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ORIGINAL ARTICLE

miR-140-5p Attenuates Neuroinflammation and Brain Injury in Rats Following Intracerebral Hemorrhage by Targeting TLR4

Shunda Wang,[1] Yujie Cui,[2] Jiaqi Xu,[3] and Heng Gao[4,5]

Abstract—The Toll-like receptor 4 (TLR4)–mediated neuroinflammation plays a key role in inducing secondary brain injury after intracerebral hemorrhage (ICH). However, how TLR4 is regulated during this pathological process is not well understood. In the present study, by taking advantage of a rat ICH model, we show that miR-140-5p is reversely correlated with TLR4 expression in the peri-hematomal striatum following ICH. *In vitro*, miR-140-5p directly targets TLR4 and suppresses its expression in a rat neuronal PC12 cell line. Moreover, an intracerebral ventricular injection of miR-140-5p mimics improves neurological function and reduces apoptotic cell death and limits the production of inflammatory cytokines following ICH, indicating that miR-140-5p attenuates brain injury and neuroinflammation *in vivo*. Furthermore, miR-140-5p suppresses TLR4 expression and inhibits the downstream MyD88/TRIF inflammatory pathway and NF-κB activity following ICH, suggesting that the inhibition of TLR4-mediated neuroinflammation at least in part accounts for the neuroprotective role of miR-140-5 against ICH-induced brain injury in rats. Collectively, these results identify miR-140-5 as a negative regulator of TLR4 and downstream inflammatory pathway following ICH, implicating that miR-140-5 might represent as a potential therapeutic target for alleviating ICH-induced brain injury.

KEY WORDS: miR-140-5p; neuroinflammation; intracerebral hemorrhage; brain injury; TLR4.

INTRODUCTION

Intracerebral hemorrhage (ICH) is a common and devastating subtype of stroke that accounts for approximately 15% of stroke-related deaths [1]. To date, there is still no effective treatment to improve the clinical outcome of ICH patients [2]. Therefore, a deeper understanding of ICH pathogenesis is urgently required for developing novel therapies. It is well-accepted that the ICH pathogenic process is divided into two major phases, including the primary brain injury arising from intraparenchymal hematoma and the secondary brain

Shunda Wang and Yujie Cui contributed equally to this work.

[1] Department of Rehabilitation Medicine, Shaanxi Provincial People's Hospital, Xi'an, 710068, Shaanxi, China
[2] Rheumatology Department, The Fifth Affiliated Hospital Sun Yat-sen University, Zhuhai, 519000, China
[3] Burn and Plastic Surgery, The Fifth Affiliated Hospital Sun Yat-sen University, Zhuhai, 519000, China
[4] Department Emergency Medicine, Shaanxi Provincial People's Hospital, No. 256 Friendship West Road, Xi'an, 710068, Shaanxi, China
[5] To whom correspondence should be addressed at Department Emergency Medicine, Shaanxi Provincial People's Hospital, No. 256 Friendship West Road, Xi'an, 710068, Shaanxi, China. E-mail: gh2637@126.com

injury, in which the neuroinflammation produced in the peri-hematomal area is a prominent pathogenic factor [3, 4]. Further, the neurological deficits caused by inflammatory responses are considered to be associated with the release of some endogenous ligands, which perform functions mainly through Toll-like receptors (TLRs) [5, 6]. In recent years, the increased expression of TLR4 has been associated with the poor outcome in ICH patients [7, 8], and the TLR4-mediated neuroinflammation has also been demonstrated to play a vital role in inducing secondary brain injury after ICH [9, 10]. On the contrary, TLR4 antagonist was found to attenuate ICH brain injury [11]. These studies indicate that TLR4 is a promising therapeutic target for ICH treatment. However, it remains largely unclear how TLR4 expression is regulated during ICH pathogenesis.

microRNAs (miRNAs) are a class of noncoding RNAs that function to suppress gene expression by interacting with the binding sites within the 3′-UTR of mRNAs [12]. The potential targets of miRNAs can be predicted by in-silico algorithms, such as TargetScan [13], miRWalk [14], and miRbase [15]. By taking advantage of these methods, we found that TLR4 may be a candidate target of miR-140-5p. Lately, miR-140-5p was reported to suppress inflammation in chondrocytes [16]. However, to our knowledge, whether miR-140-5p regulates TLR4 and affects neuroinflammation following ICH have not been explored. In the present study, we validated the regulation of TLR4 by miR-140-5p and also investigated its functional roles involved in neuroinflammation and brain injury following ICH by using a rat ICH model.

MATERIALS AND METHODS

Animals and ICH Model

Male Sprague-Dawley rats (12 weeks old, 200–220 g body weight) were purchased from the Laboratory Animal Center of Shaanxi Provincial People's Hospital. All animal experiments were conducted in accordance with the guidelines approved by the Animal Ethics Committee of Shaanxi Provincial People's Hospital. Experimental ICH was developed by intracerebral injection of collagenase [17]. Briefly, 1 μl saline containing 0.23 U bacterial collagenase type IV (Solarbio) was administrated over a 5-min period through the hole into the right striatum. Sham operation was processed by injecting 1 μl sterile saline. For intracerebral ventricular injection of negative control (NC) mimics or miR-140-5p mimics, at 3 days prior to ICH induction, 5 μl NC mimics or miR-140-5p mimics (20 nmol/L, diluted in sterile saline) were slowly injected into the right lateral ventricle as previously described [18].

Evaluation of Neurological Deficits

The neurological deficits of rats were evaluated at 1 day after ICH induction using a 24-point neurological scoring system [19]. The evaluation process was performed blindly for all rats. The body symmetry, gait, climbing, circling behavior, front limb symmetry, and compulsory circling were tested and graded from 0 to 4 points.

qRT-PCR Analysis

The total RNA was isolated using the TRIzol reagent (Invitrogen) following the manufacturer's protocol. cDNA was synthesized from mRNA and miRNA using the PrimeScriptTM RT reagent Kit and Mir-X miRNA First-Strand Synthesis Kit (Takara), respectively. qRT-PCR analysis was performed using the SYBR Premix Ex Taq II Kit (Takara) according to the manufacturer's protocols. Primers were purchased from GenePharma (Shanghai, China). Each reaction was carried out in triplicate. Data were analyzed using the $2^{-\Delta\Delta CT}$ method [20]. U6 and GAPDH were used as reference controls. The primer sequences are listed as the following: miR-140-5p sense 5′-ACACTCCAGCTGGGCAGTGGTTTTACCCTA-3′ and antisense 5′-TGGTGTCGTGGAGTCG-3′; U6 sense 5′-CTCGCTTCGGCAGCACA-3′ and antisense 5′-AACGCTTCACGAATTTGCGT-3′; TLR4 sense 5′-ATCTCAGCAAAATCCCTCAT-3′ and antisense 5′-AATCCAGCCACTGAAGTTGT-3′; GAPDH sense 5′-AGGTCGGTGTCAACGGATTT-3′ and antisense 5′-CCTTCCACGATGCCAAAGTT-3′.

Western Blot Analysis

The total protein was extracted using the RIPA lysis buffer (Beyotime). The nuclear protein was extracted from brain tissues using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). Equal amounts of protein were subjected to SDS-PAGE gel electrophoresis and transferred to PVDF membranes (Millipore). Then, PVDF membranes were blocked with 5% non-fat milk for 1 h and incubated with primary antibodies overnight at 4 °C and secondary antibodies for 1 h at room temperature. Protein bands were visualized by incubating with the Immobilon Western Chemiluminescent HRP Substrates (Millipore) and analyzed using the ImageJ software. Antibodies were purchased from the following sources: anti-

TLR4 (Proteintech, 19811-1-AP), anti-GAPDH (Abcam, ab9485), anti-cleaved caspase-3 (Cell Signaling, 9661), MyD88 (Abcam, ab2064), TRIF (Abcam, ab13810), anti-p-p65 (Cell Signaling, 3031), anti-p65 (Abcam, ab16502).

ELISA Assay

The rat peri-hematomal striatum tissues (50 mg) were collected and the supernatants were used for assessing the concentrations of TNF-α, IL-1β, and IL-6 using the Quantikine ELISA Kit (R&D systems) according to the manufacturer's instructions.

TUNEL Staining

TUNEL staining on the brain coronal sections ($n = 9$ rats in each group) was performed using the *in situ* Cell Death Detection Kit, POD (Roche, 11684817910) based on the manufacturer's protocols. The sections were counterstained with DAPI. Five random fields in the peri-hematomal striatum on each section were captured using the BX51 inverted fluorescent microscope (Olympus, Tokyo, Japan). The percentage of TUNEL-positive cells was analyzed using the ImageJ software.

Luciferase Reporter Assay

The rat TLR4 3′-UTR fragment containing the binding sites for miR-140-5p was cloned into the pmirGLO vector (Promega, Madison, WI, USA). The mutant construct was established using the Q5 Site-Directed Mutagenesis Kit (NEB, E0554S) according to the manufacturer's instructions. The PC12 cells were cultured in 24-well plates and cotransfected wild-type or mutant luciferase construct with negative control mimics or miR-140-5p mimics, or with NC inhibitor or miR-140-5p inhibitor, using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The luciferase activity in each well was measured 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega) and normalized to that of Renilla construct. Each transfection was performed in 5 replicates.

Statistical Analysis

All results were expressed as the means ± standard deviation (SD). Statistical analysis was conducted using the SPSS 11.5 (Chicago, IL, USA). The Student *t* test or one-way ANOVA followed by the *post hoc* Tukey test was used to calculate significance. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

miR-140-5p Is Reversely Correlated with TLR4 Expression Following ICH

To explore whether there exists a possible regulation between miR-140-5p and TLR4 following ICH, we checked their expression in the peri-hematomal striatum obtained from a rat ICH model, which was established by intracerebral injection of collagenase into the striatum [17]. The mRNA levels of miR-140-5p and TLR4 were determined by qRT-PCR analysis, and the results showed that in contrast to the sham-operation group, miR-140-5p expression gradually declined from 12 h to 2 days, but then displayed considerable recovery at 7 days following ICH (Fig. 1a). Additionally, just opposite to the expression change of miR-140-5p, TLR4 expression level was progressively increased from 12 h to 2 days, followed by a gradual decrease to that of sham-operation group thereafter (Fig. 1b). Moreover, similar tendency of TLR4 expression change was observed when its protein level was analyzed by Western blot assay (Fig. 1c). The induction of TLR4 expression following ICH is consistent with previous reports [10, 21], which validates the methodology used in this study. Furthermore, the Pearson correlation analysis showed that a strong reverse correlation existed between miR-140-5p level and TLR4 mRNA level (Fig. 1d). Thus, by investigating a rat ICH model, we show that miR-140-5p is downregulated following ICH, with which TLR4 expression is reversely correlated.

TLR4 Is a Direct Target of miR-140-5p in PC12 Cells

miRNAs regulate gene expression through directly targeting mRNAs [22]. The reverse correlation between miR-140-5p and TLR4 observed following ICH implies that TLR4 may be a target of miR-140-5p. This conjecture is theoretically supported by their sequence complementarity, as predicted by in-silico algorithm TargetScan (Fig. 2a) [13]. To confirm whether miR-140-5p directly targets TLR4, we carried out luciferase reporter assay in PC12 cells, a widely used rat pheochromocytoma cells modeling isolated neurons *in vitro* [23]. Indeed, we found that miR-140-5p inhibition augmented the luciferase activity of wild-type but not the mutant TLR4 3′-untranslated region (3′-UTR) construct (Fig. 2b), and conversely, miR-140-5p overexpression suppressed the luciferase activity of wild-type TLR4 3′-UTR construct, but with the mutant one unaffected (Fig. 2c). In agreement with these results, miR-140-5p inhibition markedly increased and its inhibition decreased TLR4 expression at both mRNA level (Fig.

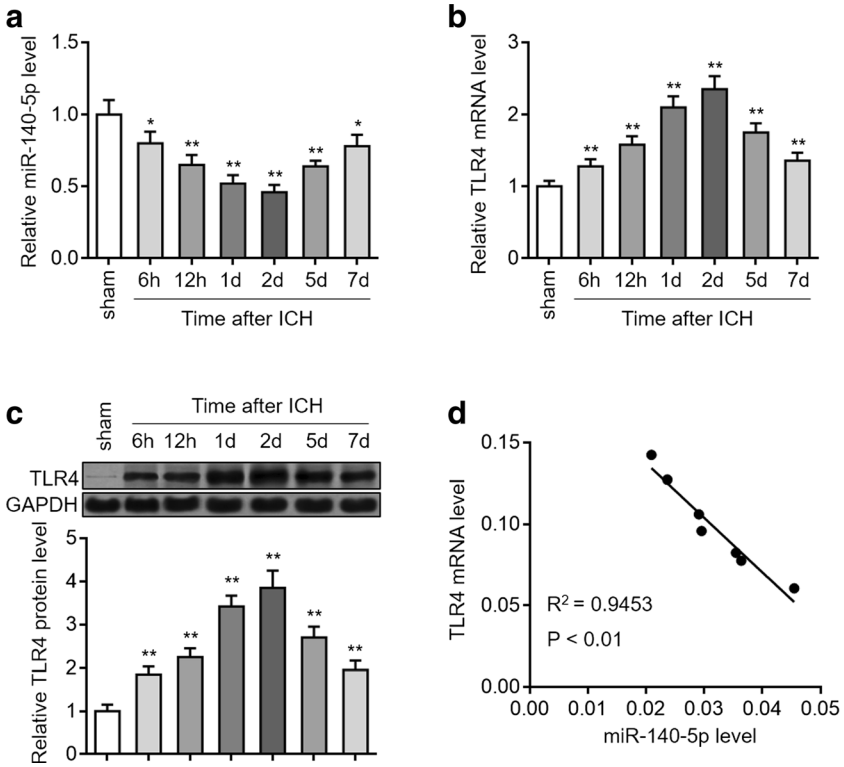

Fig. 1. miR-140-5p is reversely correlated with TLR4 expression in the peri-hematomal striatum following ICH. **a**, **b** The expression levels of miR-140-5p (**a**) and TLR4 (**b**) in rat peri-hematomal striatum at 6 h to 7 days following ICH were determined by qRT-PCR analysis. Sham-operation group was used as a control. Nine rats were included in each group. **c** The protein level of TLR4 in tissue samples as same as in (**a**, **b**) was determined by Western blotting analysis. The representative images (top) and quantitative analysis (bottom) are shown. **d** The correlation between miR-140-5p level and TLR4 mRNA level in all groups was determined by Pearson correlation analysis. GAPDH was used as a normalization control. Data are mean ± SD ($n = 9$). $^{*}P < 0.05$; $^{**}P < 0.01$ *versus* sham group.

2d) and protein level (Fig. 2e) in PC12 cells. Altogether, these data prove that miR-140-5p directly targets TLR4 and suppresses its expression in PC12 cells. Therefore, the mechanism of the negative regulation of TLR4 by miR-140-5p is associated with their reverse correlation observed following ICH (Fig. 1).

miR-140-5p Overexpression Attenuates Brain Injury and Neuroinflammation Following ICH

Next, to learn more about the role of miR-140-5p following ICH, we overexpressed miR-140-5p in rat brain *via* intracerebral ventricular injection of miR-140-5p mimic 3 days prior to ICH induction [18]. qRT-PCR analysis confirmed the overexpression of miR-140-5p in the peri-hematomal striatum as compared with injection of NC mimic (Fig. 3a). Functionally, the neurological deficits in rats were evaluated by a 24-point neurological scoring system at 24 h following ICH [19], which showed that the neurological deficits in ICH rats were significantly alleviated when miR-140-5p level was restored by overexpression (Fig. 3b). Consistent with this, TUNEL staining of the peri-hematomal striatum revealed that the induced apoptosis following ICH was also limited in rats injected with miR-140-5p mimic (Fig. 3c), which was further strengthened by the decreased expression of cleaved caspase-3, a marker of apoptosis (Fig. 3d). TLR4 plays an important role in mediating neuroinflammation following ICH [9]. Therefore, we asked whether miR-140-5p influences inflammatory response in ICH rat brain. To address it, we measured the levels of typical proinflammatory cytokines, including TNF-α, IL-1β, and IL-6 by ELISA assay. Notably, as shown in Fig. 3e, the elevated levels of all of these proinflammatory cytokines in ICH rats were decreased by miR-140-5p overexpression. Taken together, these findings suggest that miR-140-5p has a therapeutic effect on ICH in a rat model, which is associated with the attenuated brain injury and suppressed neuroinflammation.

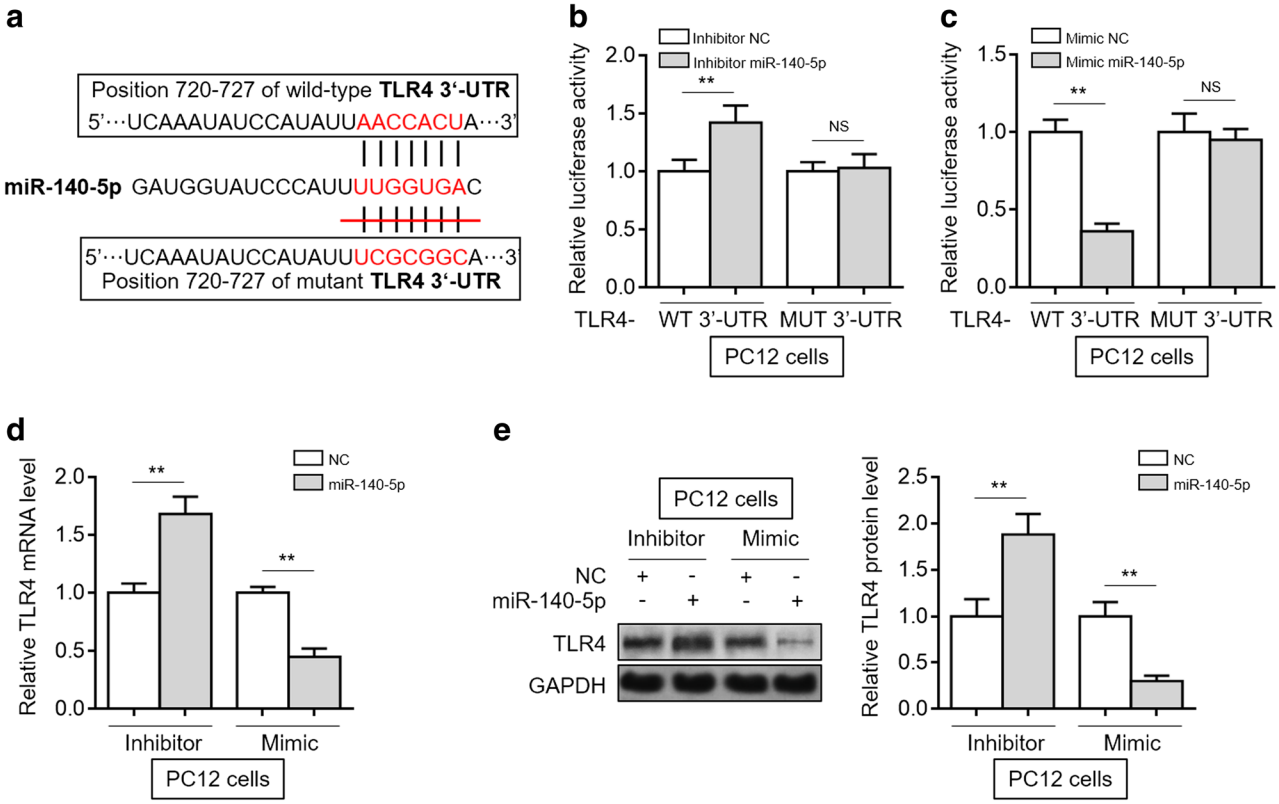

Fig. 2. miR-140-5p directly targets and suppresses TLR4 expression in PC12 cells. **a** The putative miR-140-5p binding sites within the TLR4 3′-UTR fragment are predicted by TargetScan algorithm. The designed mutant sequence of TLR4 3′-UTR is shown below. **b, c** PC12 cells were cotransfected luciferase reporter construct containing wild-type (WT) or mutant (MUT) TLR4 3′-UTR fragment with NC inhibitor or miR-140-5p inhibitor (**b**), or with negative control (NC) mimic or miR-140-5p mimic (**c**). The luciferase activity was measured at 48 h after transfection. The results are expressed as relative to NC transfection ($n = 5$). **d, e** PC12 were transfected with NC inhibitor or miR-140-5p inhibitor, or with NC mimic or miR-140-5p mimic. At 48 h after transfection, the mRNA level (**d**) and protein level (**e**) of TLR4 were analyzed ($n = 3$). Data are mean ± SD. $^{**}P < 0.01$; NS, not significant *versus* NC transfection.

miR-140-5p Overexpression Suppresses TLR4 Expression and Downstream MyD88/TRIF Inflammatory Pathway Following ICH

It has been reported that TLR4 mediates inflammatory injury following ICH through regulating the downstream MyD88/TRIF/NF-κB signaling pathway [10]. To establish whether the miR-140-5p neuroprotection in ICH rats is linked with its negative regulation of TLR4 and downstream MyD88/TRIF/NF-κB signaling pathway, we checked the impact of miR-140-5p overexpression on TLR4 induction and the activation of downstream inflammatory pathway in rat brain following ICH. We found that keeping pace with the restored expression of miR-140-5p *via* injection of mimic in the peri-hematomal striatum following ICH (Fig. 4a), not only the induced expression of TLR4 but also the increased expression of MyD88 and TRIF and elevated phosphorylation level of p65 (Fig. 4b), as well as the induced p65 nuclear translocation (Fig. 4c), in ICH rat were all remarkably suppressed, indicating that miR-140-5p overexpression inhibits ICH-induced activation of TLR4 and MyD88/TRIF/NF-κB signaling pathway. Furthermore, in concert with this concept, the transcript levels of downstream proinflammatory cytokine targets, including TNF-α, IL-1β, and IL-6, were accordingly decreased by miR-140-5p overexpression (Fig. 4d–f), coinciding with the result of their protein alteration (Fig. 3e). Overall, it could be concluded that miR-140-5p suppresses TLR4 expression following ICH in rat brain *in vivo*, leading to inhibited activation of the downstream MyD88/TRIF/NF-κB signaling pathway and limited induction of proinflammatory cytokines, which provides a mechanistic insight for explaining miR-140-5p-attenuated neuroinflammation and brain injury in ICH rat model.

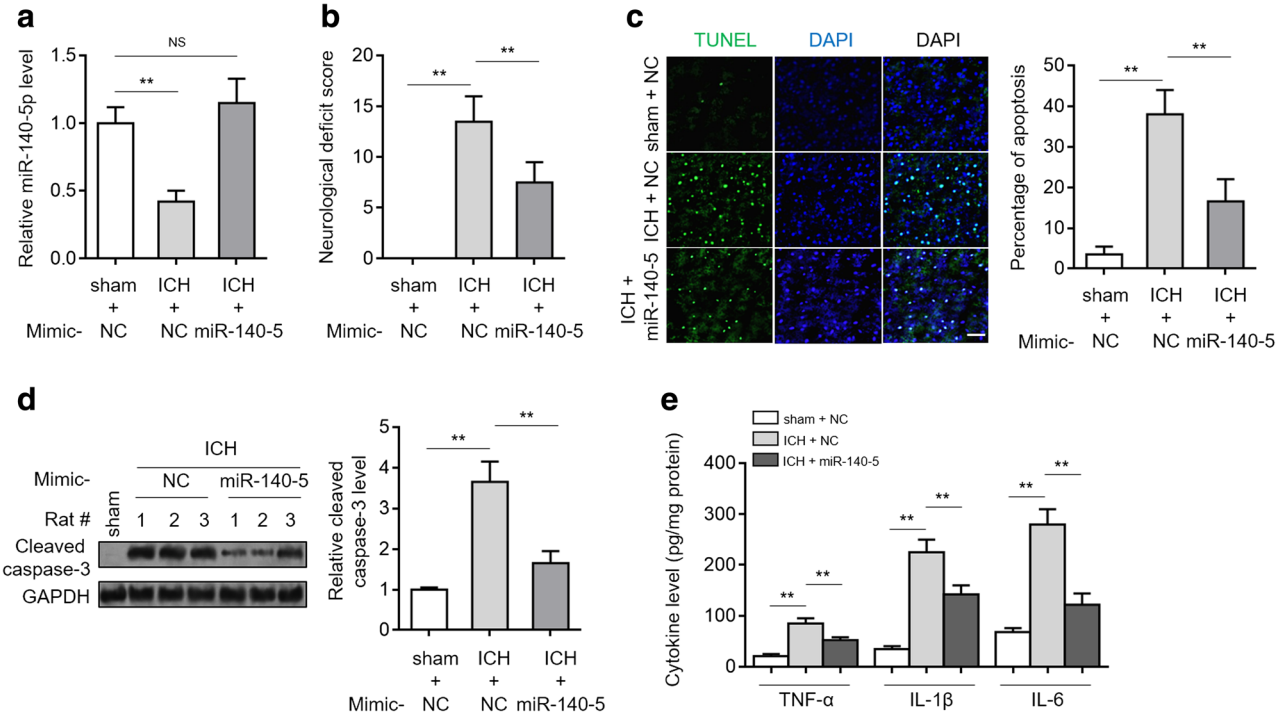

Fig. 3. Intracerebral ventricular injection of miR-140-5p attenuates brain injury and neuroinflammation following ICH. **a**–**e** NC mimic or miR-140-5p mimic were intracerebroventricularly injected into rats 3 days prior to ICH induction. The sham-operation group injected with NC mimic was utilized as a control. Nine rats were included in each group. **a** The expression level of miR-140-5p in rat peri-hematomal striatum at 24 h following ICH was determined by qRT-PCR analysis. **b** The neurological deficits in rats were assessed by a 24-point neurological scoring system at 24 h following ICH. **c** TUNEL staining of the peri-hematomal striatum at 24 h following ICH. The representative images (left) and percentage of TUNEL-positive cells (right) are shown. Scale bar, 20 μm. **d** The protein level of cleaved caspase-3 in rat peri-hematomal striatum at 24 h following ICH was determined by Western blotting analysis. The representative images (left) and quantitative analysis (right) are shown. **e** The levels of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, in rat peri-hematomal striatum at 24 h following ICH were measured by ELISA assay. Data are mean ± SD ($n = 9$). $^{**}P < 0.01$; NS, not significant.

DISCUSSION

The neuroinflammation, mediated by cellular and molecular components, is deeply involved in ICH-induced brain injury [24]. Therefore, a better understanding of the mechanisms and functional roles of ICH-induced neuroinflammation could offer profound implications for developing therapeutic approaches to ICH. Accumulating evidence from recent studies has shown that TLR4, a member of pattern recognition receptors [25], and its downstream inflammatory signaling pathway play a pivotal role in mediating neuroinflammation and brain injury following ICH in animal models [4, 26]. Moreover, an association was also found between increased expression of TLR4 and poor functional outcome and greater residual volume in ICH patients [7]. These previous findings suggest that TLR4 signaling may be a promising target for therapeutic intervention of ICH. As a support for this notion, a pre-clinical study has reported that TAK-242, a TLR4 antagonist, attenuates ICH-induced brain injury in mice [11]. Nevertheless, to date, it remains poorly clarified on how TLR4 level is regulated during the pathogenic process of ICH. In the present study, we observed a reverse correlation between the levels of TLR4 and miR-140-5p. We further demonstrated that TLR4 could be a direct target of miR-140-5p, in which its expression is suppressed by miR-140-5p. The following functional studies show that miR-140-5p overexpression attenuates brain injury and neuroinflammation following ICH, which is accompanied by decreased TLR4 expression and inhibited activation of downstream MyD88/TRIF/NF-κB inflammatory signaling pathway. Overall, these findings reveal a role of miR-140-5p in regulating TLR4 expression in ICH pathogenesis and also connect this negative regulation of TLR4 to the beneficial effects miR-140-5p exert on alleviating ICH symptoms in a rat model. Based on these presented evidence, we propose that targeting TLR4 signaling through miR-140-5p may have therapeutic effects for ICH.

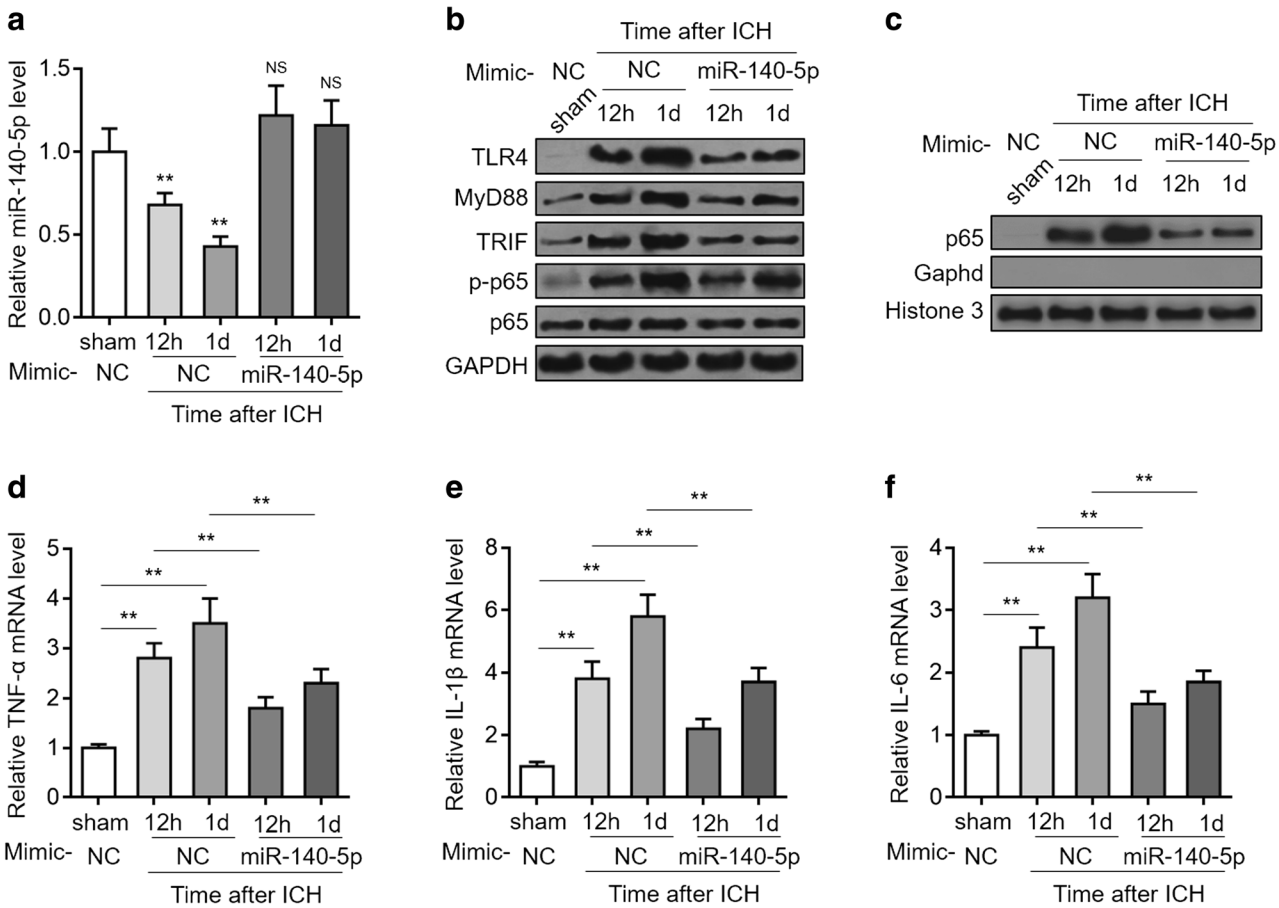

Fig. 4. Intracerebral ventricular injection of miR-140-5p suppresses TLR4 expression and downstream MyD88/TRIF inflammatory pathway following ICH. **a**–**f** NC mimic or miR-140-5p mimic were intracerebroventricularly injected into rats 3 days prior to ICH induction. The sham-operation group injected with NC mimic was utilized as a control. Nine rats were included in each group. **a** The expression level of miR-140-5p in rat peri-hematomal striatum at 12 h and 1 day following ICH was determined by qRT-PCR analysis. **b**, **c** The total protein levels of TLR4, MyD88, TRIF, p-p65, and p65 (**b**) and nuclear fraction p65 level (**c**) in tissue samples as same as in (**a**) were determined by Western blotting analysis. The representative images from 3 replicates are shown. **d**–**f** The transcript levels of proinflammatory cytokines, including TNF-α (**d**), IL-1β (**e**), and IL-6 (**f**), in tissue samples as same as in (**a**) were measured by qRT-PCR analysis. Data are mean ± SD ($n = 9$). $^{**}P < 0.01$; NS, not significant.

The studies associating miR-140-5p with neurological disorders are scarce, excepting that its expression was found to be upregulated in cerebrospinal fluid and blood samples from patients with acute ischemic stroke [27]. Yet, whether miR-140-5p is associated with ICH pathogenesis has not been explored. We found, for the first time, that miR-140-5p level was gradually declined at early phase, but then recovered thereafter in ICH rats. Coincidentally, this dynamic change of miR-140-5p is reversely correlated with that of TLR4 expression. These observations suggest that there may exist a negative feedback mechanism that controls the level of miR-140-5p. Further investigations are needed to elucidate how miR-140-5p expression pattern is modulated following the induction of ICH. The upregulation of TLR4 following ICH has been reported previously [10], which is consistent with our observation. We suspected that the upregulation of TLR4 may be associated with miR-140-5p downregulation. This conjecture is then endorsed by the following lines of evidence: (1) miR-140-5p suppresses the luciferase activity of TLR4 construct in PC12 cells; (2) miR-140-5p suppresses TLR4 expression at both mRNA and protein levels in PC12 cells; (3) miR-140-5p overexpression reduces TLR4 expression in rat brain following ICH. Altogether, these evidences indicate that TLR4 serves as a direct

target of miR-140-5p and that its expression could be modulated by miR-140-5p both *in vitro* and *in vivo*.

In truth, apart from our study, the regulation of TLR4 by miR-140-5p has also been reported by previous studies. For example, miR-140-5p suppresses TLR4 expression, which mediates its regulation of the proliferation and differentiation of human dental pulp stem cells [28]. Additionally, miR-140-5p was found to inhibit the proliferation and inflammatory cytokines secretion in synovial fibroblasts through targeting TLR4 [29]. Together with our findings, these results suggest that the negative regulation of TLR4 by TLR4 would occur in multiple scenarios. Therefore, it is very possible that miR-140-5p may participate in the regulation of other activities, in which TLR4 plays regulatory functions, such as some infectious and noninfectious diseases [25]. It is interesting to test whether this is the case in the future.

TLR4 and downstream MyD88/TRIF/NF-κB signaling pathway trigger innate inflammatory responses and increase cytokine expressions that underlie neurological impairments following ICH [10]. We observed that miR-140-5p overexpression suppressed the activation of MyD88/TRIF/NF-κB signaling pathway and reduced the expression and production of inflammatory cytokines in rat brain following ICH. Meanwhile, the expression of TLR4 was also suppressed, indicating that the attenuated brain injury and neuroinflammation by miR-140-5p overexpression following ICH are at least partially attributed to the suppressed expression of TLR4. Be mechanistically relevant, in traumatic brain injury in a rat model, the neuroprotective effects of resatorvid also reportedly involve the suppression of TLR4/MyD88/TRIF/NF-κB signaling pathway [30]. One limitation of this study is that the mechanisms by which miR-140-5p attenuates neuroinflammation and brain injury following ICH are not fully discovered. One study has reported that expect for TLR4, TLR2 is also associated with poor outcome in ICH patients [7]. Moreover, TLR2 could form a heterodimer with TLR4 and mediates ICH-induced inflammatory injury [21]. Given the important role of TLR4 we revealed in the current study, we doubt that TLR2 is one of the candidates with potential relevance to the mechanisms underlying miR-140-5p function in ICH. Further studies are needed to address this issue, which would advance our understanding of the relation between miR-140-5p and ICH.

In summary, our study reveals that miR-140-5p is a negative regulator of TLR4 in ICH pathogenesis and also highlights an important role of the modulation of the MyD88/TRIF/NF-κB signaling pathway in mediating the suppressive effects of miR-140-5p on neuroinflammation and brain injury following ICH, which may provide a molecular basis for future clinical application.

COMPLIANCE WITH ETHICAL STANDARDS

All animal experiments were conducted in accordance with the guidelines approved by the Animal Ethics Committee of Shaanxi Provincial People's Hospital.

Conflict of Interest. The authors declare that they have no conflict of interest.

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