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Original Article

Silencing lncRNA GABPB1-AS1 alleviates cerebral ischemia reperfusion injury through the miR-641/NUCKS1 axis

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Abstract: Objective: To investigate the possible mechanism of lncRNA GA binding protein transcription factor beta subunit 1 antisense RNA 1 (GABPB1-AS1) in cerebral ischemia/reperfusion (CI/R) injury. Methods: RT-qPCR was applied to determine GABPB1-AS1 expression in oxygen-glucose deprivation/reoxygenation (OGD/R) cells. The targeting relationships between GABPB1-AS1 and miR-641, as well as between miR-641 and nuclear casein and cyclin-dependent kinase substrate 1 (NUCKS1) were examined by dual luciferase reporter assay. The protein expression of caspase-3, Bax, Bcl-2 and NUCKS1 was examined by western blot. Cell apoptosis was measured by flow cytometry (FCM) and western blot. Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results: GABPB1-AS1 was significantly elevated in SH-SY5Y cells under OGD/R. Downregulation of GABPB1-AS1 accelerated cell viability and suppressed cell apoptosis. GABPB1-AS1 silencing reduced ROS and MDA levels in OGD/R-treated cells. Furthermore, miR-641 inhibitor aggravated damage from OGD/R, but GABPB1-AS1 silencing notably attenuated this effect. NUCKS1 was proven to be a target gene of miR-641. Conclusion: GABPB1-AS1 silencing alleviated CI/R injury through the miR-641/NUCKS1 axis, indicating that GABPB1-AS1 might serve as a therapeutic target for CI/R injury.

Keywords: Cerebral ischemia reperfusion, GABPB1-AS1, miR-641, NUCKS1

Introduction

The incidence of ischemic cerebrovascular disease (ICD) is increasing annually, and the onset age tends to be younger. Timely restoration of blood supply to ischemic brain tissue is an effective method to treat ICD [1]. However, while reperfusion restores blood supply, pathologic responses such as blood-brain barrier destruction, cerebral edema, cerebral hemorrhage, neurological dysfunction and cell apoptosis are prone to further aggravate the brain tissue injury. Cerebral ischemia/reperfusion (CI/R) injury refers to a series of pathologic reactions caused by blood recovery after a period of cerebral ischemia, leading to more serious secondary injury [2]. Current clinical treatments of ICD remain suboptimal. Accordingly, it is urgent to conduct an in-depth study on the mechanism of IR injury, find new targets for the intervention of ischemic stroke, and develop effective prevention and treatment drugs.

Long noncoding RNAs (lncRNAs), nucleotides with transcripts greater than 200 nt in length, regulate the gene expression and function at epigenetic, transcriptional, and post-transcriptional levels. LncRNAs can not only affect normal biological processes through various regulatory mechanisms, but also participate in the progression of various diseases [3]. It has been reported that imbalances in lncRNA regulation can lead to nervous system diseases. For example, ANRIL knockdown reduced inflammation and the viability in oxygen-glucose deprivation/reoxygenation (OGD/R) cells by regulating miR-671-5p and NF-κB [4]. MALAT1 blocked cell apoptosis in OGD/R cells, though it did not significantly affect ROS level [5]. Moreover, FOXD3-AS knockdown alleviated I/R injury through the regulation of miR-765/BCL2L13 [6]. Another lncRNA, GA binding protein transcription factor beta subunit 1 antisense RNA 1 (GABPB1-AS1), which is unique to the human genome, plays a significant role in the pathology of various dis-

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Table 1. Primer sequences for RT-qPCR

Gene	Primer sequence
GABPB1-AS1	Forward: 5'-AGGGAAAGAAAATATGCCATTTCTA-3'
	Reverse: 5'-ATCATTCCGCCGCTTTCT-3'
miR-641	Forward: 5'-GCGCGAAAGACATAGGATAGAGT-3'
	Reverse: 5'-AGTGCAGGGTCCGAGGTATT-3'
NUCKS1	Forward: 5'-TACGAGTGGATGGTCAAGAG-3'
	Reverse: 5'-ATGAACTTGCTGTGTAGGGAC-3'
U6	Forward: 5'-GCTTCGGCAGCACATATACTAA-3'
	Reverse: 5'-GCTTCACAATTTGCGTGTCAT-3'
GAPDH	Forward: 5'-GCACCGTCAAGGCTGAGAAC-3'
	Reverse: 5'-TGGTGAAGACGCCAGTGGA-3'

eases [7]. Chen *et al.* discovered that GABPB1-AS1 silencing impaired cell multiplication, migration, and EMT in osteosarcoma by regulating miR-199a-3p, SP1 and Wnt/β-Catenin [8]. Besides, GABPB1-AS1 is highly expressed in glioma, and GABPB1-AS1 knockdown notably blocked cell progression by regulating miR-330 and ZNF367 [9]. In addition, GABPB1-AS1 also responds to oxidative stress and other stimuli [7]. Nevertheless, the action of GABPB1-AS1 in CI/R injury remains underexplored.

LncRNAs are known to affect the disease progression through their competitive binding to miRNAs. miRNAs are proven to play a notable role in cell growth and development by binding to complementary mRNAs to modulate target genes [10]. Numerous miRNAs have been identified as key regulators of CI/R injury. For instance, miR-10b-3p has been shown to mitigate the CI/R injury by impairing cell apoptosis [11]. Similarly, miR-98-5p suppressed cell apoptosis and oxidative stress to reduce the CI/R injury [12]. In contrast, MiR-641 which is upregulated in bladder cancer and promotes cell proliferation in that context [13], acts as a tumor suppressor in breast cancer [14] and glioma [15]. The mechanism of miR-641 in CI/R injury remains unclear. Hence, we investigated the function of miR-641 in CI/R cells and verified the relationship between GABPB1-AS1 and miR-641.

In this study, SH-SY5Y cells were subjected to OGD/R treatment to simulate IR injury *in vitro*. The function of GABPB1-AS1 on the viability in OGD/R treated cells was explored. Furthermore, the mechanism of GABPB1-AS1/miR-641/NUCKS1 axis in CI/R injury was investigated.

Materials and methods

Cell culture

SH-SY5Y cells were acquired from Tongpai Biotechnology Co., Ltd. (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) medium supplemented with 10% fetal calf serum (FBS, Nanjing SenBeiJia Biological Technology Co., Ltd., China). The culture conditions were maintained at 37°C, 5% CO_2 and saturated humidity.

To simulate I/R conditions *in vitro*, SH-SY5Y cells were cultured in glucose free medium under hypoxia (1% O_2, 5% CO_2 and 94% N_2) at 37°C. After 12, 24 and 48 h, the cells were transferred to normal medium and incubated under normal oxygen for another 24 h.

Cell transfection

In a logarithmic growth phase, the routine culture of SH-SY5Y cells was conducted in a 6-well plate at 37°C and 5% CO_2. The transfections of si-GABPB1-AS1 (5'-AUGGCUUUCCCA-ACCUAGUTT-3'), miR-641 inhibitor (5'-GAGG-UGACUCUAUCCUAUGUCUUU-3') and corresponding negative controls in SH-SY5Y cells were performed by using Lipo™2000 (Invitrogen, USA).

Real-time quantitative polymerase chain reaction (RT-qPCR) assay

The total RNA was extracted by Trizol reagent (Vazyme Biotech, China), and the RNA concentration was determined by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer (Thermo Fisher, USA). The cDNA synthesis was performed using a reverse transcription kit (Thermo Scientific, USA). The $2^{-\Delta\Delta CT}$ method was used for data analysis. Primer sequences are presented in **Table 1**.

Cell viability assay

Cells (2×10^4/well) were seeded in a 96-well plate and treated under OGD/R conditions for 24 hours. Following this, cells were incubated with 20 μL of 3-(4,5-dimethylthiazol-2-yl)-

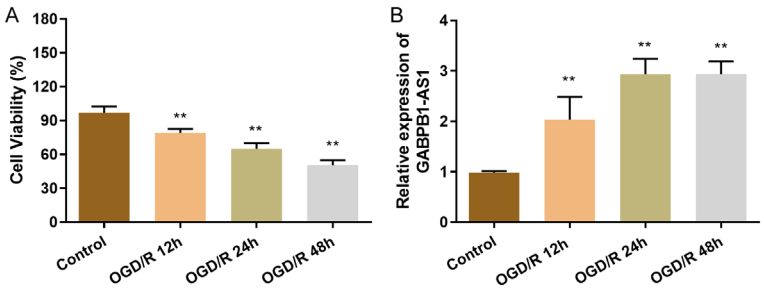


Figure 1. GABPB1-AS1 was upregulated in OGD/R treated SH-SY5Y cells. A. Cell viability in SH-SY5Y cells treated with OGD/R was detected by MTT assay. B. GABPB1-AS1 expression in SH-SY5Y cells treated with OGD/R was measured by RT-qPCR. **P<0.01. GABPB1-AS1: GA binding protein transcription factor beta subunit 1 antisense RNA 1.

2,5-diphenyltetrazolium bromide (MTT) reagent (Beyotime, Shanghai, China) for 4 hours. The original medium was then discarded, and the cells were further incubated with 150 μL of DMSO (Solarbio, Beijing, China) for 10 minutes at room temperature to dissolve the formazan crystals. The absorbance was measured at 490 nm using a SpectraMax i3x microplate reader (MD, USA). Each experimental group included five replicates per time point.

Cell apoptosis assay

The treated cells were centrifuged at 12000 r/m for 5 min and suspended by 400 μL Annexin V binding solution. Then cells were then stained with 5 μL Annexin V-FITC (Yeasen, Shanghai, China) at 4°C for 15 min in the dark. Afterwards, 10 μL PI (Yeasen, Shanghai, China) staining solution was added, and the cells were incubated for an additional 5 minutes at 4°C in the dark. The apoptosis rate of each group was tested by flow cytometry (CytoFLEX LX, Beckman Coulter, USA).

Western blot analysis

Cells were lysed on ice in cell lysate buffer for 20 min. The supernatant was collected after 10 min centrifugation at 13000 rpm/min to obtain cellular protein. The concentration of extracted protein was quantified by BCA Protein Assay Kit. After separated by SDS-PAGE gel electrophoresis (10%), the total cell protein was transferred on PVDF membrane. The membrane was blocked by 5% skim milk at room temperature for 1 hour, followed by overnight incubation at 4°C with primary antibodies targeting NUCKS1 (abs111987), Bax (abs130057), Bcl-2 (abs155595), caspase-3 (abs111175), and GAPDH (abs132004), all sourced from Absin (Shanghai, China). Then, the membrane was incubated with HRP-labeled secondary antibody (abs200002) at room temperature. The target protein was quantified by Image J (National Institutes of Health, New York, USA).

Dual-luciferase reporter assay

Mutant- and wild-type GABPB1-AS1 3'-UTR plasmids (GABPB1-AS1-Mut, GABPB1-AS1-Wt) were constructed by Zoonbio Biotechnology (Nanjing, China). SH-SY5Y cells (10^5 cells/well) were transfected with either GABPB1-AS1-Wt or GABPB1-AS1-Mut, along with miR-641 mimic or NC mimic. The luciferase activity was measured following 48 hours transfection.

Detection of oxidative stress index

Reactive oxygen species (ROS) level in SH-SY5Y cells were assessed by ELISA according to the instructions. Malondialdehyde (MDA) level was determined by thiobarbituric acid (TBA) reaction.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8.0 software (La Jolla, CA, USA). The data were expressed as mean ± standard deviation. Data analysis adopted *t* test, one way ANOVA, or repeated measures ANOVA followed by Tukey's test as appropriate. A *p*-value of less than 0.05 was considered significant.

Results

GABPB1-AS1 was upregulated in SH-SY5Y cells under OGD/R treatment

To probe into the mechanism of GABPB1-AS1 in I/R injury, OGD/R treatment was implemented in SH-SY5Y cells to simulate the I/R injury, and the efficiency was verified through MTT assay. As indicated, cell viability of SH-SY5Y cells was blocked after OGD/R treatment (**Figure 1A**). RT-qPCR showed that GABPB1-AS1 expression

was notably increased in OGD/R treated cells (**Figure 1B**). Based on these observations, we selected a 24-hour OGD treatment for subsequent experiments. These results suggest that GABPB1-AS1 is highly expressed in cells subjected to I/R, potentially playing a significant role in I/R injury.

GABPB1-AS1 silencing alleviated OGD/R-induced injury

To study the impact of GABPB1-AS1 on I/R cells, the expression of GABPB1-AS1 was down-regulated by si-GABPB1-AS1 (**Figure 2A**). Compared to OGD/R cells, cell viability was notably facilitated after transfection with si-GABPB1-AS1 (**Figure 2B**). Furthermore, cell apoptosis rate was assessed by FCM assay and western blot assay. As expected, cell apoptosis of OGD/R+si-GABPB1-AS1 cells was sharply suppressed compared with OGD/R cells (**Figure 2C**). Western blot displayed that the expression levels of Bax and Caspase-3 were reduced, while Bcl-2 expression was increased in OGD/R+si-GABPB1-AS1 cells (**Figure 2D**). Furthermore, we noticed that the levels of ROS and MDA were notably reduced by si-GABPB1-AS1 in OGD/R-SH-SY5Y cells (**Figure 2E**, **2F**). Therefore, we conclude that GABPB1-AS1 silencing can mitigate CIR injury.

GABPB1-AS1 functions as a sponge of miR-641

Analysis of the TargetScan database revealed possible binding sites for miR-641 within the 3'-UTR region of GABPB1-AS1 (**Figure 3A**). Compared to the NC group, miR-641 significantly down-regulated the luciferase activity of GABPB1-AS1-WT, but not GABPB1-AS1-MUT, confirming a binding relationship between GABPB1-AS1 and miR-641 (**Figure 3B**). In addition, miR-641 expression was clearly increased in si-GABPB1-AS1 group (**Figure 3C**). These findings suggest that GABPB1-AS1 acts as a molecular sponge for miR-641.

GABPB1-AS1 silencing slowed the damage to I/R cells by negatively regulating miR-641 expression

To further explore the functional relationship between GABPB1-AS1 and miR-641 in I/R injury, OGD/R-treated cells were transfected with either a miR-641 inhibitor or si-GABPB1-AS1 (**Figure 4A**). As indicated in **Figure 4B**, cell viability was clearly blocked by miR-641 inhibitor. However, si-GABPB1-AS1 attenuated the suppression effect of miR-641 inhibitor on cell viability (**Figure 4B**). Similarly, miR-641 inhibitor accelerated the apoptosis in OGD/R-treated cells, and GABPB1-AS1 silencing notably diminished the promoting effect (**Figure 4C**). As expected, western blot results also showed that GABPB1-AS1 silencing down-regulated Bax and Caspase-3 expression but up-regulated Bcl-2 expression in OGD/R and miR-641 inhibitor treated cells (**Figure 4D**). Furthermore, ROS and MDA levels were notably reduced in the OGD/R+inhibitor+si-GABPB1-AS1 group (**Figure 4E**, **4F**). Accordingly, GABPB1-AS1 silencing attenuated the damage of I/R cells by negatively regulating miR-641 expression.

NUCKS1 is a target gene of miR-641

As presented in **Figure 5A**, there were binding sites between NUCKS1 and miR-641. Next, the luciferase activity of NUCKS1-WT was suppressed by miR-641 mimic (**Figure 5B**). Further exploration using western blot analysis demonstrated an increase in NUCKS1 expression when miR-641 activity was inhibited (**Figure 5C**). Furthermore, GABPB1-AS1 silencing suppressed the promoting effect of miR-641 inhibitor on NUCKS1 expression (**Figure 5D**). Hence, we conclude that NUCKS1 may be a targeted gene of miR-641, and GABPB1-AS1 knockdown alleviates CI/R injury through the miR-641/NUCKS1 axis.

Discussion

Recently, the incidence of ischemic cerebrovascular disease has risen, and the age of onset has shown a younger trend [16]. CI/R injury is a complex injury process, with neuronal apoptosis as a primary contributor. Mitochondrial dysfunction, inflammatory response, oxidative stress and other factors jointly contribute to the deterioration of CI/R injury [17, 18]. CI/R injury seriously affects patients' quality of life and even endangers their lives. Therefore, developing effective treatment for CI/R injury is a critical and urgent challenge in clinical practice. In this study, we investigated the function of GABPB1-AS1 on the viability, apoptosis, and oxidative stress of OGD/R treated SH-SY5Y cells. The mechanism of GABPB1-AS1/miR-

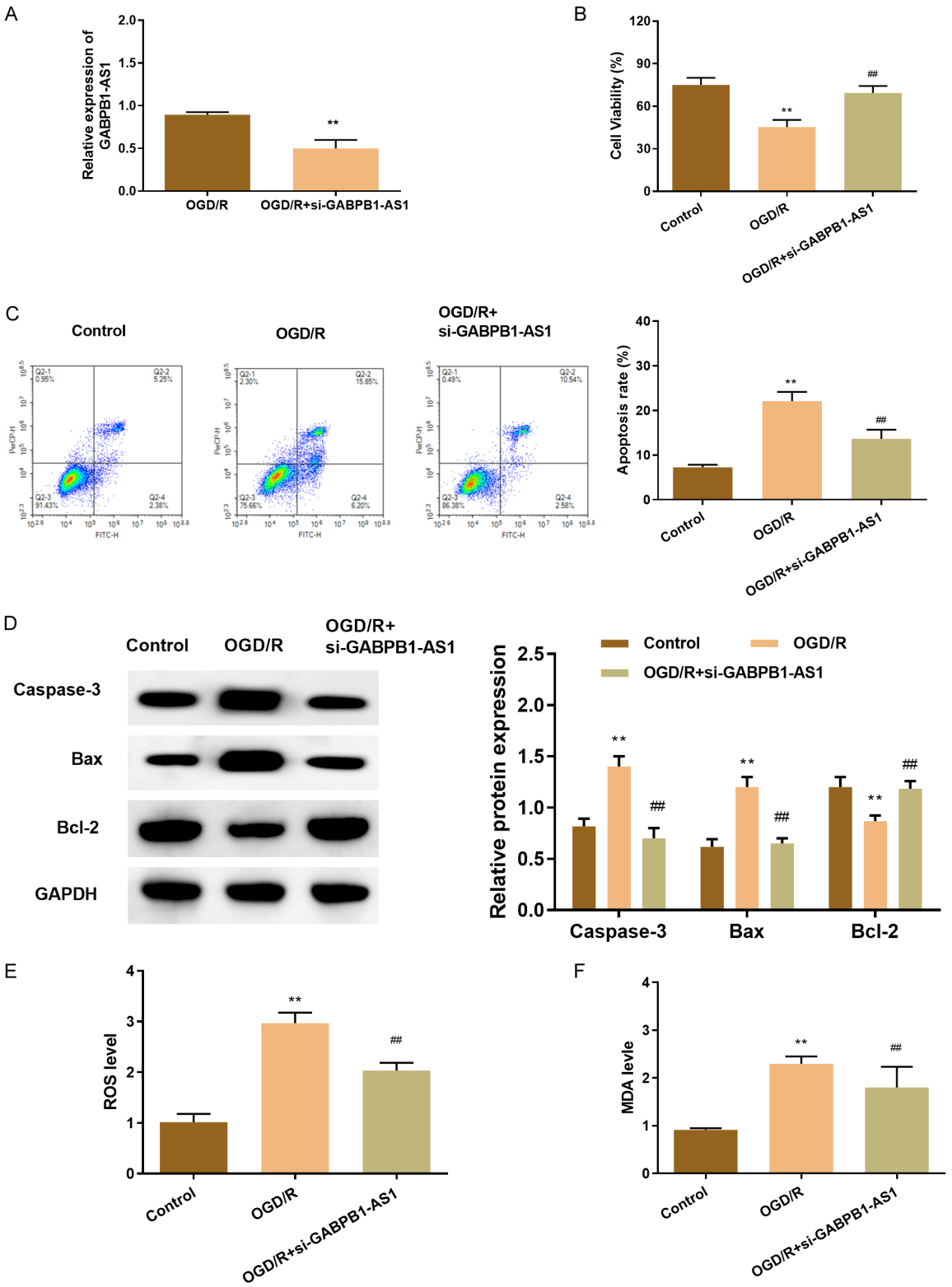


Figure 2. GABPB1-AS1 silencing alleviated OGD/R-induced injury in SH-SY5Y cells. A. The transfection efficiency of si-GABPB1-AS1 was detected by RT-qPCR. B. Cell viability in OGD/R cells or OGD/R+si-GABPB1-AS1 cells was assessed by MTT assay. C. Cell apoptosis in OGD/R cells or OGD/R+si-GABPB1-AS1 cells was detected by flow cytometry. D. The protein expression levels of Caspase-3, Bax, and Bcl-2 were measured by western blot. E, F. The level of ROS and MDA in OGD/R cells or OGD/R+si-GABPB1-AS1 cells. **P<0.01, ##P<0.01.

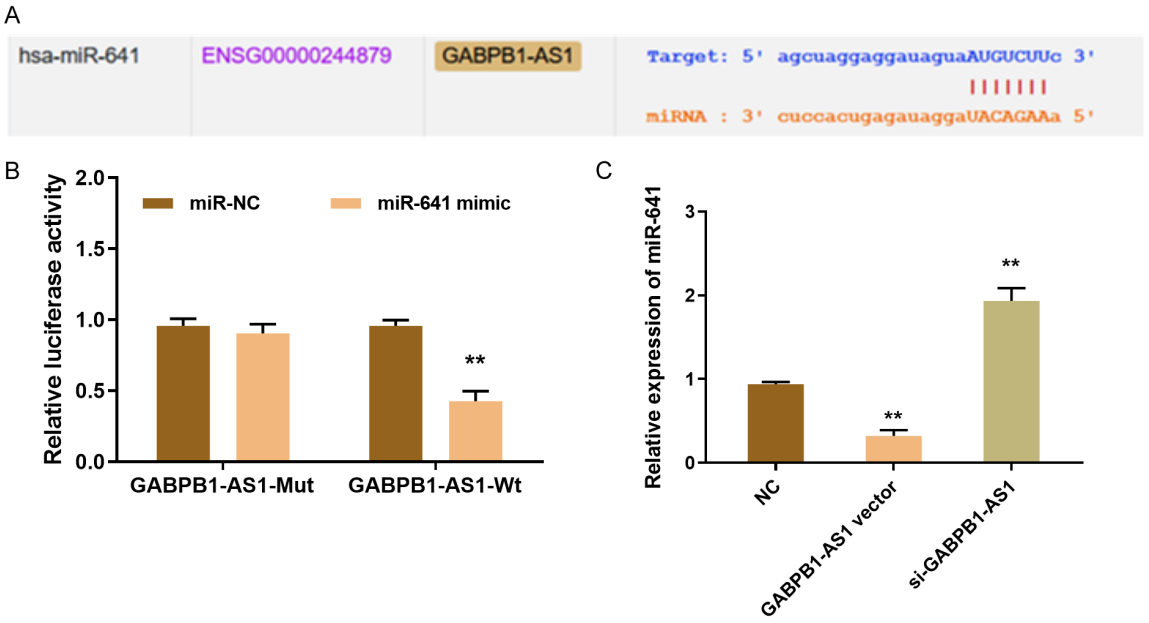


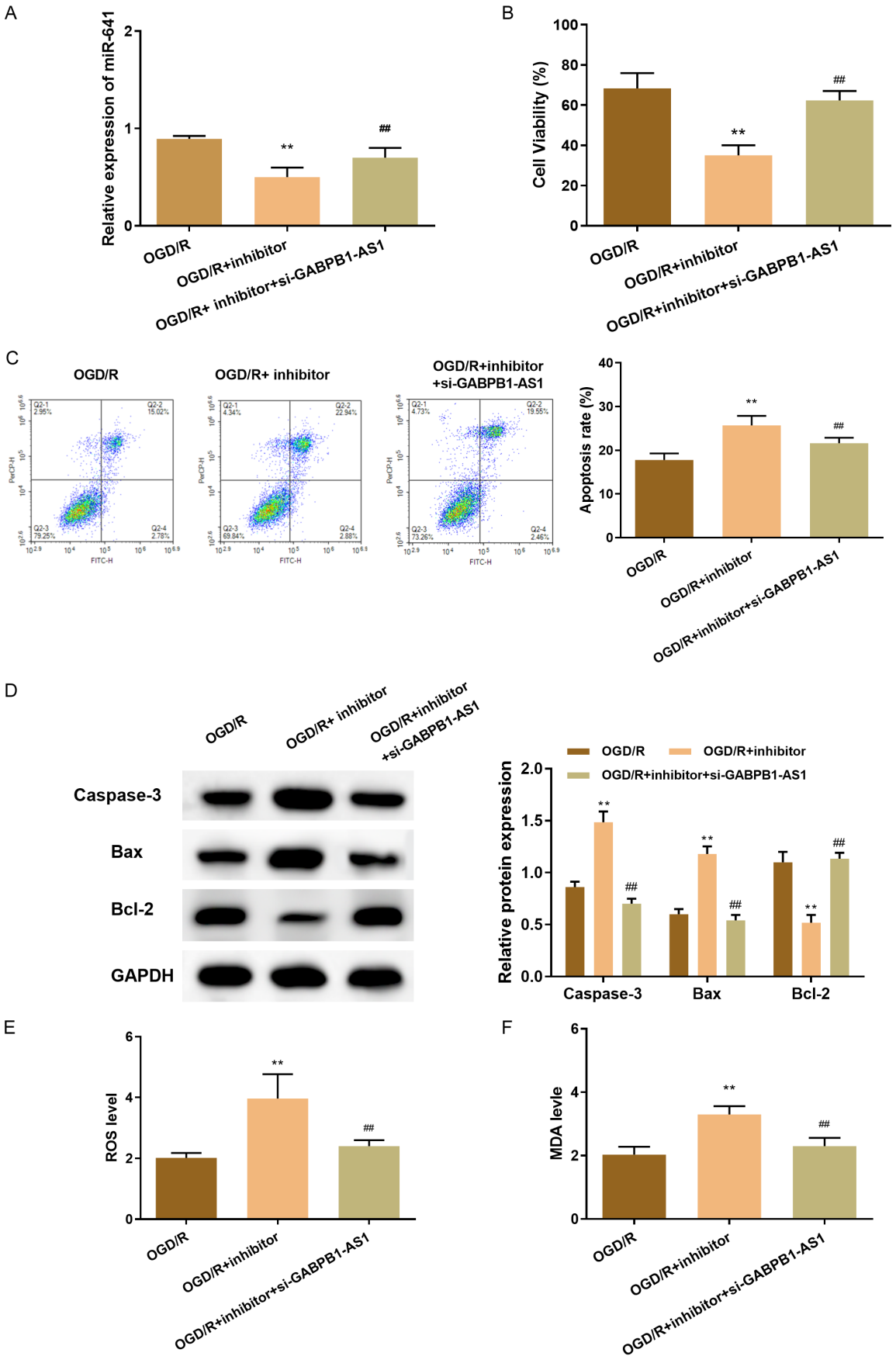
Figure 3. GABPB1-AS1 served as a sponge of miR-641. A. Predicted binding sites between GABPB1-AS1 and miR-641. B. The luciferase activity was detected by dual-luciferase reporter assay. C. The expression of miR-641 was measured by RT-qPCR. **P<0.01.

641/NUCKS1 axis in CI/R injury was also probed.

Previous research showed that various lncRNAs can either exacerbate or mitigate CI/R injury. For example, overexpression of MALAT1 inhibited OGD/R-induced cell apoptosis, promoted cell proliferation, decreased the levels of ROS and MDA, and increased the levels of SOD and CAT [19]. Conversely, ZFAS1 blocked the inflammation, cell apoptosis, and oxidative stress in CI/R injury through the miR-582/NOS3 axis [20]. TTTY15 aggravated neuronal apoptosis and brain damage induced by CI/R *in vivo* though the regulation of miR-520a-3p/IRF9 [21]. According to previous studies, GABPB1-AS1 was discovered to promote cell growth in cervical cancer [22] and glioma [9]. Moreover, Sin1 treatment of SH-SY5Y cells revealed a large number of upregulated lncRNAs, including GABPB1-AS1, associated with REDOX homeostasis and neurodegenerative diseases [23]. In the current work, GABPB1-AS1 was elevated in OGD/R-induced SH-SY5Y cells. Downregulation of GABPB1-AS1 notably promoted cell viability and suppressed cell apoptosis. During the C/I process, mitochondrial oxygen supply is impaired, leading to excessive ROS production in neurons. We found that knockdown of GABPB1-AS1 effectively reduced the levels of ROS and MDA, thereby alleviating the damage of nerve cells. Similarly, GABPB1-AS1 overexpression accelerated the production of ROS and MDA, resulting in membrane lipid peroxide damage, and therapy inducing cell apoptosis in Erastin-treated cells [24]. Our study preliminarily concluded that GABPB1-AS1 knockdown could ameliorate CI/R injury.

Certain regulatory effect are known to exist between lncRNAs and corresponding miRNAs. Bioinformatic analysis displayed that miR-641 has binding sites on the 3'UTR of GABPB1-AS1. MiR-641 has been proven, to block cell growth, invasion, and migration in glioma [25]. In our study, miR-641 inhibitor blocked cell viability, and promoted the apoptosis in OGD/R-treated cells. Furthermore, miR-641 inhibitor aggravated OGD/R-induced injury, and GABPB1-AS1 silencing disrupted this effect. Interestingly, miR-641 suppressed cell growth and metastasis in ox-LDL-treated vascular smooth muscle cells [26].

NUCKS1, a substrate for cyclin-dependent kinases, is involved in a variety of disease processes. For instance, NUCKS1 facilitated cell growth and metastasis in lung cancer [27] and pancreatic cancer [28]. Furthermore, NUCKS1 played an critical role in DNA damage response,


A
Relative expression of miR-641
OGD/R
OGD/R+inhibitor
OGD/R+ inhibitor+si-GABPB1-AS1
B
Cell Viability (%)
OGD/R
OGD/R+inhibitor
OGD/R+inhibitor+si-GABPB1-AS1
C
OGD/R
OGD/R+ inhibitor
OGD/R+inhibitor +si-GABPB1-AS1
Apoptosis rate (%)
OGD/R
OGD/R+inhibitor
OGD/R+inhibitor+si-GABPB1-AS1
D
OGD/R
OGD/R+ inhibitor
OGD/R+inhibitor +si-GABPB1-AS1
Caspase-3
Bax
Bcl-2
GAPDH
OGD/R
OGD/R+inhibitor
OGD/R+inhibitor+si-GABPB1-AS1
Relative protein expression
Caspase-3
Bax
Bcl-2
E
ROS level
OGD/R
OGD/R+inhibitor
OGD/R+inhibitor+si-GABPB1-AS1
F
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OGD/R
OGD/R+inhibitor
OGD/R+inhibitor+si-GABPB1-AS1

Figure 4. GABPB1-AS1 silencing mitigated the damage of I/R cells by regulating miR-641. A. The expression of miR-641 in OGD/R+inhibitor cells or OIGD/R+inhibitor+si-GABPB1-AS1 cells was detected by RT-qPCR. B. Cell viability in OGD/R+inhibitor cells or OIGD/R+inhibitor+si-GABPB1-AS1 cells was detected by MTT assay. C. Cell apoptosis in OGD/R+inhibitor cells or OIGD/R+inhibitor+si-GABPB1-AS1 cells was detected by low cytometry. D. The protein expression levels of caspase-3, Bax and Bcl-2 were detected by western blot. E, F. The level of ROS and MDA in OGD/R+inhibitor cells or OIGD/R+inhibitor+si-GABPB1-AS1 cells. **P<0.01, ##P<0.01.

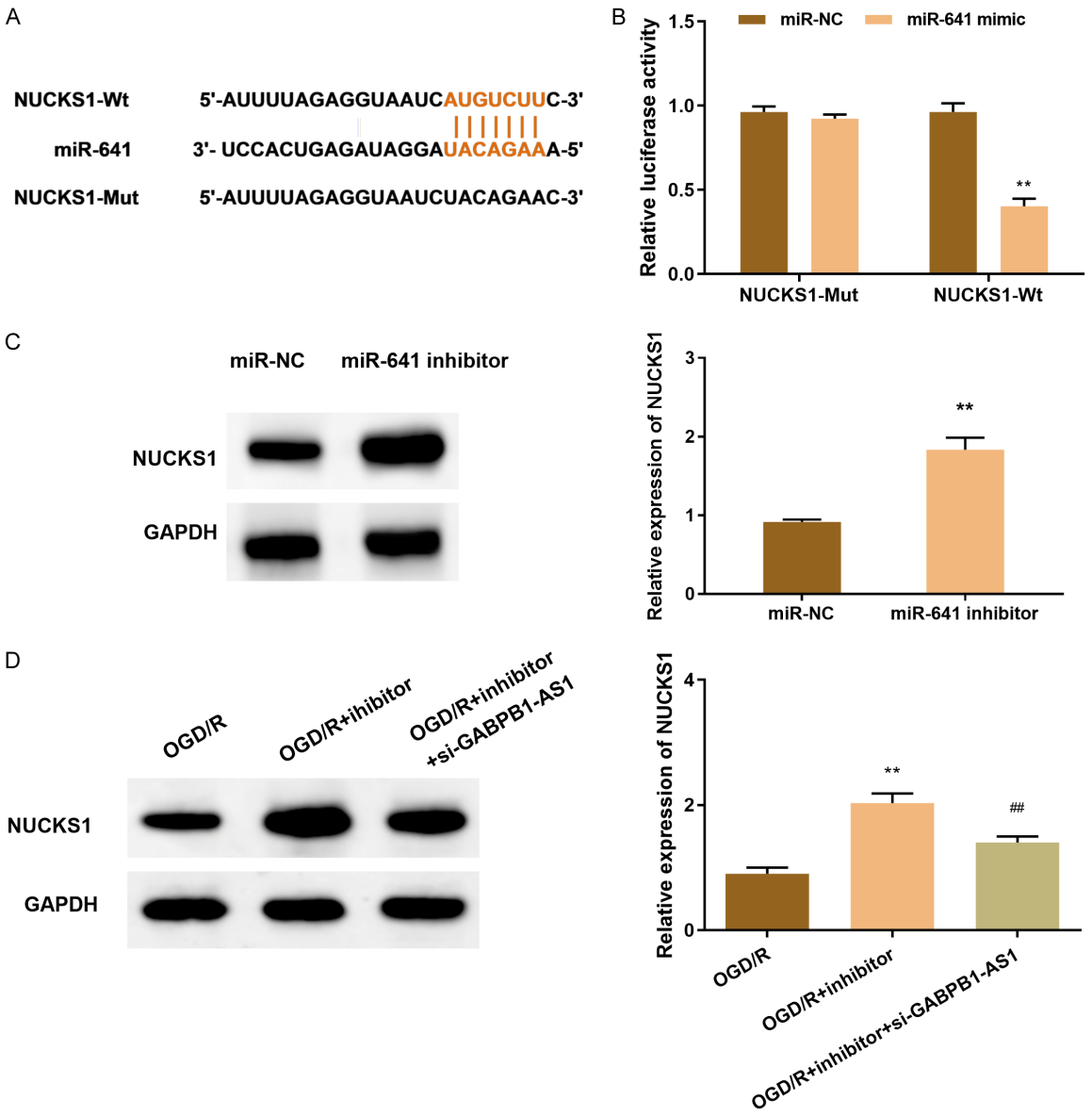

Figure 5. NUCKS1 was proved to be a target gene of miR-641. A. The predicted binding sites between NUCKS1 and miR-641. B. The luciferase activity was detected by dual-luciferase reporter assay. C, D. The protein expression of NUCKS1 was detected by western blot. **P<0.01, ##P<0.01.

inflammatory immune response, and metabolism [29]. In our study, we identified NUCKS1 as a targeted gene of miR-641, and GABPB1-AS1 knockdown alleviated CI/R injury through the miR-641/NUCKS1 axis. Nevertheless, the effect of NUCKS1 in CI/R injury is uncertain yet, and subsequent functional experimental studies are needed. In addition, the function of GABPB1-AS1 knockdown on CI/R injury in animal model is lacking. Furthermore, the role of

GABPB1-AS1 overexpression in CI/R injury and related signaling pathways needs to be further studied.

Conclusion

GABPB1-AS1 knockdown alleviates CI/R injury by the miR-641/NUCKS1 axis. Our results provide an experimental basis for further exploring the pathologic mechanism of CI/R injury, contributing to the development of new protective drugs or therapeutic strategies.

Acknowledgements

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Disclosure of conflict of interest

None.

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