Original Article
miR-142-3p promotes amyloid-β induced blood-brain barrier disruption by p38/MAPK/JNK activation

Junliang Tao1-2, Dongxian Zhang2, Yonghong Man1-2, Dongyi Zhang3, Yongyi Bi1

1Department of Occupational Health and Environmental Hygiene, School of Public Health, Wuhan University, Wuhan, Hubei, China; 2Department of Basic Medicine, Nanyang Medical College, Nanyang, Henan, China; 3Department of Cardiac Surgery, The First Hospital Affiliated to Nanyang Medical College, Nanyang, Henan, China

Abstract: This study aimed to investigate effects of abnormal expression of miR-142-3p on cerebral amyloid angiopathy (CAA) and amyloid-β induced blood-brain barrier (BBB). The BBB models were established and the Trans endothelial electrical resistance (TEER), horseradish peroxidase (HRP) and permeability in BBB were also detected. Then the expression of miR-142-3p in CAA patient and BBB model was determined by real-time PCR. Additionally, the expressions of tight junction (TJ)-related proteins including zonula occludens-1 (ZO-1), occludin, and claudin-5 were detected by western blot and real-time PCR. Furthermore, the expressions of p38/MAPK/JNK signaling pathway associated proteins p38 and JNK were also detected by western blot and real-time PCR. The present study showed that miR-142-3p was down-regulated in serum of CAA patients and BBB models. Up-regulation of miR-142-3p increased TEER, while decreased HRP and permeability significantly. Additionally, up-regulation of miR-142-3p significantly increased the expressions of TJ proteins ZO-1, occludin and claudin-5. Furthermore, overexpression of miR-142-3p could inhibit p38/MAPK/JNK signaling pathway. Overexpression of miR-142-3p could repair amyloid-β induced BBB disruption by inhibiting the p38/MAPK/JNK signaling pathway.

Keywords: Cerebral amyloid angiopathy, amyloid-β, blood-brain barrier disruption, miR-142-3p, p38/MAPK

Introduction

Cerebral amyloid angiopathy (CAA) is a common disease in older persons, which has been implicated with serious adverse neurological conditions [1, 2]. It is thought to be causative to cerebrovascular disease affecting normal brain function especially cognition in the elderly [3]. It is also an important cause of spontaneous intracranial hemorrhage in the normotensive individuals [4]. CAA is characterized by the deposition of amyloid-β peptide in the walls of leptomeningeal and cortical arterioles [5]. Specially, amyloid-β peptide can be transported bidirectionally across the blood-brain barrier (BBB), and amyloid-β peptide accumulation in capillaries may affect BBB integrity [6, 7]. BBB dysfunction has been reported in various neurological conditions, such as cerebral ischemia and Alzheimer's disease [7, 8].

MicroRNAs (miRNAs) are a class of short ribonucleic acid molecules, which play an important role in regulating gene function [9]. The miRNA expression profiles often present significant modifications in response to a disease state, therefore, miRNAs have been widely studied in human diseases, such as tumor and nervous system disease [10, 11]. Studies in miRNA research associated with human disease reveal that miRNAs may be used as potential disease-modifying agents [12, 13]. Recent study found that miR-142-3p was down-regulated in serum of CAA patients and BBB models. Up-regulation of miR-142-3p increased TEER, while decreased HRP and permeability significantly. Additionally, up-regulation of miR-142-3p significantly increased the expressions of TJ proteins ZO-1, occludin and claudin-5. Furthermore, overexpression of miR-142-3p could inhibit p38/MAPK/JNK signaling pathway. Overexpression of miR-142-3p could repair amyloid-β induced BBB disruption by inhibiting the p38/MAPK/JNK signaling pathway.

In the present study, we investigated the relative expression level of miR-142-3p in CAA patients. In addition, we further investigated the effects of dysregulated expression of miR-142-3p on cell permeability and angiogenesis related proteins expressions based on the blood-brain barrier (BBB) model. We aimed to explore the effect of abnormal expression of
miR-142-3p on CAA and to further investigate the potential mechanisms.

Materials and methods

Patients

From January 2014 to January 2016, 20 patients who were diagnosed with CAA were enrolled in this study. Additionally, 20 healthy persons who participated in health check-up in our hospital were enrolled as control. The blood samples of CAA patients and healthy persons were collected. All the patients were informed with the consent and this experiment was approved by our hospital’s protection of human ethics committee.

Cell culture

Human cerebral microvascular endothelial hCMEC/D3 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA. The cell line was cultured in T25 flasks pre-coated with 150 mg/ml rat tail collagen in endothelial basal medium-2 (EBM-2) containing 1% penicillin-streptomycin, 1 ng/ml bFGF, 1% chemically defined lipid concentrate, and 5% fetal bovine serum (FBS). Cells were cultured at density of 5×10⁴ per cm² and the medium was replaced every 3 to 4 days. Cell were detached using 0.05% trypsin (Thermo Fisher Scientific, USA), centrifuged at 1000 g for 10 min, and seeded into newpre-coated T25 flasks. Normal human astrocytes and human brain vascular pericytes, also obtained from ATCC, were prepared according to the instruction of the manufacturer. All cells were maintained in an atmosphere of 5.0% CO₂ at 37°C.

BBB model establishment in vitro

To construct various in vitro models of BBB, 1.5×10⁴ cells/cm² pericytes or astrocytes were seeded on the bottom side of the collagen-coated polyester membrane of the Trans well inserts. After overnight adhesion, 1.5×10⁵ cells/cm² endothelial cells were seeded on the upper side of the inserts placed in the well of the 12-well culture plates containing nocells, pericytes, or astrocytes. BBB models were maintained in rat brain vascular endothelial cells (RBEC) medium II. The day when the endothelial cells were plated and models were established was defined as day zero in vitro (Day 0). From Day 1, the culture medium was supplemented with 500 nM hydrocortisone. Under these conditions, in vitro BBB models were established within 3 days after setting of the cells. As negative controls, astrocytes and pericytes that did not form barrier were cultured on the inserts, respectively.

Cell transfection

For cell transfection, miR-142-3p inhibitor, miR-142-3p mimic and miR-142-3p scramble were purchased from Sangon Biotech (Shanghai, China). Cell transfection was performed using the Lipofectamine 2000 according to the manufacture’s protocol (Invitrogen, USA).

Trans endothelial electrical resistance

The Trans endothelial electrical resistance (TEER) measurement provides a quick and easy evaluation of the integrity of the BBB model, which was assessed using a millicel-ERS apparatus (Millipore, USA). TEER was recorded 30 min after the medium exchange at room temperature to ensure temperature equilibration and culture environment uniformity. Collagen coated Tranwell inserts without cells were used to correct for background resistance. The final resistance (Ωcm²) was calculated by subtracting background resistance from measured barrier resistance, then multiplying by the effective surface area of the filter membrane.

HRP flux assay

After BBB models were established, 0.1M phosphate buffer solution containing 0.5 μM horseradish peroxidase (HRP) was added into the upper compartment of the Trans well system. After one hour, the medium in the lower chamber was collected and the HRP content of the samples was assayed colorimetrically. The HRP flux was expressed as pmol passed per cm² surface area per hour.

Permeability measurement

After BBB models were established, the culture medium in the lower chamber of Trans well was replaced with the transport buffer com-
miR-142-3p in blood-brain barrier disruption

Table 1. Primers used for targets amplification

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occludin</td>
<td>5'-GCCTTTTGCTCTCATCGCTCC-3'</td>
<td>5'-AACATGATTAAAGGCAAAAG-3'</td>
</tr>
<tr>
<td>Claudin-5</td>
<td>5'-GCAGAGGACCCAGACAGAC-3'</td>
<td>5'-CAGACACAGCCCAACACC-3'</td>
</tr>
<tr>
<td>ZO-1</td>
<td>5'-CTGAAGAGGCTAGAAGGATACCT-3'</td>
<td>5'-TGAGAAGGCTGGCTG-3'</td>
</tr>
<tr>
<td>JNK</td>
<td>5'-CCAGATTGCATATTCGCCGTACG-3'</td>
<td>5'-TTCCCAGTTCCATCCTGGTG-3'</td>
</tr>
<tr>
<td>P38</td>
<td>5'-AGCCAAATCCAGTGGGAC-3'</td>
<td>5'-TTCTGCTGCTCAATGATCC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GGGAGCCAAAGGGGTGCAT-3'</td>
<td>5'-GAGTCTCTCCAGTACACAA-3'</td>
</tr>
</tbody>
</table>

posed of Hank's buffer saline solution with 10 mM HEPES and 1 mM sodium pyruvate (Thermo Fisher Scientific, Germany). The culture transport buffer was supplemented with 50 mM lucifer yellow (LY, 457 Da), and 50 mM fluorescein isothiocyanate-Dextran. After incubations in a humidified incubator at 37°C and 5% CO₂, samples were analyzed using a fluorescence multi wall plate reader (FlexStation 3, Molecular Devices, Sunnyvale, CA, USA). Permeability coefficients were calculated using the slopes of the curves which were obtained through the volume cleared plotted against time.

**Real-time PCR**

Total mRNA was isolated from cells using TRIzol Reagent (Invitrogen, CA, USA). Complementary DNA (cDNA) was produced using reverse transcriptase (iScript™ cDNA Synthesis Kit; Bio-Rad Laboratories, CA, USA). The expression level of mRNA was measured by SYBR green-based quantitative RT-PCR (SYBR Green Master mix; Thermo Scientific, Waltham, MA, USA). Primers used for targets amplification were shown in Table 1.

**Western blotting**

Protein samples were separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel at 50 μg per lane and blotted onto polyvinylidene difluoride membranes. After being blocked in PBST, the membranes were probed with primary antibodies (1:1,000 for zona occludens-1 (ZO-1), occludin, claudin-5, p-JNK, JNK, p-p38 and p38, 1:5,000 for GAPDH (internal control) at 4°C overnight. Then the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. The immunoreactive protein bands were developed by enhanced chemiluminescence.

**Statistical analysis**

All experiments were repeated 3 times independently. Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using Graph Prism 6.0 software (GraphPad Prism, San Diego, CA). The significant difference for data in different groups was analyzed using the one-way ANOVA with post-hoc test. P<0.05 was considered as statistical significant.

**Results**

**Model construction**

To detect whether BBB models were established successfully, the TEER, HRP and permeability were analyzed. As shown in Figure 1A-C, TEER significantly decreased, while HRP and permeability increased in BBB models compared with in normal controls (P<0.05), suggesting that BBB models were established successfully.

**miR-142-3p expression in CAA person and in cells**

As shown in Figure 2A, compared with normal persons, miR-142-3p was low-expressed in serum of CAA patients (P<0.01). Additionally, in BBB models, the expression of miR-142-3p was also decreased significantly in comparison with that in normal control (P<0.01), which indicated that the down-regulation of miR-142-3p may be associated with BBB rupture induced by amyloid-β.

**Effect of miR-142-3p up-regulation on BBB integrity and permeability**

After cell transfection, the expressions of miR-142-3p in control (BBB model), miR-142-3p mimic transfection and miR-142-3p scramble transfection groups were shown in Figure 3A. The expression of miR-142-3p in miR-142-3p mimic transfection group was significantly higher than that in the other two groups (P<0.01). Figure 3B-D showed that TEER significantly increased, while HRP and permeability decreased significantly in miR-142-3p mimic transfection group compared with in the other groups (P<0.05), suggesting
miR-142-3p in blood-brain barrier disruption

**Figure 1.** The values of (A) Trans endothelial electrical resistance (TEER), (B) horseradish peroxidase (HRP) and (C) permeability (Pe) in blood-brain barrier models and controls. Error bars indicated means ± SD (P value was determined by ANOVA; **P<0.01, *P<0.05).**

**Figure 2.** The expression of miR-142-3p in (A) cerebral amyloid angiopathy (CAA) patients and (B) amyloid-β induced blood-brain barrier (BBB) models assayed by real time-PCR. Error bars indicated means ± SD (P value was determined by ANOVA; **P<0.01).**

that miR-142-3p overexpression could repair BBB rupture induced by amyloid-β.

**Effect of miR-142-3p up-regulation on tight junction (TJ)-related protein expression**

The expressions of TJ-related proteins were investigated by western blot. As shown in **Figure 4**, the expression levels of ZO-1, occluding and claudin-5 were low in BBB and miR-142-3p scramble groups. However, when miR-142-3p up-regulated, the expressions of ZO-1, occluding and claudin-5 increased markedly (P<0.05), indicating that miR-142-3p overexpression repairing BBB rupture induced by amyloid-β may achieved by regulating the expressions of TJ-related proteins.

**Effect of miR-142-3p up-regulation on p38/MAPK/JNK signal protein expression**

In BBB cells, the p38/MAPK/JNK signaling pathway was activated, showing higher expressions of p-p38 and p-JNK (**Figure 5A-D**). When miR-142-3p overexpressed, the expressions of p-p38 and p-JNK decreased significantly (P<0.05), suggesting that p38/MAPK/JNK signaling pathway could be blocked by the up-regulation of miR-142-3p. Subsequently, to further investigate the role of p38/MAPK/JNK signaling pathway in BBB cells, the normal and BBB cells were treated with SB203580 that was an inhibitor of p38/MAPK. As shown in **Figure 5E**, SB203580 had no significant effect on TEER, HRP and permeability of cells. However, SB203580 could reverse the effect of miR-142-3p down-regulation on TEER, HRP and permeability of cells. Similarly, when cells were treated with JNK inhibitor, SP600125, the result showed that SP600125 had no significant effect on cell membrane integrity and permeability, but it could reverse the effect of miR-142-3p down-regulation on cell membrane integrity and permeability (**Figure 5F**). Taken together, the
miR-142-3p in blood-brain barrier disruption

Results suggested that miR-142-3p overexpression could repair BBB rupture induced by amyloid-β by regulating the p38/MAPK/JNK signaling pathway.

Discussion

The miRNAs are involved in some essential biological processes, including tumorigenesis, hormone signaling and differentiation [15]. The present study showed that miR-142-3p was down-regulated in serum of CAA patients and BBB models. Up-regulation of miR-142-3p decreased the permeability of BBB and up-regulated the expressions of TJ proteins ZO-1, occludin and claudin-5. Furthermore, overexpression of miR-142-3p could inhibit p38/MAPK/JNK signaling pathway.

miR-142-3p has been suggested to be associated with the regulation of obesity, hematopoietic lineage specification, and periodontitis by targeting diverse molecules [16, 17]. Specifically, it is also found play roles in several human tumors, such as acute lymphoblastic leukemia.

Figure 3. (A) The expression of miR-142-3p after cell transfection; (B-D) The values of (B) Trans endothelial electrical resistance (TEER), (C) horseradish peroxidase (HRP) and (D) permeability (Pe) in blood-brain barrier models not transfected and transfected by miR-142-3p mimic and scramble. Error bars indicated means ± SD (P value was determined by ANOVA; **P<0.01, *P<0.05).

Figure 4. The mRNA (A) and protein (B) expressions of zonula occludens-1 (ZO-1), occludin, and claudin-5 in blood-brain barrier models not transfected and transfected by miR-142-3p mimic and scramble. Error bars indicated means ± SD (P value was determined by ANOVA; *P<0.05).
miR-142-3p in blood-brain barrier disruption

Figure 5. Effects of miR-142-3p expression on p38/MAPK/JNK signal pathway related protein expression. The mRNA (A) and protein (B) expression of JNK and p38 in blood-brain barrier models not transfected and transfected by miR-142-3p mimic and scramble; The mRNA (C) and protein (D) expression of JNK and p38 in blood-brain barrier models when JNK and p38 were respectively treated with SB203580 and SP600125; (E) The values of Trans endothelial electrical resistance (TEER), horseradish peroxidase (HRP) and permeability (Pe) in blood-brain barrier models when JNK was treated with SB203580; (F) The values of Trans endothelial electrical resistance (TEER), horseradish peroxidase (HRP) and permeability (Pe) in blood-brain barrier models when p38 was treated with SP600125. Error bars indicated means ± SD (*P value was determined by ANOVA; *P<0.05 vs. normal; #P<0.05 vs. blood-brain barrier model).
leukemia [18], esophageal squamous cell carcinoma [19] and lung adenocarcinoma [20]. In this paper, in vitro BBB model was established successfully, and miR-142-3p was found down-regulate in serum of CAA patients and BBB models, suggesting its potential role in this disease.

In order to elucidate the effect of miR-142-3p on BBB function, we investigated the role of miR-142-3p in regulating the integrity and the permeability of BBB. The results showed that overexpression of miR-142-3p typically increased the TEER value and decreased permeability and HRP, indicating a key role of miR-142-3p in regulating BBB integrity and permeability. Previous studies have reported that the structural and functional integrity of the endothelial barrier are determined by TJ complexes [21], which are the most apical component of the intercellular junctional complex and form an essential structural component of the BBB [22]. The tight junctional complex comprises a large number of membrane-associated proteins including occludin, ZO-1 and claudin-5 proteins [21]. Occludin has a large cytoplasmic extension which may coordinate with junctional proteins in the cytosol [23]. Occludin can associate with ZO-1 that is an important linker molecule between perijunctional actin and occluding. Specially, annular contraction in perijunctional actin dynamically affect intercellular permeability [24]. Additionally, claudin-5 is a major cell adhesion molecule of tight junctions in brain endothelial cells. Increased expressions of the three TJ associated proteins after miR-142-3p overexpression in the present study indicated that miR-142-3p overexpression may repair BBB rupture by regulating the expressions of TJ-related proteins.

JNK and p38 MAPK pathways, also called stress-activated protein kinase pathways, can control cell proliferation, differentiation, survival and the migration of specific cell types, therefore, they play key roles in tissue homeostasis [25, 26]. Previous study has reported that p38MAPK or JNK inhibition can prevent the amyloid-β-induced increase in brain endothelial cells permeability suggests. As a result, the signaling pathways have been served as potential targets for therapeutic treatments in amyloid-β associated disease, such as Alzheimer’s disease [27]. In this study, the result showed that when miR-142-3p overexpressed, the expressions of p-p38 and p-JNK decreased significantly, suggesting the inhibition role of miR-142-3p up-regulation in p38/MAPK/JNK signaling pathway. Therefore, we speculated that miR-142-3p overexpression may repair amyloid-β-induced BBB rupture by inhibiting the p38/MAPK/JNK signaling pathway.

In conclusion, our results suggest the role of miR-142-3p in amyloid-β-induced BBB disruption. Overexpression of miR-142-3p could repair amyloid-β-induced BBB disruption by inhibiting the p38/MAPK/JNK signaling pathway. Therefore, miR-142-3p may be served as a potential molecular target for the treatment of amyloid-β-induced BBB disruption associated disease, such as CAA.

Acknowledgements

This work was supported by National Nature Science Foundation of China, Grant No: U1404827, and Key Science and Technology Program of Henan Province (132102310175).

Disclosure of conflict of interest

None.

Address correspondence to: Dongxian Zhang, Department of Basic of Basic Medical Sciences, Nanyang Medical College, 1439 Wolong Road, Nanyang 430061, Henan, China; E-mail: zdx990326@163.com; Yongyi Bi, Department of Occupational Health and Environmental Hygiene, School of Public Health, Wuhan University, 115 Donghu Road, Wuhan 430071, Hubei, China. E-mail: yongyibi262@sina.com

References

miR-142-3p in blood-brain barrier disruption


