

Long non-coding RNA H19 inhibition ameliorates oxygen-glucose deprivation-induced cell apoptosis and inflammatory cytokine expression by regulating the microRNA-29b/SIRT1/PGC-1 α axis

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Abstract. As one of the earliest discovered long non-coding (lnc)RNAs, lncRNA H19 imprinted maternally expressed transcript (H19) participates in regulating ischemic stroke. The present study aimed to investigate the combined roles of lncRNA H19, microRNA (miR)-29b, silent mating-type information regulation 2 homolog 1 (SIRT1) and peroxisome proliferator-activated receptor- γ co-activator-1 α (PGC-1 α) following ischemic stroke. lncRNA H19 expression levels in the middle cerebral artery occlusion (MCAO) mouse model and HT22 cells subjected to oxygen-glucose deprivation (OGD) were detected via reverse transcription-quantitative PCR (RT-qPCR). H19 small interfering RNA was used to knock-down H19 expression. Following OGD treatment, MTT, flow cytometry, ELISA, RT-qPCR and western blotting assays were performed to assess cell proliferation, cell apoptosis, inflammatory cytokine concentrations, and lncRNA H19, miR-29b, SIRT1, PGC-1 α expression levels, respectively. In the present study, MCAO model mice and OGD-treated cells displayed significantly increased lncRNA H19 expression levels compared with sham mice and control cells, respectively. lncRNA H19 knockdown ameliorated OGD-induced cell apoptosis and increases in inflammatory cytokine concentrations. Furthermore, lncRNA H19 knockdown also attenuated OGD-mediated downregulation of miR-29b, SIRT1 and PGC-1 α expression levels. Collectively, the results of the

present study demonstrated that lncRNA H19 knockdown ameliorated OGD-induced cell apoptosis and increases in inflammatory cytokine concentrations by regulating miR-29b, SIRT1 and PGC-1 α expression levels, which suggested the potential role of lncRNA H19 in ischemic stroke.

Introduction

As a major cause of death and disability worldwide, ischemic stroke is a serious clinical condition with poor prognosis (1). At present, tissue plasminogen activator is the only accepted treatment used in the clinic, but long-term use leads to reperfusion injury; therefore, the identification of novel therapeutic strategies for reperfusion injury, including neuroprotection, neurogenesis and angiogenesis, is important (2). By improving the current understanding of the epigenetic mechanism underlying ischemic stroke, novel strategies for the early diagnosis and treatment of ischemic stroke may be identified.

Long non-coding (lnc)RNAs, which were initially considered as noise from the translational process, are non-protein coding transcripts that are >200 nucleotides in length (3). Previous studies have revealed that lncRNAs are critical transcriptional and post-transcriptional regulators that participate in the regulation of protein expression in different types of diseases, such as cancer, osteoporosis and cardiovascular diseases (4-6). lncRNAs are important and effective regulators of disease progression and various biological activities, such as angiogenesis, macrophage M2 polarization and inflammatory responses (3). Increasing evidence also demonstrated that lncRNAs serve important regulatory roles in cell differentiation and tissue regeneration (7,8). lncRNAs have been reported to serve important roles in the cerebrovascular system (9). Several specific lncRNAs, including lncRNA H19 imprinted maternally expressed transcript (H19), were confirmed to be upregulated in cerebral ischemic model animals and oxygen-glucose deprived (OGD) cells (10-12), promoting cell apoptosis, angiogenesis, inflammation and cell death.

As one of the best characterized lncRNA, lncRNA H19 is a maternally imprinted gene that is primarily expressed during embryonic development (13). However, under certain pathological conditions, including tissue regeneration, carcinogenesis

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and hypoxia, H19 expression is reactivated (14-18). Hypoxia induces cerebral ischemia and reperfusion injury, followed by stimulating lncRNA H19 expression via activating hypoxia induced factor 1 α (19). In a previous study, lncRNA H19 expression levels were significantly upregulated in patients who had suffered from a stroke compared with healthy controls, displaying a high diagnostic value (9). In addition, lncRNA H19 knockdown promoted microglial M1 to M2 polarization by downregulating histone deacetylase 1 in OGD-treated BV2 microglial cells (9). Moreover, an association among the SNP in H19, rs217727 and the higher risk of ischemic stroke was identified (20).

lncRNA H19 acts on various microRNAs (miRNAs/miRs), including let-7, miR-22, miR-141, miR-183, miR-200a and miR-29b, resulting in the restoration of the target genes of the miRNAs (21,22). A previous study demonstrated that lncRNA H19 directly targeted miR-29b to activate TGF- β 1 signaling, further accelerating tenogenic differentiation and promoting tendon healing (23). Furthermore, lncRNA H19 mediates the protective effect of hypoxic post-conditioning against hypoxia-reoxygenation injury by inhibiting miR-29b-3p expression in aged cardiomyocytes (24). Therefore, it was hypothesized that there might be an association between lncRNA H19 and miR-29b in regulating ischemic stroke. As an NADC-dependent protein deacetylase, silent mating-type information regulation 2 homolog 1 (SIRT1) serves important roles in metabolic regulation and adaptation (25). SIRT1 regulates inflammation, oxidative stress, autophagy and cell apoptosis via deacetylation of various transcription factors, including peroxisome proliferator-activated receptor- γ co-activator-1 α (PGC-1 α) (26,27).

The present study investigated whether there were alterations in lncRNA H19 expression levels in the middle cerebral artery occlusion (MCAO) mouse model and OGD-treated HT22 cells. Subsequently, the effects of lncRNA H19 knockdown on OGD-induced expression levels of inflammatory cytokines, miR-29b, SIRT1 and PGC-1 α levels were also assessed.

Materials and methods

Animals. All experimental animal procedures were approved by the Animal Ethics Committee of the Tianjin Medical University. C57BL/6 mice (male; age, 10-12 weeks; weight, 21-23 g; n=8 in each group; Charles River Laboratories, Inc.) were housed at 22 \pm 2°C and 50 \pm 15% relative humidity and a 12-h light/dark cycle with adequate food and water.

MCAO model. Animals were randomly divided into the following two groups (n=10 per group): i) MCAO; and ii) sham-operated. To establish the MCAO mouse model, mice were anesthetized by the intraperitoneal injection of 45 mg/kg sodium pentobarbital (2%). An uncoated 6-0 monofilament nylon suture (diameter, 0.20 mm) was inserted to occlude the MCA for 1 h. Subsequently, the suture was removed for 24 h of reperfusion. Mice in the sham-operated group underwent the same procedure, but the suture was not inserted. Mice were euthanized 24 h following ischemia by intraperitoneal injection of 150 mg/kg sodium pentobarbital (2%). Death was verified by dilated pupils and cessation of the heartbeat. The

infarct ipsilateral hemisphere brain was isolated and used for subsequent experiments.

Transfection and establishment of the OGD model. The HT22 mouse hippocampal neuronal cell line was purchased from Procell Life Science & Technology Co., Ltd. Cells were incubated in a humidified incubator in a normal culture medium containing DMEM solution (Gibco; Thermo Fisher Scientific, Inc.) mixed with 10% fetal serum (Gibco; Thermo Fisher Scientific, Inc.) and 7.5% horse serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO₂. HT22 cells were seeded at a density of 4 \times 10⁵ cells/well were transfected with 100 mmol/l H19 small interfering (si)RNA or scrambled siRNA negative control (si-NC) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 37°C with 5% CO₂. The siRNA sequences were as follows: i) H19 siRNA, 5'-CCCUCAGAUGAAAGAAUUTTAUUCU UUCAUCUUGAGGGT-3'; ii) si-NC, 5'-UUCUCCGAA CGUGUCACGUTT-3'. Subsequently, HT22 cells were exposed to OGD to mimic ischemic-like conditions. Briefly, cells in the OGD group were cultured in a hypoxic incubator with 95% N₂ and 5% CO₂ for 3, 6 or 9 h. For reperfusion, cells were transferred to normal culture medium for 24 h and kept at 37°C in an incubator with 5% CO₂.

MTT assay. HT22 cell viability was assessed by performing an MTT assay. Briefly, HT22 cells were seeded (5 \times 10⁴ cells/ml) into 96-well plates. Following OGD and H19 siRNA transfection, 20 μ l MTT (5 mg/ml) was added to each well and incubated for 4 h at 37°C. Subsequently, 150 μ l DMSO was used to dissolve the purple formazan. The absorbance was measured at a wavelength of 490 nm using a microplate reader. Cell viability in the control group was set at 100% and cell viability in OGD and H19 siRNA transfection groups were normalized to the control group.

Flow cytometry analysis. Flow cytometry was performed to detect the rate of apoptosis. Cell apoptosis was assessed using the Annexin V-FITC/propidium iodide double staining kit (Beijing Solarbio Science & Technology, Co., Ltd.) according to the manufacturer's protocol.

ELISA. The concentrations of inflammatory cytokines, including interleukin (IL)-6, IL-1 β , TNF- α , IL-10 and TGF- β 1, were measured using ELISA kits (R&D Systems, Inc.) according to the manufacturer's protocols.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from mouse brain tissue and HT22 cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using the PrimeScript RT Master Mix kit (Takara Biotechnology Co., Ltd.). Subsequently, qPCR was performed using the SYBR Premix Ex Taq II kit (Takara Biotechnology Co., Ltd.). The following primers were used for qPCR: H19 forward, 5'-GTCAAACAGGGCAAGATGGG-3' and reverse, 5'-ATTACGGTGGGTGGGATGTT-3'; miR-29b forward, 5'-CAGACCTGTAGCACCATTGAA-3' and reverse, 5'-TATCCTTGTTACGACTCCTTCAC-3'; SIRT1 forward, 5'-TCCTTGAGACTGCGATGTT-3' and reverse, 5'-ATATGA AGAGGTGTTGGTGGC-3'; PGC-1 α forward, 5'-CAATAC

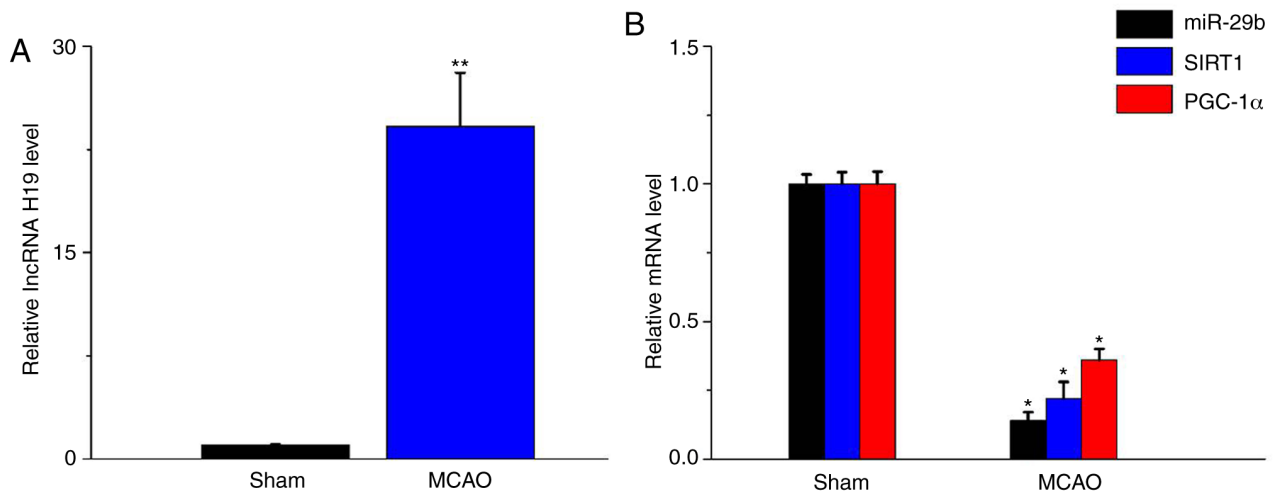


Figure 1. IncRNA H19, miR-29b, SIRT1 and PGC-1 α expression levels in the MCAO mouse model. (A) IncRNA H19, (B) miR-29b, SIRT1 and PGC-1 α expression levels were determined by performing reverse transcription-quantitative PCR. Data are presented as the mean \pm SD. * P <0.05 and ** P <0.01 vs. sham. IncRNA, long non-coding RNA; H19, H19 imprinted maternally expressed transcript; miR, microRNA; SIRT1, silent mating-type information regulation 2 homolog 1; PGC-1 α , peroxisome proliferator-activated receptor- γ co-activator-1 α ; MCAO, middle cerebral artery occlusion.

CTCATGGGACAGCG-3' and reverse, 5'-GCCTCCAGGGAAAGCAA-3'; U6 forward, 5'-CGCTTCGGCAGCACATATAC-3' and reverse, 5'-AAATATGGAACGCTTCACGA-3' and GAPDH forward, 5'-AGGTCTGGTGTGAACGGATTTG-3' and reverse 5'-TGTAGACCATGTAGTTGAGGTCA-3'. All samples were run in triplicate. miRNA and mRNA expression levels were quantified using the $2^{-\Delta\Delta C_q}$ method and normalized to the internal reference genes U6 and GAPDH, respectively.

Western blotting. Total protein was extracted from HT22 cells. Protein concentrations were determined using a Protein Assay kit (Bio-Rad Laboratories, Inc.). Proteins were separated via SDS-PAGE and transferred onto nitrocellulose membranes, which were blocked with 5% skimmed milk in TBS-Tween-20 (TBST) at room temperature for 1 h. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies targeted against: SIRT1 (1:1,000; GeneTex, Inc.), PGC-1 α (1:500; GeneTex, Inc.) and GAPDH (1:5,000; OriGene Technologies, Inc.). Following washing three times for 10 min each time, the membranes were incubated with a goat anti-rabbit secondary antibody (1:2,000; GeneTex, Inc.) at room temperature for 1 h. The membranes were washed three times with TBST for 10 min each time. Protein expression was semi-quantified via densitometry (Bio-Rad Laboratories, Inc.) with GAPDH as the loading control.

Statistical analysis. Data are presented as the mean \pm SD. Comparisons among multiple groups were analyzed using one-way ANOVA analysis followed by the Bonferroni post hoc test. P <0.05 was considered to indicate a statistically significant difference.

Results

IncRNA H19 expression levels are upregulated, whereas miR-29b, SIRT1 and PGC-1 α expression levels are down-regulated in the MCAO mouse model. To investigate whether IncRNA H19 mRNA expression level were altered in ischemic

brain tissue isolated from the MCAO mouse model, RT-qPCR was performed. The results indicated that IncRNA H19 expression levels were significantly elevated in ischemic brain tissue isolated from the MCAO mouse model compared with the sham group (P <0.05; Fig. 1A). Furthermore, miR-29b, SIRT1 and PGC-1 α expression levels were significantly decreased in the MCAO mouse model compared with the sham group (P <0.05; Fig. 1B).

OGD treatment induces cytotoxicity and increases IncRNA H19 expression levels in HT22 cells. To investigate the effect of OGD treatment on HT22 cells, cell viability and IncRNA H19 expression levels were measured following OGD treatment at different times. Following treatment for 3, 6 or 9 h, OGD significantly decreased HT22 cell viability in a time-dependent manner compared with the control group (P <0.05; Fig. 2A). The RT-qPCR results indicated that IncRNA H19 expression levels were significantly elevated in OGD-treated cells compared with control cells (P <0.05; Fig. 2B). The aforementioned results indicated that elevated IncRNA H19 mRNA expression levels may aggravate cerebral ischemia injury.

H19 knockdown relieves OGD-induced HT22 cell cytotoxicity. To investigate the role of IncRNA H19 in OGD-induced cytotoxicity, H19 siRNA was used to knock down IncRNA H19 expression. H19 siRNA significantly decreased IncRNA H19 expression levels compared with the si-NC group (P <0.05; Fig. S1). The MTT assay results demonstrated that OGD treatment for 6 h significantly reduced cell viability compared with the control group (P <0.05), which was significantly ameliorated by H19 knockdown (P <0.05; Fig. 3A). Flow cytometry was performed to evaluate the effects of IncRNA H19 on cell apoptosis. The results indicated that OGD treatment for 6 h significantly increased cell apoptosis compared with the control group (P <0.05), which was also significantly ameliorated by H19 knockdown (P <0.05; Fig. 3B). The results suggested that OGD may induce cell injury by increasing IncRNA H19 expression levels.

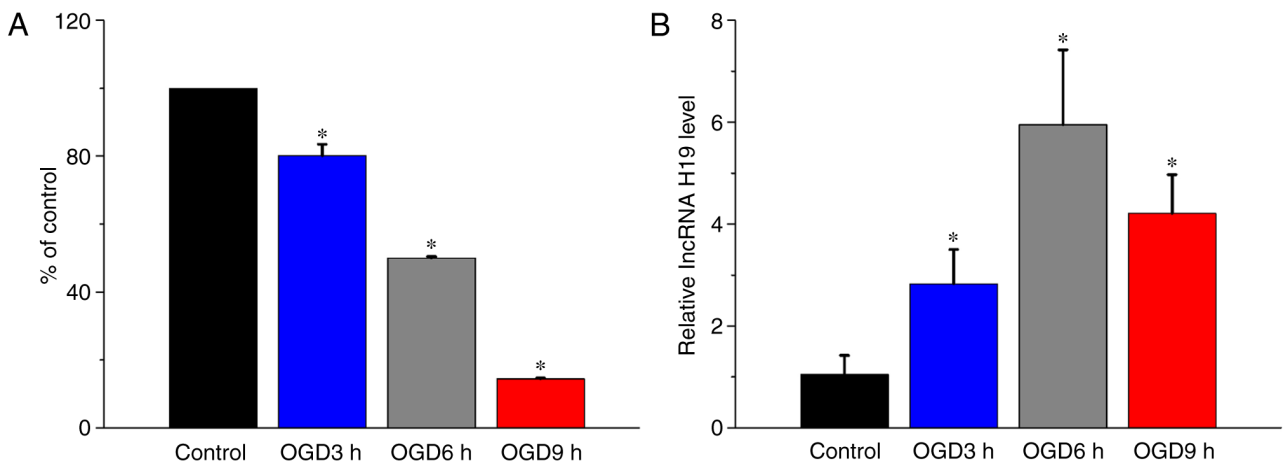


Figure 2. lncRNA H19 expression in OGD-treated HT22 cells. Effect of OGD treatment on (A) cell viability and (B) lncRNA H19 mRNA expression levels. Data are presented as the mean \pm SD. * P <0.05 vs. control. lncRNA, long non-coding RNA; H19, H19 imprinted maternally expressed transcript; OGD, oxygen-glucose deprivation.

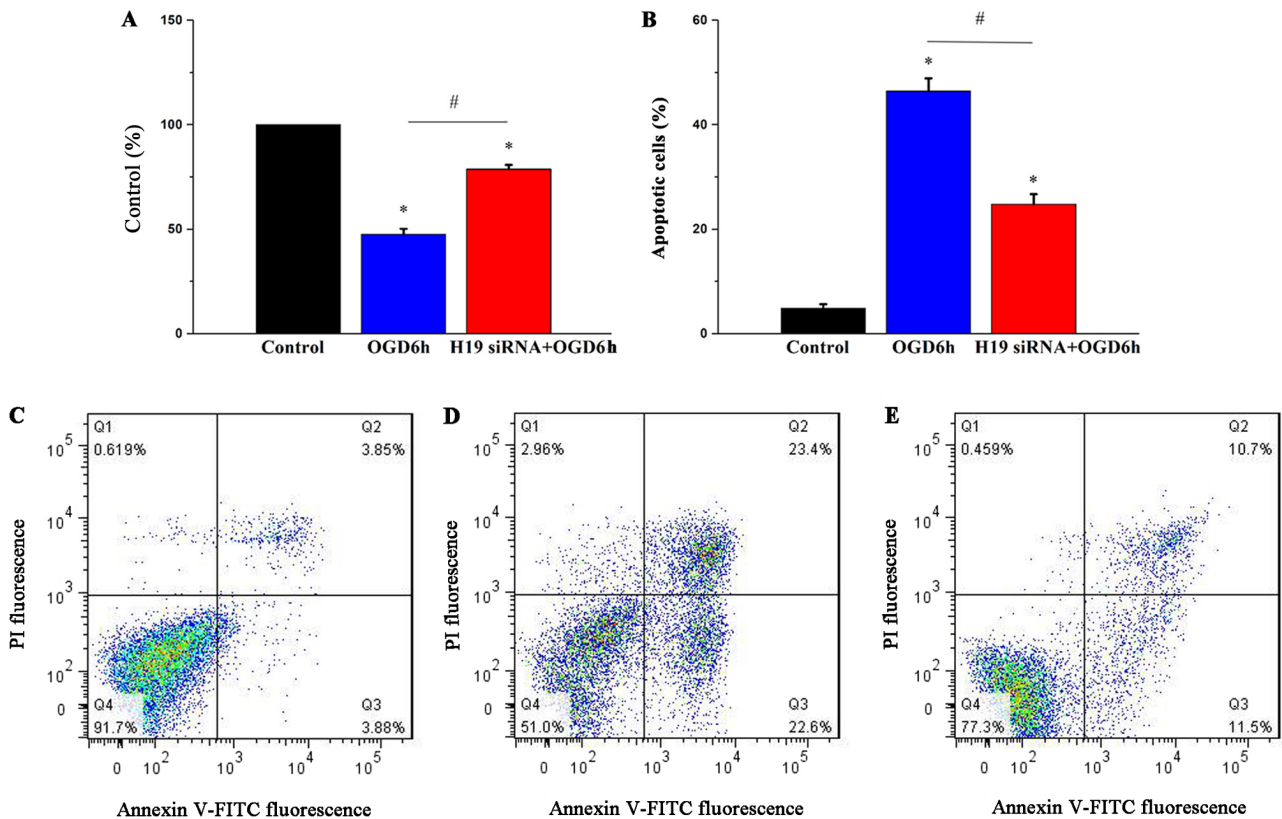


Figure 3. Effect of lncRNA H19 knockdown on cell viability and apoptosis in OGD-treated cells. Effect of lncRNA H19 knockdown on cell (A) viability and (B) apoptosis. Representative scatterplots for the (C) control, (D) OGD 6 h and (E) OGD 6 h + H19 siRNA groups. Data are presented as the mean \pm SD. * P <0.05 vs. control; # P <0.05 vs. OGD 6 h. lncRNA, long non-coding RNA; H19, H19 imprinted maternally expressed transcript; OGD, oxygen-glucose deprivation; siRNA, small interfering RNA.

H19 knockdown decreases inflammatory cytokine concentrations in HT22 cells. Increasing evidence has demonstrated that inflammatory cytokines participate in the pathogenesis of cerebral ischemia (28,29). In the present study, the concentrations of several inflammatory cytokines were detected to investigate whether H19 knockdown altered the inflammatory response in HT22 cells (Fig. 4). IL-6, IL-1 β , TNF- α , IL-10 and TGF- β 1 concentrations were significantly increased following

OGD treatment for 6 h compared with the control group (P <0.05). However, H19 knockdown significantly inhibited OGD-induced increases in IL-6, IL-1 β , TNF- α and IL-10 concentrations following treatment for 6 h (P <0.05). IL-10 is an anti-inflammatory cytokine (30). IL-10 concentrations were significantly increased by OGD treatment for 6 h compared with the control group (P <0.05), which suggested a potential self-protection or compensation mechanism. The results

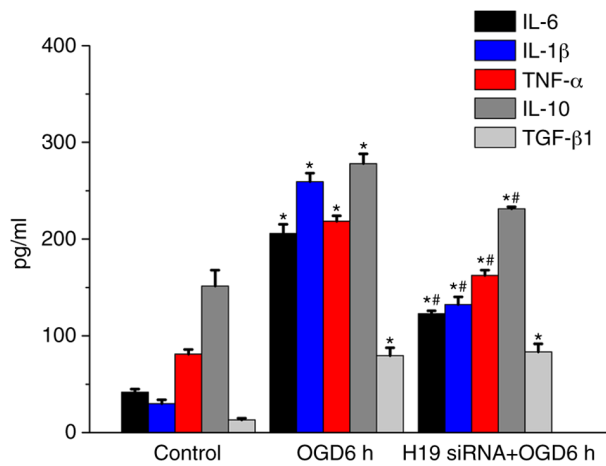


Figure 4. Effect of long non-coding RNA H19 imprinted maternally expressed transcript knockdown on inflammatory cytokine concentrations in OGD-treated cells. Inflammatory cytokine concentrations were measured by performing ELISAs. Data are presented as the mean \pm SD. * P <0.05 vs. control; # P <0.05 vs. OGD 6 h. OGD, oxygen-glucose deprivation; siRNA, small interfering RNA.

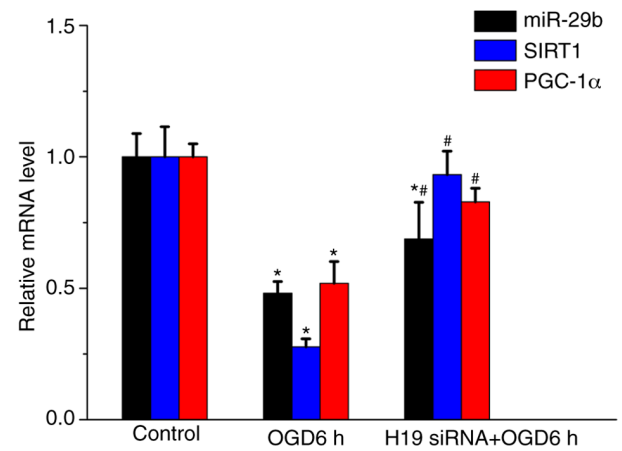


Figure 5. Effect of long non-coding RNA H19 imprinted maternally expressed transcript knockdown on miR-29b, SIRT1 and PGC-1α expression levels in OGD-treated cells. miR-29b, SIRT1 and PGC-1α expression levels were measured by performing reverse transcription-quantitative PCR. Data are presented as the mean \pm SD. * P <0.05 vs. control; # P <0.05 vs. OGD 6 h. miR, microRNA; SIRT1, silent mating-type information regulation 2 homolog 1; PGC-1α, peroxisome proliferator-activated receptor-γ co-activator-1α; OGD, oxygen-glucose deprivation; siRNA, small interfering RNA.

indicated that lncRNA H19 may prompt the inflammatory response in ischemia stroke.

H19 knockdown alters miR-29b, SIRT1 and PGC-1α expression levels in OGD-treated HT22 cells. The effects of lncRNA H19 on miR-29b, SIRT1 and PGC-1α expression levels in OGD-treated HT22 cells were investigated by performing RT-qPCR and western blotting. Following OGD treatment for 6 h, miR-29b, SIRT1 and PGC-1α expression levels were significantly decreased compared with the control group (P <0.05); however, OGD-mediated effects on expression were significantly inhibited by H19 knockdown (P <0.05; Fig. 5). Consistent with the RT-qPCR results, the western blotting results demonstrated that OGD treatment for 6 h significantly decreased SIRT1 and PGC-1α protein expression levels compared with the control group (P <0.05; Fig. 6). However, H19 knockdown significantly increased SIRT1 and PGC-1α expression levels compared with the OGD 6 h group (P <0.05).

Discussion

In the present study, lncRNA H19 expression levels in the MCAO mouse model and OGD-treated HT22 cells were investigated. The results demonstrated that lncRNA H19 mRNA expression levels in MCAO model mice and OGD-treated cells were significantly elevated compared with sham mice and control cells, respectively. Moreover, in OGD-treated cells, H19 knockdown significantly increased cell viability and significantly decreased cell apoptosis. H19 knockdown also significantly increased miR-29b, SIRT1 and PGC-1α expression levels, and significantly decreased inflammatory cytokine concentrations in OGD-treated cells. Collectively, the results of the present study indicated a potential role of lncRNA H19 in regulating the OGD-induced immune response, which may participate in subsequent pathological processes. However, the results of the present study should be verified using patient samples in future studies.

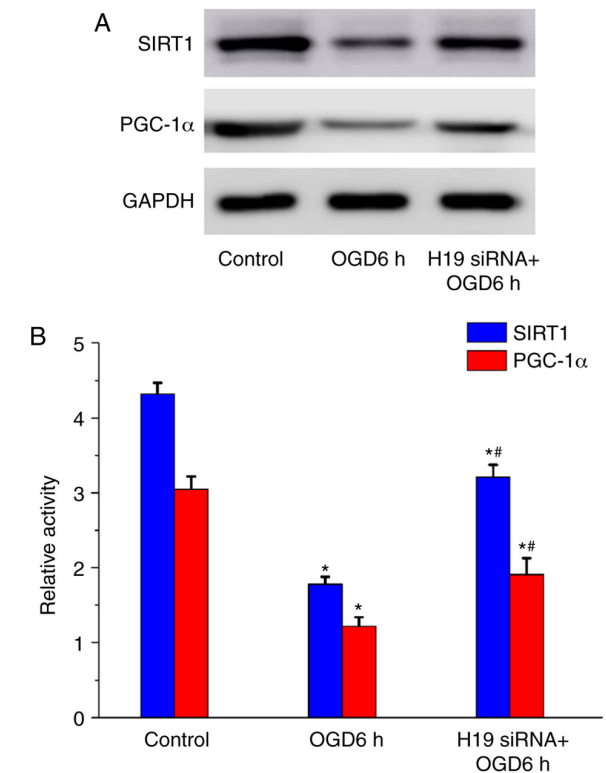


Figure 6. Effect of long non-coding RNA H19 imprinted maternally expressed transcript knockdown on SIRT1 and PGC-1α protein expression levels in OGD-treated cells. Protein expression levels were (A) determined via western blotting and (B) semi-quantified. Data are presented as the mean \pm SD. * P <0.05 vs. control; # P <0.05 vs. OGD 6 h. SIRT1, silent mating-type information regulation 2 homolog 1; PGC-1α, peroxisome proliferator-activated receptor-γ co-activator-1α; OGD, oxygen-glucose deprivation; siRNA, small interfering RNA.

Ischemic stroke remains one of the leading causes of morbidity and mortality worldwide (31), with multiple causes leading to neuronal injury. Emerging evidence indicates that

inflammation serves an important role in the pathogenesis of brain ischemia (29,32). In particular, several specific lncRNAs have been reported to participate cerebral ischemia-induced cell apoptosis, inflammation, cell death and angiogenesis (10). An example is lncRNA H19, which is abundantly expressed in embryonic development and growth control, but down-regulated following birth (14). However, the expression of lncRNA H19 can be reactivated under certain specific conditions, such as tumorigenesis and oxidative stress (17,18,33). Wang *et al* (9) reported that lncRNA H19 expression levels were elevated in patients who had suffered from a stroke compared with healthy controls. In the present study, lncRNA H19 expression was significantly upregulated in MCAO model mice and OGD-treated HT22 cells compared with sham mice and control cells, respectively. In addition, H19 knockdown protected cells against OGD-induced cell apoptosis. However, the possible regulatory mechanism underlying lncRNA H19 is not completely understood.

A previous study demonstrated that lncRNA H19 regulated autophagy via the dual specificity phosphatase 5/ERK1/2 axis in ischemic stroke (20). Wang *et al* (34) reported that H19 knockdown promotes the transcriptional activity of p53, resulting in increased Notch1 expression levels in ischemic stroke. lncRNA H19 serves as a competing endogenous RNA by sponging miRNA in several types of carcinoma, such as cholangiocarcinoma, oral squamous cell carcinoma, osteosarcoma (18,35,36). miRNAs, including the miR-29 family, serve a vital role during cerebral ischemia (37,38). In the present study, miR-29b expression levels were significantly decreased in the MCAO mouse model and OGD-treated HT22 cells compared with the sham group and control cells, respectively. In addition, H19 knockdown attenuated OGD-mediated alterations in miR-29b expression levels, which suggested that lncRNA H19 may affect ischemic stroke by regulating miR-29b.

As a key member of the sirtuin family of NAD⁺-dependent enzymes, SIRT1 serves a pivotal role in cerebral ischemia. SIRT1-overexpression mice display less hippocampal damage following severe ischemic damage (39). Several compounds exert a neuroprotective effect against cerebral ischemia by activating or upregulating SIRT1 (40,41). SIRT1 could directly phosphorylate and deacetylate PGC-1 α to form a transcription complex, which may control the expression of specific metabolic genes (42,43). Elevated PGC-1 α expression levels could protect against neuronal death during cerebral ischemia (44). Consistent with a previous study (45), SIRT1 and PGC-1 α mRNA and protein expression levels were significantly reduced in the MCAO mouse model and OGD-treated HT22 cells compared with the sham group and control cells, respectively. H19 knockdown inhibited OGD-induced cell apoptosis and suppressed OGD-mediated downregulation of SIRT1 and PGC-1 α , which suggested a neuroprotective effect against ischemic injury. The present study also indicated that elevated SIRT1 and PGC-1 α mRNA and protein expression levels may protect against neuronal death during cerebral ischemia. However, a limitation of the present study was that the expression levels of apoptosis- and inflammation-related genes were not measured using an animal model; therefore, further investigation is required.

In conclusion, the present study demonstrated that H19 knockdown ameliorated OGD-induced cell apoptosis and

increases in inflammatory cytokine concentrations, which suggested an immunomodulatory effect of lncRNA H19 in ischemic stroke. Furthermore, to the best of our knowledge, the present study demonstrated for the first time that H19 knockdown also attenuated OGD-mediated downregulation of miR-29b, SIRT1 and PGC-1 α expression levels, which indicated that lncRNA H19 may participate in neuroprotection by regulating miR-29b, SIRT1 and PGC-1 α expression levels.

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Availability of data and materials

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

Authors' contributions

JX supervised and designed the study. CW, FM and PX performed the experiments. JX revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental animal procedures were approved by The Animal Ethics Committee of Tianjin Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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