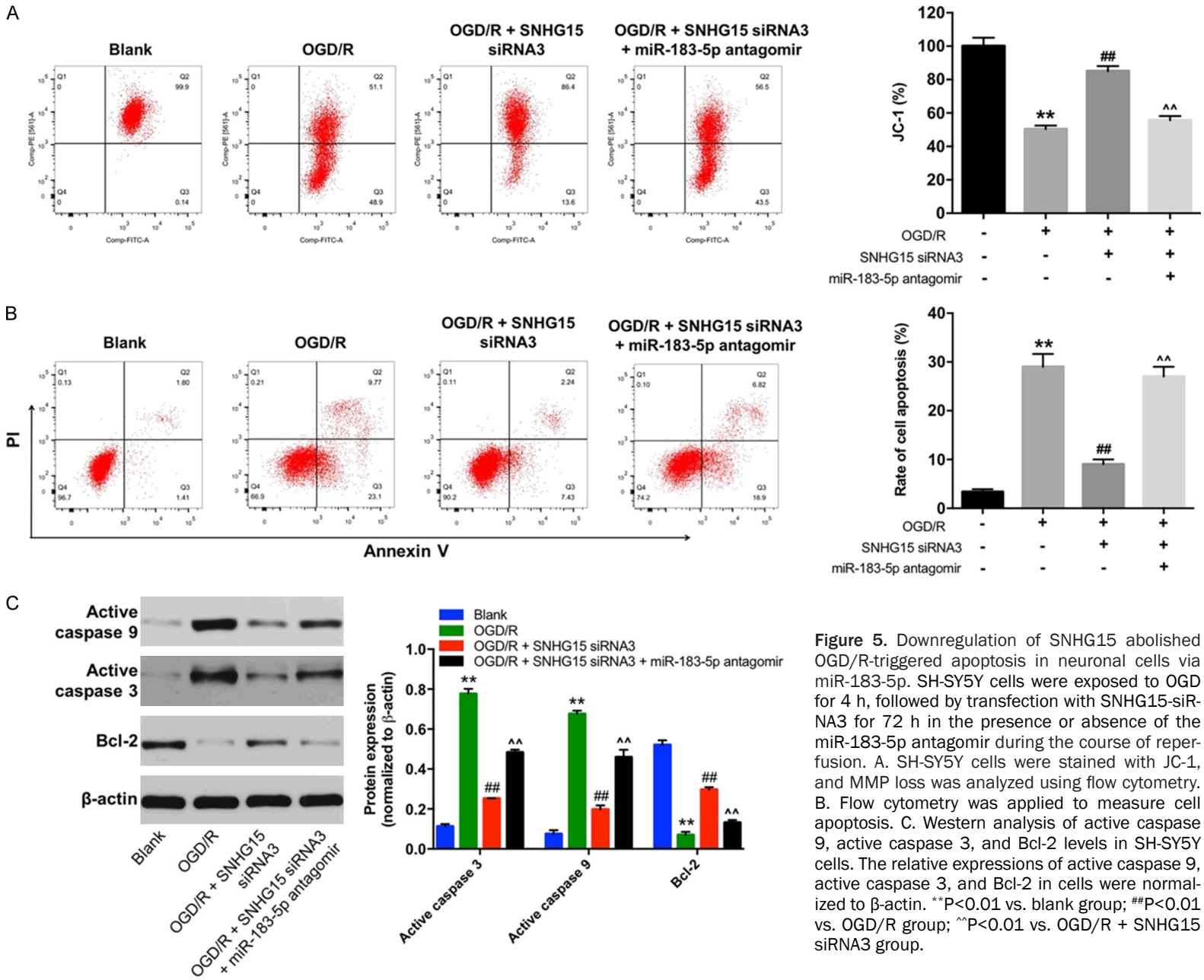


Figure 5. Downregulation of SNHG15 abolished OGD/R-triggered apoptosis in neuronal cells via miR-183-5p. SH-SY5Y cells were exposed to OGD for 4 h, followed by transfection with SNHG15-siRNA3 for 72 h in the presence or absence of the miR-183-5p antagomir during the course of reperfusion. A. SH-SY5Y cells were stained with JC-1, and MMP loss was analyzed using flow cytometry. B. Flow cytometry was applied to measure cell apoptosis. C. Western analysis of active caspase 9, active caspase 3, and Bcl-2 levels in SH-SY5Y cells. The relative expressions of active caspase 9, active caspase 3, and Bcl-2 in cells were normalized to β-actin. $^{**}P<0.01$ vs. blank group; $^{\#\#}P<0.01$ vs. OGD/R group; $^{\wedge\wedge}P<0.01$ vs. OGD/R + SNHG15 siRNA3 group.

cells; however, that effect was reversed by miR-183-5p knockdown (**Figure 5B**). Moreover, knockdown of SNHG15 downregulated the expressions of active caspase 9 and active caspase 3 proteins, and upregulated the expression of Bcl-2 in OGD/R-treated SH-SY5Y cells; however, these changes were reversed by miR-183-5p knockdown (**Figure 5C**). These data illustrated that downregulation of SNHG15 could abolish OGD/R-triggered apoptosis in neuronal cells via miR-183-5p.

Downregulation of SNHG15 ameliorated OGD/R-induced injury in neuronal cells by targeting miR-183-5p/FOXO1 axis

The data from TargetScan (http://www.targetscan.org/vert_71/) dataset revealed that FOXO1 might be a potential target of miR-183-5p (**Figure 6A**). The results of dual luciferase reporter assay showed that overexpression of miR-183-5p significantly decreased the luciferase activity in SH-SY5Y cells co-transfected with FOXO1-WT, while overexpression of miR-183-5p caused on change in luciferase activity in cells co-transfected with FOXO1-MT (**Figure 6B**). In addition, miR-183-5p agomir significantly decreased the level of FOXO1 in SH-SY5Y cells, while miR-183-5p antagomir notably increased the level of FOXO1 in cells (**Figure 6C**). Moreover, OGD/R markedly increased the expressions of FOXO1 and $p27^{Kip1}$, and notably decreased the levels of CDK2, Cyclin E1 and p-Akt in cells; however, these OGD/R-induced changes were reversed when cells were transfected with SNHG15 siRNA3 (**Figure 6D**). Conversely, additional of miR-183-5p antagomir reversed the effect of SNHG15 siRNA3 on the expressions of FOXO1, $p27^{Kip1}$, CDK2, Cyclin E1 and p-Akt in SH-SY5Y cells subjected to OGD/R insult (**Figure 6D**). Meanwhile, the results of cell cycle assay revealed that the percentage of cells were notably decreased in G0-G1 phase but increased in S and G2-M phase in OGD/R-treated SH-SY5Y cells with SNHG15 knockdown, compared with OGD/R group; however, that effect was reversed in the presence of miR-183-5p antagomir (**Figure 6E**). These results suggested that downregulation of SNHG15 could ameliorate OGD/R-induced injury in neuronal cells by targeting miR-183-5p/FOXO1 axis.

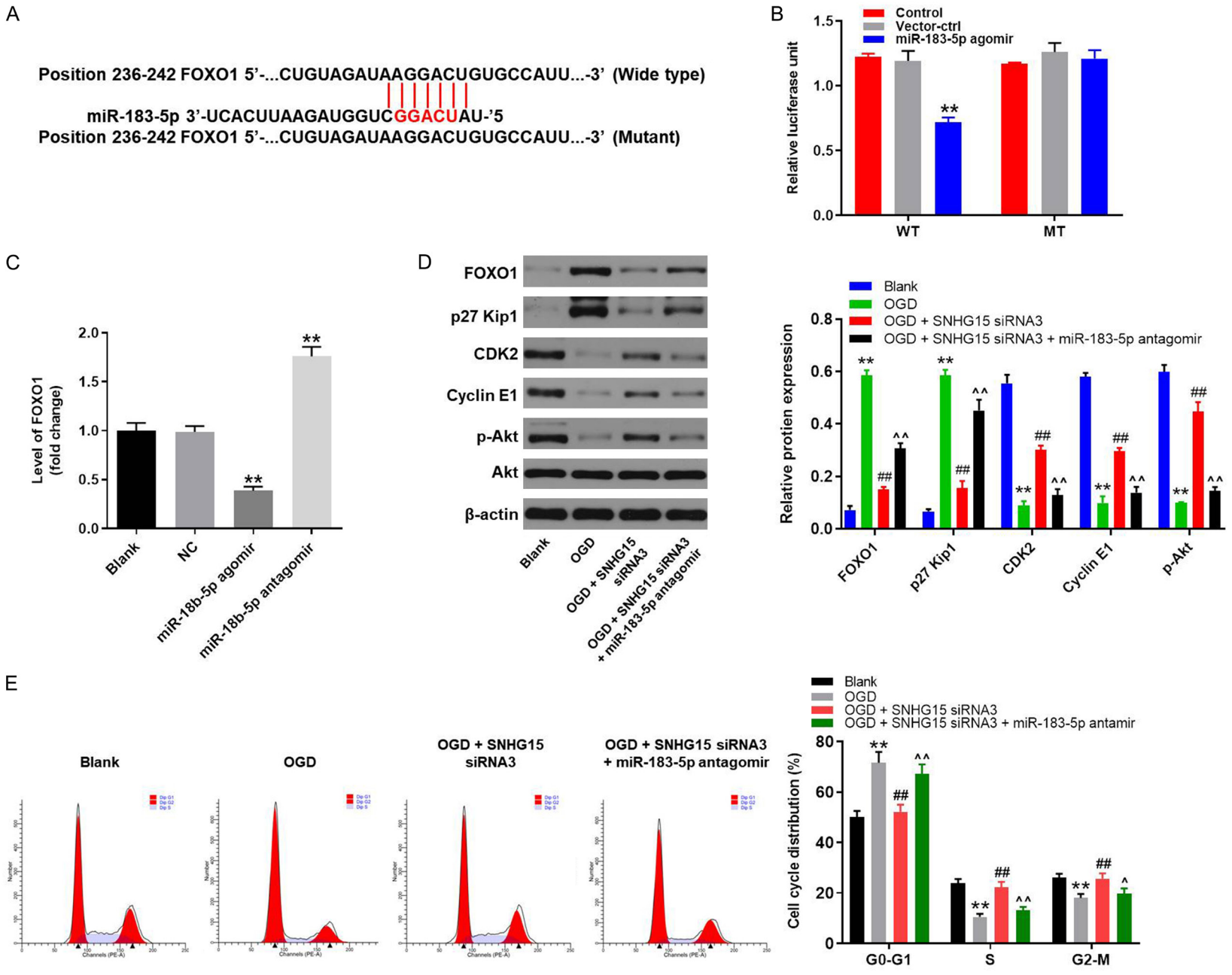
Downregulation of SNHG15 protected against brain damage after cerebral I/R in rats

To test the function of SNHG15 in I/R injury *in vivo*, TTC staining assay was applied to assess the rat focal cerebral ischemia. As indicated in **Figure 7A** and **7B**, administration of SNHG15 siRNA3 significantly decreased infarct fraction (15%) compared to I/R group (28%). In addition, compared with I/R group, SNHG15 knockdown reduced the cerebral water content in MCAO rats (**Figure 7C**). Moreover, to investigate the role of SNHG15 knockdown on neural apoptosis *in vivo*, TUNEL assay was applied. As indicated in **Figure 7D**, a significant number of TUNEL-positive cells was observed in the brain slice of rats after MCAO/R injury, compared with sham group. As expected, downregulation of SNHG15 markedly decreased MCAO-induced neuronal apoptosis in rats (**Figure 7D**). These data indicated that downregulation of SNHG15 could protect against brain damage after cerebral I/R injury in rats.

Discussion

In recent years, increasing evidences have indicated that lncRNAs were considered as novel diagnostic biomarkers and promising therapeutic targets for IS [18, 19]. Deng et al indicated that the level of SNHG15 was increased in patients with IS [13]. However, the function of SNHG15 in cerebral ischemia has not been demonstrated. To the best of our knowledge, SNHG15 has been found to act as an oncogene that inhibits apoptosis and promotes proliferation and invasion in multiple human cancer cell types [20-22]. In this study, we demonstrated the novel role of SNHG15 in IS. We found that the level of SNHG15 was upregulated in OGD/R-treated SH-SY5Y cells. In addition, downregulation of SNHG15 protected OGD/R-induced neuronal death in SH-SY5Y cells *in vitro*. Moreover, downregulation of SNHG15 protected against I/R-induced brain damage *in vivo*. These data indicated that SNHG15 knockdown might have neuroprotective effect against OGD/R-induced injury and MCAO/R-induced brain injury.

Evidence has been shown that OGD/R could mimics *in vitro* I/R injury and could result in mitochondrial damage, ROS overproduction, and the upregulation of proapoptotic factors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OGD
OGD + SNHG15 siRNA3
OGD + SNHG15 siRNA3 + miR-183-5p antamir
Cell cycle distribution (%)
G0-G1
S
G2-M

Figure 6. Downregulation of SNHG15 ameliorated OGD/R-triggered injury in neuronal cells via targeting miR-183-5p/FOXO1 axis. A. The putative binding sites of miR-183-5p on FOXO1. B. Luciferase assay of SH-SY5Y cells transfected with FOXO1-WT or FOXO1-MT reporter together with miR-183-5p or NC. **P<0.01 compared with vector-ctrl group. C. SH-SY5Y cells were transfected with NC, miR-183-5p agomir and miR-183-5p antagomir for 72 h. RT-qPCR was used to detect the level of FOXO1. **P<0.01 compared with NC group. D. SH-SY5Y cells were exposed to OGD for 4 h, followed by transfection with SNHG15-siRNA3 for 72 h in the presence or absence of the miR-183-5p antagomir during the course of reperfusion. Western analysis of FOXO1, $p27^{Kip1}$, CDK2, Cyclin E1 and p-Akt levels in SH-SY5Y cells. The expressions of FOXO1, $p27^{Kip1}$, CDK2 and Cyclin E1 in cells were normalized to β-actin. The level of p-Akt was normalized to AKT. E. Cell cycle distribution was determined using flow cytometry. **P<0.01 vs. blank group; ##P<0.01 vs. OGD/R group; ^P<0.05, ^^P<0.01 vs. OGD/R + SNHG15 siRNA3 group.

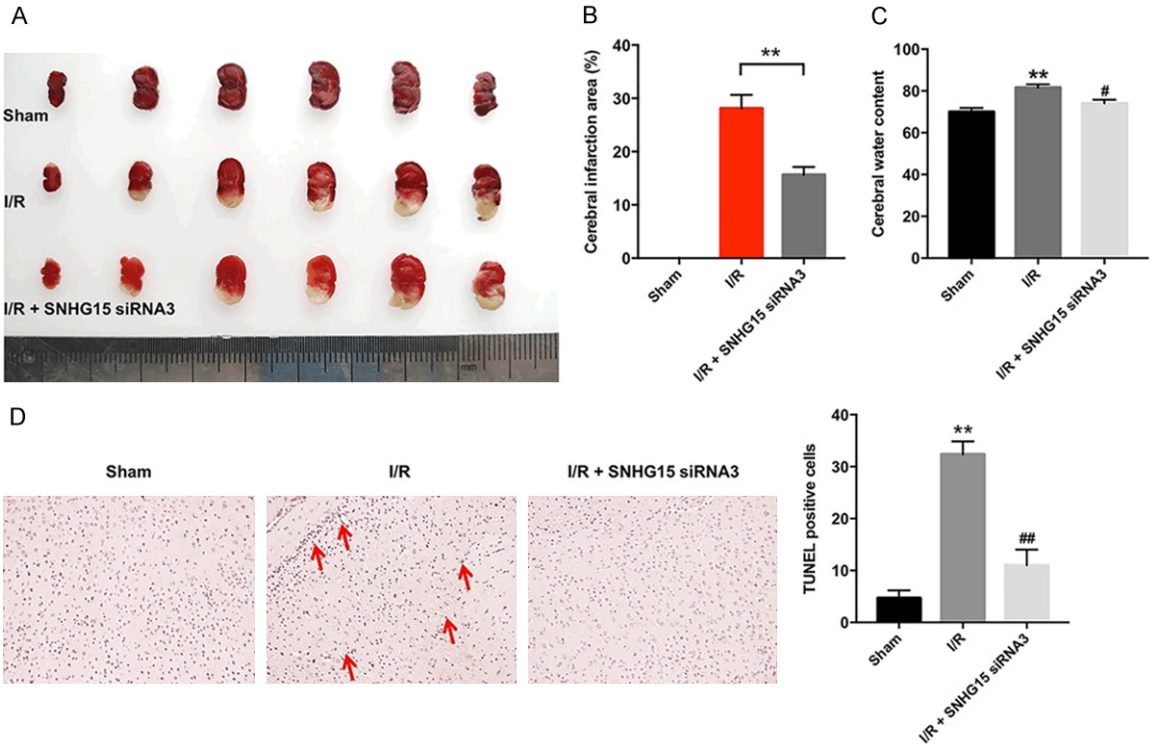

Figure 7. Downregulation of SNHG15 protected against brain damage after cerebral I/R in rats. A, B. The brain infarction was detected by TTC staining (white: infract area; magenta: healthy tissue). C. Brain water content was calculated. D. Apoptotic cell death in I/R rats challenged with MCAO was measured by TUNEL staining. **P<0.01 vs. sham group; #P<0.05, ##P<0.01 vs. I/R group.

[23]. In addition, mitochondria are the major site of ROS generation in human cells [24]. However, dysfunctional mitochondria are not only a major source of excessive production of toxic ROS but a main trigger of mitochondria-mediated apoptosis pathway [25]. MMP is a key indicator of mitochondrial function, and OGD/R significantly reduced MMP in PC12 neuronal cells [26]. In this study, downregulation of SNHG15 restrained the loss of MMP in OGD/R-treated SH-SY5Y cells, indicating that SNHG15 knockdown could improve the mitochondrial function. Additionally, OGD administration could increase formation of ROS and induce oxidative stress, leading to cerebral neuronal damage [26]. Zhang et al indicated that lncRNA ZFAS1 attenuated OGD/R-triggered cell death in neurons via inhibition of oxidative stress [27]. We found that downregulation of SNHG15 protected against OGD-triggered oxidative stress in SH-SY5Y cells evidenced by the decreased level of ROS and increased levels of GSH and SOD, indicating that downregulation of SNHG15 protected SH-SY5Y cells against OGD/R-induced oxidative stress. Moreover, knockdown of SNHG15 significantly decreased the expressions of active caspase 9, active caspase 3 proteins and increased the

expression of Bcl-2 in SH-SY5Y cells during the process of OGD/R, indicating that SNHG15 knockdown inhibited apoptosis in OGD/R-treated SH-SY5Y cells. Collectively, SNHG15 knockdown could effectively prevent OGD/R-induced MMP loss, accumulation of ROS and apoptosis in SH-SY5Y cells, suggesting that SNHG15 knockdown may exert neuroprotective effect through preventing mitochondrial dysfunction.

It has been shown that lncRNA could act as a ceRNA to compete against binding miRNAs, thus regulate post-transcriptional gene expression [28]. In this study, we have found that miR-183-5p is a potential binding target of SNHG15, and SNHG15 functions as a ceRNA of miR-183-5p in SH-SY5Y cells. Meanwhile, FOXO1 is a potential binding target of miR-183-5p. We found SNHG15 share common miRNA binding sites of FOXO1 mRNA. Roser et al indicated that the increased expression of miR-183-5p is accompanied by the decreased expression of FOXO1 and the upregulation of PI3K/Akt signaling in dopaminergic midbrain neurons [29]. FOXO1 has been found to be associated with cell apoptosis, cell cycle progression and oxidative stress [30, 31]. Knockdown of FOXO1 could promote cell proliferation and cell cycle transition [32]. In this study, SNHG15 knockdown downregulated the expressions of FOXO1 and $p27^{Kip1}$ in SH-SY5Y cells under OGD/R, indicating that downregulation of SNHG15 attenuated OGD/R-triggered cell cycle arrest. However, these changes were reversed by miR-183-5p antagomir. These data indicated that d SNHG15 knockdown could protect neuronal cells against OGD/R-induced damage by regulating the SNHG15/miR-183-5p/FOXO1 axis.

Conclusion

In conclusion, for the first time, this study revealed that SNHG15 knockdown could protect against cerebral I/R injury by targeting miR-183-5p/FOXO1 axis. Thus, targeting the SNHG15/miR-183-5p/FOXO1 axis might represent a potential therapeutic option for the treatment of IS.

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Disclosure of conflict of interest

None.

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