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Original Article

The effects of let7i on cerebral edema and hemorrhagic transformation after thrombolysis by rt-PA

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Abstract: Thrombolysis by recombinant tissue plasminogen activator (rt-PA) damages the blood-brain barrier (BBB), causing cerebral edema and hemorrhagic transformation (HT). However, the mechanism behind this process is mostly unknown. Sprague-Dawley (SD) rats were randomly divided into six groups: sham (saline treatment), model (thrombus treatment), thrombolysis (thrombolysis by rt-PA), delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis. Evans blue (EB) staining and water measurement were used to assess BBB permeability. Infarct volume, hemorrhage levels, and neurological scores were evaluated using 2,3,5-triphenyltetrazolium chloride (TTC) staining, García-Yébenes, and the revised five-point scale method, respectively. We found that let7i was downregulated in the thrombolysis group compared with the model group. Although the BBB permeability, infarct volume, hematoma volume, brain water content, and neurological scores were all elevated in the model group, the overexpressing let7i group exhibited reductions in hematoma volume brain water content, neurological scores, and improvement in BBB integrity compared with the corresponding thrombolysis and delayed thrombolysis groups, respectively. Moreover, overexpression of let7i reduced occludin expression at the protein level after thrombolysis by rt-PA, as demonstrated by qRT-PCR and western blot. These results demonstrated that let7i participated in cerebral edema and HT associated with BBB dysfunction after thrombolysis by rt-PA via the occludin signaling pathway.

Keywords: Cerebral edema, hemorrhagic transformation, thrombolysis, blood-brain barrier, let7i

Introduction

Ischemic stroke (IS) is the most common type of stroke and is associated with a high rate of disability and mortality worldwide [1]. IS is mainly caused by the occlusion of the arterial blood supply followed by cerebral thrombosis or embolism. Currently, early intravenous thrombolysis and intra-arterial thrombectomy are the two most effective strategies to treat IS. However, few patients benefit from intra-arterial thrombectomy because of stringent eligibility requirements [2]. Therefore, intravenous thrombolysis stands as the most feasible strategy to treat IS. Recombinant tissue plasminogen activator (rt-PA) is the only drug approved by the FDA to treat IS. rt-PA therapy has been shown to reduce disability and mortality by improving the prognosis of IS patients in the early stages of IS. However, rt-PA therapy should be initiated within 3-4.5 h after IS, an approach that may result in intracerebral hemorrhage, cerebral edema, and ischemia-reperfusion injury [3]. Therefore, reducing complications caused by the rt-PA therapy and prolonging the window period of thrombolysis has become a major challenge in the clinical utility of rt-PA therapy. The mechanism of the cerebral edema and hemorrhagic transformation (HT) after thrombolysis by rt-PA therapy is also an area that needs elucidation.

A growing number of studies have demonstrated that cerebral edema and HT after thrombolysis are mainly caused by a breakdown of the blood-brain barrier (BBB) and the destruction of neurovascular homeostasis [4]. The integrity of the BBB is maintained by three main elements: brain microvascular endothelial cells (BMECs), astrocytic endfeet, and pericytes [5]. Therefore, a better understanding of the regulatory mechanisms that protect the BBB may lead to a

reduced risk of HT after rt-PA. There is evidence that rt-PA may worsen BBB disruption leading to HT and is mainly caused by the destruction of the connection between BMECs and the basement membrane involved in several signaling pathways, such as Wnt/β-catenin, VEGF-MMP, and CDC42/PAK-1 [6, 7]. However, the precise molecular mechanisms underlying BBB breakdown have not been elucidated at the gene level.

MicroRNAs (miRNAs) are small noncoding RNAs with approximately 22 nucleotides that play important roles in the regulation of various pathophysiological processes by inhibiting the translation of target mRNAs [8]. There is increasing confirmatory evidence of a large number of miRNAs in the central nervous system that are involved in nerve development, axonal growth, and synaptic plasticity, and the role of these miRNAs in the progression of neuropathy and dysfunction after IS [9]. Of these, the let-7 family members are highly conserved across species and their mRNA level is most abundant in the brain and lungs [10]. The let-7 family plays important roles in the regulation of different processes, such as cell growth and differentiation, tumorigenesis, and mitochondrial apoptosis, especially for the regulation of endothelial cell functions. For instance, FGF regulates TGF-β signaling and the endothelial-to-mesenchymal transition through let-7 miRNA expression [11]. An antagomir to miRNA, let7f promotes neuroprotection in an IS model [12] and is considered a circulating potential biomarker of acute-stage IS [13]. The published data thus far suggest that let-7 plays important roles in post-IS pathological processes. Although the function of let-7 has been well described in the pathology of cancer, its role in endothelial cells is still understudied. The effect of rt-PA treatment on miRNA expression has also been reported. For example, a study found that miR-146a was upregulated in rat cerebral endothelial cells after rt-PA treatment and was capable of inactivating TLR signaling [14]. Zhao et al. also found that 31 and 11 miRNAs were upregulated and downregulated in the brain tissue after IS, respectively [15]. Unfortunately, there has been no research focusing on the effect of rt-PA on the let7 in the context of IS.

In our earlier study, we detected the downregulation of miRNA let7i expression in IS patients through miRNA microarray and real-time PCR [16]. However, the function and mechanism of let7i in cerebral edema and HT are largely unknown, especially in the process of thrombolysis through rt-PA. Therefore, the current study established a rat model of thromboembolism and thrombolysis to investigate the therapeutic potential of let7i for IS. First, the expression of let7i was determined after rt-PA thrombolysis. To explore the effects and mechanism of brain edema and HT after thrombolysis during different window periods, infarct size and BBB permeability of the rat brain were observed. Lastly, the molecular regulatory mechanism of let7i was studied. These results provide a solid foundation for supporting the clinical utility of let7i in reducing complications caused by the rt-PA therapy and prolonging the window period of thrombolysis in IS.

Materials and methods

An experimental model of middle cerebral artery occlusion (MCAO) and thrombolysis by rt-PA

Male Sprague-Dawley (SD) rats were obtained from the Guangdong Medical Experimental Animal Center. A total of 36 rats (weight range: 250-300 g) was allowed free food and water in standard cages under environmentally controlled conditions with a 12 h light-dark cycle. The SD rats were subjected to the MCAO model as described earlier [17]. First, blood was drawn from the femoral arteries of the rats and collected in PE50 tubes to form a thrombus following storage at room temperature for 2 h and at 4°C thereafter. Then, a 5-cm thrombus was cut into a PE10 tube and washed for 5 min using a saline-containing syringe, thereby forming a fibrin-rich thrombus. Finally, the thrombus was injected into the internal carotid artery with the distal end reaching the middle cerebral artery opening by a 0.3 mm PE50 tube and removed after 5 min. Before injection, each rat was anesthetized with an intraperitoneal injection of 5% chloral hydrate and subjected to a midline neck incision, where arteries were carefully separated from the adjacent tissues. We defined a successful MCAO as an approximately 70%-80% decrease in cerebral blood flow confirmed by laser Doppler flowmetry (Moor Instruments, Devon, UK).

Table 1. Primers for quantitative real-time PCR (qRT-PCR)

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	CCTCGTCTCATAGACAAGATGGT	GGGTAGAGTCATACTGGAACATG
Occludin	CCCAGACCACTATGAAACCG	CGCTCTCCTCTCTGTAGTCA
Claudin-5	GCAGCGTTGGAAATTCTGGG	AACAAAGAGTGCCACAAGCG
ZO-1	CTCATCTCCAGTCCCTTACCT	TTGTGATACGTGCGAGGTG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
Let-7i	ACACTCCAGCTGGGTGAGGTAGTAGTTTGTGC	CTCAACTGGTGTCGTGGA

The rats were randomly subdivided into six groups: the sham group (saline-treated rats); the model group (thrombus-treated rats); the model with rt-PA group (rats treated with rt-PA [10 mg/kg; Sigma, Aldrich, St. Louis, MO, USA] by way of tail vein injection at 3 h after injection with thrombus); the model with rt-PA (6 h) group (rats treated with rt-PA [10 mg/kg] by tail vein injection at 6 h after being injected with thrombus); the model with rt-PA (3 h) with let7i agomir group (rats co-treated with rt-PA [10 mg/kg] and let7i agomir [Sigma, Aldrich, St. Louis, MO, USA] simultaneously by tail vein injection at 3 h after injection with thrombus); the model with rt-PA (6 h) with let7i agomir group (rats co-treated with rt-PA [10 mg/kg] and let7i agomir simultaneously by tail vein injection at 6 h after injection with thrombus). The rats in the sham group were subjected to an identical surgical procedure. The animal study was approved by the Animal Ethics Committee of General Hospital of Southern Theater Command of PLA. For other details on the materials and methods, please see the supporting information file.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from the rat infarct cerebral cortex at 24 h after thrombolysis using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Subsequently, total RNA was reverse transcribed into cDNA by BestarTM qPCR RT kit (DBI, Denmark). To detect the expression levels of let7i, occludin, claudin-5 and ZO-1, qRT-PCR was performed using an ABI PRISM 7500 Sequence Detection System (Life Technologies, Grand Island, NY, USA) with Bestar™ qPCR Master Mix (DBI, Denmark). The reactions were performed in triplicate and contained 100 ng of cDNA, 0.5 μl of each primer (10 μm/μl), and 10 μl SYBR Green Master Mix in a final volume of 20 μl. The amplification reactions were performed under the following conditions: 95°C for 5 min, followed by 40 cycles of 94°C for 30 s, 65°C for 30 s, and 94°C for 30 s. A melting curve analysis was performed by increasing the temperature from 65°C to 94°C (0.5°C per 10 s). Relative fold differences for each sample in each experiment were calculated using the $2^{-\Delta\Delta Ct}$ method. The GAPDH gene was used as a control. All the primer sequences were synthesized by RiboBio (Guangzhou, China) and are listed in **Table 1**.

Neurologic assessment

A neurologic assessment of the rats was tested using a revised five-point scale [18] at 24 h after thrombolysis: 0 = no neurologic deficit, 1 = forelimb flexion during tailing, 2 = deceased lateral push resistance and forelimb flexion during tailing, 3 = one-way circle, 4 = one-way circle and a depressed level of consciousness.

Evaluation of the blood-brain barrier (BBB) permeability

BBB permeability was assessed at 24 h after thrombolysis using the Evans blue (EB) extravasation method, as reported previously [19]. Briefly, 2% EB (Sigma, MO, USA) dissolved in saline was administered intravenously and circulated for 2 h. Then, the rats were perfused intracardially with phosphate-buffered saline via the left ventricle under deep anesthesia. Perfusion continued until the fluid from the right atrium became colorless. The rats were then decapitated, and their brains were quickly removed and divided into ischemic and non-ischemic hemispheres. The ischemic hemisphere was weighed and placed in 3 ml formamide for 48 h in the dark at room temperature. The supernatants were collected after centrifugation at 16,000 g for 20 min for the homogenized ischemic hemisphere in 4 ml of 50% trichloroacetic acid solution. The EB concentration was determined with a spectrophotometer at 620 nm for absorbance against a standard curve. The standard curve was constructed using a dilution of EB dye in a range from 3 to 400 ng/ml.

Assessment of hemorrhage levels after cerebral artery occlusion

A hemorrhage level assessment after cerebral artery occlusion was done using the method of García-Yébenes [20], with hemorrhages macroscopically categorized into 1 of the following 5 levels: (1) non-hemorrhage; (2) hemorrhagic infarction type 1 (HI-1), characterized by small petechiae that are found in the area bordering the infarct; (3) hemorrhagic infarction type 2 (HI-2), characterized by confluent petechiae in the area of tissue damage; (4) parenchymal hemorrhage type 1 (PH-1), defined as blood clots in less than 30% of the injured parenchyma; and (5) parenchymal hemorrhage type 2 (PH-2), defined as more than 30% of the infarct having blood clots.

Evaluation of infarct volume and brain water content

Infarct volume was detected by 2,3,5-triphenyltetrazoliumchloride (TTC) staining [21]. Briefly, a brain slicer (Activational Systems, Warren, MI, USA) was used to obtain 2 mm-thick brain sections, which were then immersed in a 2% TTC solution and incubated at 37°C in the dark for 30 min. Then, the brain slices were placed in 10% formaldehyde for 24 h in the dark. The non-ischemic area appeared rose red while the infarct area was white after staining. The stained slices were photographed and quantified for the infarct area using Image J software. The corrected infarct volume was calculated as follows [22]: corrected infarct volume (%) = {[contralateral hemisphere area - (ipsilateral hemisphere area-measured infarct area)]/contralateral hemisphere area} × 100%. For the brain water content assessment, brain tissue was immediately dissected, weighed, and designated as the wet weight at 24 h after reperfusion. The tissues were then placed in an oven (105°C) and dried for 72 h to obtain the dry weight. The brain water content was calculated by the following formula: ([wet weight - dry weight]/wet weight) × 100%.

Western blot analysis

Total protein was extracted from the rat infarct cerebral cortex using a RIPA Lysis Buffer (Beyotime, Shanghai, China), and the protein concentration was determined with a BCA protein assay Kit (BioRad, CA, USA). Equal amounts of protein were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, MA, USA). After blocking with 5% nonfat milk for 1 h at room temperature, the membranes were incubated with primary antibodies against occludin (1:1000, Abcam, UK), claudin-5 (1:1000, Santa Cruz Biotechnology, Santa Cruz), ZO-1 (1:1000, Cell Signaling Technology, Danvers, MA) and GAPDH (1:10000, Cell Signaling Technology, Danvers, MA) at 4°C overnight. GAPDH was used as a loading control. After washing with Tris-buffered saline/Tween 20, the membranes were incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. The protein signal was detected and visualized using an enhanced chemiluminescence Western blotting detection kit (Thermo Fisher Scientific, Rockford, Illinois) and the net optical density value of the bands was analyzed by Image-Pro Plus 6.0.

Statistical analysis

All the results were presented as the mean ± standard deviation from at least three repetitions. All statistical analyses were calculated using SPSS 17.0. A one-way analysis of variance was used to analyze the statistical significance of the differences in BBB permeability. Infarct volume, hemorrhage levels, neurological scores, and the expressions of occludin, claudin-5 and ZO-1 were compared among the different groups. Student's *t*-test was used to evaluate the statistical significance of the differences in expression of let7i in the brains. *P* values < 0.05 were considered statistically significant and were presented as *, and *P* values < 0.001 were presented as ***.

Results

Expression level of let7i was improved in both the model group and the thrombolysis by rt-PA group compared with the sham group

To investigate the function of let7i in cerebral edema and HT after thrombolysis, the changes in the expression of let7i in the infarct cerebral cortex were measured. As shown in **Figure 1**, the expressions of let7i in both the model and thrombolysis groups were significantly elevated compared with the sham group. These results demonstrated that thrombus affected the transcriptional activity of let7i in the cerebral cor-

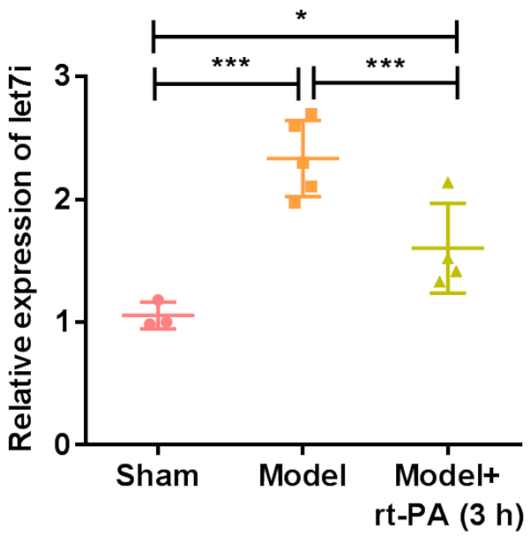

Figure 1. Relative expression of let7i in the sham, model, and model with rt-PA (3 h) groups measured using quantitative real-time PCR analysis. *Denotes the difference between the sham group and model with rt-PA (3 h) group. ***Denotes the difference in the sham group and model with rt-PA (3 h) group compared with the model group, respectively.

tex. However, the expressions of let7i were significantly decreased in the thrombolysis groups when compared with the model groups, suggesting that let7i may play an important role in the process affecting cerebral edema and HT after thrombolysis by rt-PA. However, whether let7i affects thrombolysis needs further verification.

Overexpressing let7i decreased activities of the BBB, brain edema, and neurological scores

To assess BBB permeability, EB dye extravasation was performed at 24 h after thrombolysis. An increase in EB dye extravasation was observed in the model, thrombolysis, delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis groups when compared with the sham group (**Figure 2A**). This result indicated that thrombus increased BBB permeability. Furthermore, the thrombolysis, delayed thrombolysis, and overexpressing let7i with delayed thrombolysis groups exhibited significantly increased EB dye extravasation compared with the model group, suggesting that thrombolysis by rt-PA increased BBB permeability (**Figure 2A**). Moreover, EB dye extravasation significantly decreased in the overexpressing let7i with thrombolysis and overexpressing let7i with delayed thrombolysis groups compared with the thrombolysis (thrombolysis by rt-PA) and delayed thrombolysis groups (**Figure 2A**). However, there was no difference in the BBB permeability between the thrombolysis and overexpressing let7i with delayed thrombolysis groups (**Figure 2A**), indicating that overexpressing let7i reduced BBB permeability. These results suggest that let7i protects BBB permeability caused by thrombolysis, especially in the case of overexpressing let7i at 3 h after thrombolysis by rt-PA. Second, more severe brain edema was observed in the model, thrombolysis, delayed thrombolysis, and overexpressing let7i with delayed thrombolysis groups compared with the sham group. Thrombolysis by rt-PA in both the thrombolysis and delayed thrombolysis groups aggravated brain edema compared with the model group. However, overexpressing let7i relieved brain edema in the overexpressing let7i with thrombolysis and overexpressing let7i with delayed thrombolysis groups when compared with the thrombolysis and delayed thrombolysis groups, respectively. In the overexpressing let7i with thrombolysis group, brain edema was restored to the level in the sham group (**Figure 2B**). Finally, to evaluate changes in neurological behavior, neurological scores were determined and analyzed by the revised method of Bederson et al. [23]. The SD rats in the model, thrombolysis, delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis groups exhibited severe neurological deficits compared with the rats in the sham group (**Figure 2C**). These results indicate that thrombus affects neurological function. Further analysis showed significant relief from neurological deficits in the overexpressing let7i with thrombolysis and the overexpressing let7i with delayed thrombolysis groups compared with the thrombolysis and delayed thrombolysis groups, respectively (**Figure 2C**). However, there was no difference in neurological deficits between the model group and the overexpressing let7i groups. The trend in neurological deficits in the overexpressing let7i with delayed thrombolysis group was the same as in the model group (**Figure 2C**). These results demonstrated that let7i affected the process caused by thrombolysis not for thrombolysis.

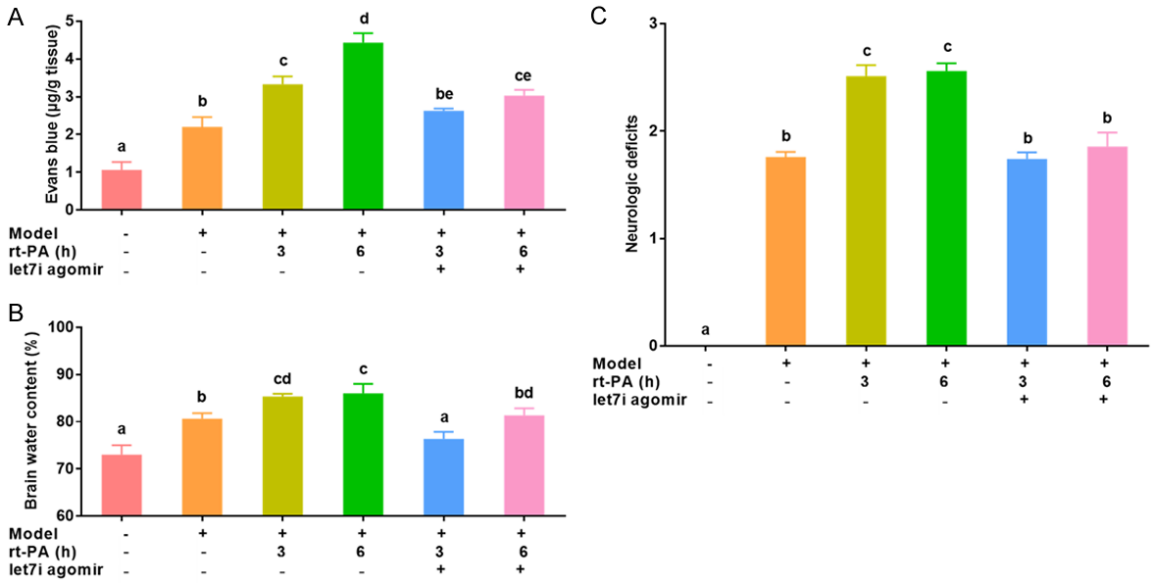

Figure 2. Overexpressing let7i reduced BBB permeability, brain edema, and neurological scores. A. BBB permeability of SD rats determined by EB in the different groups. B. Brain water content of SD rats in the different groups. C. Neurological scores of SD rats in the different groups. Different letters were used to denote statistical significance among the different groups, including the sham, model, thrombolysis, delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis groups.

Overexpressing let7i reduced hemorrhage levels after thrombolysis but did not affect infarct volume

The function of let7i in MCAO was examined using the TTC staining of brain tissue from rats in the different groups. As shown in **Figure 3A**, normal brain tissue was stained red, and the infarct lesion was stained white. There was no white region observed in the rats of the sham group. However, the levels of cerebral infarct lesions differed between the model, thrombolysis, delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis groups. Infarct volumes in the rat brains from different groups were calculated by Image J. Compared with the sham group, the model, thrombolysis, delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis groups exhibited significantly larger infarct volumes. However, thrombolysis by rt-PA (thrombolysis and delayed thrombolysis groups) and overexpressing let7i after thrombolysis by rt-PA (overexpressing let7i with thrombolysis and overexpressing let7i with delayed thrombolysis) groups ameliorated the infarct volume compared with the model group. Interestingly, overexpressing let7i did not reduce the infarct volume after treatment by rt-PA in the overexpressing let7i with thrombolysis group compared with the thrombolysis group (overexpressing let7i with delayed thrombolysis group compared with delayed thrombolysis group) (**Figure 3B**). Hemorrhage levels after cerebral hemorrhage were assessed by the method described by García-Yébenes and colleagues. Hemorrhages were exacerbated in the model, thrombolysis, delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis groups compared with the sham group. Hemorrhages were also exacerbated in the thrombolysis, delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis groups after thrombolysis compared with the model group. However, overexpressing let7i significantly reduced hemorrhage levels in the overexpressing let7i with thrombolysis group compared with the thrombolysis group. These results were also observed in the delayed thrombolysis group and the overexpressing let7i with delayed thrombolysis group (**Figure 3C**). These results demonstrated that overexpressing let7i tended to reduce hemorrhage levels after thrombolysis.

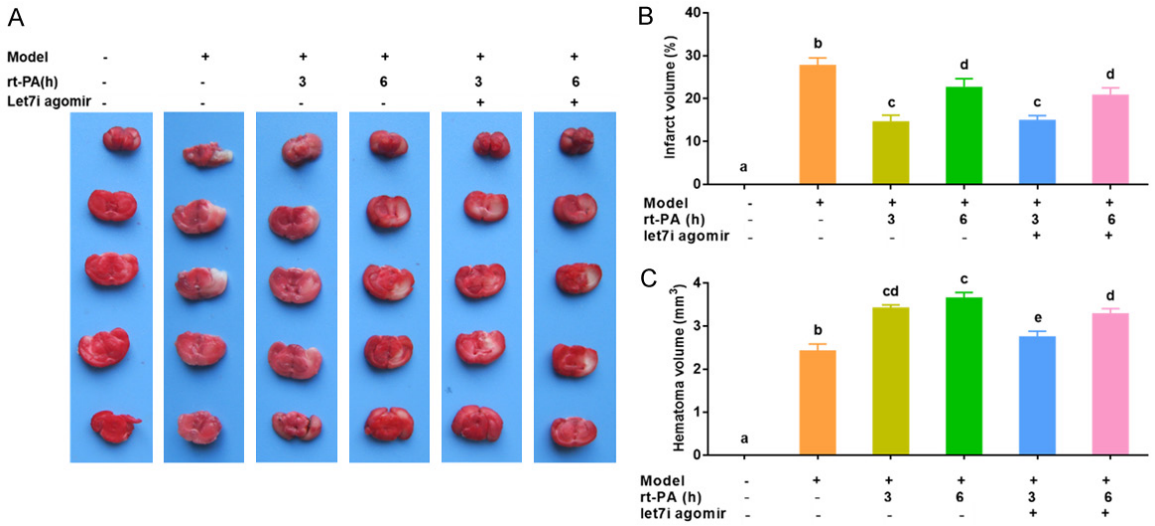

Figure 3. Overexpressing let7i reduced hematoma volume but had no effect on the infarct volume. A. Representative photographs of TTC staining in the brains of rats from the different groups. B. Infarct volumes of SD rats from the different groups were calculated by Image J. C. Hematoma volume of SD rats from the different groups. Different letters were used to denote statistical significance among the different groups, including the sham, model, thrombolysis, delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis groups. TTC = 2,3,5-triphenyltetrazolium chloride.

Overexpressing let7i increased the expression of occludin at the protein level

To understand the molecular mechanisms affected by let7i, the expressions of the proteins associated with the BBB integrity, namely, occludin, claudin-5, and ZO-1, were determined by way of qRT-PCR and Western blot. At the mRNA level, the expression of occludin was significantly decreased in the model, thrombolysis, delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis groups compared with the sham group. The expression of occludin was also significantly increased in the thrombolysis, delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis groups compared with the model group. However, there was no difference in the expression of occludin between the thrombolysis and overexpressing let7i with thrombolysis groups or the delayed thrombolysis and overexpressing let7i with delayed thrombolysis groups (**Figure 4A**). Similar trends were also observed in the expressions of claudin-5 and ZO-1 in the thrombolysis, delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis groups (**Figure 4B** and **4C**). These results demonstrated that thrombus reduced the expressions of occludin, claudin-5 and ZO-1, but thrombolysis by rt-PA increased the expressions of occludin, claudin-5, and ZO-1. However, overexpressing let7i did not affect the expressions of occludin, claudin-5, or ZO-1 (**Figure 4A-C**). These results encouraged us to further explore whether the expressions of these three proteins changed in terms of their protein levels. As shown in **Figure 4**, the expressions of the proteins claudin-5 and ZO-1 were associated with trends in the protein level corresponding to the mRNA level. The trend in the expression of occludin at the protein level was the same among the model, thrombolysis, delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis groups compared with the trend in the sham group; however, the expression of occludin at the protein level was significantly elevated in the overexpressing let7i with the thrombolysis group compared with the thrombolysis group, or between the delayed thrombolysis and overexpressing let7i with delayed thrombolysis groups. Overall, these data indicated that overexpressing let7i activated the occludin signaling pathway but not for claudin-5 and ZO-1 at the protein level.

Discussion

tPA is an FDA-approved therapy that improves outcomes in patients with IS. HT and cerebral

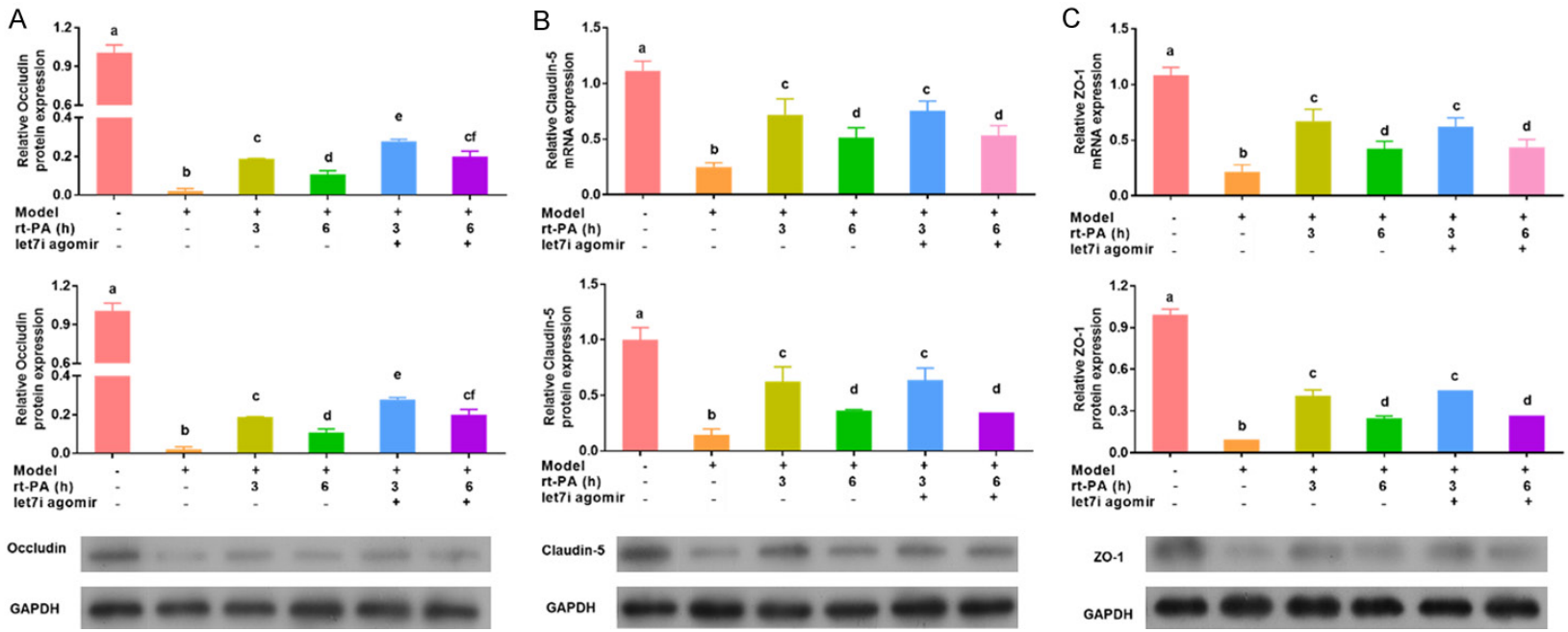

Figure 4. Relative mRNA and protein levels of occludin, claudin-5, and ZO-1 affected by let7i. A-C. Indicate the relative expressions of occludin, claudin-5, and ZO-1 at the mRNA and protein levels, respectively. Different letters were used to denote statistical significance among the different groups, including the sham, model, thrombolysis, delayed thrombolysis, overexpressing let7i with thrombolysis, and overexpressing let7i with delayed thrombolysis groups.

edema are devastating complications in patients treated with the rt-PA therapy [24]. However, the mechanism to reduce the risk of these two complications after thrombolytic therapy by rt-PA has not been extensively explored. In an earlier study, we reported that let7i was decreased in stroke patients compared with healthy controls [16]. In the present study, we successfully established the thrombolysis model to explore the effects of let7i on BBB permeability, brain water content, neurologic deficits, infarct volume, hematoma volume, and regulation of tight junction (TJ) proteins after rt-PA treatment.

First, the expression of let7i in the cerebral cortex was assessed by qRT-PCR in the sham, model, and model + rt-PA groups. As shown in **Figure 1**, the expression of let7i was significantly elevated both in the model and model + rt-PA groups compared with the sham group. However, the expression of let7i was significantly downregulated in the model with rt-PA group compared with the model group (**Figure 1**). The expression pattern of the circulating let-7 family members has been well described in stroke patients and animal models; for example, the expression levels of both let-7b and let-7e were reportedly elevated in IS patients [13, 25], but the expressions levels of let-7e and let-7i were reportedly decreased in patients with massive cerebral infarction without HT and in peripheral blood cells in IS patients, respectively [13, 26]. Another study reported that the leukocyte response was regulated by let7i in patients with acute IS [27]. Overall, we speculated that let7i may play important roles in thrombolytic treatment by rt-PA for IS.

Emerging data show that treatment with rt-PA is associated with BBB breakdown, edema, and HT, and that delayed treatment with tPA aggravates these complications [20]. In a recent study, let-7g was reported to protect the BBB in a neuroinflammatory model and to improve endothelial cell function [28]. Therefore, let7i was used to evaluate whether it could decrease rt-PA associated BBB disruption, brain water content, neurologic deficits, infarct volume, and hematoma volume in an animal model of IS. In the present study, we report that thrombolysis by rt-PA affected cerebral edema and HT-related indices (**Figures 2** and **3**); these results are consistent with earlier studies [20, 29]. Further analysis demonstrated that let7i combined with the rt-PA treatment attenuated BBB disruption, brain water content, neurologic deficits, and hematoma volume, but did not affect thrombolytic therapy. Thus, let7i may be a promising and effective therapeutic strategy against IS.

We demonstrated here that let7i plays an important role in protecting BBB permeability and reducing the risk of subsequent HT, especially for delayed rt-PA treatment. Therefore, the exploration of the mechanism of let7i in protecting BBB is quite important. The TJ proteins regarded as major structural proteins and essential factors for BBB integrity [30] including occludin, claudin-5, and ZO-1 were investigated, using qRT-PCR and Western blot. We found changes in the expressions of occludin, claudin-5, and ZO-1 (**Figure 4**) that were consistent with findings in earlier studies; for example, increases in occludin and claudin-5 at 24 h were detected after focal ischemia and were significantly higher in patients with HT than in those without HT [31, 32]. In contrast, a reduction was observed in claudin-5 at 24 h after ischemia in occludin and ZO-1 at 48 h after ischemia in mice treated with rt-PA [33]. Further analysis showed that the expression of occludin at the protein level was significantly elevated in the overexpressing let7i groups after rt-PA treatment, but no effect on the expression of claudin-5 and ZO-1 both at the mRNA and protein levels was observed (**Figure 4**). These results demonstrated that the protective role of let7i in BBB permeability was broken down mainly by regulating the expression of occludin at the protein level.

The risk of complications in patients treated with rt-PA for IS is an unaddressed issue worldwide. Thus far, the most effective strategy to protect against ischemic injury is to prevent BBB breakdown caused by rt-PA treatment. A number of agents decrease rt-PA-associated complications, including angiopoietin-1, granulocyte colony-stimulating factor, an optimized human apyrase, and progesterone experimentally. However, no therapy has been found to reduce the risk of complications clinically. In the present study, the analysis of the mechanism and function of let7i provides a solid foundation for its clinical application in patients with IS.

Conclusion

Overexpressing let7i may alleviate BBB permeability, brain edema, and hemorrhage levels, suggesting protective effects in IS patients after thrombolysis by rt-PA. The protective role of let7i after thrombolysis by rt-PA may be related to decrease occludin expression at the protein level. Our data suggested that let7i is a potentially effective target for clinical applications in IS. Further investigations are needed to evaluate dose- and time-response relationships when let7i is administered in combination with rt-PA treatment, using an animal stroke model that is more representative of the clinical population at risk for stroke.

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Disclosure of conflict of interest

None.

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