

Up-regulation of miR-499a-5p decreases cerebral ischemia/reperfusion injury by targeting PDCD4

Weifeng Shan

The 1st Affiliated Hospital, School of Medicine, Zhejiang University

Huifeng Ge

The 1st Affiliated Hospital, School of Medicine, Zhejiang University

Bingquan Chen

The 1st Affiliated Hospital, School of Medicine, Zhejiang University

Linger Huang

The 1st Affiliated Hospital, School of Medicine, Zhejiang University

Shaojun Zhu

The 1st Affiliated Hospital, School of Medicine, Zhejiang University

Yanfeng Zhou (✉ zhou_yanfeng127@126.com)

The 1st Affiliated Hospital, School of Medicine, Zhejiang University <https://orcid.org/0000-0002-3145-0093>

Research Article

Keywords: ischemic stroke, ischemia/reperfusion injury, OGD/R, miR-499a-5p, PDCD4

Posted Date: March 11th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-297082/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Cellular and Molecular Neurobiology on April 9th, 2021. See the published version at <https://doi.org/10.1007/s10571-021-01085-4>.

Abstract

MiR-499a-5p was significantly down-regulated in degenerative tissues and correlated with apoptosis. Nonetheless, the biological function of miR-499a-5p in acute ischemic stroke has been still unclear. In this study, we found the plasma levels of miR-499a-5p were significantly down-regulated in 64 ischemic stroke patients and negatively correlated with the National Institutes of Health Stroke Scale score. Then, we constructed cerebral ischemia/reperfusion (I/R) injury in rats after middle cerebral artery occlusion and subsequent reperfusion and oxygen-glucose deprivation and reoxygenation (OGD/R) treated SH-SY5Y cell model. Transfection with miR-499a-5p mimic was accomplished by intracerebroventricular injection in the *in vivo* I/R injury model. We further found miR-499a-5p overexpression decreased infarct volumes and cell apoptosis in the *in vivo* I/R stroke model using TTC and TUNEL staining. PDCD4 was a direct target of miR-499a-5p by luciferase report assay and western blotting. Knockdown of PDCD4 reduced the infarct damage and cortical neuron apoptosis caused by I/R injury. MiR-499a-5p exerted neuroprotective roles mainly through inhibiting PDCD4-mediated apoptosis by CCK-8 assay, LDH release assay and flow cytometry analysis. These findings suggest that miR-499a-5p might represent a novel target that regulates brain injury by inhibiting PDCD4-mediating apoptosis.

Introduction

Ischemic stroke is identified as a type of cerebrovascular disease accounting for nearly 90% of stroke cases with lower life quality of victims and huger public health burden (Benjamin et al. 2018; Luengo-Fernandez et al. 2013). Sudden insufficient blood flow to an area of the brain is the main characteristics of ischemic stroke, which could cause neuronal cell apoptosis, necrosis and other metabolism-related disorders (Tobin et al. 2014). Despite the advances in rapid restoration of the blood supply for ischemic stroke, blood flow reperfusion, a process termed cerebral ischemia/reperfusion (I/R) injury seriously limits its development (Chomova and Zitnanova 2016). Thus, a better understanding of the molecular mechanisms underlying the pathological process of I/R injury may improve the ischemic stroke therapy.

MicroRNAs (miRNAs/miRs), small endogenous noncoding RNA molecules (18–25 nts) regulate a various of biological processes, including cell proliferation, apoptosis and neuroinflammation primarily through the interaction with mRNAs via binding their 3'-untranslated region (3'-UTR) regions (Bartel 2004; Qian Zhang 2017; Zhong et al. 2019). Increasing evidence has indicated that aberrantly expressed miRNAs play important regulatory roles in the pathogenesis of cerebral I/R injury (Di et al. 2014; Hu et al. 2015). For instance, Yin et al (Yin et al. 2010) first reported that miR-497 aggravated ischemic neuronal death by directly binding to the predicted 3'UTR target sites of Bcl-2/Bcl-w genes. Similarly, downregulation of miR-217 significantly ameliorated oxygen-glucose deprivation and reoxygenation (OGD/R)-induced neuron injury, inflammatory responses and oxidative stress by targeting sirtuin 1 (SIRT1) (Rao et al. 2019). Inversely, miR-132 could attenuate cerebral injury by protecting blood-brain barrier disruption in ischemia stroke (Zuo et al. 2019). In recent years, miR-499a-5p has been manifested to mediate cell proliferation, migration and EMT in several tumor cells, including cervical cancer (Gu et al. 2020), osteosarcoma (Liu et al. 2018a) and lung adenocarcinoma (He et al. 2019). It's worth noting that Kang et al (Liu et al. 2019)

reported miR-499a-5p was significantly downregulated associated with myocardial I/R injury. Functionally, miR-499a-5p overexpression promotes the cardiomyogenic differentiation of Bone human marrow-derived mesenchymal stem cells (hBM-MSCs) (Neshati et al. 2018). Zhao et al (Zhao et al. 2020) further demonstrated that miR-499a-5p could relief the injury of cardiomyocytes induced by hypoxia/reoxygenation via targeting cluster of differentiation 38 (CD38). Based on these facts, we speculated that miR-499a-5p might be a crucial regulator in cerebral I/R injury.

Human programmed cell death 4 (PDCD4) was initially identified as a nuclear antigen gene and mapped at chromosome 10q24 (Soejima et al. 1999), which is further confirmed to be a novel tumor suppressor to show multi-functions inhibiting cell growth, invasion and especially inducing apoptosis (Matsushashi et al. 2019). Notably, PDCD4 was involved in several miRNAs-mediated I/R injury as follows: Knockdown of miR-21 or inhibition of hypoxia inducible factor 1 alpha (HIF-1 α) abolished the Ischemic postconditioning-mediated attenuation of intestinal injury and apoptosis and the downregulation of PDCD4 (Jia et al. 2017). MiR-21 participates in H₂O₂-mediated gene regulation and functional modulation in cardiac myocytes via its target gene PDCD4 (Cheng et al. 2009). MiR-340-5p might exert neuroprotective effects during OGD/R injury by targeting PDCD4 (Zheng et al. 2020). In addition, PDCD4 silencing can activate the Akt signaling pathway and attenuate vascular endothelial cell injury caused by ischemia-reperfusion in the lower extremities in rats (Chen et al. 2019). Our previous prediction analysis showed that PDCD4 was a target gene of miR-499a-5p. However, there was no data regarding the miR-499a-5p/PDCD4 axis in regulating cerebral I/R injury until now.

Therefore, the aims of the present study were to investigate the functional role of miR-499a-5p in neuronal injury induced by cerebral I/R using an in vivo middle cerebral artery occlusion (MCAO) rat model and in vitro OGD/R induced SH-SY5Y cellular model. Moreover, we confirmed the direct targeting of miR-499a-5p on PDCD4 in neuronal injury.

Materials And Methods

Blood samples of patients with acute ischemic stroke

Total 64 patients with acute ischemic stroke were enrolled from the 1st Affiliated Hospital, School of Medicine, Zhejiang University (Zhejiang Province, China), which were all clinically diagnosed by neurologists based on the medical imaging diagnostic methods (CT scanning and MRI), clinical symptoms and neurological examination. The baseline characteristics of enrolled stroke patients were summarized at **Table 1**. Clinical severity was assessed at the third month after the stroke onset using the National Institutes of Health Stroke Scale (NIHSS) score ([Special report from the National Institute of Neurological Disorders and Stroke. Classification of cerebrovascular diseases III 1990](#)). The patients who received thrombolytic therapy or diagnosed as renal or liver failure, tumor, infectious and hematologic disease were excluded. Equivalent number of age- and sex- matched patients without medical history of stroke were enrolled as controls. The peripheral blood sample was collected from patient/volunteer into tubes containing EDTA, which was immediately centrifuged at 1,500 g for 10 minutes at 4 °C until further

analysis. The use of the human blood samples for research purposes was approved by the Ethics Committee of the 1st Affiliated Hospital, School of Medicine, Zhejiang University (Approval number: ZU349-2; 2017.8.16), and all participants signed the written informed consent.

Construction of I/R injury rat model

Healthy male Sprague-Dawley (SD) rats at the age of 25 weeks (220-250g) were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China) and housed in a standard environment with free access to food/water. Rat model of cerebral I/R injury was established by 2 h of middle cerebral artery occlusion (MCAO) via intraluminal silicon-coated filament method following previously reported procedures (Begum et al. 2015). Briefly, rats were subcutaneously anesthetized with isoflurane (3% initially, 1 to 1.5% maintenance). Then, midline skin incision was made to carefully isolated the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA). Subsequently, a heparinized intraluminal silicon-coated filament (diameter 0.43 ± 0.02 mm) was inserted from ECA through the ICA until it reached the CCA, and finally blocked the origin of middle cerebral artery (MCA). After 2 h occlusion, the filament was slowly withdrawn, with subsequent 24 h of reperfusion. The rats in the sham group underwent the same operations with the exception of the filament insertion. Following depth of anesthesia, the brains of rats from different groups were quickly removed for further analysis.

Animal groups

All rats were randomly assigned as the following three groups with 6 rats per group: 1) Sham group; 2) I/R group; 3) I/R + mimic group; 4) I/R + siNC group; 5) I/R + siPDCD4 group, of which miR-499a-5p mimics, siNC and siPDCD4 were synthesized by GenePharma Co., Ltd. (Shanghai, China), diluted with EntransterTM *in vivo* transfection reagent (Engreen, Beijing, China), and then intracerebroventricularly (i.c.v) injected into the cerebral cortex with a stereotaxic instrument while the rats were under anesthesia 3 days prior to MCAO.

2, 3, 5-triphenyltetrazolium chloride (TTC) staining

TTC staining was conducted in accordance with a previous method to measure infarct volumes (Yang et al. 2018). In brief, the brains of rats from the above groups were quickly removed at 24 h after reperfusion, frozen at -20 °C for 30 min and then cut into 1.5-mm coronal sections with a sharp blade. The sections were incubated in 2% TTC solution (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37 °C and then fixed in 10% formalin for 2 h. After photographed, the infarct volume calculation was performed blindly with Image-Pro Plus 6.0 analysis software according to the following formula: total infarct volume/total brain volume \times 100%.

TdT-mediated dUTP Nick-end labeling (TUNEL) staining

TUNEL staining was performed to examine the apoptosis of cortical neurons in rat brains by a commercially available kit (Roche Diagnostics Crop., Indianapolis, USA). Briefly, the cortex region brain tissues were fixed with 4% paraformaldehyde, embedded in paraffin and made into 5 μ m thickness

sections. Afterwards, the sections were digested with 2% proteinase K for 30 min, washed with PBS (3 × for 5 min) and incubated with TUNEL reaction mixture at 37 °C for 30 min in darkness, followed by immersed into DAPI staining (Beyotime Biotechnology, China). Positive TUNEL staining (green) and DAPI-staining (blue) were observed under a fluorescence microscope. The percentage of positive TUNEL staining was evaluated by determining the ratio of the number of TUNEL-positive cells to that of total cells in each field.

Cell culture, transfection, and OGD/R establishment

A neuron-like human-derived neuroblastoma cell line SH-SY5Y was provided by American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, USA) with 10% fetal bovine serum (FBS, Gibco) at 37 °C. The miR-499a-5p mimic, inhibitor, corresponding negative control miR-NC, small interference RNA against PDCD4 (si-PDCD4) and siNC were purchased from GenePharma Co., Ltd. The pcDNA3.1/PDCD4 vector was generated by inserting the open reading frame of PDCD4 without 3'UTR into the pcDNA3.1 vector (Sangon Biotech, Shanghai, China). Cell transfection or co-transfection was performed in SH-SY5Y cells, which at a density of 3×10^5 cells per well were seeded in six-well plates using Lipofectamine 2000 reagent (Invitrogen). After 48 h transfection, oxygen and glucose deprivation/reoxygenation (OGD/R) was performed as briefly described as follows: SH-SY5Y cells were subjected to 5 h of glucose-free DMEM in a pre-conditioned hypoxic condition (1% O₂, 5% CO₂, and 94% N₂), followed by 24 h of reoxygenation by replacing the hypoxic medium with standard medium under normoxic conditions.

Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For quantification of miR-499a-5p, reverse transcription reactions were performed using a TaqMan™ microRNA assay kit (Applied Biosystems, Foster City, CA, USA) and RT-qPCR was performed with SYBR Premix Ex Taq™ II kit (Takara Bio, Inc., Otsu, Japan) with U6 as an internal reference. For quantification of PDCD4, reverse transcription was performed using a Prime Script™ RT reagent kit (Takara Bio, Inc.) and RT-qPCR was carried out using a SYBR Green PCR Master Mix Kit (Applied Biosystems) with β-actin as an internal reference. The gene change fold was calculated using relative quantification via the 2^{-ΔΔCt} method. The primer sequences used for RT-qPCR were as following, miR-499a-5p forward, 5'-GAGTGTGGCGGGGAATCT-3', and reverse, 5'-GCGTGGTGCAGTCGTGTG-3'; U6, forward, 5'-GCACATATACGCTTCGGCATAAAAT-3', and reverse, 5'-CATTTGCGGCTTCACGATGTCAT-3'; PDCD4, forward, 5'-TCCTGAGATGGATTGGCTAATTATG-3', and reverse, 5'-CGGGAAGTTGTTATCTGACATGCCG-3'; β-actin, forward, 5'-TGTCACCAACTGGGACGATA-3', and reverse, 5'-GGGGTGTGAAGGTCTCAA-3'. Each sample was analyzed in triplicate.

Western blot analysis

Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology Co. Ltd., Shanghai, China) was employed to extract total protein from tissues or cells and protein concentration was examined using

BCA Protein Assay reagent kit (Beyotime Biotechnology). A total of 30 µg of protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene membrane (Millipore). The membranes were blocked with 5% non-fat milk for 2 h and incubated with primary antibodies against PDCD4, caspase-3 and GAPDH overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 2 h at room temperature. Protein bands were detected using an enhanced chemiluminescent reagent (Thermo Fisher Scientific).

Cell viability assay

SH-SY5Y cells at a density of 4,000 cells per well were seeded into 96-well plates and incubated overnight at 37 °C. Next day, cells in each well were washed with PBS and incubated with 10 µl CCK-8 reagent (Dojindo Molecular Technologies, Dojindo, Japan) for 2 h at 37 °C. Afterwards, the absorbance was measured at 450 nm using a microplate reader and relative cell viability was expressed by setting the control group as "1". Each sample was analyzed in triplicate.

Cytotoxicity assay

The release of lactate dehydrogenase (LDH) was analyzed by the LDH Cytotoxicity Assay Kit (Beyotime Biotechnology) for evaluation of cell cytotoxicity. In brief, SH-SY5Y cells were seeded into 96-well plates at a density of 4,000 cells per well and incubated with LDH release reagent (150 µL per well) at 37 °C for 1 h. After determining the absorbance at 490 nm, the LDH release rate was expressed as the percentage of the maximum enzymatic activity of the control sample. Each sample was analyzed in triplicate.

Flow cytometric analysis

Apoptotic cells were quantified by flow cytometry with Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA) in accordance with the manufacturer's instructions. Briefly, SH-SY5Y cells harvested by trypsin digestion and seeded into 6-well plates at a density of 2.0×10^5 cell per well. After washed with PBS, cells were re-suspended in 200 µL binding buffer containing 10 µL Annexin V-FITC and 5 µL PI for 30 min in the dark. The apoptotic cells (Annexin V positive) were distinguished by a flow cytometry. Each sample was analyzed in triplicate.

Caspase-3 activity assay

Colorimetric Assay Kits (R&D systems Co. Germany) were utilized to measure the caspase-3 activity in SH-SY5Y cells from different groups following the manufacturer's protocol. In brief, cells were centrifuged at 10000 g for 10 min for preparation of cell lysate supernatant. Then, the supernatant was incubated with 50 µl reaction buffer and 5 µl of caspase-3 substrate at 37 °C for 1.5 h. according to the absorbance at 405 nm, relative caspase-3 activity was expressed as fold change of caspase-3 activity in experimental group divided by that of control group. Each sample was analyzed in triplicate.

Dual-luciferase reporter assay

Based on the predicted sequences of the 3' untranslated region (UTR) of PDCD4 mRNA containing miR-499a-5p binding sites (UCAGAAU) by TargetScan version 7.2 (http://www.targetscan.org/vert_72/), the putative and mutated sequences of PDCD4, which harbored the miR-499a-5p binding sites were synthesized and cloned into psiCHECK-2 vector (Promega, Madison, WI, USA) to construct PDCD4 3'UTR-wild type (WT PDCD4) and PDCD4 3'UTR-mutant type (MUT PDCD4) reporter plasmids. These recombinant plasmids (200 ng) were co-transfected with 50 nM of miR-499a-5p mimic or miR-NC into SH-SY5Y cells using Lipofectamine 2000 (Invitrogen). After culturing for 48 h, relative luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega). Each sample was analyzed in triplicate.

Statistical analysis

All quantitative data were presented as mean \pm SD of three independent experiments. Data were evaluated using SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). Comparison on the expression of miR-499a-5p between ischemic stroke patients and matched healthy controls was performed using Wilcoxon signed-rank test. Correlation between miR-499a-5p and NIHSS scores were estimated by Pearson correlation test. Differential comparison was performed using the Student's t-test for two group and one-way analysis of variance with Tukey's post-hoc test for multiple groups. A value of p less than 0.05 was thought to be statistically significant.

Results

Circulating miR-499a-5p expression level in acute ischemic stroke patients

To reveal the role of miR-499a-5p in acute ischemic stroke, we performed RT-qPCR analysis to analyze the expression level of miR-499a-5p in the blood samples derived from 64 cases of ischemic stroke patients and matched healthy controls. As shown in **Figure 1A**, circulating miR-499a-5p expression level was significantly downregulated in stroke group compared with control group. We further analyzed the association between miR-499a-5p and NIHSS scores. The results of Pearson correlation test showed that miR-499a-5p expression level was negatively correlated with NIHSS scores (**Figure 1B**, $r = -0.3802$, $p = 0.0046$).

Ischemic expression of miR-499a-5p and PDCD4 in rat brain against cerebral I/R injury

To investigate the potential role of miR-499a-5p in brain I/R injury, the rats randomly received an intracerebroventricular injection of miR-499a-5p mimic prior to MCAO treatment. RT-qPCR analysis first demonstrated that the expression of miR-499a-5p was significantly decreased in the I/R group compared with sham group, but notably increased after intracerebroventricular injection of miR-499a-5p mimic in I/R group (**Figure 2A**). Meanwhile, we found miR-499a-5p overexpression remarkably suppressed the increased expression of PDCD4 mRNA and protein in I/R group by RT-qPCR (**Figure 2B**) and western blot analysis (**Figure 2C-D**), respectively.

Overexpression of miR-499a-5p reduced the infarct damage and cortical neuron apoptosis caused by I/R injury

Based on the constructed I/R injury rat model, we further investigate the role of miR-499a-5p cerebral infarction and the apoptosis of cortical neurons. According to the results from TTC staining (**Figure 3A-B**), normal brain tissue was red, while the infarcted brain tissue was pale. The brain tissue of the Sham group was red with no signs of pale infarction identified. The infarct region was obviously observed in the brain of I/R groups in comparison with the Sham group, which was significantly reduced after intracerebroventricular injection of miR-499a-5p mimic in I/R group. TUNEL assay further showed more TUNEL-positive cells was observed in the brain cortical neurons sections from the I/R group, whereas miR-499a-5p mimic treatment induced significant decrease in the TUNEL-positive cells in I/R group (**Figure 3C**). Furthermore, pro-apoptotic caspase-3 was found to be significantly upregulated in I/R group compared with Sham group, which was obviously attenuated after miR-499a-5p mimic injection in I/R group (**Figure 3D**). These *in vivo* findings indicated that overexpression of miR-499a-5p could exert protective role against cerebral I/R injury by decreasing the infarct damage and cortical neuron apoptosis.

MiR-499a-5p expression was decreased in the *in vitro* model of OGD/R

To confirm the protective role of miR-499-5p in cellular OGD/R injury, we established OGD/R cell model in SH-SY5Y cells and performed a series of functional experiments to evaluate the constructed *in vitro* model of OGD/R. CCK-8 assay showed that cell viability was significantly reduced in OGD/R cells compared to those in normoxic control cells (**Figure 4A**). Consistent with decreased cell viability, the levels of LDH release (**Figure 4B**) and caspase-3 activity (**Figure 4C**) were both significantly elevated when the SH-SY5Y cells under OGD/R conditions. What's more, flow cytometry further manifested the percentage of apoptotic cells was remarkably increased after OGD/R treatment (**Figure 4D**). Within the successfully constructed OGD/R cell model, we consistently observed that miR-499-5p expression was significantly downregulated in OGD/R group compared with control group (**Figure 4E**).

MiR-499a-5p exerted protective effects against OGD/R-induced injury in SH-SY5Y cells

Since the expression of miR-499a-5p was downregulated in OGD/R cell model, we then performed gain-of-function assays in SH-SY5Y cells by transfecting with miR-499a-5p mimic, followed by OGD/R treatment. RT-qPCR analysis confirmed the successfully transfection of miR-499a-5p mimic in SH-SY5Y cells, as demonstrated by remarkably increased miR-499a-5p expression (**Figure 5A**). Then the effects of miR-499a-5p on cell viability, LDH release and apoptosis were evaluated in OGD/R cell model. The results from CCK-8 assay indicated that miR-499a-5p mimic transfection obviously increased the cell viability in OGD/R cells (**Figure 5B**). The LDH release was significantly reduced after miR-499a-5p overexpression in OGD/R cell model (**Figure 5C**). In addition, miR-499a-5p overexpression notably decreased the cell apoptosis in SH-SY5Y cells after OGD/R treatment, as reflected by caspase-3 activity assay (**Figure 5D**) and flow cytometry (**Figure 5E**). Moreover, we performed loss-of-function assay to confirm the protective effects of miR-499a-5p against OGD/R-induced injury. Our data demonstrated that knockdown of miR-499a-5p (**Figure 5F**) significantly decreased cell viability (**Figure 5G**) and enhanced the caspase-3 activity

(**Figure 5H**) in OGD/R cell model. To further confirm apoptosis, we measured the protein level of caspase-3. As shown in **Figure 5I**, miR-499a-5p mimic transfection obviously downregulated, while inhibitor transfection upregulated the protein level of caspase-3, compared with miR-NC transfection in OGD/R cell model.

PDCD4 was a potential target of miR-499a-5p

The online TargetScan program was first employed to predict the targets of miR-499a-5p. Among these predicted targets, we selected five targets of miR-499a-5p. As shown in **Figure 1S-A**, the binding sites between miR-499a-5p and four top targets (MRPS35, ARGLU1, PFN2 and HNRNPC) were displayed. Western blot analysis showed that the protein levels of MRPS35, ARGLU1, PFN2 and HNRNPC were downregulated in different degrees after miR-499a-5p mimic transfection compared with siNC transfection in OGD/R cell model (**Figure 1S-B**). Next, we focused on PDCD4, closely associated with the occurrence of apoptosis, has been involved in several miRNAs-mediated I/R injury ([Cheng et al. 2009](#); [Zheng et al. 2020](#)), which thus was selected as a potential target of miR-499a-5p. As shown in **Figure 6A**, the binding sites between miR-499a-5p and PDCD4 were displayed. Then, dual luciferase reporter assay was performed to confirm PDCD4 as a target of miR-499a-5p. As depicted in **Figure 6B**, the luciferase activity of WT PDCD4 in the miR-499a-5p mimic group was significantly reduced in comparison with the miR-NC group. However, there was no remarkable difference in relation to the luciferase activity of MUT PDCD4 between the miR-NC and miR-499a-5p groups. Next, we analyzed the expression of PDCD4 in OGD/R cell model. Both the RT-qPCR (**Figure 6C**) and western blot analysis (**Figure 6D**) consistently demonstrated that OGD/R induced upregulation of PDCD4 mRNA and protein expression was significantly reversed by miR-499a-5p mimic transfection in SH-SY5Y cells. MiR-499a-5p inhibitor transfection obtained the opposite results in SH-SY5Y cells (**Figure 6E-F**). These results suggested that miR-499a-5p negatively regulated PDCD4 expression via binding its 3'-UTR.

Knockdown of PDCD4 reduced the infarct damage and cortical neuron apoptosis caused by I/R injury

We next investigate the role of PDCD4 in I/R injury in vivo. According to the results from TTC staining (**Figure 7A**), intracerebroventricular injection of siPDCD4 dramatically decreased the infarct area in MCAO group compared with siNC group. TUNEL assay indicated that knockdown of PDCD4 induced significant decrease in the TUNEL-positive cells in I/R group (**Figure 7B**). Consistently, the protein level of pro-apoptotic caspase-3 was obviously attenuated after siPDCD4 injection in I/R group compared with siNC injection (**Figure 7C**).

MiR-499a-5p exerted the protective role against OGD/R-induced injury by targeting PDCD4 in SH-SY5Y cells

To explore whether PDCD4 was a downstream regulator involved in miR-499a-5p regulating OGD/R-induced injury, we first transfected siPDCD4 or siNC into SH-SY5Y cells, followed by OGD/R exposure. With the significant downregulation of PDCD4 in OGD/R model (**Figure 8A**), we observed that the cell viability was increased (**Figure 8B**), while LDH release (**Figure 8C**), cell apoptosis (**Figure 8D**) and

caspase-3 activity (**Figure 8E**) were decreased in SH-SY5Y cells after OGD/R treatment. On the other hand, we performed rescue experiments in SH-SY5Y cells by co-transfection with miR-499a-5p mimic and PDCD4 overexpression plasmid or empty vector to further confirm our hypothesis. As shown in **Figure 9A**, PDCD4 overexpression plasmid transfection increased the PDCD4 expression under miR-499a-5p mimic. Functionally, PDCD4 overexpression significantly reversed the effects of miR-499a-5p on cell viability (**Figure 9B**), LDH release (**Figure 9C**), cell apoptosis (**Figure 9D**) and caspase-3 activity (**Figure 9E**) in SH-SY5Y cells under OGD/R condition. These findings suggest that miR-499a-5p attenuated OGD/R induced injury might through inhibiting PDCD4.

Discussion

In the present study, we reported that circulating miR-499a-5p expression level was significantly downregulated in stroke patients and negatively associated with NIHSS scores. By constructing the in vivo MCAO rat model, we further confirmed that the expression of miR-499a-5p was rapidly increased in the rat brain tissues after MCAO. Subsequently, rats were given intracerebroventricular injection of miR-499a-5p mimic and the data showed that overexpression of miR-499a-5p significantly effectively reduced brain infarct size and the apoptosis of cortical neurons. Consistently, miR-499a-5p expression has been shown to be remarkably decreased after myocardial I/R injury (Liu et al. 2019). Similarly, miR-499a-5p may attenuate TNF- α -induced human nucleus pulposus cell apoptosis (Sun et al. 2019). These data indicated that miR-499a-5p suppressed the cerebral I/R injury might by decreasing MCAO-induced apoptosis.

As previously demonstrated, the neuron-like human-derived neuroblastoma cell line SH-SY5Y is a recognized as the most commonly used tool in five models of ischemia-related injury, including OGD, H₂O₂-induced oxidative stress, oxygen deprivation, glucose deprivation and glutamate excitotoxicity because of its human origin, catecholaminergic neuronal properties, and ease of maintenance (Liu et al. 2018b). Here, we further established an in vitro model of I/R injury with SH-SY5Y and determined the effect of miR-499a-5p on cell viability and apoptosis. Our data showed that overexpression of miR-499a-5p protected SH-SY5Y cells against OGD/R-induced injury, as reflected by elevated cell viability and decreased LDH release, apoptosis rate accompanied decrease in caspase-3 expression and caspase-3 activity. Knockdown of miR-499a-5p obtained the opposite results. As our best knowledge, I/R injury can induce apoptosis of tissues and organs, which is an important pathological manifestation of I/R injury affecting tissues and organs (Lopez-Neblina et al. 2005). Apoptosis usually occupies a crucial role in the pathogenesis of ischemic brain injury and the subsequent reperfusion damage (He et al. 2016; Machado et al. 2009). Similar to miR-499a-5p-induced suppression of apoptosis, miR-499a-5p could suppress the apoptosis of human nucleus pulposus cells and degradation of their extracellular matrix by targeting SOX4 (Sun et al. 2019). Downregulation of CD38 by miR-499a-5p significantly decreased the apoptosis and the LDH activity of H9C2 cells under hypoxia-reoxygenation conditions (Zhao et al. 2020). In addition, miR-499a-5p participated in circ_0079593 (Yang et al. 2020) and lncRNA secretory carrier

membrane protein 1 (SCAMP1) (Zong et al. 2019) regulating glioma cell proliferation, motility and apoptosis.

Since PDCD4 is closely associated with the occurrence of apoptosis (Matsushashi et al. 2019). In our constructed in vivo and in vitro models, we found that PDCD4 expression was significantly upregulated compared with the corresponding control groups. As expected, we confirmed that the 3'UTR region of PDCD4 contains a binding site for miR-499a-5p and miR-400a-5p negatively regulated the PDCD4 expression. Furthermore, knockdown of PDCD4 reduced the infarct damage and cortical neuron apoptosis caused by I/R injury. PDCD4 knockdown imitated, while overexpression reversed the effects of miR-499a-5p on cell viability, LDH release, cell apoptosis and caspase-3 activity in SH-SY5Y cells after OGD/R treatment. Similarly, PDCD4 silencing can attenuate vascular endothelial cell injury caused by ischemia-reperfusion in the lower extremities in rats (Chen et al. 2019). In line with our data regarding on the role miR-499a-5p/PDCD4 axis in I/R injury, PDCD4 was demonstrated to be a downstream regulator of miR-21 (Jia et al. 2017; Cheng et al. 2009) and miR-340-5p regulating ischemic postconditioning-mediated attenuation of intestinal injury and apoptosis (Zheng et al. 2020). According to these facts, we concluded that miR-499a-5p exerted the protective role against OGD/R-induced injury might by targeting PDCD4 in SH-SY5Y cells.

In summary, the present study confirmed that the expression of miR-499a-5p was decreased during cerebral I/R injury in vivo and in vitro. Our in vitro transfection experiments show that elevated miR-499a-5p expression can rescue the apoptosis of SH-SY5Y cells under OGD/R condition. We further demonstrated that miR-499a-5p exerted the protective role against OGD/R-induced injury by targeting PDCD4 in SH-SY5Y cells. The data of this study suggest that targeting miR-499a-5p/PDCD4 axis may provide a potential therapeutic option for clinical treatment of ischemic stroke.

Abbreviations

I/R, ischemia/reperfusion; OGD/R, oxygen-glucose deprivation and reoxygenation; 3'-UTR, 3'-untranslated region; PDCD4, programmed cell death 4; HIF-1 α , hypoxia inducible factor 1 alpha; MCAO, middle cerebral artery occlusion; NIHSS, National Institutes of Health Stroke Scale; TTC, 2, 3, 5-triphenyltetrazolium chloride; TUNEL, TdT-mediated dUTP Nick-end labeling; DMEM, Dulbecco's modified Eagle's medium; RT-qPCR, reverse transcription quantitative PCR; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LDH, lactate dehydrogenase;

Declarations

Ethics approval and consent to participate

The use of the human blood samples for research purposes was approved by the Ethics Committee of the 1st Affiliated Hospital, School of Medicine, Zhejiang University (Approval number: ZU349-2;

2017.8.16) and all participants signed the written informed consent, which in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Funding

Not applicable.

Availability of data and materials

The data in this study are available in this published article.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

Not applicable.

References

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116 (2):281-297. doi:10.1016/s0092-8674(04)00045-5

Begum G, Yuan H, Kahle KT, Li L, Wang S, Shi Y, Shmukler BE, Yang SS, Lin SH, Alper SL, Sun D (2015) Inhibition of WNK3 Kinase Signaling Reduces Brain Damage and Accelerates Neurological Recovery After Stroke. *Stroke* 46 (7):1956-1965. doi:10.1161/STROKEAHA.115.008939

Benjamin EJ, Virani SS, Callaway CW, Chamberlain AM, Chang AR, Cheng S, Chiuve SE, Cushman M, Delling FN, Deo R, de Ferranti SD, Ferguson JF, Fornage M, Gillespie C, Isasi CR, Jimenez MC, Jordan LC, Judd SE, Lackland D, Lichtman JH, Lisabeth L, Liu S, Longenecker CT, Lutsey PL, Mackey JS, Matchar DB, Matsushita K, Mussolino ME, Nasir K, O'Flaherty M, Palaniappan LP, Pandey A, Pandey DK, Reeves MJ, Ritchey MD, Rodriguez CJ, Roth GA, Rosamond WD, Sampson UKA, Satou GM, Shah SH, Spartano NL, Tirschwell DL, Tsao CW, Voeks JH, Willey JZ, Wilkins JT, Wu JH, Alger HM, Wong SS, Muntner P, American Heart Association Council on E, Prevention Statistics C, Stroke Statistics S (2018) Heart Disease and Stroke Statistics-2018 Update: A Report From the American Heart Association. *Circulation* 137 (12):e67-e492. doi:10.1161/CIR.0000000000000558

Chen H, Zhu H, Yang J, Zhu Y, Mei J, Shen H, Liang K, Zhang X (2019) Role of Programmed Cell Death 4 (PDCD4)-Mediated Akt Signaling Pathway in Vascular Endothelial Cell Injury Caused by Lower-Extremity

Ischemia-Reperfusion in Rats. *Medical science monitor : international medical journal of experimental and clinical research* 25:4811-4818. doi:10.12659/msm.914035

Cheng Y, Liu X, Zhang S, Lin Y, Yang J, Zhang C (2009) MicroRNA-21 protects against the H₂O₂-induced injury on cardiac myocytes via its target gene PDCD4. *Journal of molecular and cellular cardiology* 47 (1):5-14. doi:10.1016/j.yjmcc.2009.01.008

Chomova M, Zitnanova I (2016) Look into brain energy crisis and membrane pathophysiology in ischemia and reperfusion. *Stress*:1-8

Di Y, Lei Y, Yu F, Changfeng F, Song W, Xuming M (2014) MicroRNAs expression and function in cerebral ischemia reperfusion injury. *Journal of molecular neuroscience : MN* 53 (2):242-250. doi:10.1007/s12031-014-0293-8

Gu X, Dong M, Liu Z, Yang J, Shi Y (2020) MiR-499a-5p Inhibits Proliferation, Invasion, Migration, and Epithelial-Mesenchymal Transition, and Enhances Radiosensitivity of Cervical Cancer Cells via Targeting eIF4E. *OncoTargets and therapy* 13:2913-2924. doi:10.2147/ott.s241631

He G, Xu W, Tong L, Li S, Su S, Tan X, Li C (2016) Gadd45b prevents autophagy and apoptosis against rat cerebral neuron oxygen-glucose deprivation/reperfusion injury. *Apoptosis : an international journal on programmed cell death* 21 (4):390-403. doi:10.1007/s10495-016-1213-x

He S, Li Z, Yu Y, Zeng Q, Cheng Y, Ji W, Xia W, Lu S (2019) Exosomal miR-499a-5p promotes cell proliferation, migration and EMT via mTOR signaling pathway in lung adenocarcinoma. *Experimental cell research* 379 (2):203-213. doi:10.1016/j.yexcr.2019.03.035

Hu Y, Deng H, Xu S, Zhang J (2015) MicroRNAs Regulate Mitochondrial Function in Cerebral Ischemia-Reperfusion Injury. *International journal of molecular sciences* 16 (10):24895-24917. doi:10.3390/ijms161024895

Jia Z, Lian W, Shi H, Cao C, Han S, Wang K, Li M, Zhang X (2017) Ischemic Postconditioning Protects Against Intestinal Ischemia/Reperfusion Injury via the HIF-1 α /miR-21 Axis. *Scientific reports* 7 (1):16190. doi:10.1038/s41598-017-16366-6

Liu J, Huang L, Su P, Song T, Zhang W, Fan J, Liu Y (2018a) MicroRNA-499a-5p inhibits osteosarcoma cell proliferation and differentiation by targeting protein phosphatase 1D through protein kinase B/glycogen synthase kinase 3 β signaling. *Oncology letters* 15 (4):4113-4120. doi:10.3892/ol.2018.7814

Liu K, Ma L, Zhou F, Yang Y, Hu HB, Wang L, Zhong L (2019) Identification of microRNAs related to myocardial ischemic reperfusion injury. *Journal of cellular physiology* 234 (7):11380-11390. doi:10.1002/jcp.27795

Liu Y, Eaton ED, Wills TE, McCann SK, Antonic A, Howells DW (2018b) Human Ischaemic Cascade Studies Using SH-SY5Y Cells: a Systematic Review and Meta-Analysis. *Translational stroke research* 9 (6):564-

574. doi:10.1007/s12975-018-0620-4

Lopez-Neblina F, Toledo AH, Toledo-Pereyra LH (2005) Molecular biology of apoptosis in ischemia and reperfusion. *Journal of investigative surgery : the official journal of the Academy of Surgical Research* 18 (6):335-350. doi:10.1080/08941930500328862

Luengo-Fernandez R, Gray AM, Bull L, Welch S, Cuthbertson F, Rothwell PM, Oxford Vascular S (2013) Quality of life after TIA and stroke: ten-year results of the Oxford Vascular Study. *Neurology* 81 (18):1588-1595. doi:10.1212/WNL.0b013e3182a9f45f

Machado NG, Alves MG, Carvalho RA, Oliveira PJ (2009) Mitochondrial involvement in cardiac apoptosis during ischemia and reperfusion: can we close the box? *Cardiovascular toxicology* 9 (4):211-227. doi:10.1007/s12012-009-9055-1

Matsushashi S, Manirujjaman M, Hamajima H, Ozaki I (2019) Control Mechanisms of the Tumor Suppressor PDCD4: Expression and Functions. *International journal of molecular sciences* 20 (9). doi:10.3390/ijms20092304

Neshati V, Mollazadeh S, Fazly Bazzaz BS, de Vries AAF, Mojarrad M, Naderi-Meshkin H, Neshati Z, Mirahmadi M, Kerachian MA (2018) MicroRNA-499a-5p Promotes Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells to Cardiomyocytes. *Applied biochemistry and biotechnology* 186 (1):245-255. doi:10.1007/s12010-018-2734-2

Qian Zhang KZ, Chao Zhang, Hongfei Ge, Yi Yin, Hua Feng, Rong Hu (2017) MicroRNAs as Big Regulators of Neural Stem/Progenitor Cell Proliferation, Differentiation and Migration: A Potential Treatment for Stroke. *Curr Pharm Des:-*

Rao G, Zhang W, Song S (2019) MicroRNA-217 inhibition relieves cerebral ischemia/reperfusion injury by targeting SIRT1. *Molecular medicine reports* 20 (2)

Soejima H, Miyoshi O, Yoshinaga H, Masaki Z, Ozaki I, Kajiwara S, Niikawa N, Matsushashi S, Mukai T (1999) Assignment of the programmed cell death 4 gene (PDCD4) to human chromosome band 10q24 by in situ hybridization. *Cytogenetics and cell genetics* 87 (1-2):113-114. doi:10.1159/000015408

Special report from the National Institute of Neurological Disorders and Stroke. Classification of cerebrovascular diseases III (1990). *Stroke* 21 (4):637-676. doi:10.1161/01.str.21.4.637

Sun JC, Zheng B, Sun RX, Meng YK, Wang SM, Yang HS, Chen Y, Shi JG, Guo YF (2019) MiR-499a-5p suppresses apoptosis of human nucleus pulposus cells and degradation of their extracellular matrix by targeting SOX4. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 113:108652. doi:10.1016/j.biopha.2019.108652

Tobin MK, Bonds JA, Minshall RD, Pelligrino DA, Testai FD, Lazarov O (2014) Neurogenesis and inflammation after ischemic stroke: what is known and where we go from here. *Journal of cerebral blood*

flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 34 (10):1573-1584. doi:10.1038/jcbfm.2014.130

Yang G, Liu Z, Wang L, Chen X, Wang X, Dong Q, Zhang D, Yang Z, Zhou Q, Sun J, Xue L, Wang X, Gao M, Li L, Yi R, Ilgiz G, Ai J, Zhao S (2018) MicroRNA-195 protection against focal cerebral ischemia by targeting CX3CR1. *Journal of neurosurgery*:1-10. doi:10.3171/2018.5.JNS173061

Yang Z, Li C, Fan XY, Liu LJ (2020) Circular RNA circ_0079593 promotes glioma development through regulating KPNA2 expression by sponging miR-499a-5p. *European review for medical and pharmacological sciences* 24 (3):1288-1301. doi:10.26355/eurev_202001_20186

Yin KJ, Deng Z, Huang H, Hamblin M, Xie C, Zhang J, Chen YE (2010) miR-497 regulates neuronal death in mouse brain after transient focal cerebral ischemia. *Neurobiology of disease* 38 (1):17-26. doi:10.1016/j.nbd.2009.12.021

Zhao L, Wang B, Zhang W, Sun L (2020) Effect of miR-499a-5p on damage of cardiomyocyte induced by hypoxia-reoxygenation via downregulating CD38 protein. *Journal of cellular biochemistry* 121 (2):996-1004. doi:10.1002/jcb.29334

Zheng Y, Zhao P, Lian Y, Li S, Chen Y, Li L (2020) MiR-340-5p alleviates oxygen-glucose deprivation/reoxygenation-induced neuronal injury via PI3K/Akt activation by targeting PDCD4. *Neurochemistry international* 134:104650. doi:10.1016/j.neuint.2019.104650

Zhong G, Long H, Ma S, Shunhan Y, Li J, Yao J (2019) miRNA-335-5p relieves chondrocyte inflammation by activating autophagy in osteoarthritis. *Life sciences* 226:164-172. doi:10.1016/j.lfs.2019.03.071

Zong Z, Song Y, Xue Y, Ruan X, Liu X, Yang C, Zheng J, Cao S, Li Z, Liu Y (2019) Knockdown of LncRNA SCAMP1 suppressed malignant biological behaviours of glioma cells via modulating miR-499a-5p/LMX1A/NLRC5 pathway. *Journal of cellular and molecular medicine* 23 (8):5048-5062. doi:10.1111/jcmm.14362

Zuo X, Lu J, Manaenko A, Qi X, Tang J, Mei Q, Xia Y, Hu Q (2019) MicroRNA-132 attenuates cerebral injury by protecting blood-brain-barrier in MCAO mice. *Experimental neurology* 316:12-19. doi:10.1016/j.expneurol.2019.03.017

Tables

Table 1 Baseline characteristics of ischemic stroke patients

Demographic characteristics	Value
Total (N)	64
Age (median, range)	59.6 (51-74)
Sex (male/female)	34/30
Hypertension (n, %)	42 (65.6)
Hyperlipidaemia (n, %)	48 (75.0)
Diabetes (n, %)	25 (39.1)
Alcohol drinking (n, %)	20 (31.2)
NIHSS score (median, range)	6 (2-11)

Note: NIHSS, National Institute of Health Stroke Scale

Figures

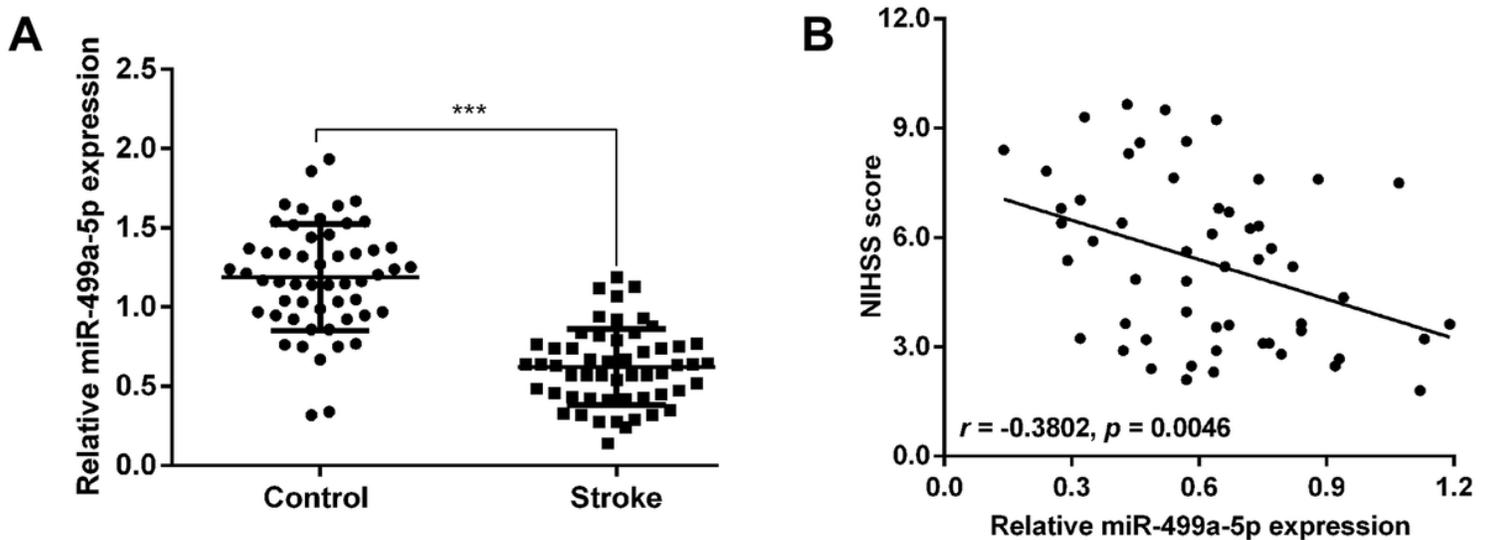


Figure 1

Circulating miR-499a-5p expression level in acute ischemic stroke patients. (A) The expression levels of miR-499a-5p in blood samples derived from 64 cases of ischemic stroke patients and matched healthy controls were determined by RT-qPCR analysis. *** $p < 0.001$ by Wilcoxon signed-rank test; (B) Correlation between miR-499a-5p expression level and NIHSS score in ischemic stroke patients.

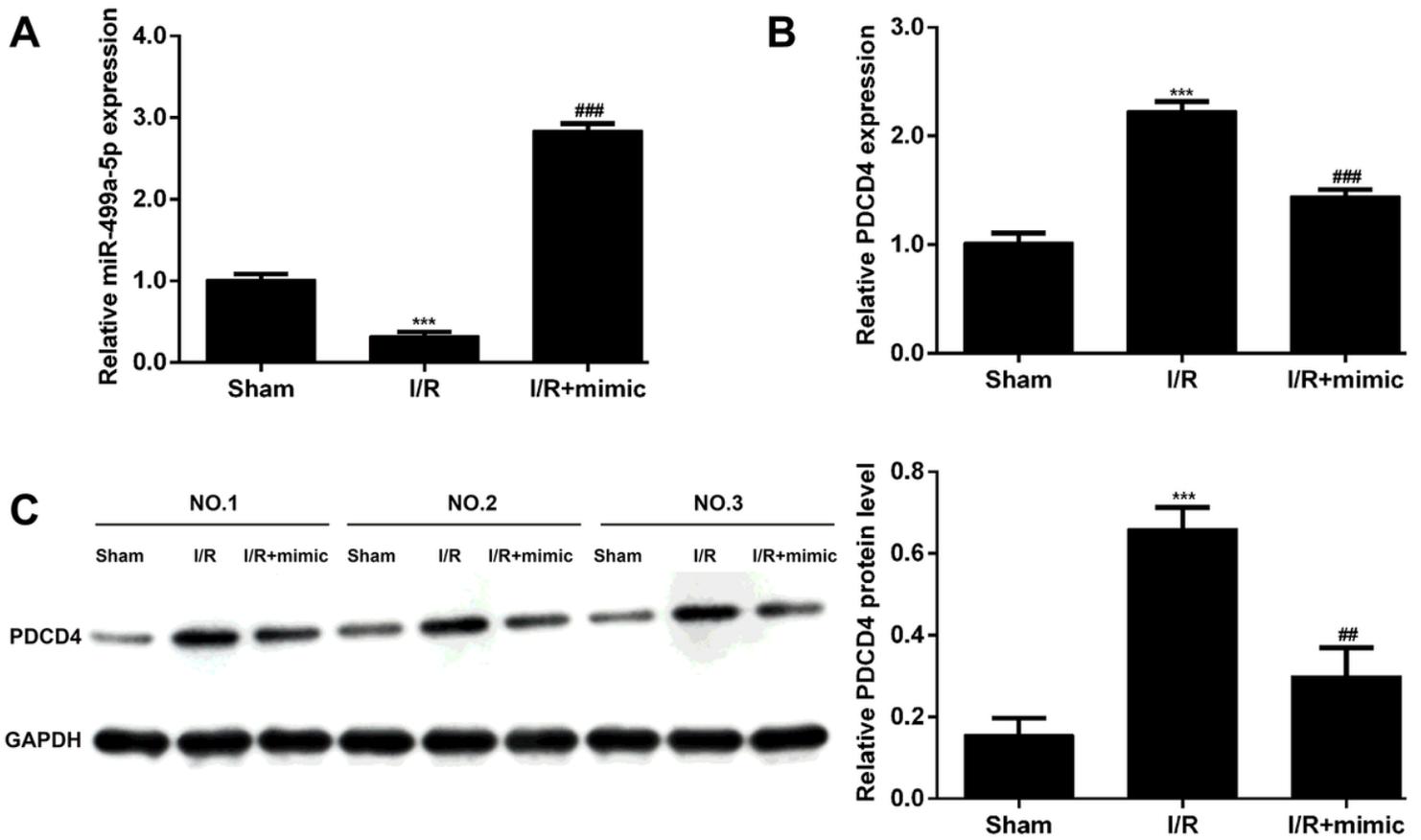


Figure 2

Ischemic expression of miR-499a-5p and PDCD4 in rat brain against cerebral I/R injury. The rats randomly received an intracerebroventricular injection of miR-499a-5p mimic prior to MCAO treatment, with Sham as control (n = 6 per group). The expression levels of miR-499a-5p (A) and PDCD4 mRNA (B) in rat brains from Sham, I/R and I/R + miR-499a-5p mimic groups were analyzed by RT-qPCR. (C-D) The protein expression of PDCD4 was detected in rat brains from Sham, I/R and I/R + miR-499a-5p mimic groups using western blot analysis. Each value represented mean \pm SD of three independent experiments. ***p < 0.001, compared with Sham; ##p < 0.01, ###p < 0.001, compared with I/R by one-way analysis of variance with Tukey's post-hoc test.

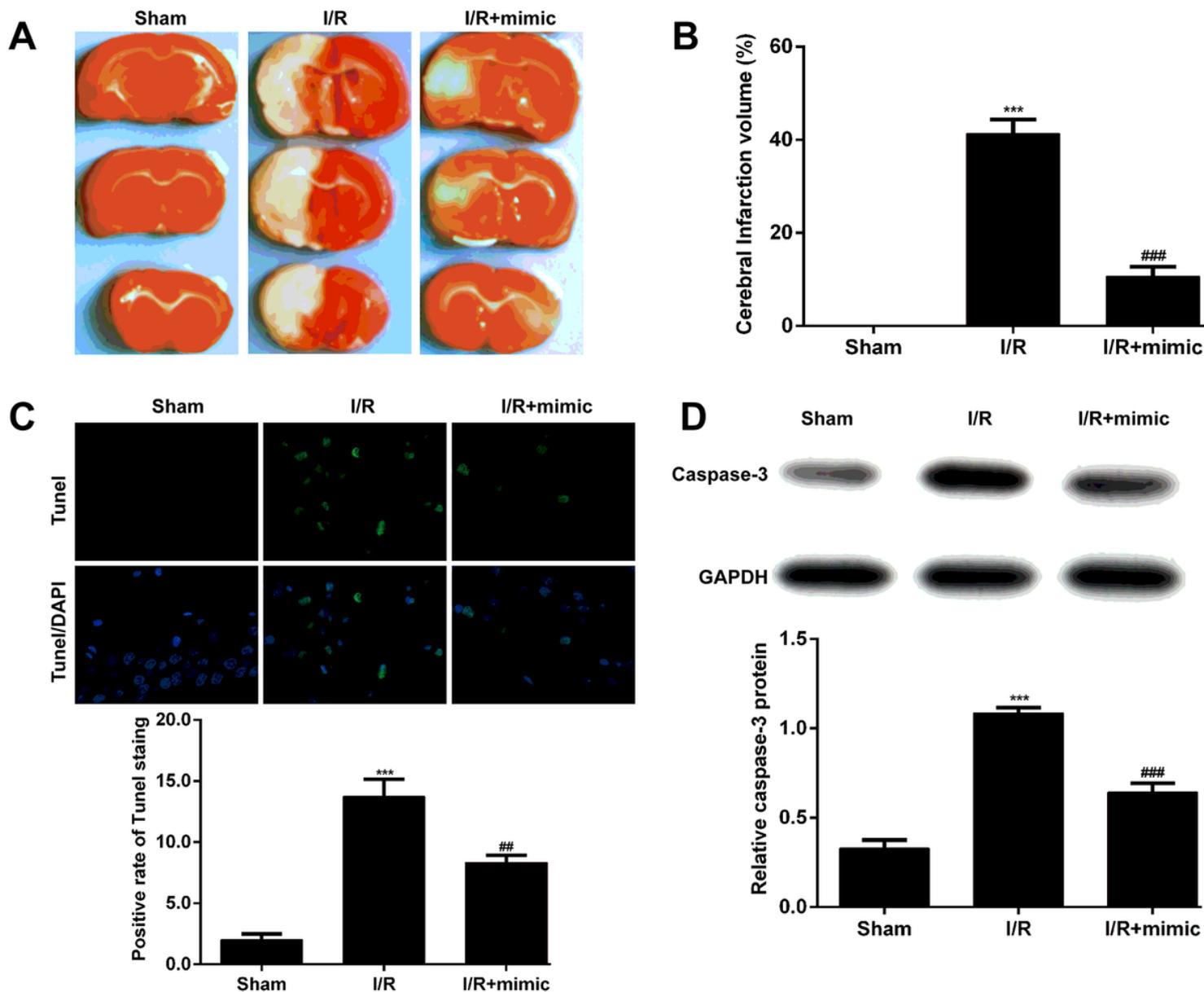


Figure 3

Overexpression of miR-499a-5p reduced the infarct damage and cortical neuron apoptosis caused by I/R injury. The rats randomly received an intracerebroventricular injection of miR-499a-5p mimic prior to MCAO treatment, with Sham as control (n = 6 per group). (A) Representative images of TTC-stained coronal sections and (B) analysis of the volumes of infarction in rat brain tissues in the above three groups. (C) The apoptosis of cortical neurons was evaluated by TUNEL staining. (D) Measurement of caspase-3 protein levels in the above three groups using western blotting. Each value represented mean \pm SD of three independent experiments. $***p < 0.001$, compared with Sham; $##p < 0.01$, $###p < 0.001$, compared with I/R by one-way analysis of variance with Tukey's post-hoc test.

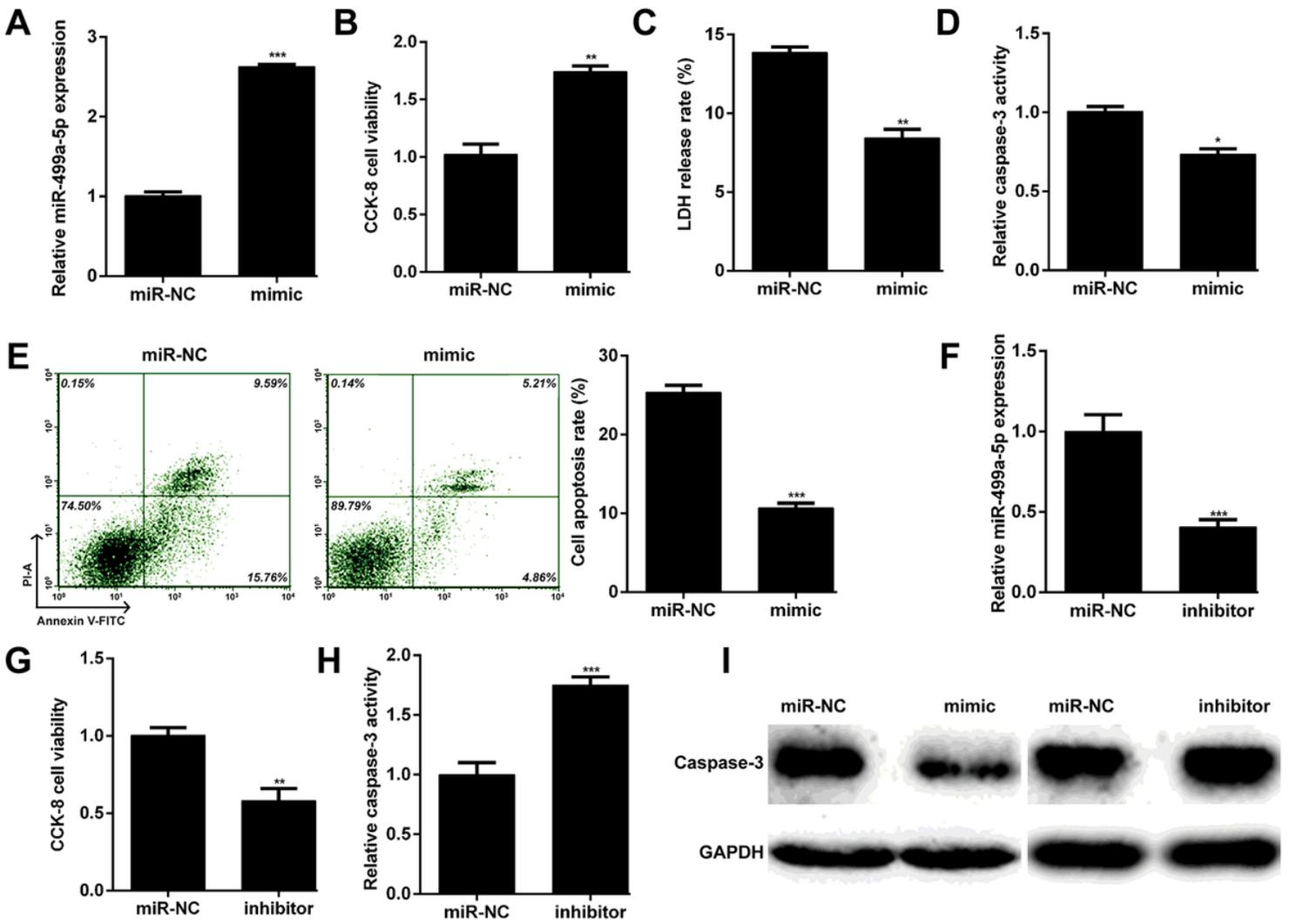


Figure 4

MiR-499a-5p expression was decreased in the in vitro model of OGD/R. SH-SY5Y cells were subjected to 5 h of oxygen and glucose deprivation (OGD), followed by 24 h of reoxygenation. (A) CCK-8 assay was utilized to analyze cell viability. (B) The release of lactate dehydrogenase (LDH) was analyzed in OGD/R group and control group. (C) Colorimetric assay of caspase-3 activity in OGD/R group and control group. (D) Flow cytometry was used to analyze cell apoptosis in SH-SY5Y cells from control and OGD/R groups. (E) The relative expression of miR-499a-5p was determined in SH-SY5Y cells from control and OGD/R groups. Each value represented mean \pm SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$, compared with Control by Student's t-test

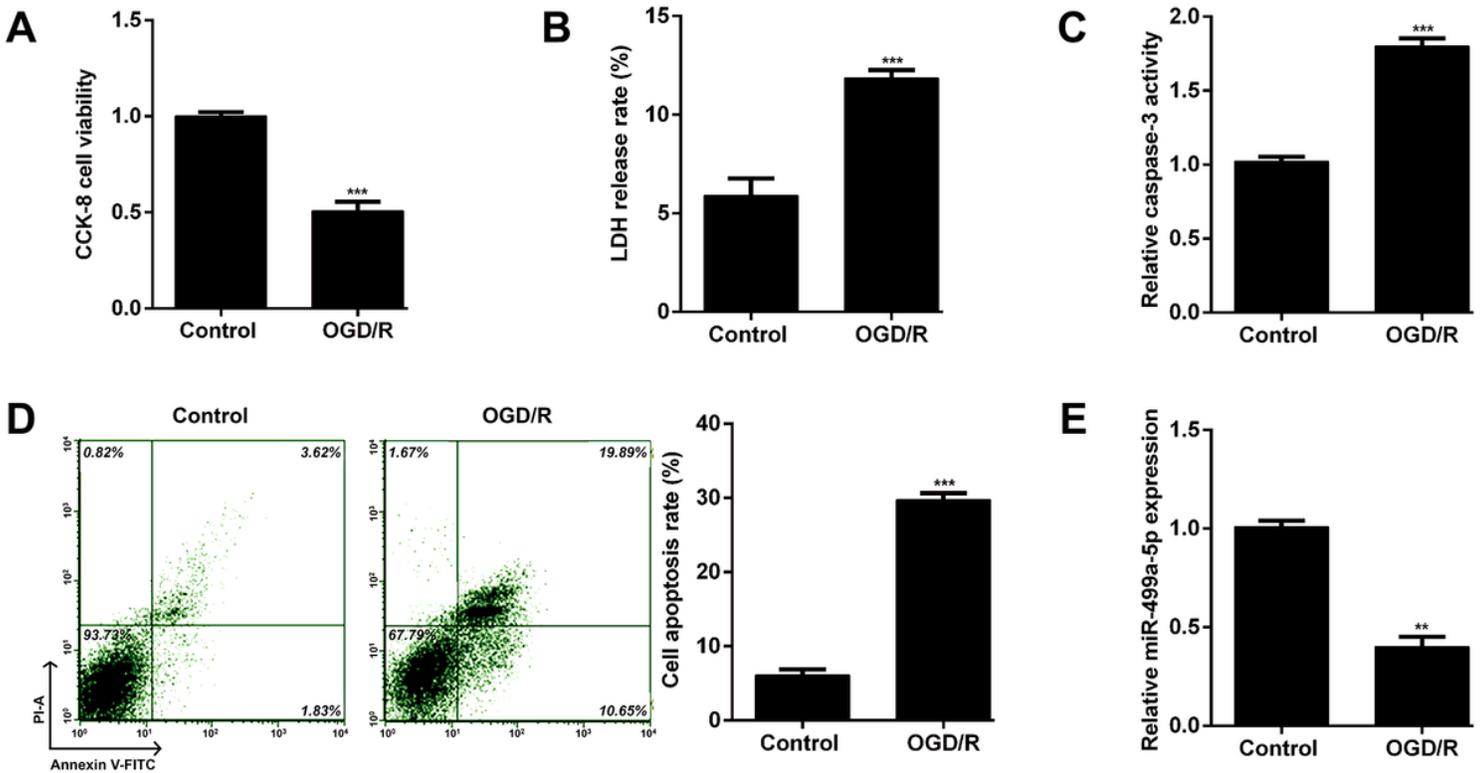


Figure 5

MiR-499a-5p exerted protective effects against OGD/R-induced injury in SH-SY5Y cells. SH-SY5Y cells were transfected with miR-499a-5p mimics or miR-NC, followed by OGD/R treatment. (A) The relative expression of miR-499a-5p was determined in the above treated SH-SY5Y cells. (B) CCK-8 assay was utilized to analyze cell viability. (C) The release of lactate dehydrogenase (LDH) was analyzed in the above treated SH-SY5Y cells. (D) Colorimetric assay of caspase-3 activity in the above treated SH-SY5Y cells. (E) Flow cytometry was used to analyze cell apoptosis in the above treated SH-SY5Y cells. (F) The relative expression of miR-499a-5p was determined in SH-SY5Y cells transfected with miR-499a-5p inhibitor or miR-NC, followed by OGD/R treatment. (G) Cell viability and (H) caspase-3 activity was analyzed in SH-SY5Y cells transfected with miR-499a-5p inhibitor or miR-NC, followed by OGD/R treatment. (I) The protein level of PDCD4 was measured in SH-SY5Y cells transfected with miR-499a-5p mimics, inhibitor or miR-NC, followed by OGD/R treatment. Each value represented mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with miR-NC by Student's t-test

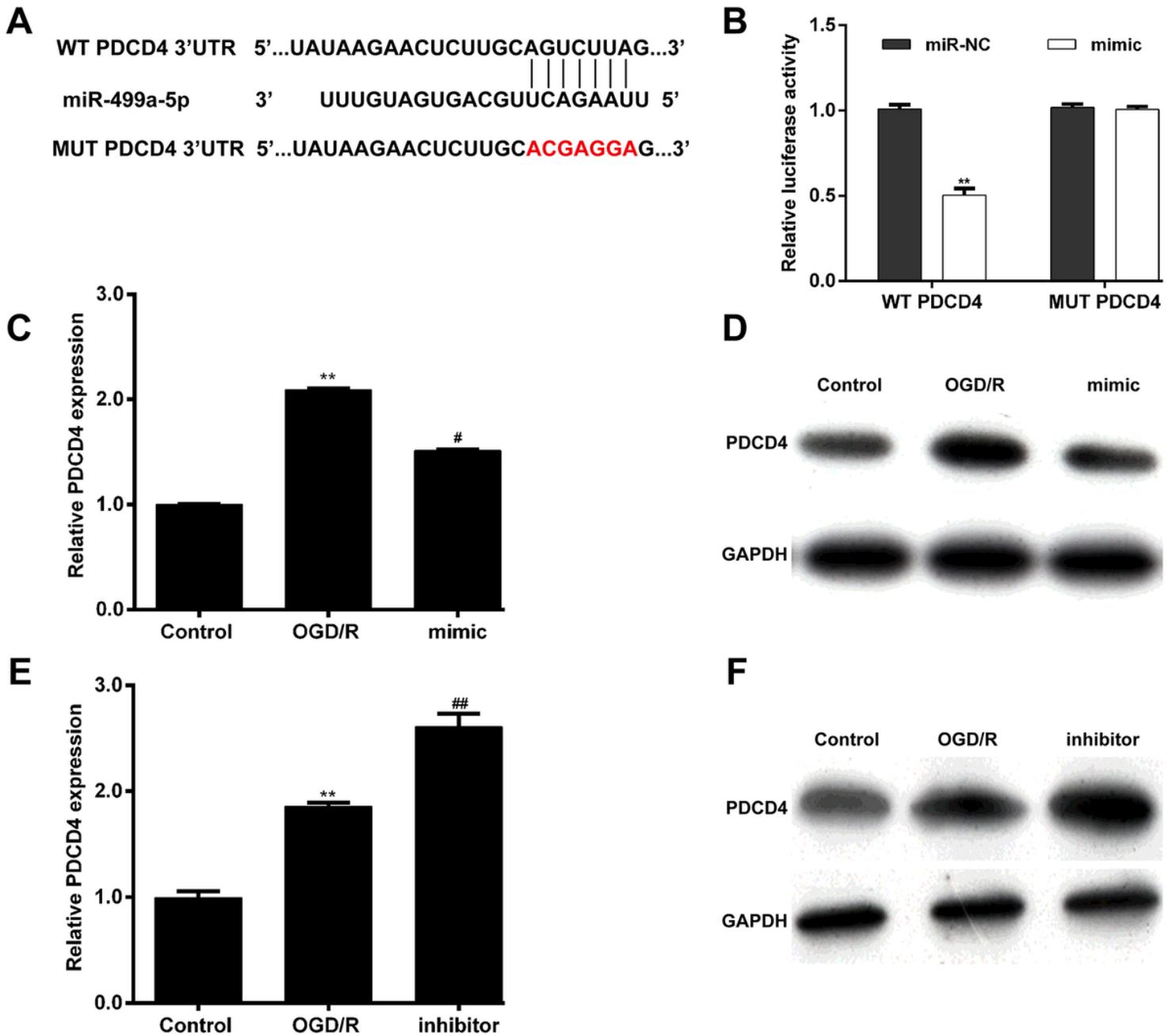


Figure 6

PDCD4 was a potential target of miR-499a-5p. (A) The potential interaction between miR-499a-5p and putative binding sites in the PDCD4 3'UTR. The mutant sequences are equivalent to the wild-type ones except mutations at the 3' end of target site is underlined. (B) Relative luciferase activities were analyzed in SH-SY5Y cells 48 h after co-transfection of miR-499a-5p mimic and either wild-type or mutant PDCD4 3'UTR. ** $p < 0.01$, compared with miR-NC by Student's t-test; (C) RT-qPCR and (D) Western blot analysis was used to determine the expression of PDCD4 in SH-SY5Y cells transfected with miR-499a-5p mimic or miR-NC, and then was exposed to OGD/R conditions. (E) RT-qPCR and (F) Western blot analysis was used to determine the expression of PDCD4 in SH-SY5Y cells transfected with miR-499a-5p inhibitor or miR-NC, and then was exposed to OGD/R conditions. Each value represented mean \pm SD of three independent

experiments. ** $p < 0.01$, compared with Control; # $p < 0.05$, ## $p < 0.01$, compared with OGD/R by one-way analysis of variance with Tukey's post-hoc test.

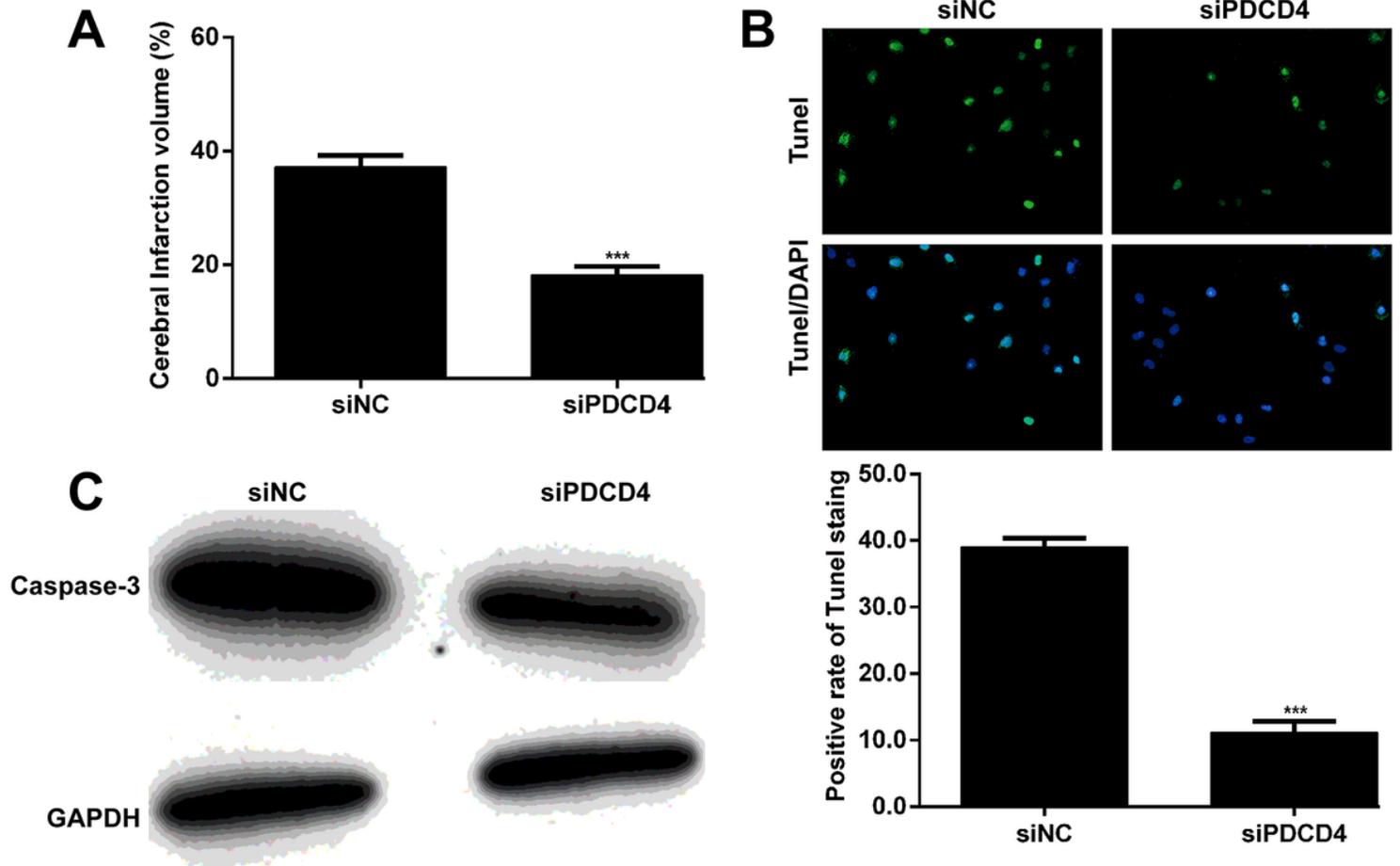


Figure 7

Knockdown of PDCD4 reduced the infarct damage and cortical neuron apoptosis caused by I/R injury. The rats randomly received an intracerebroventricular injection of siPDCD4 or siNC prior to MCAO treatment, with Sham as control (n = 6 per group). (A) Analysis of the volumes of infarction in rat brain tissues in the above two groups. (B) The apoptosis of cortical neurons was evaluated by TUNEL staining. (C) Measurement of caspase-3 protein levels in the above two groups using western blotting. *** $p < 0.001$, compared with siNC by Student's t-test.

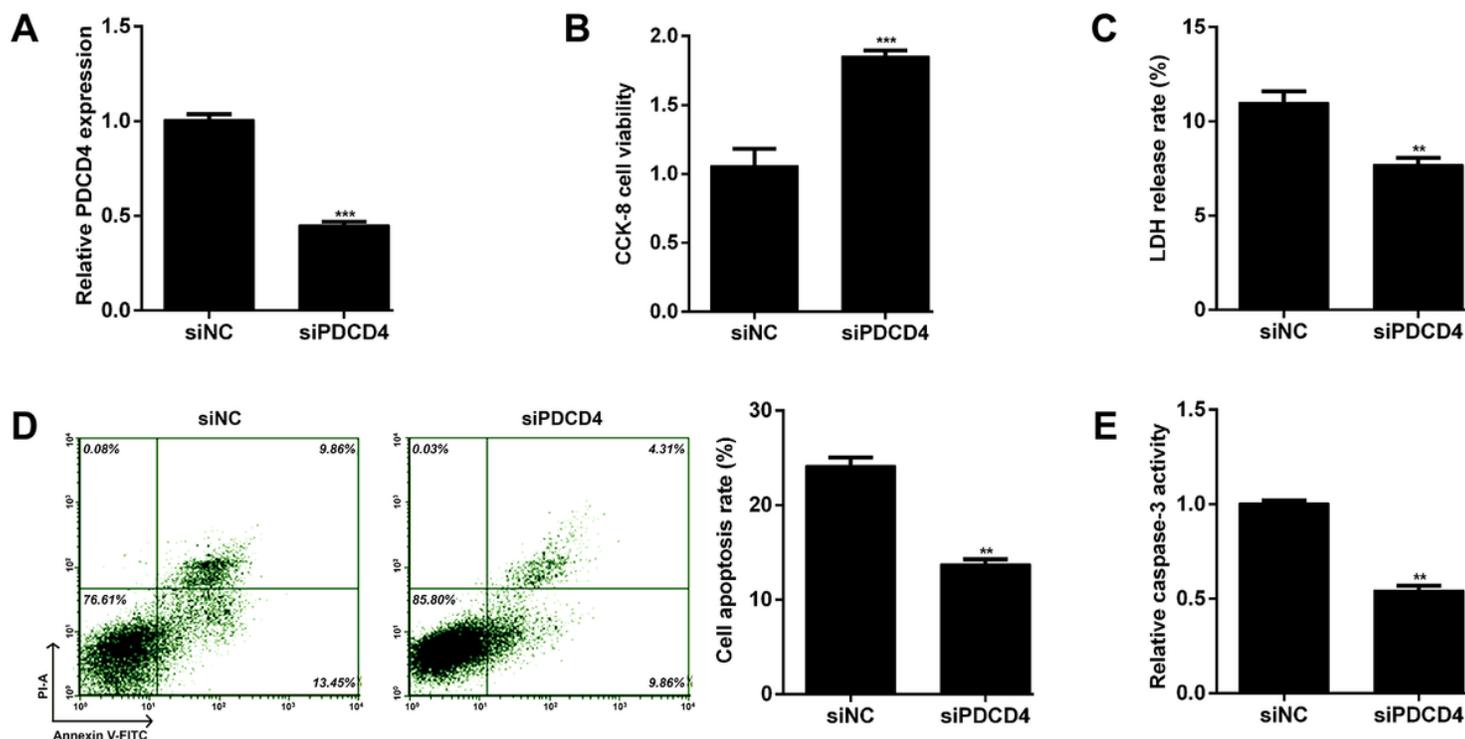


Figure 8

PDCD knockdown protected SH-SY5Y cells against OGD/R-induced injury. SH-SY5Y cells were transfected with siPDCD4 or siNC, followed by OGD/R treatment. (A) The relative expression of PDCD4 was determined in the above treated SH-SY5Y cells. (B) CCK-8 assay was utilized to analyze cell viability. (C) The release of lactate dehydrogenase (LDH) was analyzed in the above treated SH-SY5Y cells. (D) Flow cytometry was used to analyze cell apoptosis in the above treated SH-SY5Y cells. (E) Colorimetric assay of caspase-3 activity in the above treated SH-SY5Y cells. Each value represented mean \pm SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$, compared with siNC by Student's t-test

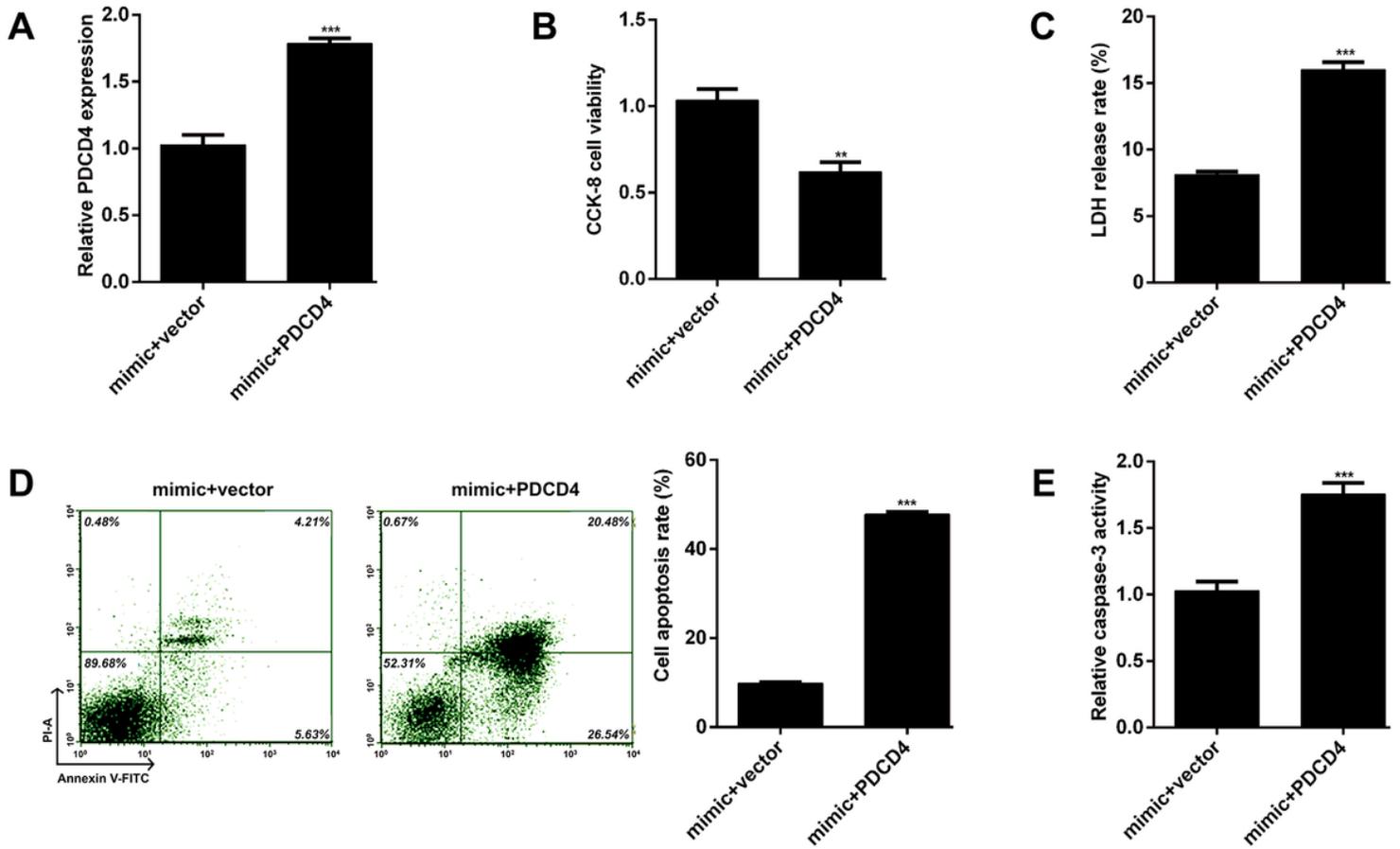


Figure 9

Overexpression of PDCD4 reversed the protective role of miR-499a-5p against OGD/R-induced injury. SH-SY5Y cells were co-transfected with miR-499a-5p mimic and PDCD4 overexpression plasmid or empty vector. (A) The relative expression of PDCD4 was determined in the above treated SH-SY5Y cells. (B) CCK-8 assay was utilized to analyze cell viability. (C) The release of lactate dehydrogenase (LDH) was analyzed in the above treated SH-SY5Y cells. (D) Flow cytometry was used to analyze cell apoptosis in the above treated SH-SY5Y cells. (E) Colorimetric assay of caspase-3 activity in the above treated SH-SY5Y cells. Each value represented mean \pm SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$, compared with mimic + vector by Student's t-test

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [RawWB.tif](#)
- [OnlineFigureS1.png](#)