Neuroprotective Effects of microRNA-140-5p on Ischemic Stroke in Mice via its Regulation on the TLR4/NF-κB Axis

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Research

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Abstract

Background and aim: Ischemic stroke is one of the main causes of death worldwide and permanent global disability. On the basis of existing literature data, we carried out studies in an effort to explore how miR-140-5p affects ischemic stroke and whether the mechanism relates to toll-like receptor-4 (TLR4) and nuclear factor-kappa B (NF-κB).

Methods: Middle cerebral artery occlusion (MCAO) was employed to establish a mouse model of ischemic stroke *in vivo*, while primary neurons were exposed to oxygen-glucose deprivation (OGD) to set up an ischemic stroke model *in vitro*. RT-qPCR was performed to detect the miR-140-5p expression and Western blot was employed to detect the expression TLR4, NF-κB, and apoptosis-related factors. Then, based gain-function of experiments using miR-140-5p mimic and TLR4 overexpression plasmid, neurological function score, TTC staining, TUNEL staining, as well as flow cytometry were carried out to evaluate the effects of miR-140-5p and TLR4 on MCAO mice and OGD neurons. Meanwhile, dual-luciferase reporter assay was used to validate the relationship between miR-140-5p and TLR4.

Results: miR-140-5p expressed at a low level and TLR4 at a high level in ischemic stroke. It was verified that miR-140-5p targeted TLR4 and downregulated its expression. miR-140-5p overexpression was observed to inhibit the apoptosis of neurons under OGD exposure and restrain the progression of ischemic stroke, while TLR4 overexpression promoted the apoptosis and disease progression. Besides, miR-140-5p overexpression led to a decrease in NF-κB protein level, which was increased by TLR4 overexpression.

Conclusion: In conclusion, from our data we conclude that miR-140-5p overexpression may be instrumental for the therapeutic targeting of ischemic stroke by alleviating neuron injury with the involvement of TLR4/NF-κB axis.

Background

Stroke, as a major cause of mortality, is the leading cause of neurological disability worldwide [1]. It remains the 5th among all causes of deaths in the US, which accounted for almost 130,000 deaths in the US in 2013 [2]. Stroke-related death has decreased over the past decade, likely due to decreased incidence of new and recurrent stroke [3]. However, this decline is not universal in all population subgroups, with significant differences still existed in stroke risk [3]. Among all strokes, up to 80% − 85% of them are ischemic strokes [4]. Aging and chronic hypertension are the two most important risk factors for acute ischemic stroke, while aging is one of the most important non-modifiable risk factors for stroke[5, 6]. A previous study suggested that the epidemiology of stroke in China not significantly differ from other developed countries. However, an increased incidence of stroke has been reported in China, largely due to the increase in aging population in China [6]. Neurological damage and cardiovascular complications have been implicated in a large majority of post-stroke mortalities [7]. The complex pathogenesis and scarcely understanding of ischemic stroke required more extensive investigations, therefore this study

sought to investigate the underlying mechanism of neuron injury caused by ischemic stroke by developing a mouse model of middle cerebral artery occlusion (MCAO).

Previous investigation had shed some light on that small, noncoding, microRNAs (miRNAs) may play important roles in the etiology and pathology of ischemic stroke, which posttranscriptionally regulate gene expression to serve as potential therapeutic targets for this disease [8, 9]. Existing literatures have suggested the involvement of miRNAs in the development of ischemic stroke. For example, miR-181c, miR-210, and miR-134 were observed to contribute to neuronal apoptosis and brain injury in acute ischemic stroke [10–12]. Interestingly, some researches also had shed their light on the neuroprotection role of several miRNAs in ischemic stroke, such as miR-124, miR-1906, and miR-381-3pd [13–15]. Moreover, it has been reported that miR-140-5p was poorly-expressed in a mouse model of MCAO and a cellular model in hypoxic conditions, and its overexpression inhibited the angiogenesis following ischemic stroke, highlighting miR-140-5p as a potential candidate for the development and treatment of ischemia [16].

Additionally, the potential effects of gene silencing mediated by miRNAs in a posttranscriptional manner on ischemic stroke have attracted the attention of many researchers [17]. For instance, miR-155-modulated TLR4/MyD88 axis regulated the inflammatory responses in cerebral ischemic stroke [18]. Toll-like receptor-4 (TLR4), as a pattern recognition receptor playing vital role in neuroinflammation, has been widely explored in strokes [19–21]. Moreover, downregulation of TLR4 and nuclear factor-kappa B (NF-κB) was indicated to involve the neuroprotective effect of Luteolin and Bicyclol on ischemia [22, 23]. In this investigation, the authors hypothesize that miR-140-5p protects against ischemic brain injury by regulation on the TLR4/NF-κB axis. This study aims to confirm this hypothesis by establishing a mouse model of MCAO and a cell model under oxygen-glucose deprivation (OGD) condition for the development of possible therapy targets for ischemic stroke.

Materials And Methods

Animal treatment [24]

A total of 75 C57/BL6 mice (aging 8–10 week old; weighing 22–25 g), were purchased from Shanghai Ruibo Biotechnology Co., Ltd.

Among the 75 mice, 60 were used to establish MCAO model, and the remaining 15 were sham-operated to serve as control. For MCAO model establishment, the mice were anesthetized with 5% pentobarbital sodium, with the right common, external (ECAs), and internal carotid arteries (ICAs) were exposed. After ligation of the common carotid arteries with sutures, a 7/0 silicone-coated monofilament nylon suture in diameter of 0.22–0.23 mm was gently introduced into the ICAs through the ECA using a rounded tip and advanced 10 ± 0.5 mm until the rounded tip reached the entrance to the right middle cerebral artery, with a slight resistance felt. Laser Doppler flowmetry (PeriFlux 5000, Järfälla, Sweden) was used to identify the successful occlusion.

Then, the 60 successfully modeled mice were randomly grouped into MCAO, MCAO + agomir negative control (NC) + overexpression (oe)-NC (agomir NC and oe-NC were stereotactically injected into the brains of MCAO mice after 1 h of modeling), MCAO + miR-140-5p agomir + oe-NC (miR-140-5p agomir and oe-NC plasmid were stereotactically injected into the brains of MCAO mice after 1 h of modeling), and MCAO + miR-140-5p agomir + oe-TLR4 (miR-140-5p agomir and oe-TLR4 plasmid were stereotactically injected into the brains of MCAO mice after 1 h of modeling) (n = 15 in each group). The stereotactic injections were employed according to a previous study [24]. In brief, mice were anesthetized with intraperitoneal injection of 3% pentobarbital sodium. According to *The Mouse Brain in Stereotaxic Coordinates: Second Edition (Deluxe)* (GeorgePaxinos, Keither B J Franklin), after 1 h of MCAO, the above mentioned lentiviruses containing plasmids (2×10^8 ifu/mL, 4 μL for each plasmid) were stereotactically injected at a ratio of 1 μL/min into the mice by using a stereotaxic apparatus (KOPF, Tujunga, California, USA) and a stepper-motorized microsyringe (Hamilton, Bonaduz, Switzerland), followed by retaining the needle for 5–10 min.

Neurological function evaluation

As a previous reference reported [24], 6 mice in each group were randomly selected for neurological function evaluation after 24 h of MCAO. The tests included motor tests (including flexion of forelimb and hindlimb, and head movement, scored on a scale of 0–6), beam balance tests (scored on a scale of 0–6), and tests for the presence of reflexes and abnormal movements (scored on a scale of 0–2). The score was blindly evaluated by three independently researchers, with 1–4 points indicated mild injury, 5–9 points indicated moderate injury, and 10–14 points indicated severe injury.

Model identification by triphenyltetrazolium chloride (TTC) staining

Six mice in each group were euthanized after evaluating neurological function. Brains were collected and cut to 2 mm coronal sections. Each section was stained with 2% TTC solution for 30 min at 37 °C. Infarcted areas were white-stained and non-infarct zones were stained as red, which were analyzed by NIH ImageJ software.

Primary culture and treatment of neurons

The primary culture of neurons in the cerebral cortex was obtained from newborn C57BL/6 mice, which was conducted as previously reported [25]. Lentiviral vector LV5-GFP (#25999, Addgene, Cambridge, MA, USA) used for gene overexpression, miR-140-5p mimic, and its negative control (mimic-NC) (Shanghai Genepharma, Shanghai, China). The lentivirus package was used HEK293T cells, which were then incubated in a Roswell Park Memorial Institute (RPMI)-1640 complete medium supplemented with 10% fetal bovine serum (FBS) and passed every other day. Then the lentiviruses (1×10^8 TU/mL) were added in the neurons for infection. After that, the infected neurons were induced as ischemic injury cell model by OGD treatment according to a previous report [24]. In brief, neurons were washed by phosphate buffered saline (PBS) for three times, and added into a glucose-free Dulbecco's modified Eagles Medium (DMEM)

at 37 °C. The neurons were subsequently incubated in a 5% CO_2 and 95% N_2 hypoxic incubator at 37 °C for 2 h, followed by incubation a 5% CO_2 normoxic incubator at 37 °C.

RNA isolation and quantification

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was employed according to the manufacturer's instructions of TaqMan MicroRNA Assays Reverse Transcription primer (4427975, Applied Biosystems, Foster City, CA, USA). After measurement of quality and concentration using an ultraviolet spectrophotometry (Nanodrop ND1000, Thermo Scientific, Waltham, MA, USA), the isolated RNA was reversely transcribed to cDNA using PrimeScript™ RT Reagent Kit (Takara, Kusatsu, Japan). Subsequently, RT-qPCR was conducted using the SYBR Premix Ex Taq™ II (TliRNaseH Plus) kit (Takara, Kusatsu, Japan). Primers were synthesized by Shanghai Sangon Biotech (Shanghai, China), as listed in Table 1. The $2^{-\Delta\Delta CT}$ method was applied to calculate the fold changes, with U6 served as internal reference.

Table 1
Primer sequences for RT-qPCR

Gene of interest	Sequences
miR-140-5p	F: 5'-GAGTGTCAGTGGTTTTACCCT-3'
	R: 5'- GCAGGGTCCGAGGTATTC-3'
U6	F: 5'- TGCGGGTGCTCGCTTCGCAGC-3'
	R: 5'- CCAGTGCAGGGTCCGAGGT-3'
Note: F, forward; R: reverse.	

Protein quantification by Western blot assay

Neurons were lysed in lysis buffer containing protease and alkaline phosphatase inhibitors (C0481, Sigma-Aldrich, St. Louis, USA) at 4℃ for 30 min, followed by centrifugation at 10000 r/min for 15 min. The supernatant was collected, and the proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was subsequently blocked and incubated at 4℃ overnight with diluted primary rabbit antibodies from Abcam (Cambridge, UK): rabbit anti-Bcl-2 (ab196495, 1: 1000), mouse anti-Bax (ab32503, 1: 1000), rabbit anti-cleaved caspase 3 (ab49822, 1: 500), TLR4 (ab13356, 1: 1000), NF-κB-p65 (ab207297, 1: 1000), and GAPDH (ab9485, 1: 2000, as internal control). After incubation, the membrane was washed with Tris Buffered saline Tween (TBST) buffer for 5 min three times, and incubated with horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG secondary antibody (ab205718, Abcam, Cambridge, UK) for 1 h at room temperature. The immunoblots were visualized with enhanced chemiluminescence (ECL) reagent, and the images were captured and analyzed by ImageJ to calculate the grey value. All experiments were repeated three times.

Dual-luciferase reporter assay

TLR4 3’-UTR sequences with mutant (MUT) or wildtype (WT) miR-140-5p binding sites were constructed by Shanghai Ruibo Biotechnology Co., Ltd. The promoter sequence was cloned into the pGL-3 luciferase reporter vector. In the 24-well plate, after 24 h of cell incubation, pGL-3-TLR4 WT or pGL-3-TLR4 MUT was co-transfected with miR-140-5p mimic or mimic negative control (mimic-NC) into the HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fluorescence intensity was measured using a GeneCopoeia dual-luciferase reporter assay kit (D0010, Solarbio, Beijing, China) on a Promega Glomax20/20 luminometer (E5311, Shaanxi Zhongmei, Shaanxi, China). All experiments were repeated three times.

Flow cytometry for cell apoptosis

Cells ere titrated into cell suspension and seeded in a 6-well plate. After 48 h transfection, medium was aspired before the cells were washed with PBS and titrated into a single cell suspension. Then, the cells were stained with 5 μL of Annexin-V-FITC and 5 μL of propidium iodide (PI) for 15 min avoiding exposure to light. Next, the cells were re-suspended by 200 μL of 1 × binding buffer followed by cell apoptosis detection on a flow cytometry.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining

Brain tissues of mice were harvested and fixed in 4% paraformaldehyde overnight, which were then sectioned. Five randomly selected sections were dewaxed by xylene, dehydrated by gradient alcohol, and treated with 50 μL of 1% proteinase K at 37 °C for 30 min. The sections were then treated with 3% H_2O_2 methanol solution at 37 °C for 30 min to eliminate endogenous peroxidase activity. Following this, the sections were incubated with a prepared TUNEL working solution in a wet box at 37 °C for 1 h in dark, followed by adding with 50 μL of conversion-peroxidase solution for 30 min. After staining with diaminobenzidine and counterstaining with hematoxylin, the sections were dehydrated with gradient ethanol (50%, 70%, 90%, and 100%), cleared by xylene, and sealed with neutral balsam. Under a 40 × optical microscope, the positively stained neurons in 10 randomly selected visual fields of each section were observed as yellowish-brown, and neurons with blue nuclei were normal neurons. The neuronal apoptosis rate was calculated as the apoptosis ratio = the number of positive neurons with yellowish-brown/the number of total neurons × 100%, while the number of total neurons = neurons with yellowish-brown + neurons with blue nuclei.

Statistical analyses

Statistical data was processed by SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Normal distribution and variance test were carried out, and measurement data conforming to the Normal distribution was expressed as mean ± standard deviation. Data comparisons between the two groups were analyzed by unpaired *t*-test, while comparisons among multiple groups were performed by one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test. A value of $p < 0.05$ indicated a significant difference.

Results

miR-140-5p expresses lowly in ischemic stroke

It has been reported the involvement of miR-140-5p in ischemic stroke [16]. In order to further investigate the role of miR-140-5p in ischemic stroke, we established a mouse model of ischemic stroke by MCAO, followed by neurological performance evaluation (Fig. 1A) and identification by TCC staining (Fig. 1B). As illustrated in Fig. 1A and B, the sham-operated mice exhibited no functional impairment in nerves and no infracted area, while the MCAO mice showed neuron functional impairment and obvious infracted areas. Then, the expression of miR-140-5p in MCAO mice was detected using RT-qPCR, which revealed that MCAO mice exhibited lower expression of miR-140-5p than sham-operated mice ($p < 0.05$, Fig. 1C). This result was subsequently validated in cellular experiments of OGD-induced primary neurons. We employed flow cytometry to measure the cell apoptosis, which found that cell apoptosis of neurons was increased after OGD induction (Fig. 1D). Moreover, RT-qPCR analysis displayed that OGD-treated neurons exhibited reduced expression of miR-140-5p ($p < 0.05$, Fig. 1E). These results highlighted a poorly-expressed miR-140-5p in ischemic stroke.

Overexpression of miR-140-5p inhibits neuron apoptosis under the OGD condition

As lowly-expressed miR-140-5p has been identified in ischemic stroke, this investigation further explored whether and how miR-140-5p affects neuron apoptosis. After OGD induction, the neurons were treated with miR-140-5p mimic, which significant elevated the expression of miR-140-5p in neurons relative to mimic NC treatment ($p < 0.05$, Fig. 2A). Following this, flow cytometry was carried out and showed that neuronal apoptosis ability was restrained in OGD-induced neurons with miR-140-5p mimic treatment when compared with those with mimic NC treatment ($p < 0.05$, Fig. 2B). Meanwhile, as shown in Fig. 2C, miR-140-5p mimic was observed to decrease the protein level of Bax and cleaved caspase 3 and increased the Bcl-2 protein level, in comparison to mimic NC, as detected by Western blot analysis. Above mentioned data suggested that miR-140-5p overexpression exerted inhibitory role in neuronal apoptosis after OGD treatment.

miR-140-5p targets TLR4 and negatively regulates its expression

A binding site between miR-140-5p and TLR4 was predicted online (Fig. 3A). Interestingly, a high expression of TLR4 was found in ischemic stroke in a previous report [26]. Then, with the attempt to validate the relationship between miR-140-5p and TLR4, dual-luciferase reporter assay was performed. The results are displayed in Fig. 3B. As compared to the cells co-transfected with mimic NC, the cells co-transfected with TLR4 WT and miR-140-5p mimic exhibited remarkable decreased fluorescence intensity ($p < 0.05$), while the fluorescence intensity of cells co-transfected with TLR4 MUT and miR-140-5p mimic showed no significant changes ($p > 0.05$). In addition, the protein levels of TLR4 in MCAO mice and OGD-induced neurons were quantified by Western blot. It was demonstrated that the protein level of TLR4 was

elevated in MCAO mice relative to sham-operated mice ($p < 0.05$, Fig. 3C), and also increased in the neurons under OGD condition ($p < 0.05$, Fig. 3D). Then, miR-140-5p was overexpressed in the OGD-treated neurons using miR-140-5p mimic ($p < 0.05$, Fig. 3E), with a descended level of TLR4 protein observed using Western blot analysis ($p < 0.05$, Fig. 3F). The results uncovered the targeting relationship between miR-140-5p and TLR4, and miR-140-5p could negatively regulate the expression of TLR4.

miR-140-5p inhibits cell apoptosis of OGD-induced neurons via the TLR4/NF-κB axis in vitro

Subsequently, for the purpose to discover the function of miR-140-5p and TLR4 on neuronal apoptosis, both of miR-140-5p and TLR4 were upregulated in the neurons under OGD condition. RT-qPCR analysis demonstrated that, in comparison with the co-treatment of mimic NC and oe-NC, the OGD-induced neurons following co-treatment of miR-140-5p mimic with oe-NC or with oe-TLR4 were found to have increased expression of miR-140-5p ($p < 0.05$, Fig. 4A). Then, Fig. 4B sketches the results of Western blot detection on TLR4 and NF-κB-p65 protein levels. The protein levels of TLR4 and NF-κB-p65 were reduced in the OGD-treated neurons with miR-140-5p overexpression and elevated in those with TLR4 overexpression, while the OGD-treated neurons following co-overexpression of miR-140-5p and TLR4 also had increased protein levels of TLR4 and NF-κB-p65 ($p < 0.05$). Next, we conducted flow cytometry to explore the effect on neuron apoptosis, supplemented with Western blot detection on the protein levels of apoptosis-related factors (Fig. 4C and D). It was observed that miR-140-5p mimic suppressed the cell apoptosis of OGD-induced neurons, accompanied by decreased protein levels of Bax and cleaved caspase 3 and increased protein level of Bcl-2, while TLR4 had opposite effects on the neurons ($p < 0.05$). Additionally, the co-upregulation of miR-140-5p expression and TLR4 expression also promoted neuron apoptosis, elevated protein levels of Bax and cleaved caspase 3, as well as declined Bcl-2 protein level ($p < 0.05$). The above diagrams from *in vitro* cellular experiments served to illustrate that miR-140-5p could regulate the TLR4/NF-κB axis to inhibit neuron apoptosis under OGD condition.

miR-140-5p suppresses the development of ischemic stroke via the TLR4/NF-κB axis in vivo

Finally, we paid our attention to how miR-140-5p and TLR4 affect MCAO mice. Figure 5A presents the results of TTC staining, suggesting that miR-140-5p agomir reduced the infracted areas, while oe-TLR4 plasmid reversed this effect to increase the infracted areas ($p < 0.05$). Then, as shown in Fig. 5B, miR-140-5p agomir decreased the neuron function score and oe-TLR4 plasmid increased the neuron function score ($p < 0.05$), indicating miR-140-5p improved the neuron function but TLR4 impaired the neuron function. Then, TUNEL staining was employed to observe the cell apoptosis in brain tissues of MCAO mice. The results revealed that miR-140-5p overexpression inhibited cell apoptosis and TLR4 overexpression reversed this effect to promote cell apoptosis (Fig. 5C). The data summarized that miR-140-5p could restrain the development of ischemic stroke through regulation on the TLR4/NF-κB axis in MCAO mice.

Discussion

Stroke is regarded as the second commonest cause of death and as a major cause of disability worldwide. Compared with white populations, a slightly higher overall incidence of stroke and proportion of intracerebral hemorrhage are reported among Chinese [27]. With the attempt to discover the function miR-140-5p/TLR4/NF-κB axis has in acute ischemic stroke, a series experiments were carried out based on MCAO mouse model and OGD-induced cell model. With the research, the author's endeavor is to explain the possible mechanism underling miR-140-5p and TLR4 in ischemic stroke. Finally, we found that miR-140-5p could bind to TLR4 gene and regulate its downstream NF-κB, thereby protecting neurons against ischemic injury.

As previously investigated, poorly-expressed miR-140-5p was identified in MCAO mice and hypoxia-treated cells, while its overexpression alleviated the development of ischemic stroke [16]. This is in lines with our experiments, which uncovered that both in mice treated with MCAO and cells induced by OGD, miR-140-5p expressed at a low level. In addition to the ischemic stroke, many other diseases also exhibited dysregulated expression of miR-140-5p, such as hepatocellular carcinoma, colorectal cancer, and pulmonary arterial hypertension [28–30]. Previous reports also discovered various biological activities of miR-140-5p, including adipocyte differentiation regulation, cancer growth inhibition, as well as Alzheimer's disease modulation [31–33]. It is generally known that neuron protection is an important therapeutic target in ischemic stroke [34]. Thereby, we subsequently focused our attention on the effects of miR-140-5p on the neuron injury following ischemic stroke.

During the current study, we obtained data demonstrating that miR-140-5p upregulation exerted inhibitory effects on neuron apoptosis under OGD induction and infracted areas of MCAO mice. It suggested that miR-140-5p could inhibit neuronal injury, improve neuron function, and suppress the development of ischemic injury. The effects of miRNAs on the regulation of experimental stroke and in the development of carotid artery stroke, as well as functioning as promising biomarkers of stroke have been reported [35]. Besides, miRNAs have emerged as potential diagnostic and prognostic biomarkers in ischemic stroke has also been discussed [36]. For example, miR-7a-5p was demonstrated to therapeutically reduce the brain damage and disability caused by ischemic stroke [37]. Additionally, consistent with the observations of our study, miR-140-5p has been highlighted due to its promotion role in the cerebral protective effects of dexmedetomidine against hypoxic–ischemic brain damage (HIBD) in neonatal rats [38]. Furthermore, there is existing data that suggests that miR-140-5p suppresses the tumor growth and progression of hepatocellular carcinoma, tongue cancer, and cervical cancer [28, 39, 40]. This brings insight into the positive role of miR-140-5p in the management and treatment of many diseases.

As the potential of miRNAs as mediators of gene silencing at posttranscriptional level has been demonstrated to contribute to the pathogenic and pathological aspects of ischemic stroke [8]. Through online website, we predicted the target gene of miR-140-5p, revealing the binding relationship between miR-140-5p and TLR4. Then, dual-luciferase reporter assay was employed and verified that miR-140-5p bound to TLR4. Most interestingly, TLR4 has been suggested to play the crucial role of in stroke, which

could mediate several pathways related to inflammation or migration of neutrophils into the parenchyma that induced by ischemia [41]. Hence, we guessed that TLR4 may be involved in the ischemic stroke affected by miR-140-5p, which was validated by means of RT-qPCR, Western blot, flow cytometry, and TTC staining, as well as TUNEL staining. It was observed that miR-140-5p negatively regulated the expression of TLR4 and overexpression of TLR4 might reverse the inhibition of miR-140-5p in neuronal apoptosis, functional damage of nerves, and development of stroke. Xiang Y, *et al.* highlighted that the silencing of TLR4 could protect brain against ischemic stroke associated with eicosanoid downregulation [42]. Moreover, we observed that NF-κB was also downregulated by miR-140-5p overexpression and upregulated by TLR4 overexpression. NF-κB, a central transcription factor of inflammatory response, is required for the transcriptional induction of many pro-inflammatory mediators, which was implicated to potentially regulate the inflammatory processes in ischemic stroke [43]. In consistent with our data, downregulation of TLR4 and NF-κB p65 contributed to the ameliorated ischemic brain injury by Rab7b in rats after transient tMCAO treatment [44].

Conclusion

Returning to the hypothesis posed at the beginning of this study, it is now possible to state that miR-140-5p could ameliorate neuron injury following ischemic stroke via suppression of TLR4 and NF-κB. Overall, our findings illuminate how overexpression of miR-140-5p confers a protective effect on ischemic stroke-induced injury that may offer a novel theranostic marker in this disease.

Declarations

Ethics approval

Animal use and all procedures of animal experiments were carried out approved by the Experimental Animal Ethics Committee of the Second Affiliated Hospital of Lanzhou University, which were in line with the guidelines.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

The datasets generated/analysed during the current study are available.

Competing interests

The authors declare no competing financial interests.

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Authors' contributions

Wenjun Song, Tiancheng Wang and Bei Shi performed experiments, contributed to experiment design, and assisted in data interpretation; Zhijun Wu and Wenjie Wang helped design experiments and assisted in data interpretation and statistical analysis; Wenjun Song, Tiancheng Wang and Yanhong Yang supervised planning of experiments and data interpretation and contributed to the preparation of the manuscript; Wenjun Song wrote the manuscript. All authors have read and approved the final submitted manuscript.

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Figures

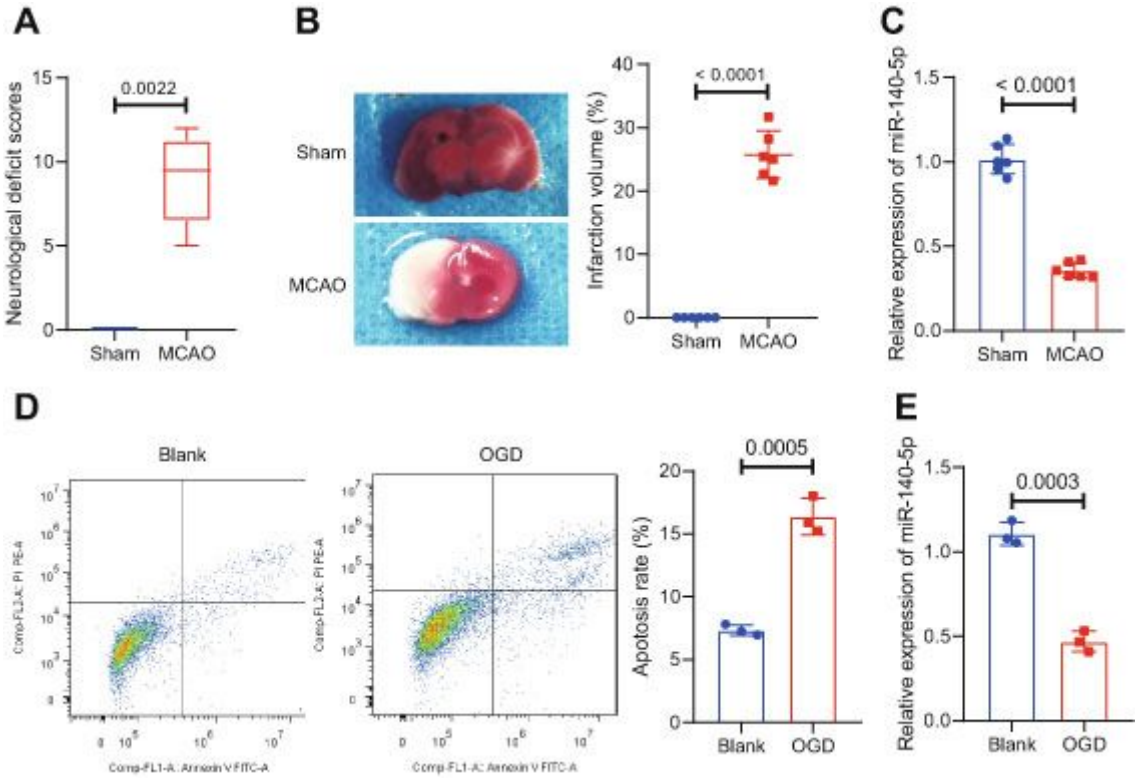

Figure 1

miR-140-5p is lowly-expressed in ischemic stroke; A, neurological function evaluation of mice; B, infracted areas in brain tissues of mice by TTC staining; C, RT-qPCR results of miR-140-5p expression of mice; D, cell apoptosis of neurons by flow cytometry; E, RT-qPCR results of miR-140-5p expression in neurons. *p < 0.05, vs. the sham group or the blank group. Measurement data expressed as mean ± standard deviation, and data comparisons between two groups were analyzed by unpaired t-test. The experiments were repeated three times.

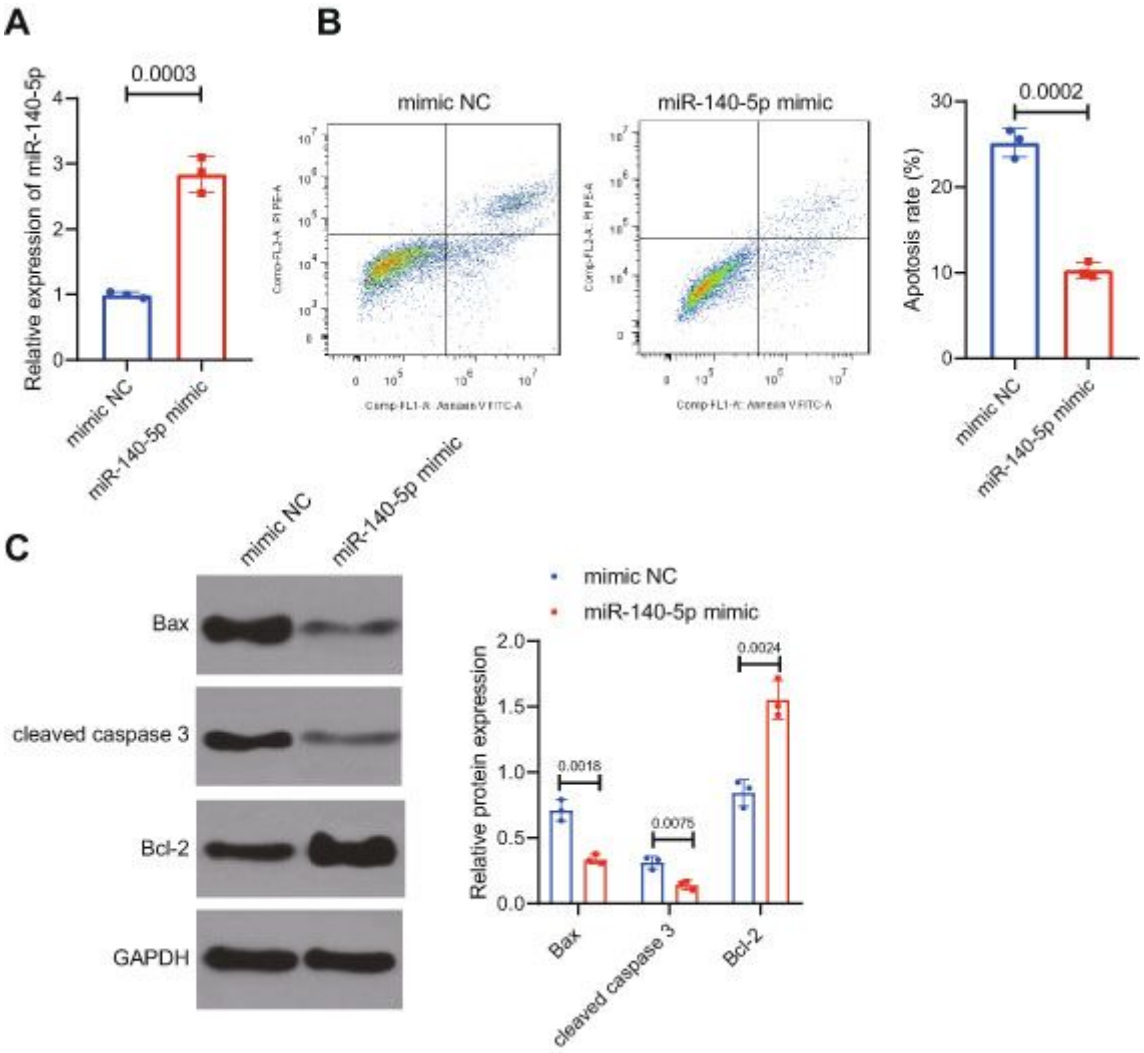

Figure 2

miR-140-5p overexpression inhibits neuronal apoptosis after OGD induction; A, RT-qPCR results of miR-140-5p expression in neurons with different treatments; B, cell apoptosis of neurons with different treatments by flow cytometry; C, protein level of apoptosis-related factors (Bax, Bcl-2, and cleaved caspase 3) expression in neurons with different treatments by Western blot analysis. *p < 0.05, vs. the mimic NC group. Measurement data expressed as mean ± standard deviation, and data comparisons between two groups were analyzed by unpaired t-test. The experiments were repeated three times.

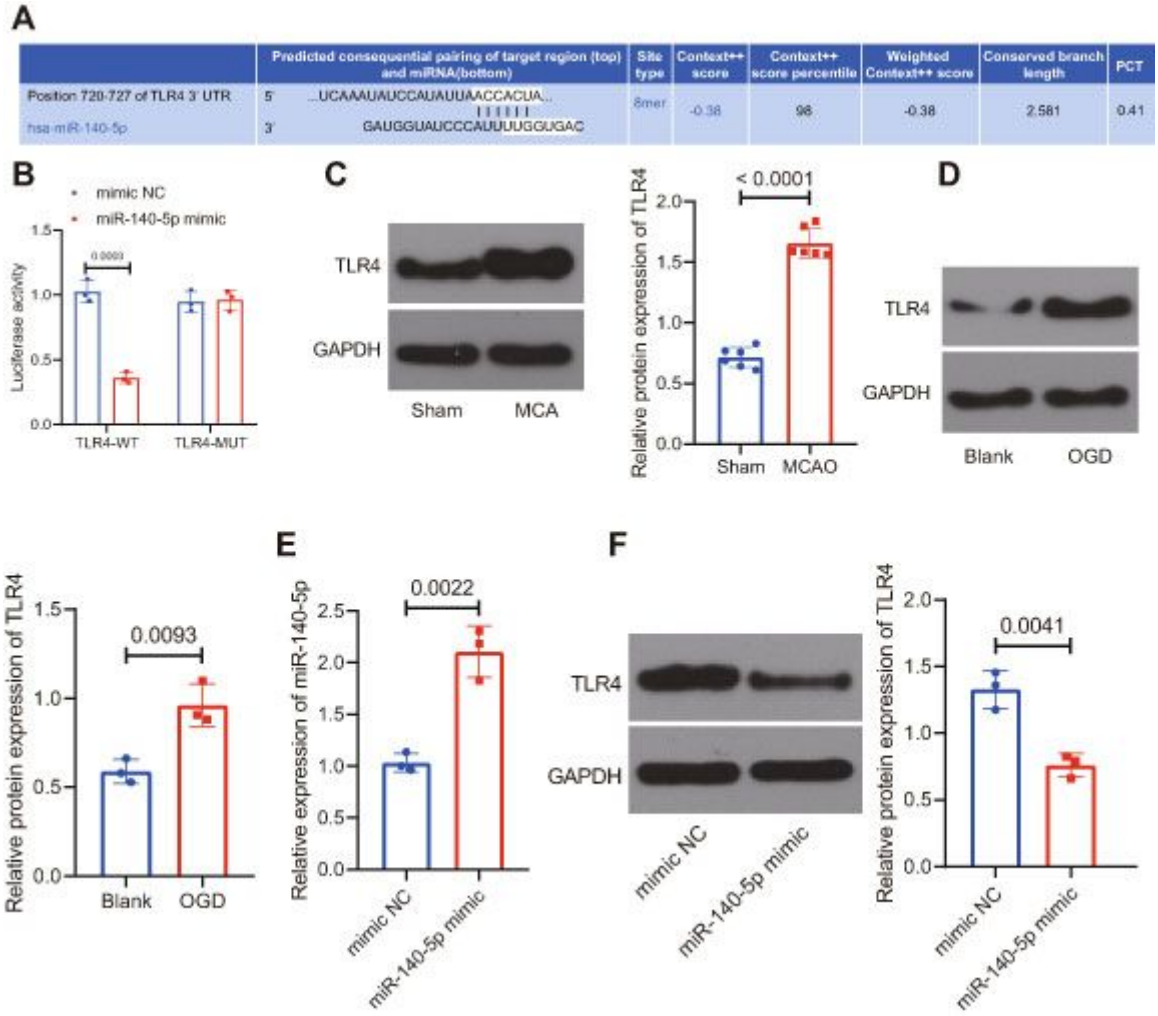

Figure 3

miR-140-5p targets and downregulates TLR4; A, online prediction on the binding site of miR-140-5p and TLR4; B, fluorescence intensity of cells detected by dual-luciferase reporter assay; C, Western blot results of TLR4 protein level in mice; D, Western blot results of TLR4 protein level in neurons; E, RT-qPCR results of miR-140-5p expression in neurons; F, Western blot results of TLR4 protein level in neurons with different transfection. *p < 0.05, vs. the mimic NC group. Measurement data expressed as mean ± standard deviation, and data comparisons between two groups were analyzed by unpaired t-test. The experiments were repeated three times.

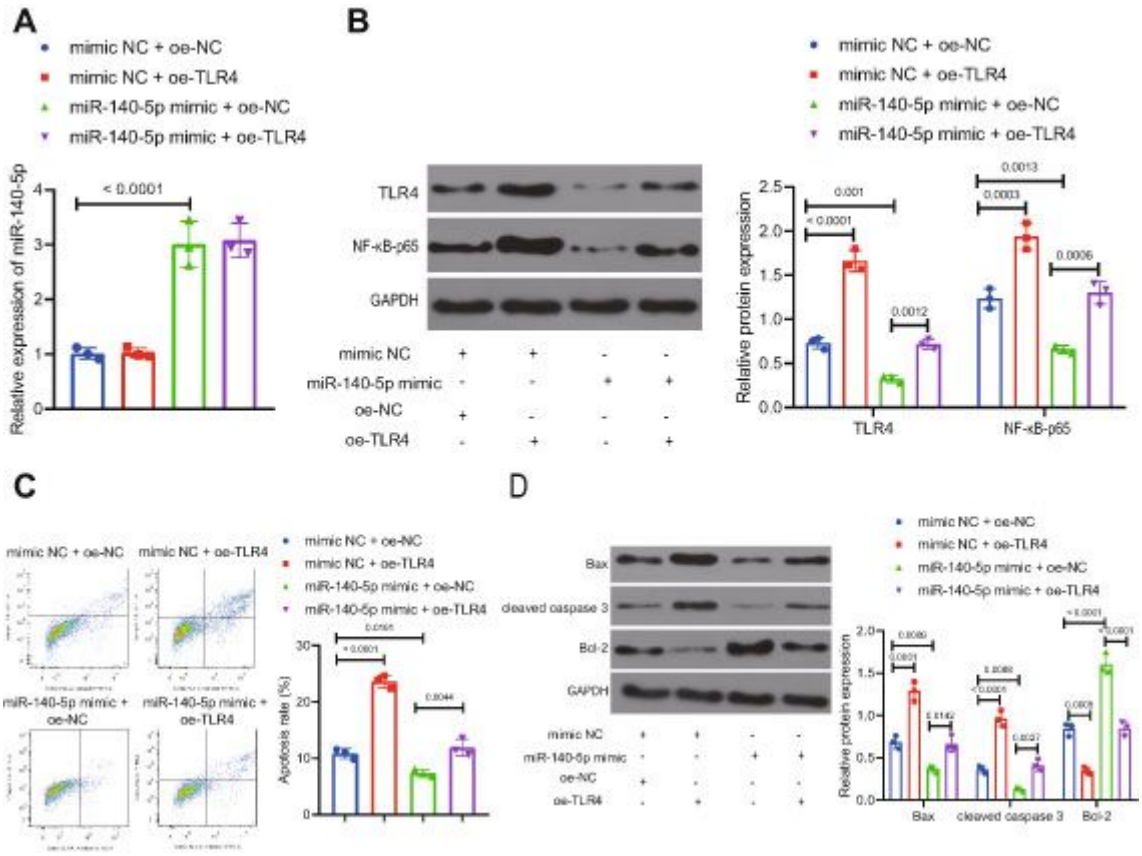

Figure 4

Inhibitory effects of miR-140-5p/TLR4/NF-κB axis on neuronal apoptosis after OGD induction; A, RT-qPCR results of miR-140-5p expression in neurons; B, protein levels of TLR4 and NF-κB-p65 in neurons measured by Western blot analysis; C, cell apoptosis of neurons detected by flow cytometry; D, protein levels of apoptosis-related factors in neurons determined by Western blot analysis. *p < 0.05. Measurement data expressed as mean ± standard deviation, and data comparisons between two groups were analyzed by unpaired t-test. The experiments were repeated three times.

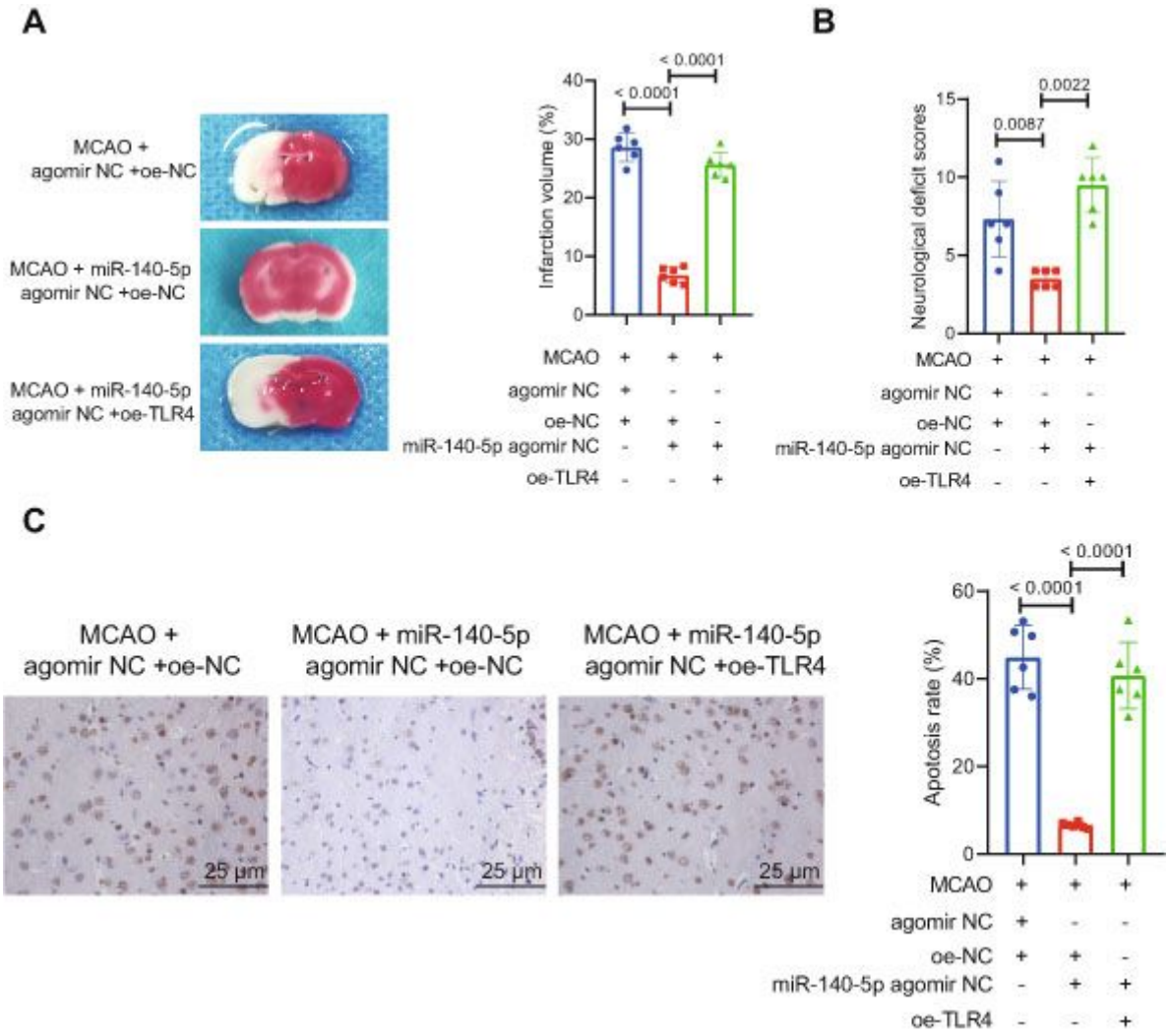

Figure 5

Inhibitory effects of miR-140-5p/TLR4/NF-κB axis on the development of ischemic stroke in MCAO mice; A, infracted areas in brain tissues of mice by TTC staining; B, neurological function evaluation of mice; C, cell apoptosis in brain tissues by TUNEL staining. *p < 0.05, vs. the MCAO + agomir NC + oe-NC group; #p < 0.05, vs. the MCAO + miR-140-5p agomir + oe-NC group. Measurement data expressed as mean ± standard deviation, and data comparisons between two groups were analyzed by unpaired t-test. The experiments were repeated three times.