MiR-16 exacerbates neuronal cell growth and inhibits cell apoptosis by targeting AKT3 in cerebral ischemia injury

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INTRODUCTION

Cerebral ischemia, a common cerebrovascular problem, is a refractory disease that seriously endangers human health, and it is a major cause of permanent disability in adults [1]. It is associated with high morbidity, mortality and recurrence, as well as slow recovery [2]. In the pathogenesis of stroke, ischemia-reperfusion causes serious damage to the affected neurons [3]. Neuronal apoptosis is a major cause of cerebral ischemic injury. Thus, inhibition of apoptosis is important in cerebral ischemic injury treatment. MicroRNAs (miRNAs) affect gene expression through inhibition of translation or induction of mRNA degradation by hybridizing with the 3′-UTR of target genes at post-transcriptional level [4]. Studies have indicated that the expressions of miRNAs are altered in cerebral ischemia [5].
It has been reported that miR-16 acted as a tumor suppressor by inhibiting tumor progression in several cancers, including OSCC, breast cancer and HCC [6]. However, miR-16 also improved radiosensitivity, or inhibited cell apoptosis and enhanced cell proliferation in prostate cancer and polycystic ovarian syndrome [7,8]. Thus, it may be hypothesized that miR-16 expression is tissue-specific.

The AKT kinases are considered as cell signaling regulators in response to insulin and growth factors [9]. In particular, AKT serine/threonine kinase 3 (AKT3) relates to cell proliferation, differentiation, apoptosis and tumorigenesis [10]. In this study, the roles of miR-16 and AKT3, and the potential mechanism involved in cerebral ischemia injury were investigated.

**EXPERIMENTAL**

**Cell culture**

Human brain cortical neuron HCN-2 cell line was purchased from ATCC, and cultured in DMEM (Sigma) containing 10 % FBS at 37 °C incubator with 5 % CO₂.

**OGD model**

To simulate ischemia-like conditions *in vitro*, an OGD model was established using HCN-2 cell line. The cells were exposed to 1 % O₂/94 % N₂/5 % CO₂ conditions in glucose-free Hanks’ balanced salt solution (Invitrogen) for 4 h.

**Cell transfection**

The AKT3 small interfering RNA (siRNA-AKT3), miR-16 mimic and inhibitor, as well as controls were obtained from ThermoFisher Scientific (Shanghai, China). The AKT3 overexpressed plasmid (pcDNA3.1-AKT3) and the control plasmid (pcDNA3.1-NC) were obtained from Shanghai GenePharma. The transfected cells were cultivated in 6-well plate overnight. Lipofectamine 2000 (Invitrogen) was performed for cell transfection.

**Cell counting kit-8 (CCK-8) assay**

Cells were cultured in 96-well plate and divided into 5 groups: OGD, OGD + NC mimic, OGD + NC inhibitor, OGD + miR-16 mimic, and OGD + miR-16 inhibitor groups. Then, each group was added with cell counting kit-8 (CCK-8) reagent (Solarbio). After 1.5 h of incubation, and absorbance (450 nm) was measured with a microplate reader.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

The TRizol reagent (Thermo Fisher Scientific) was selected to extract total RNA. Subsequently, TaqMan microRNA assay kit (Applied Biosystems) and Prime Script RT reagent kit (Takara) were used for reverse transcription of the RNAs to cDNAs which were then subjected to qRT-PCR using SYBR Green PCR kit (Toyobo). Primer sequences were listed in Table 1. The mRNA expressions of AKT3 and miR-16 were normalized with U6 and GAPDH, and calculated with the 2⁻ΔΔCt method.

**Table 1: Primer sequences used for qRT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
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<tbody>
<tr>
<td>miR-16</td>
<td>F: TAGCAGCACGTAATATTGGCGG&lt;br&gt;R: TGGCGTGTCGGAGACTC</td>
</tr>
<tr>
<td>U6</td>
<td>F: CTGGCTCGGGACAC&lt;br&gt;R: AACGCTTCACGATTTTCGTA</td>
</tr>
<tr>
<td>AKT3</td>
<td>F: TCCTTCCAGACAAAGACCTC&lt;br&gt;R: CGCTCATGATGACTCCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: AGAAGGCTGGGCTTCATTG&lt;br&gt;R: AGGAGCCCATCCACAGTCTC</td>
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**Luciferase reporter assay**

Targets of miR-16 were predicted through TargetScan online software (http://www.targetscan.org). The AKT3 was predicted as a potential target gene. Wild type (WT) and mutant type (MUT) dual luciferase reporter vectors (Promega) of AKT3-3’UTR were constructed. Then, the co-transfection of miR-16 mimic and the WT or MUT were performed by Lipofectamine 2000 (Invitrogen). After transfection for 48 h, luciferase activities were assayed with a dual-luciferase assay kit (Promega).

**Western blot assay**

Total protein was extracted with RIPA buffer. Proteins were separated using 10% SDS-PAGE, and transferred onto PVDF membrane. The membrane was then blocked in 5 % skimmed milk at room temperature. Thereafter, the membrane was incubated with primary antibodies (Bax, Bcl-2 and GAPDH), followed by incubation with horseradish peroxidase-labeled secondary antibody. Finally, signals were detected using an enhanced chemiluminescence kit.

**Statistical analysis**

All data are presented as mean ± standard deviation (SD). Statistical analysis was performed with SPSS 16.0 software (IBM).
Student's t-test or one-way analysis of variance (ANOVA) was used to compare the differences. P < 0.05 was considered statistically significant.

RESULTS

MiR-16 was up-regulated following OGD treatment

The expression of miR-16 was increased in OGD-exposed neurons (Figure 1 A). The use of mimic or inhibitor to upregulate or inhibit miR-16 expression in OGD-exposed neurons revealed that that increased miR-16 expression may contribute to OGD-induced neuronal injury (Figure 1 B).

MiR-16 enhanced cell viability and inhibited cell apoptosis

From the results of CCK-8 assay, it was discovered that OGD treatment decreased cell proliferative capacity (Figure 2 A). In contrast, miR-16 mimic improved cell viability, while miR-16 inhibitor suppressed same after OGD exposure (Figure 2 B). In addition, cell apoptotic ability was determined based on the expressions of Bax and Bcl-2. It was found that Bax expression was enhanced, while Bcl-2 expression was reduced, on exposure of the cells to OGD (Figure 2 C). Bax and Bcl-2 expressions were also calculated after overexpression or knockdown of miR-16. Results indicated that miR-16 mimic promoted Bcl-2 expression, while it inhibited Bax expression in OGD-exposed cells (Figure 2 D). In contrast, Bcl-2 was downregulated, while Bax was upregulated by miR-16 inhibitor of OGD-treated cells (Figure 2 E). Western blotting showed results similar to those from RT-qPCR, i.e., miR-16 mimic promoted Bcl-2 protein expression, while it suppressed Bax protein expression, with the reverse trend for miR-16 inhibitor (Figure 2 F). These results suggest that miR-16 reduced OGD-induced apoptosis. Thus, miR-16 regulated OGD-induced neuronal damage.

Figure 1: MiR-16 expression after OGD treatment. A: miR-16 expression in OGD-exposed cells, as determined using RT-qPCR. B: After miR-16 mimic or inhibitor transfection, miR-16 expression in OGD-exposed cells

MiR-16 could target AKT3

To identify the molecular mechanism involved in the role of miR-16 in cerebral ischemia, potential targets of were predicted by Targetscan online software. Interestingly, binding site were existed between miR-16 and AKT3 (Figure 3A). As expected, luciferase activity was reduced by WT and miR-16 mimic co-transfection, but there were no changes in others (Figure 3 B). To confirm AKT3 as a downstream gene of miR-16, AKT3 expression was measured after altering miR-16 in HCN-2 cells. It was found that AKT3 expression was reduced by miR-16 mimic, whereas it was increased by miR-16 inhibitor in HCN-2 cells (Figure 3 C). These data demonstrate that miR-16 regulated AKT3 expression via targeted binding to AKT3.

AKT3 suppressed neuronal cell viability and promoted cell apoptosis

AKT3 expression was measured in cells after OGD treatment using qRT-PCR. In contrast to miR-16, AKT3 expression was inhibited in OGD-treated cells compared with normal cells (Figure 4 A). Furthermore, AKT3 expression was evaluated after overexpressing or inhibiting miR-16 in OGD-treated cells. As shown in Figure 4 B, pcDNA3.1-AKT3 enhanced AKT3 expression, while siRNA-AKT3 reduced the expression of AKT3 in OGD-treated cells (Figure 4 B).
viability was determined after AKT3 overexpression or inhibition in OGD-treated cells. As expected, AKT3 upregulation inhibited viability, while AKT3 downregulation enhanced viability of OGD-exposed cells (Figure 4 C). The expression of Bax was promoted by overexpression of AKT3, while it was impaired by knockdown of AKT3 in OGD-treated cells (Figure 4 D). In contrast, Bcl-2 expression was suppressed by AKT3 overexpression, while it was enhanced by siRNA-AKT3 in OGD-treated cells (Figure 4 E), suggesting that AKT3 may enhance HCN-2 cell apoptosis. Moreover, the protein expression level of AKT3 was consistent with qRT-PCR results (Figure 4 F). These results indicate that AKT3 may suppress the viability of HCN-2 cells and promote their apoptosis.

**AKT3 reversed the effect of miR-16 on cerebral ischemia**

To further confirm whether miR-16 regulated neuronal cell viability and apoptosis via AKT3, AKT3 was downregulated in HCN-2 cells that overexpressed miR-16 (Figure 5 A). Results from CCK-8 assay demonstrated that AKT3 counteracted miR-16 mimic’s enhancing effect on cell proliferation in OGD-exposed cells (Figure 5 B). In addition, Bax and Bcl-2 expressions were measured. It was found that Bax expression was enhanced, while that of Bcl-2 was inhibited after co-transfection with miR-16 mimic and pcDNA3.1-AKT3 (Figures 5 C and 5 D), indicating that AKT3 overexpression reversed the enhancing effect of miR-16 mimic on cerebral ischemia. These findings indicate that miR-16 regulated cerebral ischemic injury through targeting of AKT3.

**DISCUSSION**

Cerebral ischemia induces various pathological pathways, and eventually leads to irreversible injury in the ischemic region [11]. More and more attention has been focused on apoptosis in...
cerebral ischemia [12]. Thus, an understanding of the mechanisms associated with neuronal apoptosis is a very important issue at present.

It is known that miRNAs related to cerebral ischemia may provide new insights into the treatment of stroke. For example, it has been reported that the potential of miR-145 as a biomarker was upregulated in cerebral ischemia [13]. Moreover, inhibition of miR-429 attenuated neuronal injury induced by OGD/R via regulation of GATA4 [14]. Moreover, a previous study revealed that downregulation of miR-30a-induced neuroprotection from ischemic damage by regulating HSPAS expression [19].

In addition, studies have shown that miR-16 acts as a diagnostic, stratification, and prognosis biomarker of hyperacute cerebral infarction [16]. Consistent with these findings, miR-16 was found to be upregulated in HCN-2 neuronal cells after OGD treatment in the present study. Importantly, miR-16 significantly aggravated ischemic injury via enhancement of cell proliferation. Moreover, miR-16 suppressed HCN-2 cell apoptosis by enhancing Bcl-2 expression and inhibiting Bax expression, suggesting that miR-16 might be a therapeutic target of cerebral infarction.

It is known that AKT3, also named PKB, is involved in various biological processes [17]. It has been reported that in stroke-induced neuronal injury, AKT3 activated phosphorylation of AKT and mTOR compared with AKT1 [18]. In this study, AKT3 was predicted as a target for miR-16 gene in HCN-2 cells. Additionally, AKT3 was downregulated in cells after OGD treatment. Overexpression of AKT3 inhibited HCN-2 cells proliferation and enhanced their apoptosis via regulating Bax and Bcl-2 expression after OGD treatment. Moreover, AKT3 overexpression partially offset functions of miR-16 on cells proliferation and apoptosis after OGD treatment.

**CONCLUSION**

The findings of this study show that miR-16 is upregulated in OGD-treated cortical neurons and that it promotes the proliferation of neuronal cells, while inhibiting apoptosis. In addition, the results indicate that miR-16 targets AKT3, and also that AKT3 expression is regulated by miR-16. Therefore, miR-16 may promote cortical neuronal proliferation and inhibit cortical neuronal apoptosis via regulation of AKT3 expression. However, there is need for further studies to provide in vivo evidence for the relationship between miR-16 and AKT3 in ischemic brain injury.

**DECLARATIONS**

**Conflict of Interest**

No conflict of interest associated with this work.

**Contribution of Authors**

We declare that this work was done by the authors named in this manuscript, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yijun Song conceived and designed the study, and drafted the manuscript. Bo Wang collected, analyzed and interpreted the experimental data. Yijun Song revised the manuscript for important intellectual content. Both authors read and approved the final manuscript. Yijun Song and Bo Wang contributed equally to this work.

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**REFERENCES**


