MicroRNA-23a-3p promotes the perihematomal edema formation after intracerebral hemorrhage via ZO-1

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Abstract. – OBJECTIVE: To explore the relationship between microRNA-23a-3p expression and perihematomal edema (PHE) in patients with acute intracerebral hemorrhage (ICH) and its underlying mechanism.

PATIENTS AND METHODS: Clinical data and blood samples on the 3rd day after ICH onset were collected. Head CT was performed in each subject when admitted. Serum expressions of microRNAs were detected by quantitative real-time PCR (qRT-PCR). The relationship between hematoma volume and perihematomal edema was analyzed by correlation analysis. The direct binding of microRNA-23a-3p and zonula occcludens-1 (ZO-1) was verified by luciferase activity assay and Western blot, respectively. Moreover, in vitro experiments were carried out by flow cytometry and CCK-8 assay, respectively.

RESULTS: Serum levels of microRNA-23a-3p and microRNA-130a in ICH patients were remarkably higher than those in the control group, microRNA-26a and microRNA-146a, however, were significantly decreased. A positive correlation was observed between the microRNA-23a-3p expression and the volume of relative perihematomal edema (rPHE) on the 3rd day after ICH (r²=0.3985; p=0.0002). Up-regulation of microRNA-23a-3p significantly decreased ZO-1 expression in hCMEC/D3 cells. Results of luciferase activity assay further indicated that microRNA-23a-3p directly targets the wild-type of ZO-1. In vitro results suggested that microRNA-23a-3p expression markedly affects the proliferation and apoptosis of hCMEC/D3 cells. Similar results were obtained after overexpression or knockdown of ZO-1.

CONCLUSIONS: Up-regulated microRNA-23a-3p in ICH patients promotes the apoptosis of cerebral vascular endothelial cells by down-regulating ZO-1, thus participating in the perihematomal edema formation after intracerebral hemorrhage.

Key Words: MicroRNA-23a-3p; Perihematomal edema; Intracerebral hemorrhage; ZO-1.

Introduction

Spontaneous intracerebral hemorrhage (ICH) is one of the most serious types of stroke, with high morbidity and mortality. Perihematoma edema (PHE) forms rapidly after ICH onset, which remarkably increases within 7-11 days and continues for a long time. Progressive expansion of PHE further increases the hematoma space occupying effect, which leads to intracranial hypertension and hernia. Although the relationship between edema volume and disease prognosis remains controversial, accurate assessment and timely treatment of cerebral edema are still the key factors to cure ICH. Two large clinical studies, VISTA-ICH and INTERAC, have shown that PHE volumes in patients with deep ICH are closely related to the disease prognosis. Currently, CT and MRI are common clinical measures to assess hematoma volume and edema volume. Results of CT/MRI have indicated that there is a positive correlation between edema volume and hematoma volume in the late stage of ICH. It is reported that the correlation coefficient (r²) between edema volume and hematoma size is between 0.46 and 0.72 within 24 h after ICH, indicating an exact individual difference in the formation and development of PHE after ICH. Therefore, sensitive biomarkers are urgently needed to evaluate the ICH prognosis.
inhibiting mRNA translation or reducing mRNA stability. MiRNA expression and its regulation effect on target genes not only occur in cells, but also in serum and other extracellular fluid. Studies have demonstrated that extracellular miRNAs protect themselves from nuclease degradation by two mechanisms, including encapsulation of lipid vesicles and binding to RNA-binding proteins. Among various diseases, similar miRNA expression profiles are found in both peripheral blood and lesion tissues, including cerebral hemorrhage. Previous studies have identified that specific miRNAs in peripheral blood can serve as biomarkers in various diseases, including lung cancer, breast cancer, cardiovascular disease, hepatitis, etc.

Accumulated evidence has demonstrated that microRNA-23a-3p is involved in the disease pathogenesis by regulating cell proliferation. Furthermore, PHE occurrence is considered to be associated with the proliferation and apoptosis of vascular endothelial cells constituting the blood-brain barrier. Thus, we explored whether microRNA-23a-3p could contribute to the development of cerebral edema by regulating proliferation and apoptosis of cerebrovascular endothelial cells.

**Patients and Methods**

**Basic Characteristics of Subjects**

This study was approved by the Medical Ethics Committee at Beijing Luhe Hospital, Capital Medical University and all subjects were consent informed. All hospitalized patients with acute spontaneous ICH treated in our hospital from 2015 to 2016 were included. Inclusion criteria were applied in ICH patients who were older than 18 years and admitted within 24 h after ICH onset. Exclusion criteria were applied in ICH patients with infratentorial intracerebral hemorrhage, intraventricular hemorrhage, hematoma enlargement, secondary cerebral hemorrhage (hemorrhage caused by a potential brain tissue lesion, cerebral infarction or thrombotic drugs), hematoma aspiration after intracerebral hemorrhage, recent cerebral infarction (within the last 3 months), and refusal of participation. Another 30 cases matched with age, sex, and basic diseases were enrolled in the control group. Blood samples were collected from ICH patients and controls 3 days after admission, which were centrifuged at 1500 r/min for 10 min. Serum samples were finally obtained and then preserved in a -80°C refrigerator.

**Image Examination**

Non-contrast enhanced head CT (128-MSCT, Siemens, Berlin, Germany) was performed on the 3rd day after ICH onset. Perihematoma edema volume was calculated by subtracting the high-density portion volume based on the high-density and low-density CT shadow. Additionally, the rPHE (relative perihematoma edema) was calculated via dividing the perihematoma edema volume by the hematoma volume. Image examination was independently performed by 3 radiologists who were blinded to the experimental design. Average data were recorded.

**RNA Extraction and qRT-PCR**

The extraction of total RNA was performed according to the instructions of RNA Rapid Extraction Kit. Briefly, 800 μL of TRIzol Lysis Buffer was added to 200 μL of serum. The upper liquid phase was collected after centrifugation. 10 μL of isopropanol was then added for another centrifugation. Finally, 20-30 μL of Diethyl pyrocarbonate (DEPC) was added to dissolve RNA precipitation. Reverse transcription was performed according to the instructions of TaKaRa One Step PrimeScript® miRNA cDNA Synthesis Kit. SYBR Green I fluorescent dye method was used for PCR detection. The relative concentration was calculated by the 2−ΔΔCT method. Primer sequences used in the study were showed in Table I.

**Luciferase Activity Assay**

Target Scan website was used to predict the binding sites for microRNA-23a-3p and ZO-1. ZO-1 luciferase reporter vectors containing wild or mutant predicted binding sites were conducted by Ruizhen Bio Co., Ltd. (Nanjing, China). MicroRNA-23a-3p mimic and inhibitor were purchased from Gima Bio Co., Ltd. (Shanghai, China). The relative fluorescence value after plasmid transfection was then measured by a standardized method. All experiment was independently performed for 3 times.

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**Table I.** Primer sequences used in the study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>ZO-1</td>
<td>F: 5’-CAACATACAGTGACGCTTCACA-3’</td>
<td>R: 5’-CACTATTGACGTTTCCCCACTC-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5’-GCACCGTCAAGGGCTAGAAC-3’</td>
<td>R: 5’-GGATCTCGCTCCTGGAAGATG-3’</td>
</tr>
<tr>
<td>U6</td>
<td>F: 5’-CTCGCTTCGGCAGCAACA-3’</td>
<td>R: 5’-AACGCTTCAGAATTTCGCT-3’</td>
</tr>
</tbody>
</table>

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**Western Blot**

The total protein was extracted by the TRIzol reagent. Protein samples were then separated by 12% sodium dodecyl sulphate (SDS) protein electrophoresis after the concentration of each sample was adjusted to the same level. Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane and routinely immunostained at 4°C overnight (anti-ZO-1, diluted in 1:500). After washed with TBST for 3 times, membranes were incubated with the secondary antibody (1:1000) at room temperature for 1 h. All membranes were exposed by enhanced chemiluminescence (ECL) method.

**Cell Culture**

Cerebrovascular endothelial cell line CMEC/D3 was purchased from ATCC and cultured in EBM-2 medium containing 1% penicillin-streptomycin, 1.4 μM hydrocortisone, 5 μg/mL ascorbic acid, 1/100 lipid concentrate, 10 mM HEPES, 1 ng/mL basic fibroblasts cell growth factor (bFGF) and 5% fetal bovine serum. Cells were maintained in a 5% CO₂ incubator at 37°C. Culture medium was changed every 1 to 2 days.

**Cell Transfection**

Transfection reagents were purchased from Gene Pharma Co., Ltd (Shanghai, China), including microRNA-23a-3p mimics, microRNA-23a-3p inhibitor, ZO-1 siRNA and ZO-1 overexpression plasmid. Cell transfection was performed based on the instructions of Lipofectamine 2000. Briefly, cells were seeded into 6-well plates at a density of 4×10⁵/mL and then transfected with the above-mentioned plasmids when cell confluence was up to 80%-90%. Transfection efficiency was measured 24 h later. All experiments were independently repeated for three times.

**Cell Counting kit-8 (CCK-8) Assay**

Cells in logarithmic growth phase were seeded in 96-well plates at a density of 1×10⁴/mL, with 100 μL of EBM-2 medium in each well. 10 μL of CCK-8 solution was added to each well 24 h later. Absorbance at 450 nm was measured after another 1 h-incubation.

**Flow Cytometry**

Cells were collected by trypsin digestion and then prepared for single cell suspension. After washed twice with pre-cooled phosphate-buffered saline (PBS), cell density was adjusted to 1×10⁶/mL. 100 μL of cell suspension and 10 μL of Annexin-V were added to each tube, respectively. Before flow cytometry determination, 380 μL of flow cytometry buffer and 10 μL of propidium iodide (PI) were mixed in each tube for 15-min incubation.

**Statistical Analysis**

Statistical product and service solutions (SPSS 19.0, Armonk, NY, USA) software was used for statistics analysis. Category variables were expressed as numbers and percentages. Continuous variables were shown as mean ± standard deviation. The independent sample t-test was used to compare the data between two groups, and chi-square test was used to compare categorical variables. p<0.05 indicated the difference was statistically significant.

**Results**

**Differentially Expressed Serum miRNAs in ICH Patients**

To assess the diagnostic value of miRNAs in PHE after ICH, we examined serum expressions of miRNAs in ICH patients. Our study indicated that ICH patients were remarkably older than those of the control group, while no remarkable differences in sex and hypertension were found (Table II). Based on the previous studies, serum expressions of microRNA-23a-3p, microRNA-130a, microRNA-26a, and microRNA-146a in all sub-

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**Table II. Basic characteristics of enrolled patients.**

<table>
<thead>
<tr>
<th>Indicators</th>
<th>Controls (N=30)</th>
<th>ICH patients (N=30)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y, SD)</td>
<td>61.00(2.360)</td>
<td>60.13(3.720)</td>
<td>0.8454*</td>
</tr>
<tr>
<td>Hypertension (n %)</td>
<td>83.33%</td>
<td>96.67%</td>
<td>0.1611^</td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15(50)</td>
<td>17(56.67)</td>
<td>0.6048^</td>
</tr>
<tr>
<td>Female</td>
<td>15(50)</td>
<td>13(43.33)</td>
<td></td>
</tr>
</tbody>
</table>

*Student’s t-test; ^Two-sided chi-squared test.
Subjects were detected\textsuperscript{19,20}. QRT-PCR results showed that microRNA-23a-3p and microRNA-130a were upregulated in ICH patients, while microRNA-26a and microRNA-146a were down-regulated in ICH patients (Figure 1A-D).

Figure 1. Serum miRNA expressions in ICH patients and controls. Serum expressions of four candidate miRNAs in ICH patients and controls were explored by qRT-PCR. MicroRNA-23a-3p and microRNA-130a expressions in ICH patients were significantly higher than those of control group, whereas microRNA-26a and microRNA-146a in ICH patients were significantly lower (\(*\)\(p<0.01\)).

Correlation Between Serum Expressions of miRNAs and PHE Volumes in ICH Patients

Correlation analysis was performed to assess the diagnostic value of miRNAs in PHE after ICH. A significant association was observed between PHE volumes and serum expressions of microRNA-23a-3p in ICH patients on the 3\textsuperscript{rd} day after onset (\(p<0.05\)). However, no correlation between PHE volumes and the other miRNAs was observed (Figure 2A-D).

Effect of microRNA-23a-3p on Functions of hCMEC/D3 cells and ZO-1 Expression

Transfection efficacy of microRNA-23a-3p mimics or inhibitor in hCMEC/D3 cells was verified by qRT-PCR (Figure 3A). Overexpression of microRNA-23a-3p resulted insignificantly decreased cell proliferation and increased cell apoptosis (Figure 3B-C). Meanwhile, mRNA level of ZO-1
was reduced in cerebrovascular endothelial cells after microRNA-23a-3p knockdown (Figure 3D). The protein level of ZO-1 was also regulated by microRNA-23a-3p (Figure 3E). Results of luciferase activity assay showed that microRNA-23a-3p directly binds to ZO-1 (Figure 3F). The above results suggested that microRNA-23a-3p regulates proliferation and apoptosis of cerebrovascular endothelial cells via ZO-1.

**Effect of ZO-1 on hCMEC/D3 Cell Function**

ZO-1 overexpression plasmid and siRNA were transfected into cerebrovascular endothelial cells, respectively. Transfection efficacy was verified by qRT-PCR and Western blot (Figure 4A-B). In addition, ZO-1 siRNA inhibited the proliferation and promoted the apoptosis of vascular endothelial cells (Figure 4C-D). Our data further suggested that microRNA-23a-3p might affect proliferation and apoptosis by regulating ZO-1 expression.

**Discussion**

Intracerebral hemorrhage (ICH) is usually accompanied by perihematomal edema (PHE), which aggravates clinical symptoms of ICH pa-
patients. A certain correlation between the severity of clinical symptoms and hematoma volume of ICH has been well recognized. It is reported\textsuperscript{21,22} that hematoma volume, hyperglycemia, clotting factors and statins application could affect the formation and development of PHE after ICH. Previous studies\textsuperscript{19,20} have indicated that microRNA-26a, microRNA-146a, and microRNA-130a might participate in the formation of brain edema by regulating expressions of specific target genes. In this study, we examined expressions of candidate miRNAs in clinical samples of ICH patients by qRT-PCR and found that serum level of microRNA-23a-3p is positively correlated with PHE volume.

Apoptosis is a process of programmed cell death that maintains a stable internal environment. Unlike necrosis, apoptosis is an active process involving the activation, expression, and regulation of a series of genes. It is not a phenomenon of autologous injury under pathological conditions but rather a death process pursued to better adapt to the living environment\textsuperscript{23}. PHE occurs mainly due to the integrity destruction of blood-

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**Figure 3.** High expression of microRNA-23a-3p promoted brain endothelial cell apoptosis. **A**, hCMEC/D3 cells were transfected with microRNA-23a-3p mimics and inhibitor. **B-C**, Cell proliferation and apoptosis after microRNA-23a-3p overexpression. **D**, ZO-1 expression was detected after microRNA-23a-3p knockdown. **E**, Protein level of ZO-1 after transfected with microRNA-23a-3p mimics and inhibitor. **F**, The Luciferase activity assay was used to verify the direct binding between microRNA-23a-3p and ZO-1.
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brain barrier caused by cell apoptosis. Our data indicated that the proliferation and apoptosis of brain microvessel epithelial cells are regulated by microRNA-23a-3p.

To further explore potential regulatory mechanisms of microRNA-23a-3p, we found that ZO-1 is a potential target for miRNA-23a-3p through the bioinformatics analysis. ZO-1 is an important tight junction protein involved in the blood-brain barrier formation, which was firstly identified in 1986. It is involved in the maintenance and regulation of epithelial hedge and barrier function, regulation of cellular transport, epithelial polarity, cell proliferation and differentiation, and tumor cell metastasis. In most cases, structure destruction of ZO-1 leads to the change of tight junctions. Therefore, ZO-1 is often served as an indicator of the barrier function and permeability function in closely connected tissues, such as the blood-brain barrier, intestinal epithelial cells, retinal pigment epithelial cells, corneal endothelial cells, diabetic nephropathy, cochlear hair cell precursor cells, cytoskeletal reconstruction, etc. In this study, we found that microRNA-23a-3p directly binds to the 3’UTR of ZO-1. Overexpression or knockdown of ZO-1 in brain microvascular cells also significantly affected the apoptosis and proliferation of vascular endothelial cells, demonstrating that microRNA-23a-3p promotes the formation of perihematoma edema by regulating ZO-1 translation.

Figure 4. Down-regulated ZO-1 promoted brain endothelial cell apoptosis. A-B, The mRNA and protein expressions of ZO-1 were detected after transfected with ZO-1 siRNA and overexpression plasmid. C-D, Proliferation and apoptosis changes in cerebrovascular endothelial cells.
Conclusions

We showed that microRNA-23a-3p disrupts the tight junctions and increases the apoptosis of cerebral vascular endothelial cells via ZO-1, thus participating in cerebral edema after intracerebral hemorrhage.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References