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MicroRNA-128 exerts protective effects on intracerebral hemorrhage and blood-brain barrier by upregulation of Connective tissue growth factor (CTGF)

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Abstract

Connective tissue growth factor (CTGF) is a cellular protein essential for tissue repair. MicroRNA (miR)-128 can alleviate myocardial ischemia/reperfusion injury by promoting CTGF expression, but its effect on cerebral hemorrhage injury is not yet known. Herein, we intend to identify the interaction between miR-128 and CTGF in intracerebral hemorrhage (ICM). A model of ICM was established through injection of autologous blood. siRNA and miR-128 mimic were injected into the rats to assess the effect of miR-128 and CTGF on ICM by measuring neurological severity score. ELISA was carried to detect the release of inflammatory factors and Evans Blue staining assessed the permeability of the blood-brain barrier (BBB) of brain tissue. CTGF level was decreased in ICM tissues of human and rats and reached a minimum value at 24h after establishment of ICM model. Silencing of CTGF aggravated cerebral injury, which was manifested by elevated neurological severity score, increased brain water content, Evans blue extravasation, and elevated MPO cells. miR-128 knockdown inhibited CTGF expression and aggravated inflammatory response, while miR-128 overexpression alleviated the damage induced by ICM and improved BBB, and increased CTFG expression. In conclusion, miR-128 enhances damaged wound healing and protects BBB integrity in ICH by promoting CTGF expression, which provides a novel insight into the development of targeted therapy for this disorder.

Keywords: MiR-128; Intracerebral hemorrhage; Connective tissue growth factor; Blood-brain barrier; fibroblast

Introduction

Intracerebral hemorrhage (ICH) causes 10-30% of all strokes with a high morbidity and mortality rate and it affects more than 2 million people worldwide every year. Stroke survivors often suffer from chronic disabilities [1, 2]. However, without a clear understanding of the etiology of brain injury, there is no effective clinical treatment approaches at present. Connective tissue growth factor (CTGF) is a candidate extracellular matrix (ECM) protein and its specific function in vascular matrix has not been elucidated [3].

CTGF was first cloned from cDNA libraries[4] and has mitogenic activity on fibroblasts and cultured endothelial cells [5]. It is also called Cell Communication Network 2 (CCN2) for its structure similar to the CCN protein family [6]. CTGF mainly produces a 38 kDa protein that consists of four different structural modules. CTGF binds to the surface receptors of cells, such as HSPGs and LRP[7, 8]. It is believed that this interaction enables CTGF to affect cellular processes such as cell invasion, apoptosis and growth[9]. The N-terminal and C-terminal parts of CTGF are linked with various molecules including growth factors, however this interaction in vivo is not clear yet [10]. CTGF is mainly originated from endothelial cells (ECs) in central nervous system [11]. Mice with decreased expression of CTGF in vascular endothelial cells showed delayed growth and morphogenesis, as well as damaged blood barrier, and even severe cerebral hemorrhage [7]. CTGF is a cytoskeleton and extracellular matrix protein and transcription co-regulatory gene. For instance, CTGF is a YAP target gene mediating angiogenesis and the transcription of the blood-brain barrier (BBB) factors. YAP overexpression attenuates angiogenesis and improves blood-brain barrier formation in the brain tissue of CTGF mutant mice [12]. Therefore, CTGF-YAP axis is essential is for brain blood vessel development and barrier function. CTGF is involved

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in wound healing and its protein levels are associated with the progression of vascular diseases [13]. In glomerulonephritis, extracapillary hyperplasia, peri-glomerular fibrosis, and interstitial lesions are accompanied with the elevated CTGF levels [14]. Although these studies have shown that CTGF physiologically and pathologically affects angiogenesis, its role in cerebral hemorrhage has not been investigated yet.

MicroRNAs (miRNAs) (19-25 nucleotides long) regulate gene expression by binding to the mRNA 3'-UTR. The gene encoding the primary transcript of miR-128 (pro-miR-128) is located in the intron of TMEM49 [15]. pro-miR-128 transcription is initiated by the pro-miR-128 promoter and terminated by the poly-A and matured into miR-128 [16]. miR-128 is mainly secreted from cells (osteocyte, neuron, MSCs), and regulates intercellular communication through exosomal complexes. miR-128 is also present in the central nervous system and vascular system, and contributes to remodeling of the BBB by regulating the differentiation of astrocytes and microglia [17]. A recent study has pointed out [18] that miR-128 protects against ICH by inhibiting the release of inflammatory factors and has a certain up-regulation effect on CTGF. This study aims to explore whether CTGF functions in cerebral hemorrhage in rat models.

Materials and methods

Tissue collection

Human brain tissues were collected from ICH patients (12 males, average age: 52.6±4.9 years old, and 17 females, average age: 56.3±5.2 years old) by hematoma removal after obtaining approval from the Review Committee of Children's Hospital of Anhui Medical University.

Animals

A total of 36 SD adult rats (male, 250~300g weight) were raised in the Experimental Animal Center of Anhui Medical University under a 12h dark/light cycle, with free access to food and water. The animals were randomly divided into 6 groups: ICH group (n=6), sham-operated group (n=6), ICH + normal control group (n=6), ICH + miR-128 mimics group (n=6), ICH + si-CTGF group (n=6), cerebral hemorrhage + miR-128 inhibitor group (n=6).

Establishment of ICH model [19]

Rats were anesthetized and fixed. The autologous blood was collected from the right femoral artery, and kept in the micro-injection pump, and then injected into the right striatum (dorso-abdominal 5.8 mm, 0.2 mm posterior and anterior, 3.0 mm lateral to bregma). 10 minutes after administration, the needle was removed. Rats in the sham-operated group were injected with normal saline.

Injection of si-CTGA and miR-128 mimics

CTGF siRNA (Reverse: 5′-UAGCAGAGUCGAAUCCAGCTT-3′; Forward: 5′-GCUGGAUCGACUCUGCUATT-3′) or miR-128 mimics (Reverse: 5′-UUUCGAACUCCAUACAUGCTT-3′; Forward: 5′-GCAUGUGAGUCGAAATT -3′) was dissolved in 10 μl DEPC water, then shaken and centrifuged 3 times. Subsequently, the solution was injected into rats (dorso-abdominal 3.5 mm, 1 mm posterior and anterior, 2.0 mm lateral to bregma). 24 hours after injection of siRNAs, the efficiency was detected.

Modified neurological severity score (MNSS)[20]

The neurological function was evaluated using MNSS in terms of exercise, sense, balance beam and reflex. The scores ranged from 0 to 18. The higher the score, the more severe the neurological damage.

Brain water content

24 hours after induction of ICH, rats were euthanized and brain tissue was excised and the wet weight was measured. The tissue then was dried at 120°C until weight stopped declining, and then the dry weight was recorded. Water content was calculated as following equation: brain water content = (wet weight-dry weight)/wet weight x 100%.

Evans Blue staining

Evans Blue (EB) dye (Sigma, USA) was diluted with normal saline and administered via femoral vein (5ml/kg). After anesthesia, rat heart was perfused with normal saline and brains were sliced into coronal sections (40 mm thick) using cryostats (Leica) and observed. The bilateral cerebral hemispheres were weighed, cut, and immersed in formamide to extract EB. The solution was centrifuged at 1000 rpm. With supernatant collected, the content of EB in the supernatant was detected by a spectrophotometer at a wavelength of 620 nm.

Western blot

The tissue was lysed in ice-cold IP lysis buffer. The lysate was centrifuged at 4°C at 12,000g for 15 minutes to isolate proteins, whose concentration was determined with a BCA kit (Beyotime, Jiangsu, China). Proteins were loaded onto SDS-PAGE gel, separated by electrophoresis, and then transferred to a PVDF membrane (Millipore, Massachusetts,

USA). After blocked with 5% skim milk, the membrane was incubated with anti-CTGF antibody (H3413, 1:500, Sigma, USA) at 4°C for 2 hours, and then washed with TBST. The membrane was developed with enhanced chemiluminescence and band density was normalized to bactin and analyzed by ImageJ.

Statistical analysis

Data were analyzed by GraphPad Prism 7.0 software and presented as Mean ± SD. Differences between two groups were analyzed by t test and differences among multiple groups were compared by two-way ANOVA. $p<0.05$ indicates a statistical significance.

Results

The expression of CTGF is down-regulated following ICH

The CTGF expression in the brain tissue was detected. Compared with normal group, the expression of CTGF in ICH patients was significantly reduced (Figure 1A, B). We further determined the CTGF level in rat brain tissues and found that its level was dramatically decreased after ICH, and reached a lowest value 24 hours after induction (Figure 1C, D).

Silencing CTGF aggravates brain injury

To elucidate the role of CTGF in ICH, we injected CTGF siRNA to ICH rats, and the transfection efficiency was verified by Western blot with siRNA-2 being more effective (Figure 2A-B). After treatment with CTGF siRNA-2, the mNSS score increased and higher than those treated with NC or that of sham-operated rats (Figure 2C and 2D). Additionally, 24 hours after establishment of animal model, ICH rats had more EB exudation and cerebral water content than sham-operated rats. Silencing of CTGF resulted in the increased EB exudation and cerebral water content (Figure 2E).

Overexpression of miR-128 relieves cerebral hemorrhage injury

The role of miR-128 in cerebral hemorrhage was studied with miR-128 mimics, and the transfection efficiency was verified by RT-qPCR as shown by the increased level of miR-128 after transfection (Figure 3A). Treatment with miR-128 mimics alleviated brain injury and decreased mNSS score (Figure 3B and 3C). Besides, as EB exudation and brain water content of rats in the intracerebral hemorrhage group and intracerebral hemorrhage + normal control group was higher than those in sham operation group, the presence of miR-128 mimics significantly reduced EB exudation and brain water content (Figure 3D).

Silencing miR-128 stimulates inflammatory response in ICH

As revealed by Western blot, cleaved caspase-1, IL-18, and IL-1b level in the ICH group and the ICH+NC group were higher than those in sham-operated group (Figure 4A-B). Treatment with miR-128 inhibitor decreased the expression of CTGF in ICH tissues and importantly, it upregulated the expression of inflammatory factors (Figure 4C-G). This evidence suggests that downregulation of miR-128 may aggravate the inflammatory response after ICH.

miR-128 elevates the expression of CTGF

Western blot analysis showed that treatment with miR-128 mimics could reverse the inhibition of CTGF expression induced by ICH in rats, and after treatment with miR-128 mimics, CTGF protein level increased (Figure 5).

Discussion

Stromal cell protein CTGF is known to function in diverse biological processes and directly regulates cell microenvironment [21]. CTGF regulates biological processes such as cell proliferation, wound healing, and angiogenesis [19]. These effects are mediated through interaction of CTGF with growth factors or ECM. CTGF-integrin interaction induces functional angiogenesis in rat brain tissue. However, although CTGF has been frequently studied for many years, its role in ICH is unclear. In the present study, we found that the expression of CTGF was down-regulated following ICH, and reached a minimum level 24 hours later. Importantly, miR-128 is indicated to enhance the expression of CTGF in a time-dependent manner. CTGF siRNA impairs neurological function, increases brain water content and EB exudation. These results support that miR-128 reduces inflammation and alleviates brain damage after ICH possibly through regulating CTGF expression.

The innate immune system and the BBB system determines the response of the central nervous system to tissue damage or pathogen invasion. CTGF is located in the cytoplasm and responds to damage-related patterns during injury and molecules binding to bacteria to prevent harmful substances from invading brain tissues [20]. In this study, Western-blot indicated that the expression of CTGF was decreased in rat and human cerebral hematoma tissues, suggesting that the low expression of CTGF may be involved in the pathogenesis and development of ICH. Previous evidence has unveiled that CTGF is crucial to

neuroinflammation and brain damage [22], and upregulation of CTGF reduces inflammation and brain damage. Silymarin and cordycepin promote the expression of CTGF and have a protective effect on cerebral hemorrhage [23]. CTGF has a leucine-rich domain related to its biological effect. Mechanism analysis shows that CTGF participates in the inflammatory stimulation of temporomandibular joint synovial MSCs. However, few studies have reported its function in ICH. Our work demonstrated that CTGF levels decreased in ICH, and its silencing aggravated cerebral hemorrhage injury. miR-128 has been reported to negatively regulate the expression of CTGF [24] through binding the 3'UTR of CTGF. Consistently, in this study, our results also indicated that miR-128 promoted the expression of CTGF. The low expression of CTGF causes the infiltration of caspase-1 and IL-1b and IL-18 by damaging the integrity of BBB, which causes inflammation and promotes the expansion of the edema area around the hematoma, thereby exacerbating hemorrhagic brain injury [25]. After establishment of ICH animal model, the integrity of BBB was assessed by EB staining. After cerebral hemorrhage injury, the brain water content increased significantly and the permeability EB increased, suggesting the destruction of BBB. After treatment with CTGF siRNA, the water content of brain tissue and the level of EB penetration further increased, suggesting that CTGF may have protective effect on ICH. Further analysis of inflammatory factors showed that with the destruction of BBB induced by brain tissue injury, the release of inflammatory factors was significantly increased. After miR-128 inhibitor treatment, the level of inflammatory factors continued to increase. However, administration of miR-128mimic finally decreased the water content and EB exudation.

In conclusion, this study demonstrates that CTGF and miR-128 have a protective effect on ICH. miR-128 alleviates the destruction of BBB by upregulating the expression of CTGF, thereby alleviating the release of inflammatory factors and brain damage. Our study indicates that miR-128 might be a novel target for the treatment of ICH. However, this study also has certain limitations. The exact mechanism by how miR-128 specifically regulates CTGF expression is not yet known and requires further investigations in the future.

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Figure legends

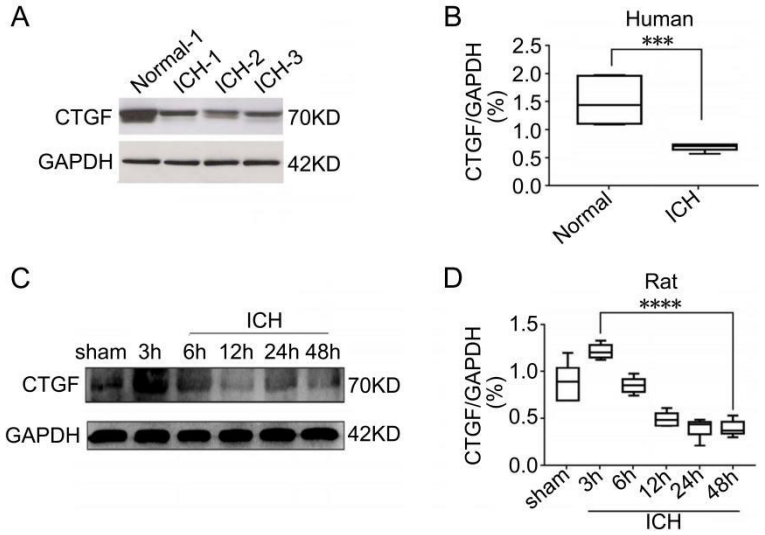

Figure 1. **The expression of CTGF was down-regulated after intracerebral hemorrhage (ICH).**

(A) Western blot of CTGF expression in brain tissues of healthy people and patients with ICH. (B) Quantification of the relative expression ratio of CTGF. (C) Western blot of CTGF expression in brain tissues of sham-operated rats and ICH model rats at 3, 6, 12, 24, 48 hours. (D) Quantification of the relative expression ratio of CTGF in the sham-operated group and ICH group. *p <0.001 and *p <0.0001

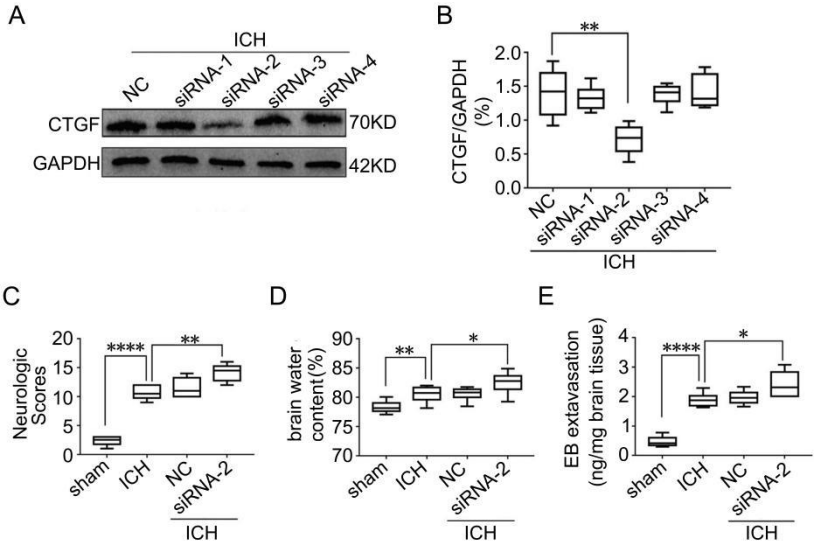

Figure 2. **Silencing CTGF aggravates ICH.**

(A, B) RT-qPCR of CTGF expression following treatment with siRNAs. (C) Quantification of MNSS of neurological function upon treatments. (D) Quantification of brain water content of sham operation group, ICH + NC group, ICH group and ICH + CTGFsiRNA group. (E) Quantification of EB exudation content of each group, *p <0.05, **p <0.01 and *p <0.0001.

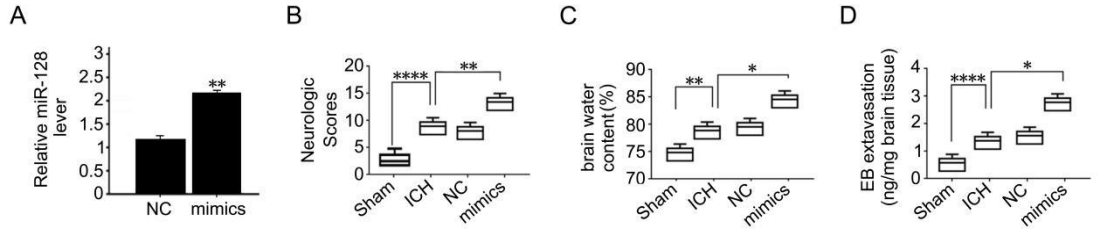

Figure 3. **Overexpression of miR-128 relieves ICH damage.**

(A) RT-qPCR analysis of transfection efficiency of miR-128 mimics. (B) Quantification of MNSS upon treatments. (C) Quantification of brain water content of each group (D) Quantification of EB exudation content of each group *p<0.05, * *p <0.01 and *p <0.0001.

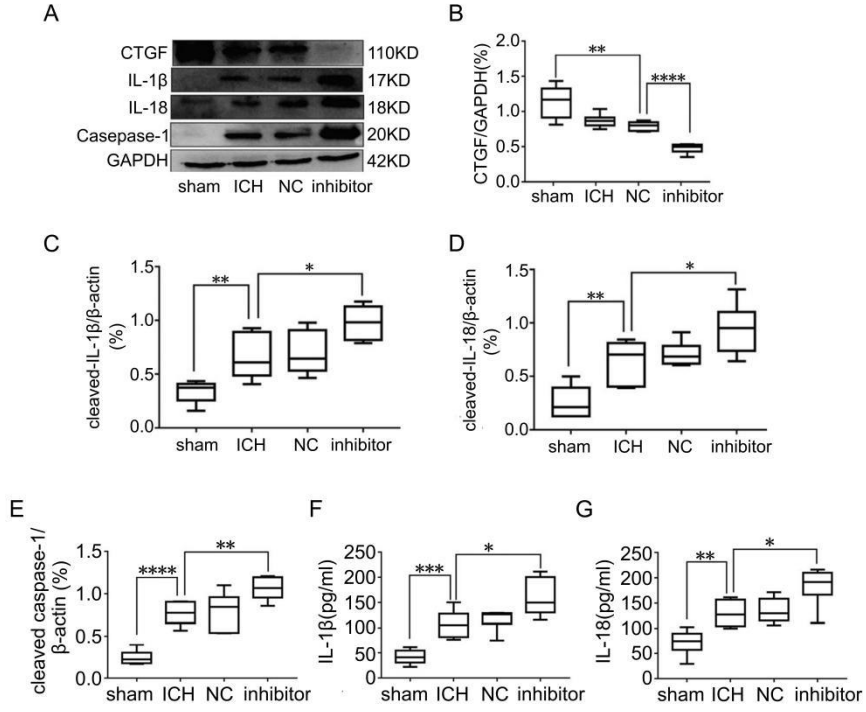

Figure 4. **miR-128 downregulation enhances the inflammatory response after ICH.**

(A-B) Western blot analysis of CTGF, cleaved caspase-1, IL-18, and IL-1b protein levels in sham group, ICH group, ICH + NC group and ICH + inhibitor group. (C-G) ELISA of CTGF (B), cleaved IL-1β (C), cleaved IL-18 (D), and cleaved caspase-1 (E), serum IL-1, β(F) and IL-18(G) levels. *p <0.05, **p <0.01, *p <0.001 and *p <0.0001.

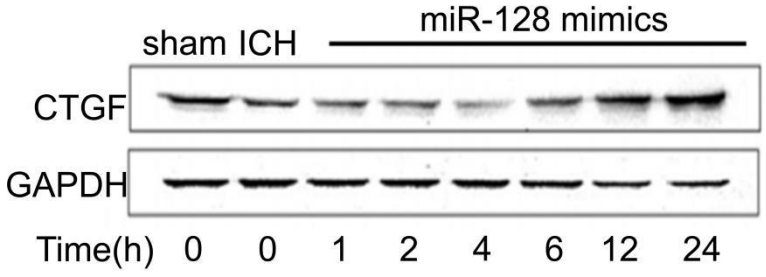

Figure 5. **miR-128 increases the expression of CTGF.**