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Protective effect of silencing lncRNA HCP5 against brain injury after intracerebral hemorrhage by targeting miR-195-5p

Zhanhua Lu[1] and Kun Huang[1*]

Abstract

Background Intracerebral hemorrhage (ICH) is a common subtype of stroke, characterized by a high mortality rate and a tendency to cause neurological damage. This study aims to investigate the role and mechanisms of lncRNA HCP5 in ICH.

Methods We simulated ICH in vivo by injecting collagenase into rats and established an in vitro model using hemoglobin-treated BV2 cells. HCP5 and miR-195-5p levels were quantified by RT-qPCR. mNSS score was used to evaluate neurological deficits in the rats. The dry-wet weight method assessed the degree of brain edema. Cell viability and apoptosis rates were determined using the CCK-8 assay and flow cytometry, respectively. The targeting relationship between HCP5 and miR-195-5p was confirmed using dual-luciferase reporter assays and RNA immunoprecipitation. ELISA was utilized to measure inflammatory factors, and commercial kits were used to detect MDA and ROS levels.

Results In the ICH model rats, HCP5 levels were significantly elevated. It was also found that silencing HCP5 significantly alleviated brain edema and neurological deficits in the ICH rats, and there was a marked improvement in ICH-induced neuroinflammation and oxidative stress. Moreover, HCP5 was found to sponge miR-195-5p, and inhibiting miR-195-5p could counteract the neuroprotective effects of silencing HCP5. Similar results were obtained in the in vitro experiments with BV2 cells.

Conclusions Silencing HCP5 can alleviate brain edema, neurological dysfunction, neuroinflammation, and oxidative stress caused by ICH via miR-195-5p.

Keywords ICH, HCP5, miR-195-5p, Inflammation, Oxidative stress

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Background

Intracerebral hemorrhage (ICH) is a prevalent cerebrovascular disease, representing 12–20% of all strokes [1]. ICH is associated with a high incidence and mortality rate, as well as a generally unfavorable prognosis [2]. Approximately 2 million people worldwide suffer from ICH each year, and the risk of ICH increases with age [3]. Given the increasing severity of population aging, it is foreseeable that the number of patients with ICH will rise rather than decrease. The occurrence of ICH not only results in hematoma formation and increased intracranial pressure, causing primary damage to the brain [4], but the components of the hematoma can also induce secondary damage, including neuronal apoptosis, cell inflammation, oxidative stress, and blood-brain barrier disruption [5]. Consequently, even if patients survive an ICH event, the likelihood of disability is high, and the risk of recurrent ICH increases. Therefore, post-stroke prevention of ICH is crucial for improving the long-term prognosis of these patients [6].

With the advancement of science and technology, especially in high-throughput sequencing technology, we are observing a growing identification of non-coding RNAs, including long non-coding RNAs (lncRNAs) [7]. LncRNAs are defined by their length, exceeding 200 nucleotides, and do not participate in protein coding [8, 9]. Although the expression levels of lncRNAs are relatively low, more and more evidence suggests that the abundance, spatiotemporal expression, and mechanisms of action of lncRNAs differ in different cells and tissues, leading to functional diversity [10]. LncRNAs are involved in various diseases, and their abnormal expression is also associated with cardiovascular and cerebrovascular diseases [11, 12]. LncRNAs can not only serve as diagnostic biomarkers [13], but regulating their levels may also impact disease treatment. For example, knocking out lncRNA H19 can inhibit neuroinflammation after traumatic brain injury and protect neurons [14]. Earlier research has indicated that lncRNA HCP5 is aberrantly expressed in the serum of patients with ICH and may serve as a potential biomarker for predicting the progression of the condition [15]. However, there have been no studies to date on the specific role of HCP5 in ICH-induced brain injury.

Therefore, this study conducted an in-depth investigation into the role and potential mechanisms of HCP5 in ICH through the establishment of ICH model rats and hemoglobin (Hb)-induced BV2 cells.

Methods

Establishment of a rat ICH model

This study was conducted with the approval of the Animal Ethics Committee of Nantong Haimen District People's Hospital. All animal experiments were carried out in strict compliance with the NIH Guide for the Care and Use of Laboratory Animals. Our study adhered to the 3R principles (Replacement, Reduction, and Refinement). We minimized the number of rats used in our experiments to achieve statistically significant results. Additionally, we implemented anesthesia and postoperative care to reduce suffering in the rats during the experiments.

Adult SPF-grade male SD rats were purchased from Shanghai Hengrui Pharmaceutical Co., with a weight of 220–240 g. They were housed in an environment of 22℃ ± 3℃, under a 12-hour light/dark cycle, and were allowed free access to food and water for 1 week to acclimatize before the experiments. To establish the ICH model, the rats were anesthetized with 30 mg/kg of pentobarbital sodium and then fixed onto the stereotaxic apparatus. Next, 0.23U of type VII collagenase was dissolved in 1 μl of saline and injected into the rats' striatum at a rate of 0.5 μl/min (0.2 mm posterior to the bregma, 3.0 mm lateral to the midline, and 6 mm deep to the skull). For the sham group, the procedure was the same as the surgical group, except that the injected solution was saline. The design of the animal experiment was shown in Fig. 1.

Grouping

The rats were divided into two batches. One batch was used to analyze the role of HCP5 in ICH and was divided into 4 groups: sham, ICH, ICH + lentivirus (LV)-shNC, and ICH + LV-shHCP5. Another batch was used to analyze the cooperative regulation of the HCP5/miR-195-5p axis in ICH and was divided into 6 groups: sham, ICH, ICH + LV-shNC, ICH + LV-shHCP5, ICH + LV-shHCP5 + antagomir NC, and ICH + LV-shHCP5 + miR-195-5p antagomir. Each group consisted of 10 rats. The transfection reagents were injected into the rat brain 1 h after ICH induction. After the mNSS assessment, all the rats were euthanized. Based on similar past studies, the rats were given an intraperitoneal injection of 30 mg/kg pentobarbital sodium for anesthesia, and after anesthetization, their heads were severed from their necks using surgical scissors to collect brain tissue [16].

RT-qPCR

Total RNA was first extracted from cells and tissues using a Trizol reagent. HCP5 was reverse-transcribed into cDNA using the PrimpScript RT reagent kit, while miR-195-5p was reverse-transcribed into cDNA using the Mir-X miRNA First-Strand Synthesis Kit. Finally, RT-qPCR was performed using TB Green Premix Ex Taq II. In the experiments, GAPDH was used as the internal control for HCP5, and U6 was used as the internal control for miR-195-5p. HCP5 and miR-195-5p levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

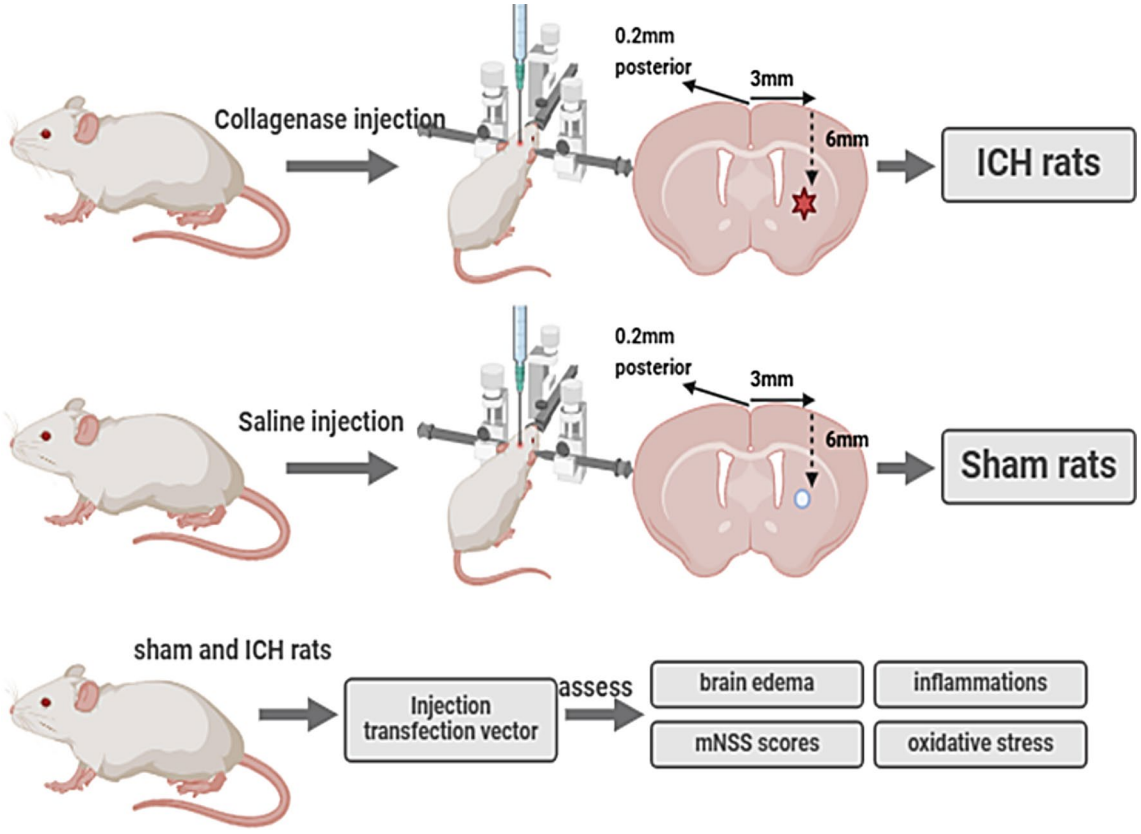

Fig. 1 Schematic diagram of animal experiment design

Evaluation of brain edema

Based on previous experience, the wet-dry weight method was used to assess brain edema. At the end of the experiment, the brains from each group of rats were extracted and immediately weighed (wet weight). The fresh brain tissue was then placed in an oven at 100℃ for 24 h and weighed again (dry weight). The water content of the tissue (BWC) was calculated as follows: BWC = (wet weight - dry weight) / wet weight × 100%.

Modified neurological severity score (mNSS) test

The mNSS was employed to evaluate the neurological function of rats in each group after treatment. The assessments were conducted on days 0, 1, 3, and 7 following the establishment of the ICH model. The test results were quantified based on scores from 4 components: motor function, sensory function, beam balance, reflex disappearance, and abnormal movement. The normal score was 0, with higher scores indicating more severe neurological impairment, and the maximum score was 18.

Cell culture and treatment

Mouse microglial BV2 cells were cultured at 37℃ with 5% CO_2 in a DMEM culture medium containing 10% FBS. To simulate ICH conditions, BV2 cells were treated with 20μM Hbfor 16 h, while the control group was treated with the same volume of PBS. Following the manufacturer's instructions, the transfection reagents LV-shNC, LV-shHCP5, inhibitor NC, and miR-195-5p inhibitor were transfected into BV2 cells using Lipofectamine 3000. After incubating BV2 cells with the transfection reagents at 37℃ for 6 h, they were transferred to a standard culture medium and continued to culture for an additional 48 h.

Cell viability and apoptosis detection

Cell viability was assessed using the CCK-8 assay. Cells in the logarithmic phase were resuspended and seeded at a density of 1.5×10^3 cells in a 96-well plate. After routine culture for 0, 24, 48, and 72 h, CCK-8 reagent was added, followed by an additional 4 h of incubation. The OD value at 450 nm was measured using a microplate reader.

Apoptosis of cells was detected using the Annexin V-FITC/Propidium Iodide (PI) apoptosis detection kit. BV2 cells were cultured in a 6-well plate, collected, washed, and resuspended. A final addition of 5 μl of PI and 5 μl of Annexin V was made, followed by dark incubation for 15 min. Apoptosis rates were evaluated using flow cytometry.

Subcellular localization

To detect the localization of HCP5 within cells, the A SurePrep Nuclear or Cytoplasmic RNA Purification Kit was used to isolate nuclear RNA and cytoplasmic RNA from BV2 cells. After reverse transcription, RT-qPCR was performed to examine the relative expression levels of HCP5 in both the nucleus and cytoplasm, with GAPDH and U6 used as internal controls.

Dual luciferase reporter gene assay

LncRNASNP2 predicted potential binding sites for HCP5 and miR-195-5p, which were validated using a dual-luciferase reporter gene assay. Dual-luciferase reporter gene plasmids were constructed for HCP5, including the wild-type plasmid HCP5-WT and the mutant plasmid HCP5-MUT. After BV2 cells were cultured to the logarithmic phase, they were seeded into a 6-well plate. Using the Lipofectamine 3000 reagent kit, the above plasmids along with miR-195-5p mimic, mimic NC, miR-195-5p inhibitor, and inhibitor NC were transfected into the BV2 cells. After 48 h of culture, luciferase activity was measured using the Dual-Glo luciferase detection system, following the manufacturer's instructions.

RIP analysis

RIP analysis was performed using the Magna Nuclear RIP (Native) Kit. BV2 cells were lysed in a complete RIPA buffer containing a cocktail of protease inhibitors and RNase inhibitors. The lysates were incubated with magnetic beads coated with Anti-Ago2 and Anti-IgG control at 4℃ for 8 h. The complexes were then purified, and the enrichment of HCP5 was detected using RT-qPCR.

Cytokine assay

Inflammatory cytokines in the cells and rat brain tissue were detected using an ELISA method. Rat brain tissues and transfected BV2 cells were collected, washed with cold PBS, and homogenized. The supernatant was collected for analysis of IL-6, IL-1β, and TNF-α using an ELISA kit.

MDA and ROS detection

According to the manufacturer's instructions, tissues and cells were collected, washed, and homogenized. MDA and ROS levels in the samples were measured using MDA and ROS etection kits.

Data analysis

Experimental data were statistically analyzed and graphed using GraphPad Prism 9.0. All tests were repeated at least 5 times. Numerical data were presented as mean ± SD. Comparison between two groups was conducted using the Student's t-test, while comparison among multiple groups was performed using ANOVA, followed by Bonferroni or Tukey post hoc tests. $P < 0.05$ was considered statistically significant.

Results

HCP5 levels significantly increase in the ICH rat model

To validate the abnormal expression of HCP5 in ICH, an ICH model was established by collagenase injection, and HCP5 levels in the brain tissues of ICH model rats and sham rats were analyzed using RT-qPCR. As shown in Fig. 2A, the level of HCP5 in the ICH group was remarkably higher than in the sham group ($P < 0.001$).

Silencing HCP5 alleviates neurological dysfunction and cerebral edema in ICH rats

Following this, we conducted functional experiments to further explore the role of HCP5 in ICH. After transfection with LV-shHCP5, the level of HCP5 was considerably reduced ($P < 0.001$, Fig. 2B). Subsequently, the mNSS method was used to confirm the effect of LV-shHCP5 transfection on rat neurological function. The results, shown in Fig. 2C, indicated that the neurological dysfunction score in the ICH-induced group was significantly higher than that in the sham group, while the mNSS score was significantly reduced after LV-shHCP5 transfection ($P < 0.01$). Furthermore, using the wet-dry weight method to assess cerebral edema, we found that the degree of cerebral edema in the ICH group was significantly greater than that in the sham group. After HCP5 inhibition, the degree of cerebral edema was significantly alleviated ($P < 0.001$, Fig. 2D).

Silencing HCP5 alleviates oxidative stress and neuroinflammation in ICH rats

We also evaluated the effect of HCP5 on oxidative stress and neuroinflammation in ICH rats. The results showed that oxidative stress markers ROS and MDA levels were elevated in ICH rats, while LV-shHCP5 transfection inhibited the increases in MDA and ROS levels ($P < 0.001$, Fig. 3A **and B**). Additionally, inflammatory factors IL-6, IL-1β, and TNF-α were significantly elevated in ICH rats, and after HCP5 inhibition, ICH-induced neuroinflammation was alleviated ($P < 0.001$, Fig. 3C). These results confirmed that ICH induces oxidative stress, neuroinflammation, and neurological dysfunction, and silencing HCP5 alleviates these symptoms.

HCP5 acted as a sponge for miR-195-5p

To explore the specific pathway by which HCP5 participates in ICH, we first conducted subcellular localization detection for HCP5 and found that HCP5 is predominantly enriched in the cytoplasm (Fig. 4A). Next, we used the LncRNASNP2 online database to identify HCP5's target miR-195-5p and the binding sites between them (Fig. 4B). To confirm the targeting relationship between

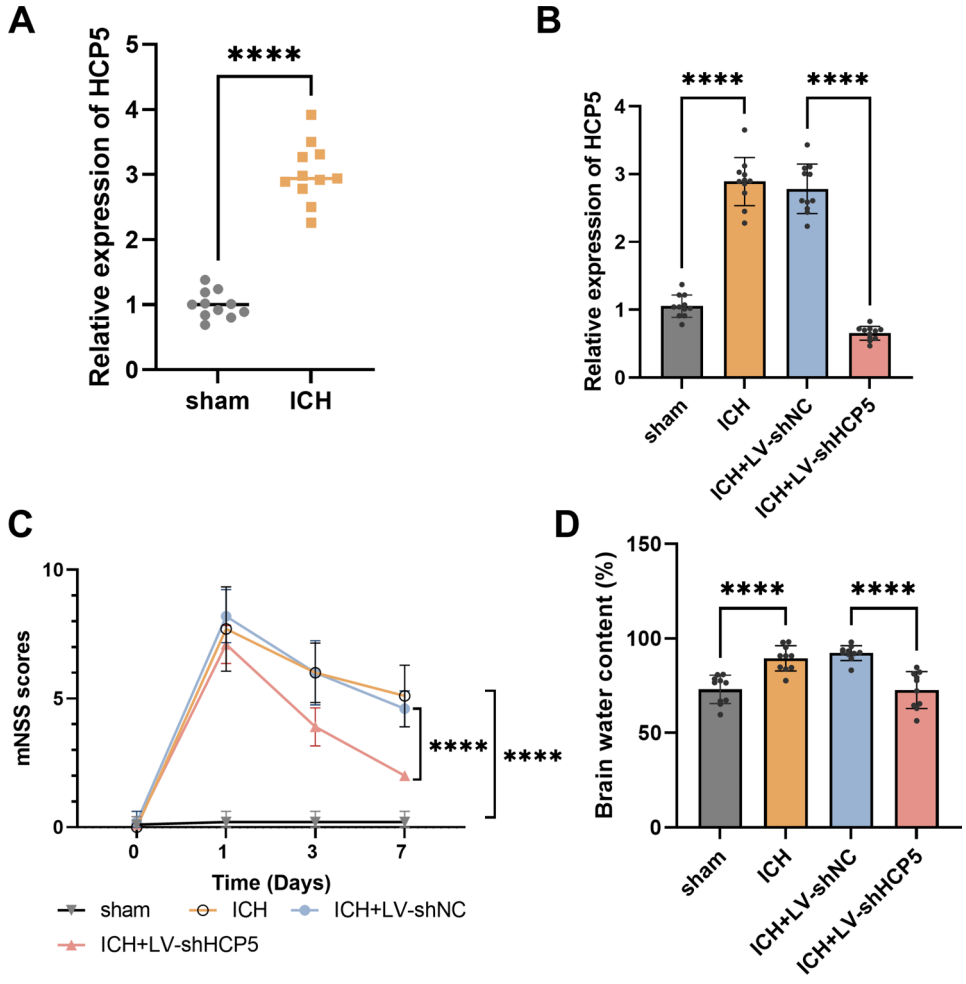

Fig. 2 Silencing HCP5 significantly alleviated neurological dysfunction and brain edema in ICH rats. **A**. RT-qPCR was performed to measure HCP5 levels in ICH rats. **B**. HCP5 levels in ICH rats after injection of LV-shHCP5. **C**. Evaluation of neurological deficits in rats using the mNSS. **D**. Assessment of brain edema in rats

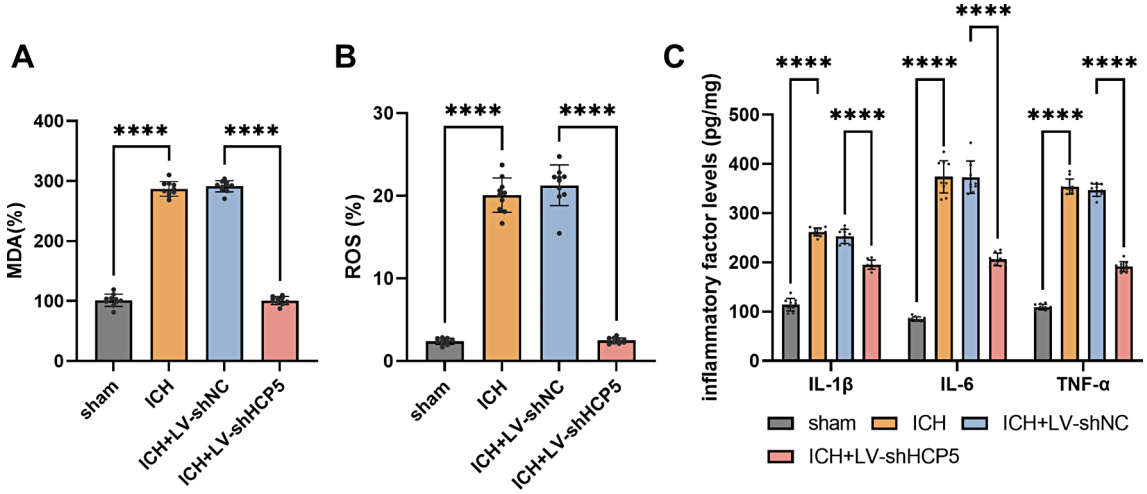

Fig. 3 Silencing HCP5 also significantly reduced neuroinflammation and oxidative stress in ICH rats. After injecting LV-shHCP5 into the rat brains, levels of MDA (**A**) and ROS (**B**) were measured using commercial kits. Inflammatory factors IL-1β, IL-6, and TNF-α were detected using ELISA(**C**)

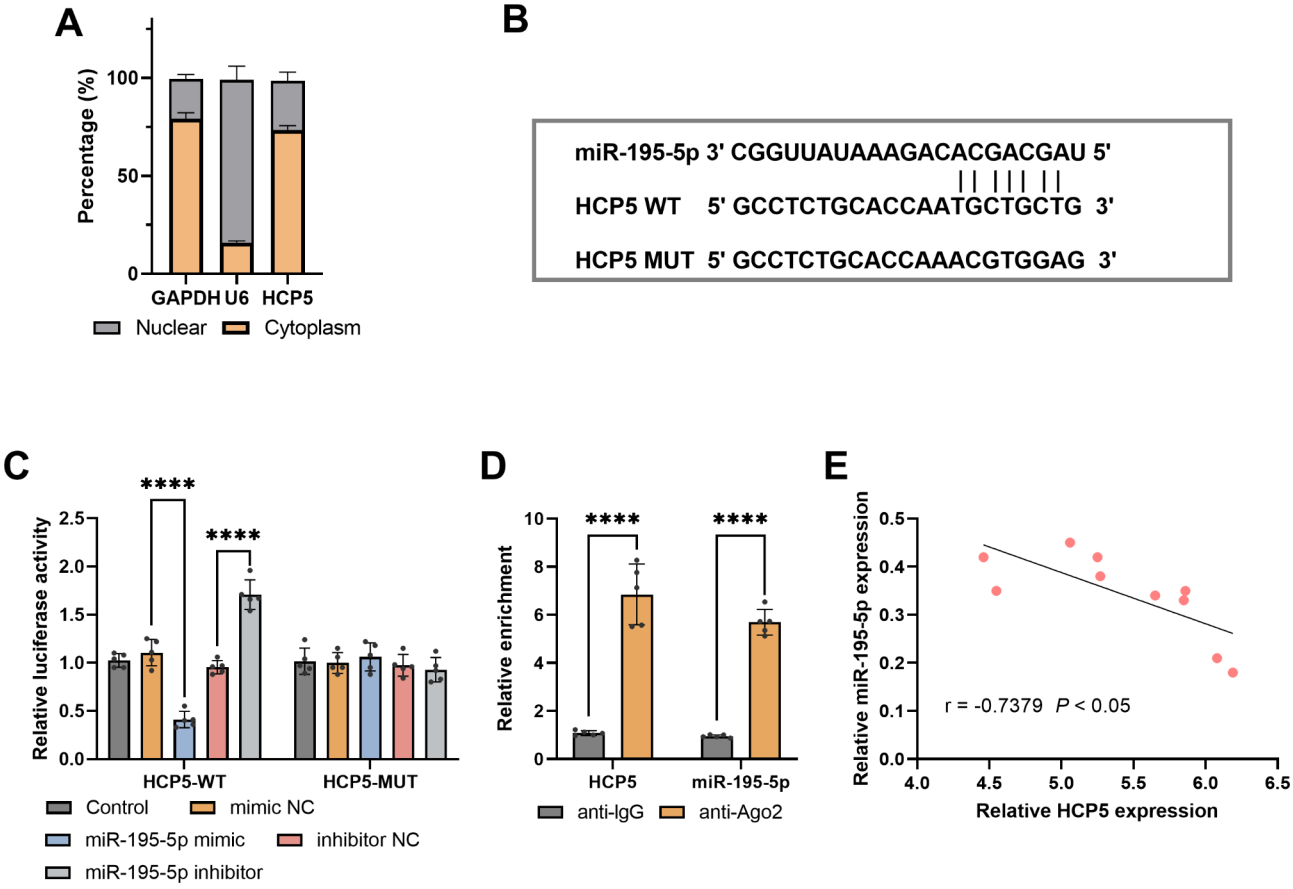

Fig. 4 HCP5 acted as a sponge for miR-195-5p. **A**. Subcellular localization analysis of HCP5 in cells. **B**. Binding sites of HCP5 and miR-195-5p. **C**. Dual-luciferase reporter assay. **D**. RIP experiment. **E**. A negative correlation was observed between HCP5 and miR-195-5p levels in ICH rats

the two, we conducted dual-luciferase reporter assays and RIP experiments. The results showed that miR-195-5p mimic reduced the activity of HCP5-WT luciferase, while miR-195-5p inhibitor increased luciferase activity ($P<0.001$). In contrast, HCP5-MUT was less affected by miR-195-5p mimic and miR-195-5p inhibitor ($P>0.05$, Fig. 4C). The results from the RIP experiment demonstrated that both HCP5 and miR-195-5p were enriched on anti-Ago2 beads ($P<0.001$, Fig. 4D). More importantly, there was a negative correlation between HCP5 and miR-195-5p levels in ICH rats (r = -0.7379, $P<0.05$, Fig. 4E).

Inhibition of miR-195-5p can counteract the protective effect of HCP5 silencing on neurological injury in ICH rats

Based on the targeting relationship between HCP5 and miR-195-5p, we further investigated the potential mechanisms of the HCP5/miR-195-5p axis in ICH. As shown in Fig. 5A, silencing HCP5 led to a significant increase in miR-195-5p levels, and transfection with miR-195-5p antagomir resulted in a decrease in miR-195-5p levels ($P<0.01$). Correspondingly, the administration of miR-195-5p antagomir reversed the reduction in neurological dysfunction scores resulting from HCP5 silencing ($P<0.05$, Fig. 5B). Additionally, the level of cerebral edema in rats simultaneously injected with LV-shHCP5 and miR-195-5p antagomir was significantly higher than that in the rats only subjected to HCP5 silencing ($P<0.05$, Fig. 5C). Meanwhile, the levels of oxidative stress markers and inflammatory factors in the rats also changed accordingly, as shown in Fig. 5D-F. The reduction of miR-195-5p partially countered the inhibitory effects of HCP5 silencing on oxidative stress and neuroinflammation in ICH rats ($P<0.001$).

The effect of the HCP5/miR-195-5p Axis on microglial cell injury

To further confirm the involvement of the HCP5/miR-195-5p axis in ICH, we employed Hb-induced BV2 cells to model ICH in vitro. First, we assessed the impact of transfection reagents on miR-195-5p levels via RT-qPCR. The results showed that miR-195-5p levels remarkably decreased after Hb induction, while transfection with LV-shHCP5 increased miR-195-5p levels. However, simultaneous transfection with miR-195-5p inhibitor caused a partial decrease in miR-195-5p levels ($P<0.01$,

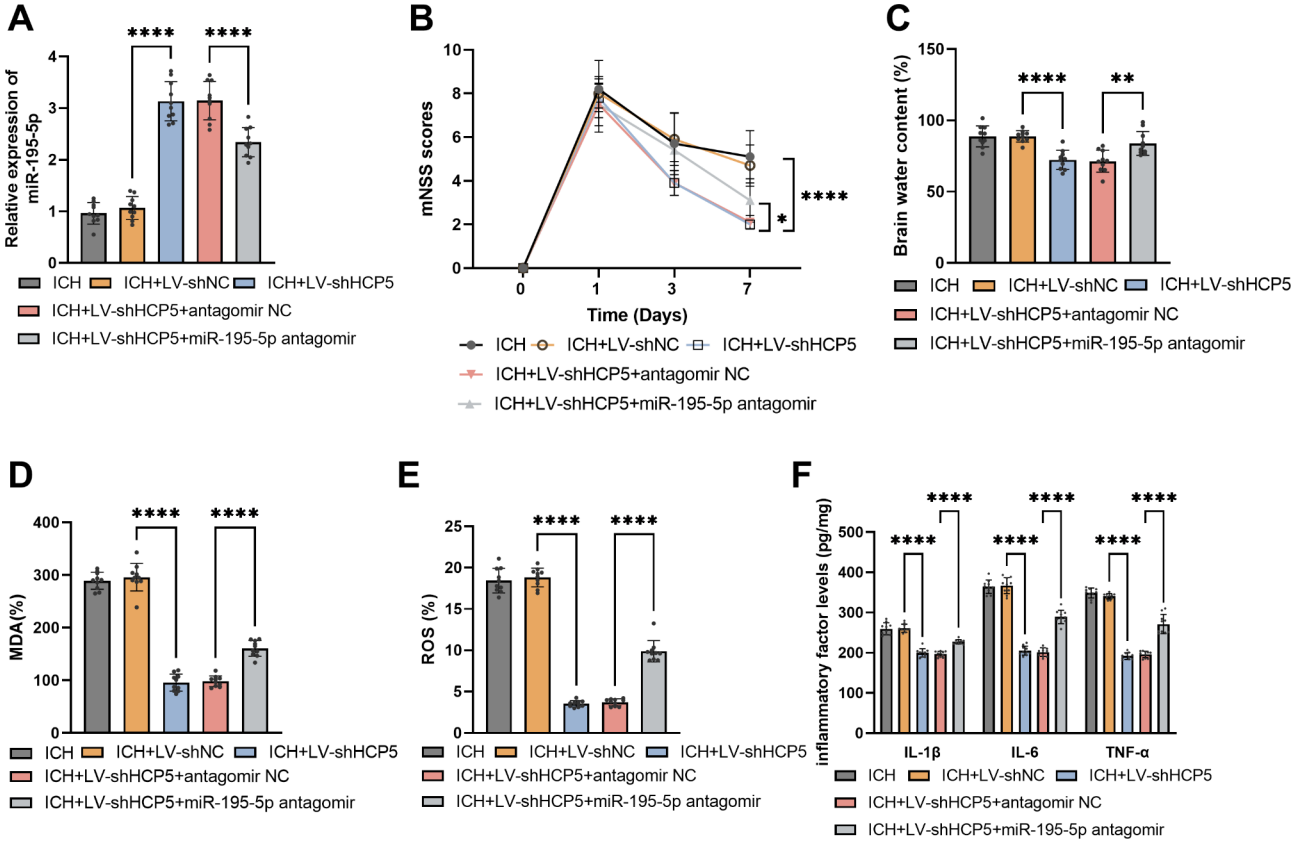

Fig. 5 The effects of regulating HCP5 and miR-195-5p on miR-195-5p levels (**A**), brain edema (**B**), degree of neurological deficits (**C**), MDA (**D**), ROS (**E**), and inflammatory factors (**F**) in ICH rats

Fig. 6A). Additionally, compared to the control group, the BV2 cells in the Hb treatment group exhibited reduced cell viability and increased apoptosis, along with significantly elevated oxidative stress markers ROS and MDA, and inflammatory factors ($P < 0.001$, Fig. 6B-F). More importantly, after transfection with LV-shHCP5, the decline in cell viability, oxidative stress, and inflammation induced by Hb was significantly alleviated, whereas co-transfection with miR-195-5p inhibitor counteracted the protective effects of LV-shHCP5 on BV2 cell neuronal injury ($P < 0.001$, Fig. 6B-F).

Discussion

ICH represents a significant public health issue [17]. With advancements in technology, we have gained deeper insights into ICH, whose severity is associated with factors such as hemorrhagic brain injury and edema around the hematoma [18]. Moreover, treatment methods for ICH are also progressing, including blood pressure control and minimally invasive surgery to remove blood clots [19], which can improve clinical outcomes and reduce mortality rates. However, the occurrence of hematoma causes further nerve damage [20], often leading to long-term neurological dysfunction in patients [21]. Currently, there are no effective therapeutic approaches to improve neurological impairments caused by ICH [22]; hence, further research into the processes and mechanisms of ICH will pave the way for developing new diagnostic and therapeutic measures.

In recent years, non-coding RNAs have garnered extensive attention in various diseases [23], and numerous ncRNAs with diagnostic and prognostic functions have been discovered. Long non-coding RNA (lncRNA) is a type of ncRNAs that plays a vital role in bodily functions [24] and is abundantly present in the central nervous system of mammals [25]. Studies have shown that lncRNA exhibits abnormal expression in neurodegenerative diseases [26] and are closely related to traumatic brain injury [27]. LncRNA HCP5 is involved in the development of various cancers [28] and, importantly, has been found to be upregulated in ICH patients, participating in the pathological process of glioma [29]. Our research found that HCP5 expression significantly increased in ICH model rats. To further validate the mechanism of HCP5 in ICH, we constructed ICH model rats using collagenase and conducted functional deficiency experiments. The results showed that silencing HCP5 alleviated brain edema, neuroinflammation, oxidative stress, and neurological dysfunction induced by ICH, indicating that silencing HCP5 can reduce nerve damage caused by ICH

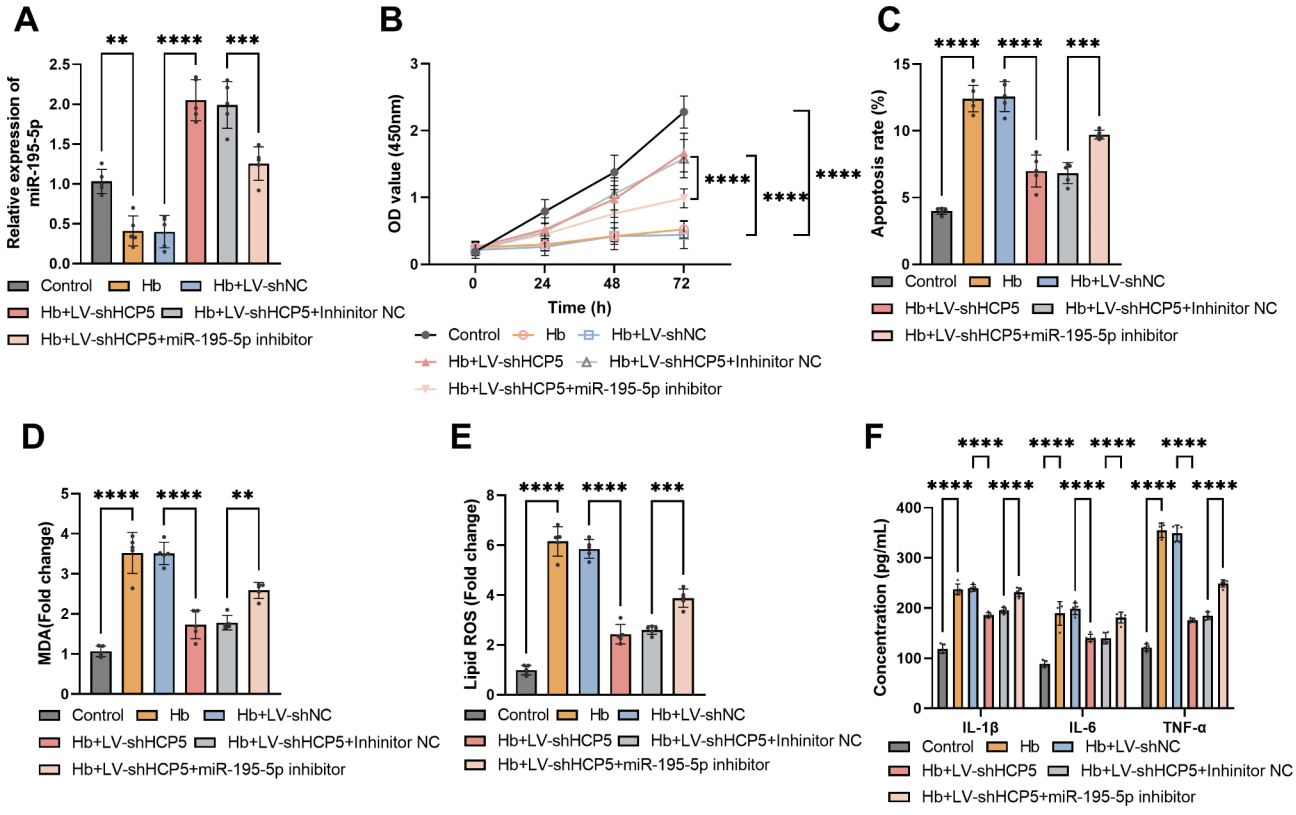

Fig. 6 The effects of regulating HCP5 and miR-195-5p on miR-195-5p levels (**A**), cell viability (**B**), apoptosis (**C**), MDA (**D**), ROS (**E**), and inflammatory factors (**F**) in Hb-treated BV2 cells

stimulation. Gao et al. also found that HCP5 is involved in cerebral ischemia-reperfusion injury, with silencing HCP5 capable of restoring neuronal viability [30], which aligns with our findings.

Based on the primary regulatory mechanism of lncRNAs acting as "sponges" for miRNAs, we identified the target miRNA of HCP5, miR-195-5p, using online database. Reports indicated that overexpression of miR-195-5p can mitigate hypoxia-reperfusion-induced brain damage by modulating the PTEN-AKT signaling pathway [31]. Notably, miR-195-5p can also alleviate brain edema and motor dysfunction caused by ICH, affecting enzymes related to secondary brain damage [32]. Our study also confirmed that HCP5 can bind to miR-195-5p, and the levels of both showed a negative correlation in ICH rats. To further validate the role of the HCP5/miR-195-5p axis in ICH, we injected ICH rats with LV-shHCP5 and miR-195-5p antagomir together. Our results revealed that decreased levels of miR-195-5p diminished the neuroprotective effects associated with HCP5 silencing. Specifically, the inhibition of miR-195-5p expression partially counteracted the effects of HCP5 silencing on the alleviation of brain edema and neurological deficits in ICH rats.

Given that microglia are among the first cells to respond to brain injuries such as ICH, this study simulates ICH in vitro by treating BV2 cells with Hb. Previous research has shown that ICH affects microglia, triggering cascades of inflammation, oxidative stress, and cytotoxicity, which result in secondary brain damage [33]. After Hb treatment, BV2 cells exhibited decreased cell viability, increased levels of oxidative stress markers and inflammatory factors, along with elevated expression of HCP5 and decreased levels of miR-195-5p. Furthermore, silencing HCP5 inhibited the adverse inflammatory responses induced by Hb, while lowering miR-195-5p levels countered the effects of HCP5 silencing on BV2 cells. In summary, both in vivo and in vitro experiments indicate that the HCP5/miR-195-5p axis is involved in the progression of ICH. However, our study has some limitations. First, the collagenase-induced ICH rats model we employed is a relatively reliable animal model for studying the effects of hematoma and cerebral edema on the brain [34]; however, this model is quite simplistic and cannot fully replicate the pathological features of ICH, and its clinical translational potential is limited. Second, our research was currently limited to the role of the HCP5/miR-195-5p axis in ICH, lacking exploration of the downstream targets and signaling pathways of the HCP5/miR-195-5p axis. This is also a focus of our future research, aiming to reveal the broader role of the

HCP5/miR-195-5p axis in cellular responses and disease progression in ICH.

Conclusions

In conclusion, HCP5 was significantly elevated in ICH, and mechanistically, silencing HCP5 can alleviate brain damage caused by ICH, with its effects mediated through the sponge activity of miR-195-5p. Our findings provided deeper insights into ICH and suggested that the HCP5/miR-195-5p axis may serve as a potential therapeutic target for ICH and related brain injuries.

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Author contributions
Zhanhua Lu: Conceptualization, Data curation, Resources, Software, Writing – original draft; Kun Huang: Data curation, Investigation, Methodology, Project administration, Writing – review and editing.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study was conducted with the approval of the Animal Ethics Committee of Nantong Haimen District People's Hospital. All animal experiments were carried out in strict compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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