LncRNA WT1-AS Attenuates hypoxia/ischemia Induced Neuronal Injury in Cerebral Ischemic Stroke via miR-186-5p/XIAP Axis

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Abstract

Background

Cerebral ischemic stroke was a nervous system disease with high occurrence rate and mortality rate. This study aimed to investigate the role and mechanism of lncRNA WT1-AS in cerebral ischemic stroke.

Materials and methods

Starbase and dual luciferase reporter gene assay were used to analyze the target relationship between lncRNA WT1-AS and miR-186-5p. qRT-PCR analysis was used to detect lncRNA WT1-AS and miR-186-5p expression. OGD-induced SH-SY5Y cells injury model was conducted, and cell viability and cell apoptosis were determined by MTT and flow cytometer assay. Caspase3 ability was determined using Caspase3 activity detection kit.

Results

miR-186-5p was a target of lncRNA-WT1-AS. lncRNA WT1-AS was down-regulated and miR-186-5p was up-regulated in blood samples of patients with ischemic stroke and in OGD-induced SH-SY5Y cells. We found that WT1-AS-plasmid promoted OGD-induced cell viability, reduced cell apoptosis and decreased caspase3 ability, and these changes were reversed by miR-186-5p mimic. Subsequently, our results proved that XIAP was a target of miR-186-5p. Similarly, miR-186-5p inhibitor reduced OGD-induced neuronal damage by up-regulating XIAP expression.

Conclusion

lncRNA-WT1-AS/miR-186-5p/XIAP might be a new target for cerebral ischemic stroke treatment.

Introduction

Stroke is the leading cause of dysfunction and death worldwide [1]. In the United States, the number of strokes approximately reaches 795,000 each year, but more than 140,000 people die from strokes [2]. Strokes are generally divided into three categories, including transient ischemic attack, ischemic stroke and hemorrhagic stroke. 80% of them are ischemic strokes [3]. The main feature of ischemic stroke is interruption of blood flow, lack of oxygen and glucose in brain cells, which leads to nerve cell function impaired. At present, ischemic stroke has a high morbidity and mortality. The current treatment plan mainly focuses on the following aspects: resolve the infarction in time to limit the continuous damage caused by hypoxic stress and the resulting neuronal damage; and stimulate nerve replacement [4]. Clinically, the use of recombinant tissue plasminogen activator (rt-PA) for intravenous thrombolysis can treat ischemic stroke [5,6]. However, the time window of tPA is very short, and there is a risk of hemorrhagic transformation, so the clinical progress is limited [7-9]. So far, the mechanism and treatment of neuronal damage induced by ischemia and hypoxia are still difficult. Recently, more and more studies

have shown that lncRNA and miRNA play an important role in the mechanism of neuronal damage induced by ischemia and hypoxia.

lncRNA contains about 200 nucleotides. The DNA of the genome is transcribed into RNA, but it cannot be translated into protein [10]. Exercising their biological functions at the RNA level. lncRNA can exert a crucial role in many biological processes, including epigenetic regulation, cell cycle regulation and cell differentiation regulation [11,12]. At present, there are more and more reports about lncRNA in various diseases. Many literature reports that the expression of lncRNA is elevated in many diseases, but decreased in some diseases, including cancer, neurological diseases and diabetes [13,14]. WT1-AS is also called Wilms tumor1 antisense RNA, this RNA encodes a zinc finger transcription domain [15]. An increasing evidence showed that WT1-AS is involved in many cancers development, including gastric cancer, non-small cell lung cancer, cervical cancer and glioma [16-19]. Here, we investigated that the role of WT1-AS in cerebral ischemic stroke.

MiRNAs are a general term for a class of small-molecule non-coding RNAs that are about 20 to 22 nucleotides in length, as opposed to mRNA-transcribed proteins. MiRNA did not encode proteins, and inhibited target genes expression [20-22]. Previous research demonstrated that miR-186-5p, as a cancer-related miRNA, is involved in many cancers occurrence and development [23]. Zhu et al. showed that miR-186-5p that acted as a tumor suppressor gene was negatively expressed in neuroblastoma [24]. Jones et al. showed that miR-186-5p downregulation inhibited cell proliferation, growth and invasion in metastatic cancer cells [25]. Tao et al. indicated that miR-186-5p participated in atherosclerosis development [26]. However, there was no report about the role of miR-186-5p in cerebral ischemic stroke. In our study, through bioinformatics software, we found that there was certain relationship between lncRNA WT1-AS, miR-186-5p and XIAP. Therefore, our aim is to explore the mechanism of three moleculars in cerebral ischemic stroke.

Materials And Methods

Clinical samples

30 blood samples of patients with ischemic stroke were collected within 3 hours after the onset of stroke. 30 blood samples of healthy volunteers were collected as the control group. The study procedures were approved by Ethics Committee of Taizhou People's Hospital. All patients agreed in writing to use their specimens in this study.

Dual luciferase reporter analysis

The wild-type or mutant of WT1-AS was cloned into the pmiRGLO vector (Promega, USA) to verify the relationship between WT1-AS and miR-186-5p. They were co-transfected with miR-186-5p mimic or mimic control and Renilla luciferase into 293T cells. After transfection for 48 h, luciferase activity was tested by using Promega kits.

In order to investigate the reaction of miR-186-5p and XIAP, we constructed WT-XIAP and MUT-XIAP 3'-UTR luciferase reporter gene plasmids. 293T cells were transfected with Renilla luciferase, luciferase reporter gene plasmids and miR-186-5p mimic or mimic control for 48 h. Luciferase activity was tested by using Promega kits according to the instruction.

Cell culture and transfection

Neuroblastoma cell lines SH-SY5Y cells were acquired from American Tissue Culture Collection (ATCC). The cell line was cultured in DMEM (Gibco, NY, USA) medium with 10% FBS (Gibco) and cultured at 37°C in a 5% CO₂ incubator. SH-SY5Y cells were transfected with WT1-AS-plasmid, control-plasmid, mimic control, miR-186-5p mimic, WT1-AS-plasmid+mimic control, WT1-AS-plasmid+miR-186-5p mimic, inhibitor control, miR-186-5p inhibitor, control-siRNA, XIAP-siRNA, miR-186-5p inhibitor+control-siRNA, or miR-186-5p inhibitor+XIAP-siRNA using Polyplus reagent transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 hours according to the operation steps. After 48 h, based on transfected cells, we performed following experiments.

qRT-PCR assay

We obtained total RNA using TRIzol (TaKara) following SOP. Total RNA was transformed into cDNA by reverse transcription using cDNA (Vazyme, Nanjing, Jiangsu). Subsequently, we performed qPCR with SYBR Green PCR kit (Vazyme) followed with the reference manual. GAPDH (for mRNA) or U6 (for miRNA) was used as endogenous control. Gene expression was calculated by $2^{-\Delta\Delta Ct}$ formula.

Administration of Oxygen Glucose Deprivation/ Reoxygenation (OGD/R)

In order to establish an oxygen glucose deprivation/reoxygenation (OGD/R) cell model *in vitro*, we changed the medium for SH-SY5Y cells and replaced the high-glycemic DMEM medium with the glucose-free Dulbecco's modified Eagle medium. And the cells were grown in a 37 °C, 1% O₂, 94% N₂ and 5% CO₂ environment for 4 h; then, we restored the cells to a normal culture state (95% air and 5% CO) and cultured for another 24 h.

Western blot assay

The cells were lysed using RIPA buffer (Solarbio, Beijing). The protein concentration was acquired by using bicinchoninic acid assay kit (Pierce, Appleton, WI, USA). Equal amount of proteins were isolated by 12% SDS-PAGE, and then transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk to prevent nonspecific binding and then incubated with primary antibodies including anti-cleaved-caspases3 (1:1000, abcam) and anti-caspases3 (1:1000, abcam) at 4°C overnight. The next day, they were incubated with secondary antibody for 2 h. The protein signals were visualized by ECL method (Applygen Technologies, Inc).

Flow cytometer assay

Cells apoptosis analysis was performed by using the Annexin-V/propidium iodide (PI) Apoptosis Detection Kit (BD Bioscience). Briefly, after treatment, cells were collected, **disposed** and centrifuged with low temperature at high speed, and re-suspended in 100 μ L of FITC-binding buffer. Subsequently, we added approximately 5 μ L ready-to-use Annexin V-FITC and 5 μ L PI into the buffer. Cells were incubated for 30 min at room temperature in dark. Annexin V-FITC and PI fluorescence were assessed by BD FACSCalibur flow cytometer (BD Technologies).

MTT assay

Cells were treated with specific methods and then plated into a 96-well plates and incubated for 24 h. Then, we added 20 μ L MTT (5 mg/ml, Sigma) into wells and the cells were further cultured for 4 h. Next, we removed the supernatant and added 200 μ L dimethyl sulfoxide to the wells. The absorbance were measured at 570 nm.

Caspase3 activity detection

We detect caspase3 activity by using a caspase3 activity detection kit (Beyotime Biotechnology). Briefly, we collected transfected cells into a EP tube. The cells were centrifuge at 600 xg for 5 minutes at 4 °C to collect the cells. We added lysate to the cells in a certain ratio, re-suspended, and lysed for 15 minutes in an ice bath. After centrifuged for 10 minutes, the supernatant was transferred to an ice-bath pre-cooled centrifuge tube. The caspase3 enzyme activity was measured immediately at 405 nm.

Statistical analysis

Results were analyzed by GraphPad Prism 6.0 software. Statistical significance of difference between groups was determined by Student's t-test or ANOVA followed by Tukey's post-hoc test. Data was showed as mean $\pm$ SD from three independent experiments. $p < 0.05$ was regarded as statistically significant.

Results

Determination of the binding site of lncRNA WT1-AS and miR-186-5p

Firstly, we predicted the relationship between WT1-AS and miR-186-5p by bioinformatics tool Starbase. Starbase prediction result showed that miR-186-5p and lncRNA-WT1-AS have mutual binding sites (Figure 1A). Based on software predictions, we found that miR-186-5p is partially complementary to the WT1-AS 3'-untranslated region. Then, 293T cells were co-transfected with WT1-AS-WT, WT1-AS-MUT, miR-186-5p mimic or mimic control for 48 h. We performed dual-luciferase reporter assay to detect the activity of luciferase. Dual-luciferase reporter assay indicated that miR-186-5p mimic could inhibit the activity of WT1-AS-WT. However, miR-186-5p mimic could not inhibit the activity of WT1-AS-MUT (Figure 1B). Taken together, miR-186-5p is the target gene of lncRNA-WT1-AS. Next, we collected 30 cases blood samples from ischemic stroke patients within 3 hours after the onset of stroke. qRT-PCR assay results demonstrated that compared with the healthy control group, lncRNA WT1-AS expression was

substantially down-regulated (Figure 1C), while miR-186-5p expression was up-regulated (Figure 1D) in the blood samples from ischemic stroke patients.

LncRNA WT1-AS was negatively expressed and miR-186-5p was positively expressed in OGD-induced SH-SY5Y cells

Next, we established OGD-induced SH-SY5Y cell model. LncRNA WT1-AS expression and miR-186-5p expression were detected by qRT-PCR assay. Our results showed that compared with the control group, LncRNA WT1-AS expression was significantly reduced in OGD-induced SH-SY5Y cells (Figure 1E); while miR-186-5p expression was obviously increased (Figure 1F).

WT1-AS negatively regulated miR-186-5p expression in SH-SY5Y cells

SH-SY5Y cells were plated into a 6-well plate (1×10^6 cells/well) and cultured at 37°C for 24 hours. Then WT1-AS-plasmid, miR-186-5p mimic, WT1-AS-plasmid+mimic control or WT1-AS-plasmid+miR-186-5p mimic was transfected into SH-SY5Y cells strictly following the lipofectamine®2000 (Invitrogen; Thermo Fisher Scientific, Inc.) kit instructions. Next we use qRT-PCR analysis to evaluate transfection efficiency. Compared with control-plasmid group, WT1-AS-plasmid increased WT1-AS expression in SH-SY5Y cells (Figure 2A). Compared with mimic control group, miR-186-5p mimic increased miR-186-5p expression (Figure 2B). Compared with control-plasmid group, WT1-AS-plasmid reduced miR-186-5p expression, and this reduction was significantly reversed by miR-186-5p mimic (Figure 2C).

LncRNA WT1-AS decreased OGD-induced neuronal damage by reducing miR-186-5p expression

After cell transfection for 24 hours, SH-SY5Y cells were incubated for 4 hours in serum-free and sugar-free artificial cerebrospinal fluid at 37 °C, 1% O₂, 94% N₂, and 5% CO₂, and then replaced DMEM medium to continue to incubate for 24 h under normoxia. Our experiment is divided into these groups: control group; OGD group; OGD+control-plasmid group; OGD+WT1-AS-plasmid group; OGD+WT1-AS-plas-

mid+mimic control group; OGD+WT1-AS-plasmid+miR-186-5p mimic group. Subsequently, we carried out a series of experiments. Compared with control group, miR-186-5p expression was obviously improved in OGD group; compared with OGD+control-plasmid group, miR-186-5p expression was significantly reduced in OGD+WT1-AS-plasmid group, and the reduction was reversed by miR-186-5p mimic (Figure 3A). qRT-PCR analysis showed that LncRNA WT1-AS expression in the OGD group was reduced; compared with the OGD+control-plasmid group, LncRNA WT1-AS expression was increased in the OGD+WT1-AS-plasmid group (Figure 3B). MTT and FCM assay showed that cell viability was decreased (Figure 3C) and cell apoptosis was increased (Figure 3D and E) in the OGD group. Caspase3 activity was increased in the OGD group (Figure 3F). Compared with OGD+control-plasmid group, cell viability was significantly increased (Figure 3C) and cell apoptosis was reduced (Figure 3D and E) in the OGD+WT1-AS-plasmid group, Furthermore, Caspase3 viability was reduced (Figure 3F), and all the effects were reversed by miR-186-5p mimic.

Determination of the relationship between miR-186-5p and XIAP

Then, through literature analysis and bioinformatics software (TargetScan) analysis, we found that there is a direct binding site between miR-186-5p and XIAP (Figure 4A); the dual luciferase report experiment confirmed the relationship between miR-186-5p and XIAP (Figure 4B). Subsequently, SH-SY5Y cells were seeded into a 6-well plate (1×10^6 cells/well) and cultured at 37°C for 24 hours. Inhibitor control, miR-186-5p inhibitor, control-siRNA, XIAP-siRNA, miR-186-5p inhibitor+control-siRNA, or miR-186-5p inhibitor+XIAP-siRNA was transfected into SH-SY5Y cells for 24 h. We performed qRT-PCR assay to assess transfection efficiency. Compared with the inhibitor control group, miR-186-5p inhibitor reduced miR-186-5p expression in SH-SY5Y cells (Figure 4C). Compared with the control-siRNA group, XIAP-siRNA decreased XIAP expression at mRNA level (Figure 4D). Compared with the inhibitor control group, miR-186-5p inhibitor significantly increased XIAP expression at mRNA and protein level. This increase was obviously reversed by XIAP-siRNA transfection (Figure 4E and F). Taken together, XIAP was target gene of miR-186-5p.

Effect of miR-186-5p inhibitor on OGD-induced nerve cell damage

After cell transfection for 24 h, SH-SY5Y cells were treated as OGD-induced cell injury model. Our experiment is divided into these groups, including OGD+inhibitor control; OGD+miR-186-5p inhibitor; OGD+miR-186-5p inhibitor+control-siRNA; OGD+miR-186-5p inhibitor+XIAP-siRNA. Compared with the control group, miR-186-5p was up-regulated (Figure 5A) and XIAP mRNA (Figure 5B) was reduced in OGD group; compared with the OGD+inhibitor control group, miR-186-5p was down-regulated and XIAP mRNA was increased in OGD+miR-186-5p inhibitor group. Compared with the OGD+inhibitor control group, cell viability was improved in OGD+miR-186-5p inhibitor group (Figure 5C), cell apoptosis was reduced (Figure 5D and E), Caspase3 viability (Figure 5F), and these effects were reversed by XIAP-siRNA.

Discussion

Acute cerebral ischemia stroke leads to higher mortality in humans, and also causes some complications, such as behavioral, social, attention, cognitive and functional motor dysfunction [27,28]. The pathological process of ischemic stroke is more complicated, and the contributing factors are diverse. Refers to previous literature description, neuroinflammation and oxidative stress are important factors of ischemic brain injury [29,30]. Although there have been many treatments in the past period of time, including thrombolytic therapy, anticoagulation therapy, carotid endarterectomy, and neuroprotective agents, there are still many problems with current treatment programs [31-33]. Therefore, we need to explore new approach to treat cerebral ischemic stroke.

LncRNA has now attracted more people's attention in various diseases including cerebral ischemic stroke. Shan et al. showed that lncRNA TUG1 participated in cerebral ischemic stroke development, and TUG1 down-regulation reduced OGD/R-induced damage [34]. A report from Wen's team indicated that lncRNA Gm4419 up-regulation promoted TNF- α , IL-1 β and IL-6 expression, thereby increasing OGD/R damage [35]. Hu et al. showed that knocking down lncRNA SNHG15 could improve ischemia/hypoxia-induced

neuronal damage and microglia inflammation by targeting miR-302a-3p/STAT1 [36]. Xiao et al. suggested that lncRNA H19 was highly expressed in ischemic stroke patients and H19 siRNA decreased neuronal apoptosis in OGD neuronal cells through targeting miR-19a [37]. In our experiment, we researched the role of lncRNA WT1-AS in cerebral ischemic stroke. Firstly, we employed bioinformatics analysis and dual luciferase reporter analysis to show that there was binding site between lncRNA WT1-AS and miR-186-5p.

Previous research demonstrated that lncRNA WT1-AS was involved in cancer development. WT1-AS expression was decreased in cervical carcinoma, papillary thyroid carcinoma (PTC) and cervical squamous cell carcinoma (CSCC). In addition, Cui et al. showed that WT1-AS overexpression suppressed cell viability by miR-330-5p/p53 axis in cervical carcinoma [38]. Le et al. reported that WT1-AS inhibited IHH-4 cell proliferation through miR-203 and survivin in PTC [39]. Similarly, WT1-AS suppressed CSCC cell proliferation by p53 in Zhang's team research [40]. However, there was basically no report on the study of lncRNA WT1-AS in ischemic stroke. Same as the previous result, our results showed that WT1-AS expression was reduced in blood sample of patients, while miR-186-5p expression was increased. Consistent with the expression in the patient, WT1-AS was up-regulated and miR-186-5p was down-regulated in OGD-induced SH-SY5Y cells in vitro.

Next, we explored the action mechanism of WT1-AS and miR-186-5p in OGD-induced SH-SY5Y cell injury. Du et al. showed that LINC01705 is targeted by miR-186-5p and LINC01705 participated in BC development by miR-186-5p [41]. Zhu et al. reported that HCP5 promoted neuroblastoma cell proliferation by down-regulated miR-186-5p [42]. In osteosarcoma, miR-186-5p suppressed cell proliferation, invasion and metastasis by targeting FOXK1 [43]. In our research, WT1-AS overexpression decreased miR-186-5p expression in SH-SY5Y cells and WT1-AS reduced OGD-induced neuronal damage by reducing miR-186-5p expression. Subsequently, we found that XIAP was target of miR-186-5p.

XIAP, X-linked inhibitor of apoptosis protein, is an important member of the apoptotic protein (IAP) family and is a cytoplasmic inhibitor of caspases-3, -7 and -9 [44,45]. As a target for cancer treatment, XIAP was abnormally expressed in cancer and played an important role in regulating the resistance to death [46]. Zhang et al. indicated that miR-130a promoted cisplatin resistance of cells in ovarian cancer through regulating XIAP [47]. A report from Deng's team demonstrated that XIAP participated in ischaemic stroke development and XIAP expression was decreased in MCAO rats. XIAP siRNA reversed the effect suppressed miR-130a on neurological function and angiogenesis in MCAO rats. In this study, miR-186-5p inhibitor alleviated OGD-induced SH-SY5Y cells injury. These findings indicated that WT1-AS exerted a crucial role in cerebral ischemic stroke through miR-186-5p-XIAP axis.

In conclusion, lncRNA WT1-AS reduced neuronal damage caused by hypoxia/ischemia in cerebral ischemic stroke through miR-186-5p/XIAP axis.

Declarations

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Competing interests

The authors declare that they have no competing interests.

Availability of data and material

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

Code availability

Not applicable.

Ethics approval

The study procedures were approved by Ethics Committee of Taizhou People's Hospital. All patients agreed in writing to use their specimens in this study.

Consent to participate

All patients agreed in writing to use their specimens in this study.

Consent for publication

All patients agreed for publication.

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Not applicable.

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Figures

Figure 1

The expression levels of lncRNA WT1-AS and miR-186-5p in ischemic stroke patients and OGD-induced SH-SY5Y cells. (A). Interaction between miR-186-5p and 3'UTR of WT1-AS was predicted using Starbase prediction software. (B). Dual luciferase reporter gene assay that verification of the interaction between WT1-AS and miR-186-5p when 293T cells were co-transfected with miR-186-5p mimic and wild-type or mutant WT1-AS 3'-UTR reporter. (C and D) Expression of WT1-AS and miR-186-5p in the blood samples from ischemic stroke patients and healthy control. (E). qRT-PCR analysis detected lncRNA WT1-AS expression at mRNA level. (F). qRT-PCR analysis detected miR-186-5p expression at mRNA level. ** $p < 0.01$ vs. mimic control; ## $p < 0.01$ vs. Healthy control; && $p < 0.01$ vs. Control.

Figure 2

lncRNA WT1-AS negatively regulated miR-186-5p expression in SH-SY5Y cells. (A). qRT-PCR analysis of WT1-AS expression when SH-SY5Y cells were transfected with control-plasmid or WT1-AS-plasmid. (B). qRT-PCR analysis of miR-186-5p expression when SH-SY5Y cells were transfected with mimic control or miR-186-5p mimic. (C). qRT-PCR analysis that miR-186-5p expression when SH-SY5Y cells were transfected with control-plasmid, miR-186-5p mimic, WT1-AS-plasmid+mimic control or WT1-AS-plasmid+miR-186-5p mimic. ** $p < 0.01$ vs. Control-plasmid; ## $p < 0.01$ vs. Mimic control; && $p < 0.01$ vs. WT1-AS-plasmid+mimic control.

Figure 3

The effect of LncRNA WT1-AS on nerve cell injury induced by OGD. (A). qRT-PCR analysis of miR-186-5p expression. (B). qRT-PCR analysis of lncRNA WT1-AS expression. (C). MTT assay of cell viability. (D). FCM assay of cell apoptosis. (E). The apoptosis ratio were calculated and presented. (F). The caspase3 activity was detected by specific kit. ** $p < 0.01$ vs. Control; ## $p < 0.01$ vs. OGD+mimic control; && $p < 0.01$ vs. OGD+WT1-AS-plasmid+mimic control.

Figure 4

XIAP was target of miR-186-5p and miR-186-5p negatively regulated XIAP expression in SH-SY5Y cells. (A). Interaction between miR-186-5p and 3'UTR of XIAP was predicted using TargetScan. (B). Dual luciferase reporter gene assay that verification of the interaction between miR-186-5p and XIAP when 293T cells were co-transfected with miR-186-5p mimic and wild-type or mutant XIAP 3'-UTR reporter. (C). qRT-PCR assay of miR-186-5p expression in SH-SY5Y cells transfected with inhibitor control or miR-186-

5p inhibitor. (D). qRT-PCR assay of XIAP expression in SH-SY5Y cells transfected with control-siRNA or XIAP-siRNA. (E). qRT-PCR assay of XIAP expression in SH-SY5Y cells transfected with miR-186-5p inhibitor+control-siRNA, or miR-186-5p inhibitor+XIAP-siRNA. (F). Western blot analysis of XIAP expression at protein level. \$\$ p<0.01 vs. mimic control; **p<0.01 vs. Inhibitor control; ##p<0.01 vs. Control-siRNA; &&p<0.01 vs. miR-186-5p inhibitor+control-siRNA.

Figure 5

MiR-186-5p inhibitor reduced OGD-induced neuronal damage by up-regulating XIAP expression. (A). qRT-PCR analysis of miR-186-5p expression. (B). qRT-PCR analysis of XIAP expression. (C). MTT assay detected cell viability. (D). FCM assay determined cell apoptosis. (E). The apoptosis ratio were calculated and showed. (F). The Caspase3 activity was detected by specific kit. **p<0.01 vs. Control; ##p<0.01 vs. OGD+inhibitor control; &&p<0.01 vs. OGD+miR-186-5p inhibitor+control-siRNA.