Knockdown of lncRNA TTTY15 alleviates ischemia/reperfusion-induced inflammation and apoptosis of PC12 cells by targeting miR-766-5p

CHENGUANG HAO[1] and SHIBAO CHEN[2]

[1]Department of Neurology, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang 830054; [2]Department of Neurology, Bayingolin Mongolian Autonomous Prefecture People's Hospital, Korla, Xinjiang 841000, P.R. China

Received October 26, 2020; Accepted February 22, 2021

DOI: 10.3892/etm.2021.9942

Abstract. The pathogenesis of ischemic stroke is extremely complex and has a significant impact on the quality of life of the patients. Accumulating studies have reported that long non-coding RNAs (lncRNAs) may be associated with the progression of ischemic stroke. However, the role and underlying mechanism of action of the lncRNA testis-specific transcript Y-linked 15 (TTTY15) in ischemic stroke remains unknown. The present study analyzed the expression levels of TTTY15 in PC12 cells injured by oxygen-glucose deprivation/reperfusion (OGD/R). The effects of the knockdown of TTTY15 expression on the levels of the inflammatory cytokines TNF-α, IL-1β, IL-18 and IL-10, cell apoptosis and the expression levels of the apoptosis-associated proteins Bcl-2, Bax, cleaved caspase-3, caspase-3, cleaved caspase-9 and caspase-9, were subsequently analyzed in OGD/R-treated PC12 cells using ELISA, flow cytometry and western blotting, respectively. In addition, the downstream target gene of TTTY15 was verified using a dual luciferase reporter assay. The effects of TTTY15 on the inflammation and apoptosis of PC12 cells treated with OGD/R were determined by targeting miR-766-5p. The results of the present study revealed that TTTY15 expression was upregulated in OGD/R-treated PC12 cells. The knockdown of TTTY15 significantly decreased the concentrations of the proinflammatory factors TNF-α, IL-1β and IL-18, while it increased the concentration of the anti-inflammatory cytokine IL-10 in OGD/R-treated PC12 cells. Apoptosis was also suppressed following gene silencing of TTTY15. Subsequently, miR-766-5p was identified as a target gene of TTTY15 using a dual luciferase reporter assay and the expression levels of TTTY15 and miR-766-5p were found to be negatively correlated. The overexpression of miR-766-5p alleviated the stimulatory effect of TTTY15 overexpression on the inflammation and apoptosis of PC12 cells treated with OGD/R. Therefore, the present study revealed that TTTY15 knockdown improved the OGD/R-induced injury of PC12 cells by upregulating miR-766-5p expression.

Correspondence to: Dr Chenguang Hao, Department of Neurology, The First Affiliated Hospital of Xinjiang Medical University, 137 South Liyu Shan Road, Urumqi, Xinjiang 830054, P.R. China
E-mail: zhao_tang666@163.com

Key words: ischemic stroke, testis-specific transcript Y-linked 15, microRNA-766-5p, inflammation, apoptosis

Introduction

Stroke is one of the most common types of cerebrovascular disease and has become a major neurological concern worldwide due to its high morbidity, disability and mortality rates (1). Ischemic stroke is the main subtype of stroke, accounting for ~70% of the cases (2). Although reperfusion after cerebral ischemia can rescue dying cells, it may also aggravate ischemic cell injury. Therefore, the prevention and treatment of ischemia-reperfusion (I/R) injury (IRI) may represent an important strategy for the treatment of ischemic cerebrovascular disease. Currently, thrombolysis is the only approved treatment for ischemic stroke; however, the clinical effect remains unsatisfactory, as the reperfusion of ischemic vessels after thrombolysis promotes secondary injury. Previous studies that have attempted to target the mechanisms underlying ischemic stroke have mainly focused on the excitotoxicity of neurons, oxidative stress and inflammatory injury (3-6). Among these pathways, the activation of the immune-mediated inflammatory response following a stroke has been widely investigated, with promising results to date (7,8). Therefore, the present study aimed to determine the anti-inflammatory and antiapoptotic molecular targets of ischemic stroke, which may provide further treatment options.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs of >200 nucleotides in length. lncRNAs can regulate the expression of protein-coding genes at the pre-transcriptional, mid-transcriptional and post-transcriptional levels. lncRNAs have been a focus of research in recent years, and numerous previous studies have reported that lncRNAs may be implicated in the pathological process of IRI in several vital organs, such as the heart, brain, liver, kidney and vascular endothelial tissue (9). For example, Li *et al* (10) demonstrated that the

expression levels of the lncRNA metastasis-associated lung adenocarcinoma transcript 1 were significantly upregulated in cerebral microvascular endothelial cells of I/R model mice, which promoted autophagy and cell survival by downregulating the expression levels of microRNA (miRNA/miR)-26b. In addition, the knockdown of lncRNA AK139328 reduced myocardial IRI through inhibiting autophagy by targeting miR-204-3p expression (11). Testis-specific transcript Y-linked 15 (TTTY15), which is 5,263 base pairs in length, is located on chromosome Yq 11.21 of the male-specific region of the Y chromosome. TTTY15 has only been discovered as a new lncRNA in recent years. Although few studies have reported the role of the TTTY15 gene family in different diseases, the results to date appear to be promising. For example, with regards to cancer diagnosis, the fusion of TTTY15 with ubiquitin-specific peptidase 9 Y-linked was found to be able to predict the biopsy results of lung cancer, hepatocellular carcinoma and prostate cancer (12,13). In addition, a previous study reported that the overexpression of TTTY15 significantly inhibited the proliferation and metastasis of non-small cell lung cancer cells (14). Huang *et al* (15) also reported that TTTY15 could attenuate hypoxia-induced cardiomyocyte injury by targeting miR-455-5p. Therefore, based on these aforementioned findings, it was hypothesized that TTTY15 may exert a protective effect against neuronal injury induced by I/R. miR-766-5p has been demonstrated to be overexpressed in colorectal cancer and to promote the cancer process of colorectal cancer. However, the role of miR-766-5p in IRI has not been reported to date.

The present study employed an *in vitro* model of neuronal IRI to determine the role and specific regulatory mechanism of TTTY15 in I/R-induced neuronal injury.

Materials and methods

Cell culture and establishment of the oxygen-glucose deprivation/reperfusion (OGD/R) model. PC12 neurons (rat adrenal pheochromocytoma cells) were obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The cells were cultured in DMEM supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and maintained at 37˚C in a humidified atmosphere with 5% CO_2. The proliferation of the cells was observed regularly, and cells were passaged when required. Cells in the logarithmic phase were collected for use in subsequent experiments.

Treatment of PC12 cells with OGD/R was performed to simulate cerebral IRI *in vitro*. The detailed treatment protocol is described in a previously published study (16). Briefly, PC12 cells were cultured in glucose-free DMEM without FBS at 37˚C in an incubator containing 95% N_2 and 5% CO_2 for 3 h. Subsequently, the medium was replaced with glucose-containing DMEM supplemented with 10% FBS and reoxygenated for 24 h under normoxic conditions. Following successful establishment of the cell model, the cells were used for subsequent experiments.

Cell transfection. Short hairpin RNA (shRNA/sh) targeting TTTY15 (shRNA-TTTY15-1 and shRNA-TTTY15-2) and the corresponding negative control (NC; shRNA-NC) were constructed by Shanghai GenePharma Co., Ltd. The TTTY15 overexpression plasmid (pcDNA3.1-TTTY15) and empty vector (pcDNA-NC) were produced by OBiO Technology (Shanghai) Corp., Ltd. miR-766-5p mimics and miR-NC were purchased from Guangzhou RiboBio Co., Ltd. The aforementioned plasmids were transfected into PC12 cells at a final concentration of 20 nM using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The transfection efficiency was verified using reverse transcription-quantitative PCR (RT-qPCR) at 48 h post-transfection.

RT-qPCR analysis. Briefly, total RNA was extracted from transfected cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.) and reverse-transcribed into cDNA using a SuperScript™ IV First-Strand Synthesis system (Thermo Fisher Scientific, Inc.). The procedure was performed according to the manufacturer's protocol. qPCR was subsequently performed using a TaqMan assay (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The following thermocycling conditions were used for the qPCR: 35 cycles at 95˚C for 1 min, 55˚C for 30 sec and 72˚C for 30 sec. The following primer sequences were used for the qPCR: TTTY15 forward, CAC CCAACCAGTCATCTGAGTA and reverse, 5'-GGTTGC AGTGGGCTATGACT-3'; miR-766-5p forward, 5'-TCGAGT ACTTGAGATGGAGTTTT-3' and reverse, 5'-GGCCGCGTT GCAGTGAGCCGAG-3'; GAPDH forward, 5'-CTGGGCTAC ACTGAGCACC-3' and reverse, 5'-AAGTGGTCGTTGAGG GCAATG-3'; and U6 forward, 5'-CTCGCTTCGGCAGCA CA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (17) and normalized to either GAPDH or U6.

Analysis of the secretion of inflammatory factors. The secretion of TNF-α (cat. no. ml002859-1), IL-1β (cat. no. ml058228-1), IL-10 (cat. no. ml037888-1) and IL-18 (cat. no. ml063131-1) into the cell culture supernatant was analyzed using ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd.), according to the manufacturers' protocols. Briefly, the treated cells were harvested by centrifugation at 4˚C, 12,000 x g for 10 min. Then, the supernatant was collected and plated into ELISA microplates to measure the absorbance of each well at a wavelength of 450 nm using an automatic microplate reader (Syngene). Standard curves were drawn, and the concentrations of the inflammatory factors were expressed as pg/ml.

Flow cytometric analysis of apoptosis. The levels of cell apoptosis were analyzed using an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD Biosciences), according to the manufacturer's protocol. Briefly, the transfected cells were at a density of $1x10^5$ cells/ml and incubated with 5 μl Annexin V-FITC and 5 μl PI in the dark for 15 min. Following incubation, 20 μl Annexin V binding solution was added to the cell suspension and incubated for 1 h. Apoptotic cells were subsequently analyzed using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest 3.0.1 software (BD Biosciences).

Dual luciferase reporter assay. StarBase v2.0 (http://starbase.sysu.edu.cn/index.php) was used to predict whether miR-766-5p

may be a direct target of TTTY15. Subsequently, a dual luciferase reporter assay was performed to validate the relationship between TTTY15 and miR-766-5p. The wild-type (WT) 3'-untranslated region (UTR) of TTTY15 containing the potential binding site for miR-766-5p was amplified and cloned into a pGL3-Basic vector (Promega Corporation). A mutant (Mut) 3'-UTR sequence was also generated and cloned into the pGL3-Basic vector. Then, PC12 cells were co-transfected via Lipofectamine® 3000 (cat. no. L3000001; Thermo Fisher Scientific, Inc.) with miR-NC or miR-766-5p mimics and TTTY15-WT or TTTY15-Mut according to the manufacturer's protocol. The relative luciferase activity was measured using a Dual Luciferase Reporter assay kit (Promega Corporation).

Western blotting. The expression levels of the apoptotic proteins Bcl-2, Bax, cleaved caspase-3, caspase-3, cleaved caspase-9 and caspase-9 were analyzed using western blotting. Total protein was extracted from cells using RIPA lysis buffer (Beyotime Institute of Biotechnology). Total protein was quantified and 35 μg protein per lane was separated via 12% SDS-PAGE. The separated proteins were subsequently transferred onto PVDF membranes (Thermo Fisher Scientific, Inc.) and blocked at room temperature with blocking solution (Thermo Fisher Scientific, Inc.) for 1 h. The membranes were then incubated with the following primary antibodies at 4˚C overnight: Anti-Bcl-2 (1:1,000; cat. no. 15071; Cell Signaling Technology, Inc.), anti-Bax (1:1,000; cat. no. 5023; Cell Signaling Technology, Inc.), anti-cleaved caspase-3 (1:1,000; cat. no. 9661; Cell Signaling Technology, Inc.), anti-caspase-3 (1:1,000; cat. no. 9662; Cell Signaling Technology, Inc.), anti-cleaved caspase-9 (1:1,000; cat. no. 20750; Cell Signaling Technology, Inc.) and anti-caspase-9 (1:1,000; cat. no. 9502; Cell Signaling Technology, Inc.). Following primary antibody incubation, the membranes were incubated with HRP-conjugated secondary antibody (1:1,000; cat. no. K4003; Dako; Agilent Technologies, Inc.) at room temperature for 2 h. Protein bands were visualized using an ECL reagent (cat. no. 6883; Cell Signaling Technology, Inc.) and densitometric analysis was performed using ImageJ software (version 1.4.3; National Institutes of Health). GAPDH was used as the internal reference protein.

Statistical analysis. Statistical analysis was performed using GraphPad Prism, version 6.0 (GraphPad Software, Inc.). Data are presented as the mean ± SD of three independent experiments. Unpaired Student's t-tests or a one-way ANOVA followed by Tukey's post hoc test were performed to determine the statistical significance of the differences between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

lncRNA TTTY15 expression is upregulated in PC12 cells following OGD/R. First, the expression levels of TTTY15 in PC12 cells were analyzed before and after OGD/R treatment. As shown in Fig. 1A, TTTY15 expression was upregulated in OGD/R-treated PC12 cells compared with the control group, indicating that TTTY15 may be involved in OGD/R-induced neuronal cell injury. Subsequently, cells were transfected with shRNA-TTTY15 plasmids to knock down the expression of TTTY15. As shown in Fig. 1B, the expression levels of TTTY15 in the shRNA-TTTY15-1 group were downregulated to the greatest extent. Thus, shRNA-TTTY15-1 was selected for use in further experiments.

Knockdown of TTTY15 relieves the inflammatory response in OGD/R-induced PC12 cell injury. The secretory levels of inflammatory cytokines in PC12 cells following OGD/R were determined using ELISA. The results revealed that the levels of inflammation in PC12 cells treated with OGD/R were significantly increased compared with the control group. The secretory levels of the proinflammatory factors TNF-α, IL-1β and IL-18 were decreased in PC12 cells following OGD/R compared with the shRNA-NC + OGD/R group (Fig. 2A-C). Conversely, the secretory levels of the anti-inflammatory factor IL-10 were increased in PC12 cells following OGD/R compared with the shRNA-NC + OGD/R group (Fig. 2D). These results suggested that TTTY15 knockdown may reduce the inflammatory response in OGD/R-induced PC12 cell injury.

Knockdown of TTTY15 inhibits the apoptosis of PC12 cells following OGD/R. Subsequently, the effects of TTTY15 on the apoptosis of PC12 cells following OGD/R were analyzed using flow cytometry and western blotting. The results from the flow cytometric analysis revealed that TTTY15 silencing significantly decreased the apoptotic rate in PC12 cells following OGD/R (Fig. 3A). The western blotting results demonstrated that the expression levels of the proapoptotic proteins Bax, caspase-3 and caspase-9 were downregulated, while the expression levels of the anti-apoptotic protein Bcl-2 were upregulated following TTTY15 gene silencing (Fig. 3B). Taken together, these findings suggested that the knockdown of TTTY15 in PC12 cells may exert a neuroprotective effect in PC12 cells following OGD/R.

TTTY15 negatively regulates miR-766-5p expression. To investigate the mechanism of action of TTTY15 in OGD/R-induced injury, the potential target of TTTY15 was predicted using StarBase. Through screening, miR-766-5p was predicted to be the direct target of TTTY15 (Fig. 4A). Subsequently, a TTTY15 overexpression plasmid (pcDNA-TTTY15) and miR-766-5p mimic were constructed and successfully transfected into PC12 cells (Fig. 4B and C). The results of the dual luciferase reporter assay revealed that the relative luciferase activity of PC12 cells co-transfected with the TTTY15-WT 3'-UTR and miR-766-5p mimics was significantly decreased compared with the cells co-transfected with the TTTY15-WT 3'-UTR and miR-NC. Notably, the relative luciferase activity of the PC12 cells transfected with TTTY15-Mut reporter was not altered (Fig. 4D). In addition, the overexpression of TTTY15 significantly downregulated the expression levels of miR-766-5p in PC12 cells treated with OGD/R (Fig. 5E). Taken together, these data suggested that TTTY15 may negatively regulate miR-766-5p.

TTTY15 promotes OGD/R-induced inflammation and apoptosis by downregulating miR-766-5p expression. To further determine the effects of TTTY15 on inflammation and PC12

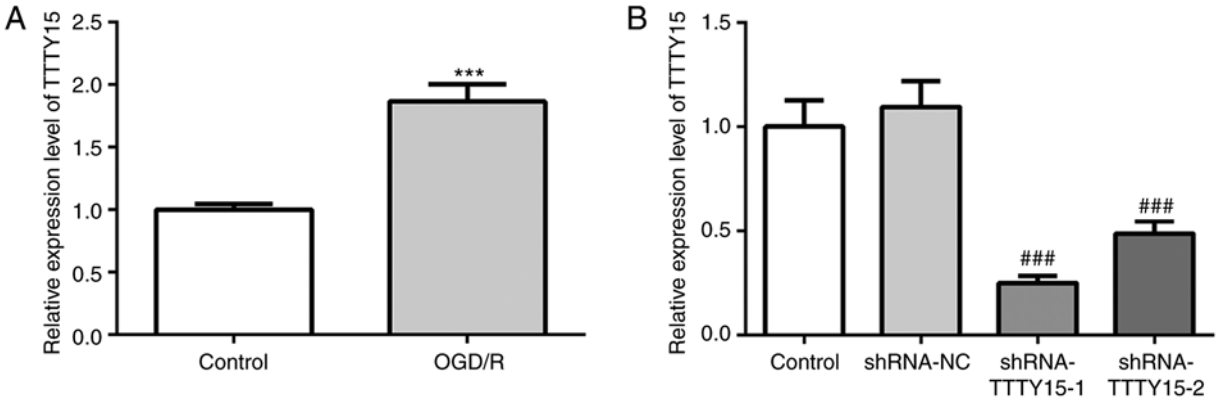

Figure 1. TTTY15 was upregulated in PC12 cells after OGD/R. (A) RT-qPCR showed the expression level of TTTY15 in PC12 cells under OGD/R treatment. (B) The transfection efficiency of TTTY15 knockdown was detected by RT-qPCR. ***P<0.001 vs. control; ###P<0.001 vs. shRNA-NC. TTTY15, testis-specific transcript Y-linked 15; OGD/R, oxygen-glucose deprivation/reperfusion; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR.

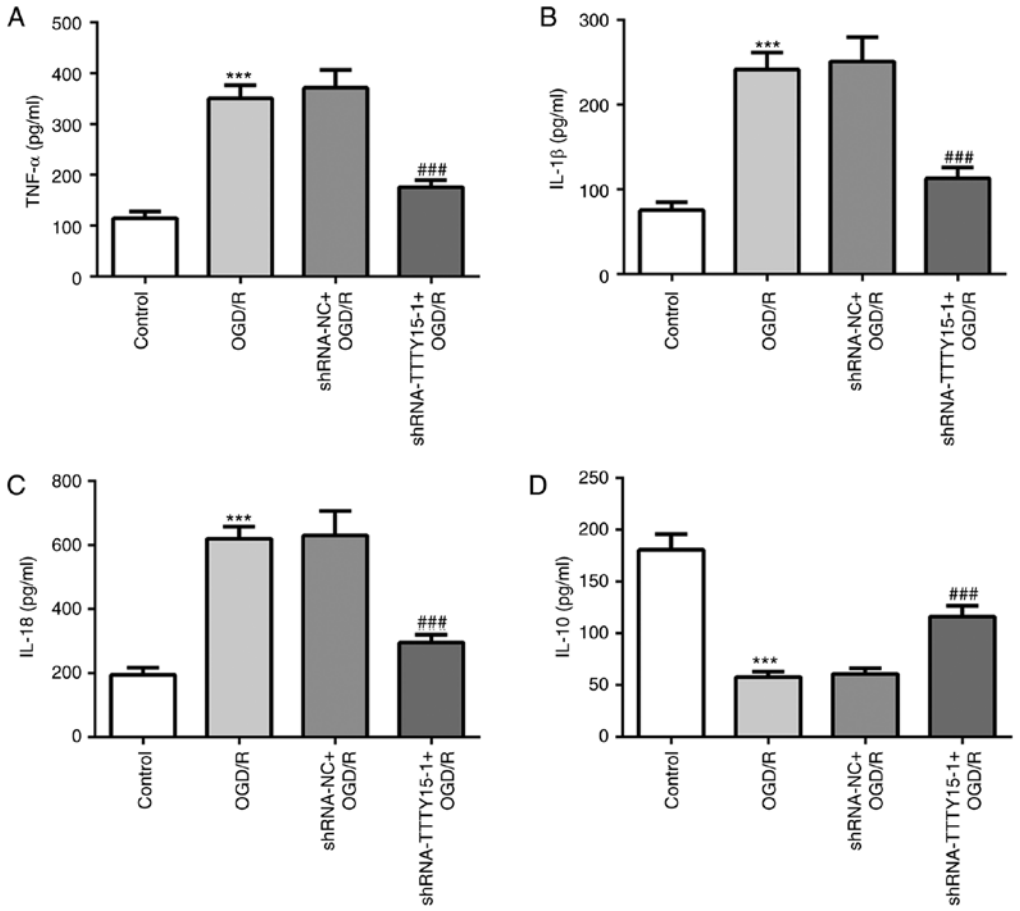

Figure 2. Downregulation of TTTY15 relieved inflammatory response in OGD/R-induced PC12 cell injury. ELISA was used to measure the expression levels of inflammatory factors, including (A) TNF-α, (B) IL-1β, (C) IL-18 and (D) IL-10. ***P<0.001 vs. control; ###P<0.001 vs. shRNA-NC + OGD/R. TTTY15, testis-specific transcript Y-linked 15; OGD/R, oxygen-glucose deprivation/reperfusion; NC, negative control.

cell apoptosis following OGD/R injury, the TTTY15 overexpression plasmid and miR-766-5p mimic were co-transfected into PC12 cells following OGD/R. The results of the ELISA demonstrated that miR-766-5p overexpression inhibited OGD/R-induced PC12 cell injury by decreasing the levels of the proinflammatory factors TNF-α, IL-1β and IL-18, and increasing the levels of the anti-inflammatory factor IL-10. Subsequent transfection with the TTTY15 overexpression plasmid attenuated the miR-766-5p overexpression-induced changes (Fig. 5A-D). In addition, miR-766-5p overexpression significantly reduced OGD/R-induced apoptosis, which could be alleviated through the overexpression of TTTY15 (Fig. 5E). The results of the western blotting experiments revealed that the overexpression of miR-766-5p significantly upregulated the expression of the anti-apoptotic protein Bcl-2 and downregulated the expression of the proapoptotic proteins

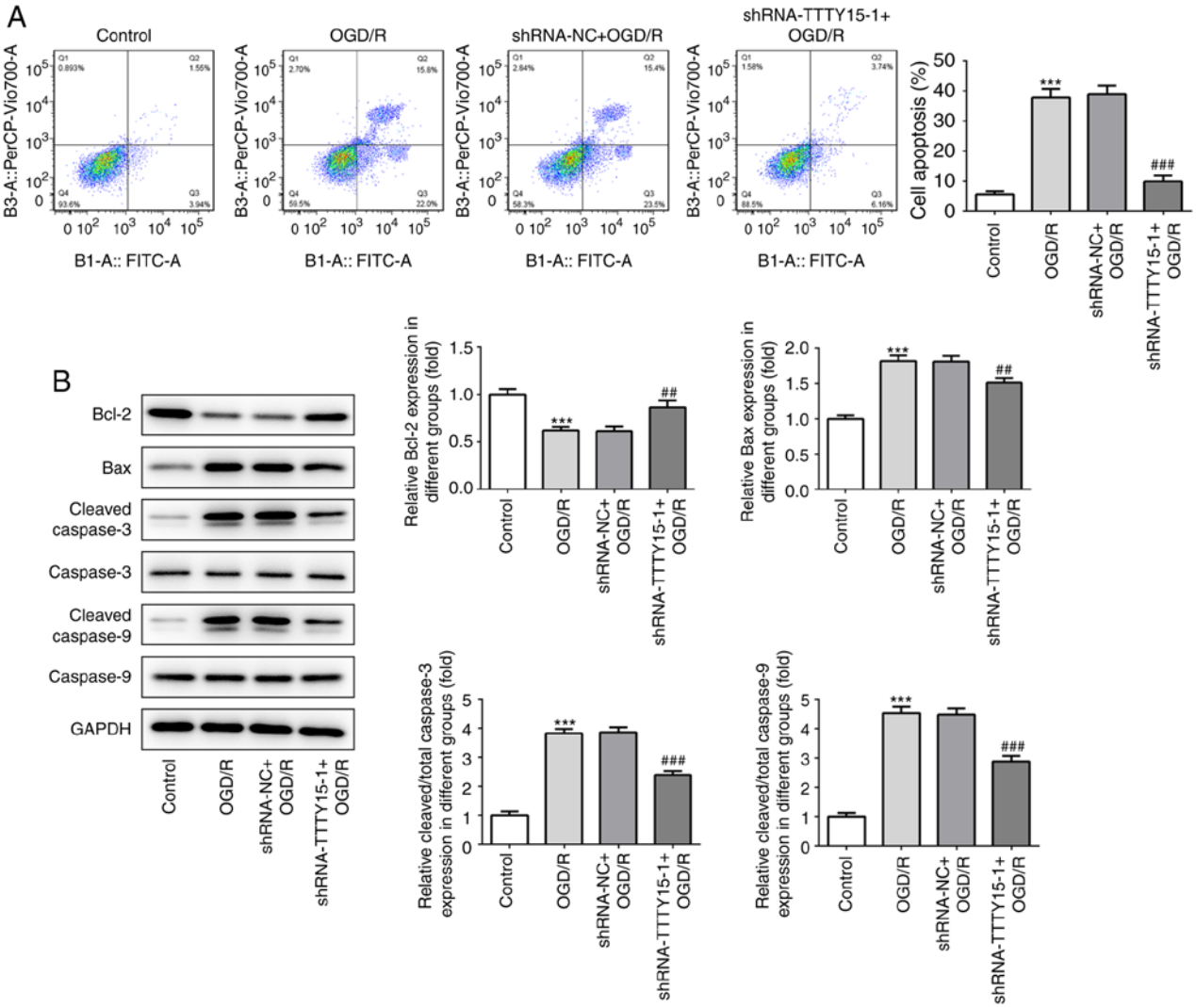

Figure 3. Downregulation of TTTY15 inhibited apoptosis in PC12 cells after OGD/R. (A) Apoptotic cells were analyzed by flow cytometry. (B) Apoptosis-related protein expression was detected by western blotting. ***P<0.001 vs. control; ##P<0.01 and ###P<0.001 vs. shRNA-NC + OGD/R. TTTY15, testis-specific transcript Y-linked 15; OGD/R, oxygen-glucose deprivation/reperfusion; NC, negative control.

Bax, caspase-3 and caspase-9. Similar to the previous results, the overexpression of TTTY15 attenuated the miR-766-5p overexpression-induced changes in the expression levels of the aforementioned proteins (Fig. 5F). Taken together, these results suggested that TTTY15 may promote OGD/R-induced inflammation and apoptosis by downregulating miR-766-5p expression.

Discussion

I/R is a common pathological process that is involved in numerous pathological conditions, such as myocardial infarction, acute ischemic stroke, acute renal injury, trauma and circulatory failure, among which ischemic and hypoxic cerebrovascular diseases, including cerebral stroke, account for the highest morbidity and mortality rates worldwide (18). Blood reperfusion is the preferred treatment option for these conditions; however, the accompanying IRI poses a major concern to clinicians and researchers. Therefore, reducing IRI in the clinical setting is a difficult problem and should be urgently addressed. An increasing number of studies have reported that the inflammatory response plays an important role in the process of cerebral IRI (19,20). Numerous different proinflammatory cytokines are produced and released during the process of inflammation, including TNF-α, IL-1β and IL-18. These proinflammatory cytokines have been discovered to activate neurons and vascular endothelial cells, lead to the death of neurons and glial cells, and aggravate the infiltration of immune cells during cerebral ischemia, thus promoting the inflammatory response (21,22). In the present study, the expression levels of TTTY15 were found to be upregulated in PC12 cells following OGD/R, whereas TTTY15 gene knockdown significantly inhibited the inflammatory response and OGD/R-induced apoptosis of PC12 cells by upregulating miR-766-5p expression.

lncRNAs are a class of non-coding RNA of >200 nucleotides in length that lack an open reading frame (23). In recent years, accumulating studies have reported that lncRNAs serve as key regulators in epigenetics and various types of disease, including cancer, nervous system diseases, cardiovascular diseases and mental disorders (24,25). Ischemic stroke is mediated by a variety of mechanisms, including oxidative

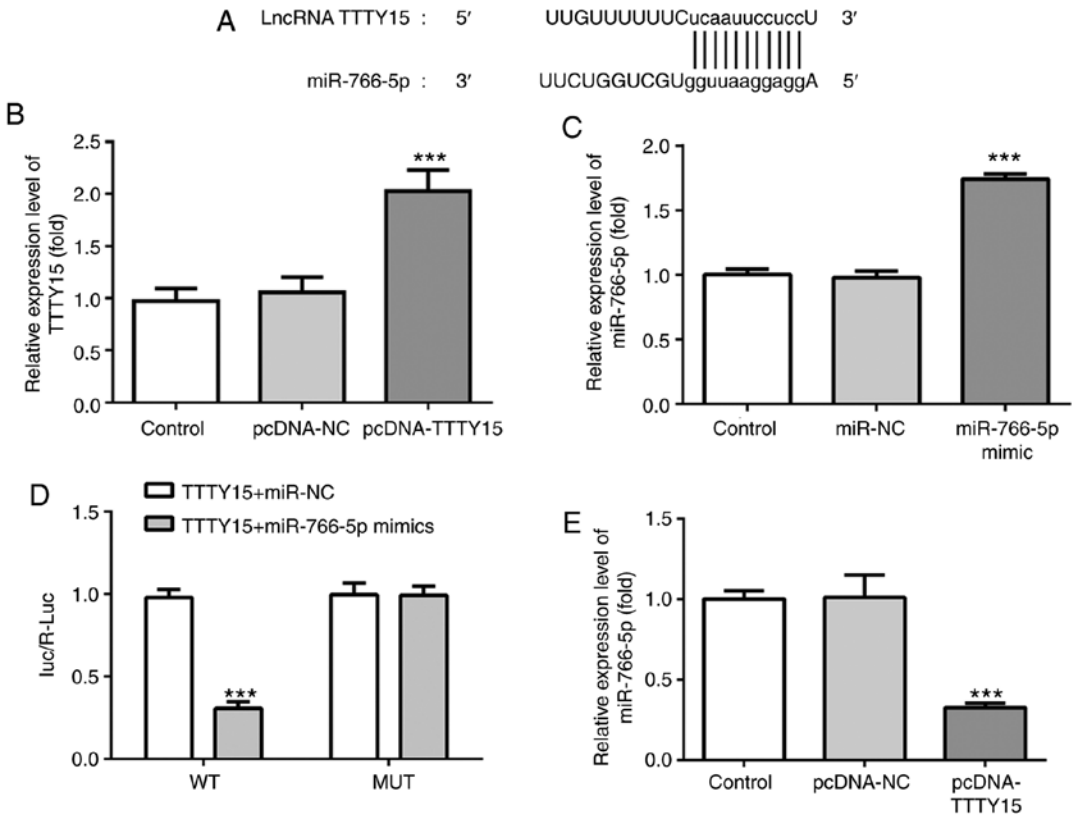

Figure 4. TTTY15 negatively regulated miR-766-5p. (A) Predicted binding site between TTTY15 and miR-766-5p. (B) TTTY15 overexpression transfection efficiency was confirmed by RT-qPCR. ***P<0.001 vs. pcDNA-NC. (C) miR-766-5p mimic transfection efficiency was confirmed by RT-PCR. ***P<0.001 vs. miR-NC. (D) Luciferase reporter assay was performed to detect the relationship between TTTY15 and miR-766-5p. ***P<0.001 vs. TTTY15 + miR-NC. (E) miR-766-5p expression was low in the TTTY15-overexpressing PC12 cells treated with OGD/R. ***P<0.001 vs. pcDNA-NC. TTTY15, testis-specific transcript Y-linked 15; OGD/R, oxygen-glucose deprivation/reperfusion; NC, negative control; RT-q PCR, reverse transcription-quantitative PCR; WT, wild-type; MUT, mutant.

stress, inflammation, vascular dysfunction, apoptosis and autophagy (26). An increasing number of studies have revealed that lncRNAs play an important role in the pathophysiological mechanisms implicated in ischemic stroke. For example, the analysis of lncRNAs in the cerebral cortex of rats revealed that the expression levels of chromatin-modifying protein-related lncRNAs after a stroke may regulate the epigenetics following ischemia (27). In addition, Deng *et al* (28) found that the expression levels of a large number of lncRNAs were significantly upregulated in patients with ischemic stroke compared with healthy individuals (29). These results revealed the potential of targeting lncRNAs in ischemic injury.

TTTY15 is a newly discovered lncRNA and, to the best of our knowledge, few studies to date have investigated the effect of TTTY15 in IRI. In addition to its reported important role in cancer diagnosis, a previous study demonstrated that the silencing of TTTY15 could protect cardiomyocytes against IRI by targeting miR-455-5p (15). In addition, TTTY15 was discovered to protect vascular endothelial cells from IRI by targeting miR-186-5p (30). These results confirmed the protective role of TTTY15 in IRI. However, to the best of our knowledge, the role of TTTY15 in nerve cell injury induced by OGD/R remains unknown. Therefore, to understand the effect of TTTY15 on I/R-induced nerve cells, the present study aimed to determine the biological function and underlying mechanisms of TTTY15 in OGD/R-induced PC12 cell injury. The results revealed that TTTY15 expression levels were upregulated in PC12 cells following OGD/R, and TTTY15 silencing significantly inhibited the inflammatory reaction and apoptosis of PC12 cells treated with OGD/R. These results suggested the potential protective role of TTTY15 against OGD/R-induced neuronal damage.

miRNAs are small non-coding RNA molecules of 18-25 nucleotides in length that can regulate gene expression by targeting specific mRNAs. miRNAs have been found to exert a wide range of biological effects on nerve cell growth, apoptosis and fat metabolism (31,32). Previous studies have reported differences in the expression levels of miRNAs in the peripheral blood of patients with stroke compared with healthy individuals (33,34). In addition, an increasing number of studies have demonstrated that certain miRNAs may exert important cerebroprotective effects against IRI (35,36). miR-766-5p has been reported to exert anti-inflammatory effects on human rheumatoid arthritis fibroblast-like synoviocyte MH7A cells (37). However, it remains unknown whether miR-766-5p, which was predicted as a potential target of TTTY15 through bioinformatics analysis, is involved in IR-induced nerve injury. In the present study, miR-766-5p was predicted and verified to be a target of TTTY15 using bioinformatics software and a dual luciferase reporter assay. Furthermore, TTTY15 overexpression significantly alleviated the inhibitory effect of miR-766-5p overexpression on the OGD/R-induced inflammation and

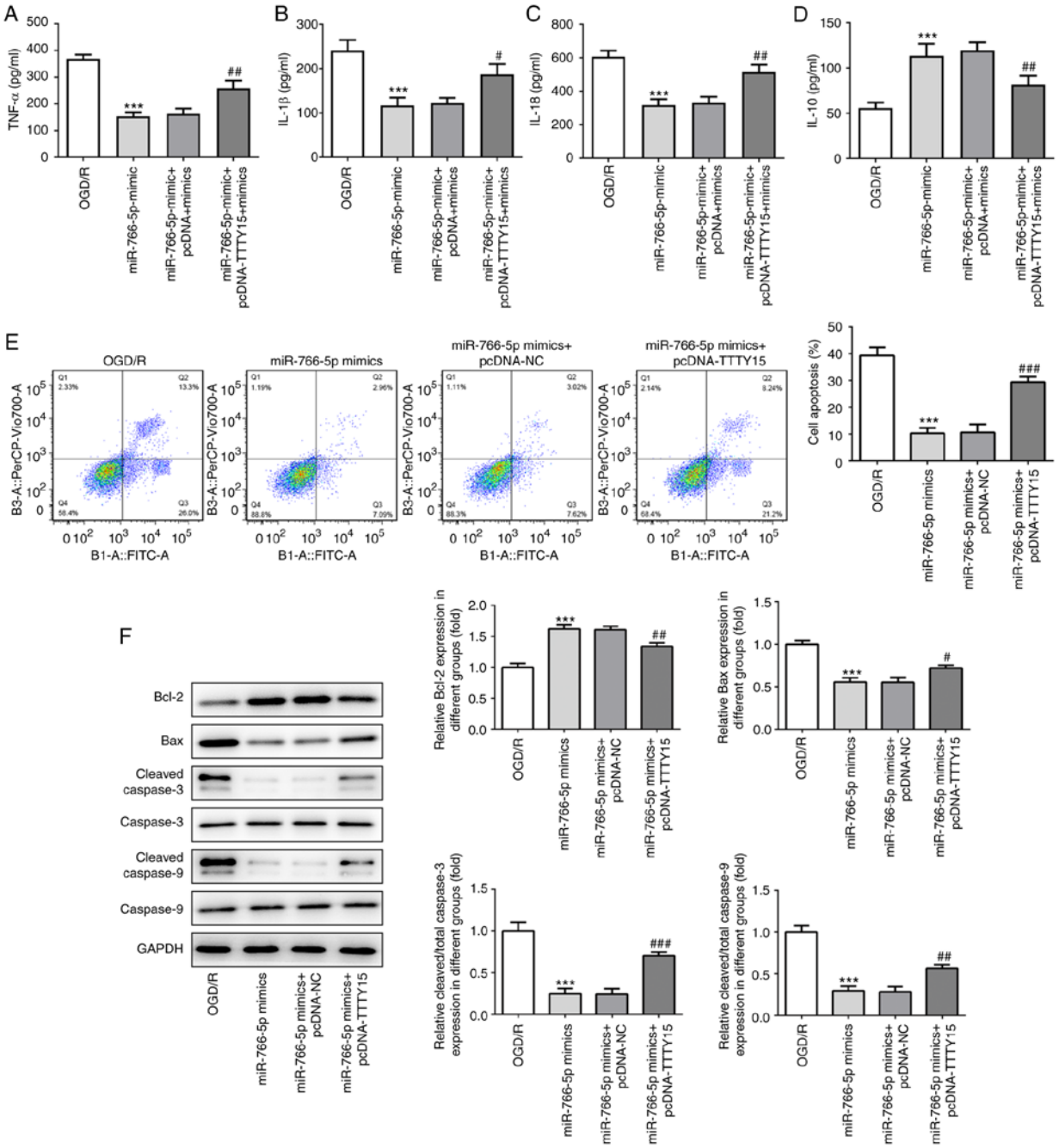

Figure 5. TTTY15 promoted OGD/R-induced inflammation and apoptosis by downregulating miR-766-5p. ELISA was conducted to determine the expression levels of inflammatory markers, including (A) TNF-α, (B) IL-1β, (C) IL-18 and (D) IL-10. (E) Apoptosis was analyzed by flow cytometry. (F) Apoptosis-related protein expression was detected by western blotting. ***P<0.001 vs. OGD/R; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. miR-766-5p mimics + pcDNA-NC. TTTY15, testis-specific transcript Y-linked 15; OGD/R, oxygen-glucose deprivation/reperfusion; NC, negative control.

apoptosis of PC12 cells. These data suggested that the effects of TTTY15 on OGD/R-induced injury in PC12 cells may be mediated by miR-766-5p.

Of note, the incidence of sex-unspecific tumors in men is significantly higher compared with in women; however, the reason for this remains unclear (33). While the Y chromosome is a male-specific chromosome with a unique evolutionary process, the function of the genes located on the Y chromosome are not sufficient to fully explain the high incidence of cancers in men (38). A previous study reported that the inflammatory response in men may be enhanced by testosterone, which subsequently increases the susceptibility of renal tissue to IRI; however, the specific differences between men and women should be further studied (39). At present, there are few studies reporting the relationship between the Y chromosome and I/R, and whether the results of the present study may be applicable to female mice remains to be examined. Thus, the effects of TTTY15 on OGD/R-induced nerve injury should be further verified *in vivo*. Future experiments should focus on the functions of TTTY15 in female subjects, which may help to elucidate the role of TTTY15 as a potential target for the treatment of neuronal IRI in both sexes.

In conclusion, the findings of the present study revealed that the expression levels of TTTY15 were upregulated in

PC12 cells following OGD/R. The knockdown of TTTY15 improved the OGD/R-induced injury of PC12 cells by upregulating miR-766-5p expression. These results suggested that TTTY15 may be considered as a potential target for the treatment of I/R.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

CH and SC conceived and designed the study; conducted the experiments; analyzed the data and wrote the paper. Both authors have read and consent to the publication of the final version of the manuscript, and confirm the authenticity of the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. GBD 2015 Mortality and Causes of Death Collaborators: Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: A systematic analysis for the Global Burden of Disease Study 2015. Lancet 388: 1459-1544, 2016.
2. Piao JM, Wu W, Yang ZX, Li YZ, Luo Q and Yu JL: MicroRNA-381 favors repair of nerve injury through regulation of the SDF-1/CXCR4 signaling pathway via LRRC4 in acute cerebral ischemia after cerebral lymphatic blockage. Cell Physiol Biochem 46: 890-906, 2018.
3. Chamorro Á, Dirnagl U, Urra X and Planas AM: Neuroprotection in acute stroke: Targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. Lancet Neurol 15: 869-881, 2016.
4. Hill MD, Martin RH, Mikulis D, Wong JH, Silver FL, Terbrugge KG, Milot G, Clark WM, Macdonald RL, Kelly ME, *et al*: Safety and efficacy of NA-1 in patients with iatrogenic stroke after endovascular aneurysm repair (ENACT): A phase 2, randomised, double-blind, placebo-controlled trial. Lancet Neurol 11: 942-950, 2012.
5. Feng S, Yang Q, Liu M, Li W, Yuan W, Zhang S, Wu B and Li J: Edaravone for acute ischaemic stroke. Cochrane Database Syst Rev: CD007230, 2011.
6. Di Menna L, Molinaro G, Di Nuzzo L, Riozzi B, Zappulla C, Pozzilli C, Turrini R, Caraci F, Copani A, Battaglia G, *et al*: Fingolimod protects cultured cortical neurons against excitotoxic death. Pharmacol Res 67: 1-9, 2013.
7. Iadecola C and Anrather J: The immunology of stroke: From mechanisms to translation. Nat Med 17: 796-808, 2011.
8. Moskowitz MA, Lo EH and Iadecola C: The science of stroke: Mechanisms in search of treatments. Neuron 67: 181-198, 2010.
9. Yoon JH, Abdelmohsen K and Gorospe M: Functional interactions among microRNAs and long noncoding RNAs. Semin Cell Dev Biol 34: 9-14, 2014.
10. Li Z, Li J and Tang N: Long noncoding RNA Malat1 is a potent autophagy inducer protecting brain microvascular endothelial cells against oxygen-glucose deprivation/reoxygenation-induced injury by sponging miR-26b and upregulating ULK2 expression. Neuroscience 354: 1-10, 2017.
11. Yu SY, Dong B, Fang ZF, Hu XQ, Tang L and Zhou SH: Knockdown of lnc RNA AK 139328 alleviates myocardial ischaemia/reperfusion injury in diabetic mice via modulating miR-204-3p and inhibiting autophagy. J Cell Mol Med 22: 4886-4898, 2018.
12. Zhu Y, Ren S, Jing T, Cai X, Liu Y, Wang F, Zhang W, Shi X, Chen R, Shen J, *et al*: Clinical utility of a novel urine-based gene fusion TTTY15-USP9Y in predicting prostate biopsy outcome. Urol Oncol 33: 384.e9-e20, 2015.
13. Ren S, Peng Z, Mao JH, Yu Y, Yin C, Gao X, Cui Z, Zhang J, Yi K, Xu W, *et al*: RNA-seq analysis of prostate cancer in the Chinese population identifies recurrent gene fusions, cancer-associated long noncoding RNAs and aberrant alternative splicings. Cell Res 22: 806-821, 2012.
14. Lai IL, Chang YS, Chan WL, Lee YT, Yen JC, Yang CA, Hung SY and Chang JG: Male-specific long noncoding RNA TTTY15 inhibits non-small cell lung cancer proliferation and metastasis via TBX4. Int J Mol Sci 20: 3473, 2019.
15. Huang S, Tao W, Guo Z, Cao J and Huang X: Suppression of long noncoding RNA TTTY15 attenuates hypoxia-induced cardiomyocytes injury by targeting miR-455-5p. Gene 701: 1-8, 2019.
16. Zhong Y, Yu C and Qin W: LncRNA SNHG14 promotes inflammatory response induced by cerebral ischemia/reperfusion injury through regulating miR-136-5p/ROCK1. Cancer Gene Ther 26: 234-247, 2019.
17. Mavridis K, Stravodimos K and Scorilas A: Downregulation and prognostic performance of microRNA 224 expression in prostate cancer. Clin Chem 59: 261-269, 2013.
18. Eltzschig HK and Eckle T: Ischemia and reperfusion-from mechanism to translation. Nat Med 17: 1391-1401, 2011.
19. Carbone F, Bonaventura A and Montecucco F: Neutrophil-related oxidants drive heart and brain remodeling after ischemia/reperfusion injury. Front Physiol 10: 1587, 2020.
20. Zhuo Y and Zhuo J: Tranilast treatment attenuates cerebral ischemia-reperfusion injury in rats through the inhibition of inflammatory responses mediated by NF-κB and PPARs. Clin Transl Sci 12: 196-202, 2019.
21. Allan SM and Rothwell NJ: Cytokines and acute neurodegeneration. Nat Rev Neurosci 2: 734-744, 2001.
22. Chamorro A and Hallenbeck J: The harms and benefits of inflammatory and immune responses in vascular disease. Stroke 37: 291-293, 2006.
23. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, Oyama R, Ravasi T, Lenhard B, Wells C, *et al*: The transcriptional landscape of the mammalian genome. Science 309: 1559-1563, 2005.
24. Leung A and Natarajan R: Non-coding RNAs in vascular disease. Curr Opin Cardiol 29: 199-206, 2014.
25. Li J, Xuan Z and Liu C: Long non-coding RNAs and complex human diseases. Int J Mol Sci 14: 18790-18808, 2013.
26. Kalogeris T, Baines CP, Krenz M and Korthuis RJ: Cell biology of ischemia/reperfusion injury. Int Rev Cell Mol Biol 298: 229-317, 2012.
27. Dharap A, Pokrzywa C and Vemuganti R: Increased binding of stroke-induced long non-coding RNAs to the transcriptional corepressors Sin3A and coREST. ASN Neuro 5: 283-289, 2013.
28. Deng QW, Li S, Wang H, Sun HL, Zuo L, Gu ZT, Lu G, Sun CZ, Zhang HQ and Yan FL: Differential long noncoding RNA expressions in peripheral blood mononuclear cells for detection of acute ischemic stroke. Clin Sci (Lond) 132: 1597-1614, 2018.
29. Zheng J, Zhuo YY, Zhang C, Tang GY, Gu XY and Wang F: LncRNA TTTY15 regulates hypoxia-induced vascular endothelial cell injury via targeting miR-186-5p in cardiovascular disease. Eur Rev Med Pharmacol Sci 24: 3293-3301, 2020.
30. Chang HL, Wang HC, Chunag YT, Chou CW, Lin IL, Lai CS, Chang LL and Cheng KI: miRNA expression change in dorsal root ganglia after peripheral nerve injury. J Mol Neurosci 61: 169-177, 2017.

31. Lefai E, Blanc S, Momken I, Antoun E, Chery I, Zahariev A, Gabert L, Bergouignan A and Simon C: Exercise training improves fat metabolism independent of total energy expenditure in sedentary overweight men, but does not restore lean metabolic phenotype. Int J Obes (Lond) 41: 1728-1736, 2017.
32. Stary CM, Xu L, Sun X, Ouyang YB, White RE, Leong J, Li J, Xiong X and Giffard RG: MicroRNA-200c contributes to injury from transient focal cerebral ischemia by targeting Reelin. Stroke 46: 551-556, 2015.
33. Xue Y, Yin P, Li G and Zhong D: Genome-wide integration study of circulating miRNAs and peripheral whole-blood mRNAs of male acute ischemic stroke patients. Neuroscience 380: 27-37, 2018.
34. Liu G, Cao C and Zhu M: Peripheral blood miR-451 may serve as a biomarker of ischemic stroke. Clin Lab 65, 2019.
35. Liu P, Zhao H, Wang R, Wang P, Tao Z, Gao L, Yan F, Liu X, Yu S, Ji X and Luo Y: MicroRNA-424 protects against focal cerebral ischemia and reperfusion injury in mice by suppressing oxidative stress. Stroke 46: 513-519, 2015.
36. Hayakawa K, Kawasaki M, Hirai T, Yoshida Y, Tsushima H, Fujishiro M, Ikeda K, Morimoto S, Takamori K and Sekigawa I: MicroRNA-766-3p contributes to anti-inflammatory responses through the indirect inhibition of NF-κB signaling. Int J Mol Sci 20: 809, 2019.
37. Forsberg LA, Rasi C, Malmqvist N, Davies H, Pasupulati S, Pakalapati G, Sandgren J, Diaz de Ståhl T, Zaghlool A, Giedraitis V, *et al*: Mosaic loss of chromosome Y in peripheral blood is associated with shorter survival and higher risk of cancer. Nat Genet 46: 624-628, 2014.
38. Prensner JR and Feng FY: 'Lincing' the Y chromosome to prostate cancer: TTTY15 takes center stage. Eur Urol. Sep 76: 327-328, 2019.
39. Kang KP, Lee JE, Lee AS, Jung YJ, Kim D, Lee S, Hwang HP, Kim W and Park SK: Effect of gender differences on the regulation of renal ischemia-reperfusion-induced inflammation in mice. Mol Med Rep 9: 2061-2068, 2014.