Neuroprotective effects of p53/microRNA-22 regulate inflammation and apoptosis in subarachnoid hemorrhage

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Abstract. The present study aimed to investigate whether the neuroprotective effects of p53/microRNA-22 regulate inflammation and apoptosis in subarachnoid hemorrhage (SAH). In a mouse model of SAH, microRNA-22 expression was upregulated. In addition, downregulation of microRNA-22 in HEB cells increased the mRNA expression levels of interleukin (IL)-6, induced cysteine rich angiogenic inducer 61 (Cyr61) expression, and suppressed the protein expression levels of B-cell lymphoma 2-associated X protein (Bax) and caspase-3 activity. Treatment with the p53 inhibitor, pifithrin-α, suppressed p53 protein expression, increased IL-6 mRNA expression, decreased microRNA-22 expression, Bax protein expression and caspase-3 activity, and induced Cyr61 expression in mice with SAH. Furthermore, p53 expression was knocked down using p53 small interfering RNA, which suppressed microRNA-22 expression and increased IL-6 mRNA expression, inhibited Bax protein expression and caspase-3 activity, and induced Cyr61 expression in HEB cells. The present study demonstrated that the neuroprotective effects of p53/microRNA-22 may regulate inflammation and apoptosis in SAH. Reverse transcription quantitative polymerase chain reaction (qPCR) was used to analyze the expression of microRNA-22, western blot analysis was used to analyze the protein expression of Bax and Cyr61.

Introduction

Subarachnoid hemorrhage (SAH) is a fatal and disabling disease that accounts for ~6% of stroke cases; annually, ~10/100,000 patients develop aneurysmal SAH worldwide (1). The interval between the occurrence of SAH and the time when patients are admitted to hospital for treatment is associated with a mortality rate of ~12%, whereas the mortality rate increases to ~40% after patients are admitted to hospital for treatment, and the disability rate for survivors of SAH is ~30%. SAH places a heavy burden on patients themselves, their families and society; therefore, the dangers of SAH cannot be underestimated (2,3). Although surgical technology, radiographic techniques and anesthesia have attained considerable achievements with regards to time, the mortality and disability rates associated with SAH have exhibited no marked alterations in recent years (4). A previous study has mainly focused on vasospasm and the resulting injury, and have considered it the major cause of SAH-associated mortality and disability (3). However, it has been reported that the prognosis for patients with SAH is not markedly improved following the prevention of cerebral vasospasm (CVS) generation. In addition, patients with no vasospasm also develop ischemic injury in the late stage of SAH; consequently, doubts have begun to surface regarding the importance of CVS in injury following SAH, and it is now considered one of numerous pathogenic factors, rather than the previously believed priority (4). Therefore, further etiological and therapeutic research is required; and the research focus has shifted to concentrate on all types of injury mechanisms during early brain injury (EBI). Previous studies have indicated that relieving all types of injury during EBI may improve the prognosis of patients (5,6).

Notably, p53 has been reported to not only serve an important role in the mechanisms underlying CVS and post-SAH apoptosis, but to also participate in the development of hydrocephalus; the reason for this is that the increased p53 following SAH can upregulate the expression and activity of matrix metalloproteinase-9, thus leading to the destruction of blood-brain barrier integrity (7). Therefore, p53 is considered to serve a role in the pathophysiological mechanism underlying SAH, and it has been suggested that targeting p53 may relieve nerve injury following SAH. Notably, progress has been achieved regarding the mode of post-transcriptional regulation of p53 (8). The post-transcriptional regulation of p53 can be divided into acetylation and phosphorylation: it has been confirmed that the protein levels of acetylated p53, which has a proapoptotic role, are notably increased in the hippocampal region of rats following cerebral ischemia (9). In addition, it has been reported that the proapoptotic effects of acetylated p53 may be relieved by deacetylation by the regulatory factor sirtuin 1 (SIRT1); p53 is a non-histone substrate of SIRT1 and SIRT1 may inhibit apoptosis via deacetylation of p53 (10).

MicroRNAs are endogenous non-coding RNAs, approximately 18-25 nucleotides in length, which regulate transcriptional gene expression through binding the 3′-untranslated region of mRNAs and the non-translation region in the d-terminus (11). The majority of microRNA genes are located in the exonic, intronic and non-coding regions of the genome, and are transcribed

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into the microRNA primary transcript by RNA polymerase II, which results in the addition of a poly(A) tail (12). MicroRNAs serve important roles in numerous pathophysiological processes, including oxidative stress, the inflammatory response and cell apoptosis (13). It is well known that microRNAs depend on two principles of sequence complementarity to negatively regulate target gene expression: i) The microRNA is completely complementary to the target gene mRNA, thus resulting in its degradation; ii) the microRNA is not completely complementary to the target gene mRNA, thus inhibiting target gene mRNA expression at the protein translation level (14). At present, >1,000 microRNAs have been discovered, which regulate ≥30% of gene expression and form a complex regulatory network; however, to the best of our knowledge, there is no information regarding the regulation of vascular smooth muscle cell (VSMC) apoptosis by microRNAs after SAH (15). Therefore, investigating the effects of microRNAs on the apoptotic mechanism of VSMCs post-SAH is promising and may result in the generation of novel knowledge. The present study aimed to determine the role of p53/microRNA-22 in the regulation of inflammation and apoptosis in SAH.

Materials and methods

Animals and SAH model. C57BL/6J male mice (weight, 19-20 g; age, 5-6 weeks old; n=12) were purchased from Chongqing Medical University (Chongqing, China), and were maintained at 23°C±1°C and 55% humidity under a 12-h light/dark cycle with free access to food and water. The present study was approved by the Animal Care and Use Committee of Chongqing Medical University.

Mice were injected with pentobarbital sodium (30 mg/kg) and were positioned in a stereotactic frame. Subsequently, mice were disinfected and a midline scalp incision was made; a hole (1x1 mm) was made in the midline 7.5 mm anterior to the bregma. Subsequently, 300 µl blood was collected from the cistern. The wound was sutured and sterilized, after which 50 µl saline at 100 µl/10 g. Pifithrin-α was purchased from Beyotime Institute of Biotechnology (Haimen, China), and was dissolved in dimethyl sulfoxide to 2 mg/kg and diluted with normal saline at 50 ng/ml LPS (Beyotime Institute of Biotechnology) for 2 h. Reverse transcription-quantitative polymerase chain reaction (qPCR). Total RNA was extracted from tissue and cell samples using RNAasy Total RNA kit (DP419; Tiangen Biotech Co., Ltd., Beijing, China). cDNA was synthesized using Super M-MLV reverse transcriptase (RP6502; BioTeke Corporation, Beijing, China). qPCR was performed to measure the expression levels of microRNA-22 and interleukin (IL)-6 mRNA using SYBR Premix kit (Takara Biotechnology Co., Ltd., Dalian, China) on an ABI 7300 real-time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). cDNA was synthesized using Super M-MLV reverse transcription kit (RP6502; BioTeke Corporation) according to the manufacturer's protocol. Amplification parameters were as follows: Denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The primer sequences used were as follows: miR-22 forward, 5'-TCAATGCTTCAGGACAGATTCT-3' and reverse, 5'-CCATGTCGAGGTGCAGTTT-3'; U6 forward, 5'-CGCTTCCGAGCAGCATAAC-3' and reverse, 5'-AAATATGGAACGGTCTTACGA-3'. The final extension step was as follows: Denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec; 4°C for 10 min. The comparative Cq method (2⁻ΔΔCq) was used for relative quantification (16).

Western blot analysis. Proteins were extracted from tissue and cell samples using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology) at 4°C for 15-30 min. Protein content was measured using the bichinchoninic acid (BCA) assay and 50 µg total protein was separated by 10% SDS-PAGE. Subsequently, proteins were transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 and incubated with corresponding primary antibodies overnight at 4°C. Following incubation, membranes were washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Signals were visualized using enhanced chemiluminescence reagent (ECL; Beyotime Institute of Biotechnology) and captured by a chemiluminescence array (DNR, Beijing, China). The optical density (OD) was measured using Image J software. All antibodies used for Western blot analysis were purchased from Beyotime Institute of Biotechnology.

Transfection. MicroRNA-22 (5'-CTCAACCTGGTGTGCGTGGATTGACGACAGATTCT-3'), microRNA-22 inhibitor (5'-GCGAAACCATTTGCAAGAGAATG-3' and 5'-CATCACAGAAGCTTCTTG-3'), small interfering RNA (si)-p53 (5'-ctcgatctggttcgctgatttc-3' and 5'-ggcggctgtagctgccgctgaa-3') and negative controls (5'-CAATTTCAGTGGACAGCTTCT-3' and 5'-ACUGAGACGGUUCAAGATT-3') were synthesized by Guangzhou Ribobio Co., Ltd. (Guangzhou, China). The forward primer of microRNA-22 mimics was 5'-CTCAACCTGGTGTGCGTGGATTGACGACAGATTCT-3' and the reverse primer was 5'-CAATTTCAGTGGACAGCTTCT-3'; the forward primer of anti-microRNA-22 was 5'-UUCUUCCAAGUGUCACUGTT-3' and the reverse primer was 5'-ACUGAGACGGUUCAAGATT-3'. HEB cells were treated with lipopolysaccharide (LPS) and were seeded into 6-well plates at a density of 1.5-2.0x10⁵ cells/well. Subsequently, the cells were transfected with the oligonucleotides using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, the cells were transfected with microRNA-22, microRNA-22 inhibitor, small interfering (si)RNA-p53 and negative controls using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 4 h. After treatment for 4 h, old medium was removed and new Dulbecco's Modified Eagle's Medium was added at 37°C. HEB cells were treated with 50 ng/ml LPS (Beyotime Institute of Biotechnology) for 2 h.
Figure 1. MicroRNA-22 expression in SAH mice. *P<0.01 compared with normal mice. SAH, subarachnoid hemorrhage.

0.1% Tween (TBST) for 1 h at 37°C and were then incubated with the following primary antibodies: p53 (1:1,000), cysteine rich angiogenic inducer 61 (Cyr61; 1:1,000), B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax; 1:1,000) and GAPDH (5174) (all Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight. After four washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated immunoglobulin G secondary antibodies (1:5,000; 7074; Cell Signaling Technology, Inc.) for 45 min at 37°C, and blots were developed using Enhanced Chemiluminescence Plus reagent (Beyotime Institute of Biotechnology) and analyzed using Image-ProPlus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Caspase-3 activity assay. Proteins were extracted from tissue and cell samples using RIPA buffer at 4°C for 15-30 min. Protein content was measured using the BCA assay and 10 µg total protein was incubated with the caspase-3 assay kit (BioVision, Inc., Milpitas, CA, USA) for 1 h at 4°C. The absorbance was measured at 405 nm using a multi-well spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Statistical analysis. Data are presented as the means ± standard deviation (n=3) using SPSS 19.0. Data were analyzed by one-way analysis of variance followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

MicroRNA-22 expression in a mouse model of SAH. The present study demonstrated that microRNA-22 expression was significantly higher in SAH mice compared with in normal mice without SAH (Fig. 1). These results suggested that microRNA-22 expression may be associated with SAH.

Effects of microRNA-22 on IL-6 mRNA expression in HEB cells. The present study transfected microRNA-22 and microRNA-22 inhibitor plasmids into HEB cells, which were treated with LPS. Transfection with the microRNA-22 plasmid increased microRNA-22 expression, whereas the microRNA-22 inhibitor plasmid inhibited microRNA-22 expression in LPS-treated HEB cells compared with in the negative control group (Fig. 2A). Furthermore, microRNA-22 overexpression significantly inhibited IL-6 mRNA expression, whereas downregulation of microRNA-22 significantly increased IL-6 mRNA expression in LPS-treated HEB cells compared with in the negative control group (Fig. 2B).

Effects of microRNA-22 on caspase-3 activity in HEB cells. In order to analyze the apoptotic mechanism in the present study, caspase-3 activity was detected. As presented in Fig. 4, overexpression of microRNA-22 significantly increased caspase-3 activity, whereas downregulation of microRNA-22 significantly suppressed caspase-3 activity compared with in the negative control group.

p53 inhibitor suppresses p53 protein expression and induces IL-6 mRNA expression in SAH mice. To analyze apoptosis, a p53 inhibitor was used to suppress p53 protein expression in SAH mice. Pifithrin-α was able to suppress the protein expression levels of p53 and increase IL-6 mRNA expression in SAH mice compared with in the control group (Fig. 5).

p53 inhibitor suppresses microRNA-22 expression in SAH mice. The present study explored whether suppression of p53 expression may affect microRNA-22 expression in SAH mice. Pifithrin-α significantly suppressed microRNA-22 expression in SAH mice compared with in the control group (Fig. 6).

p53 inhibitor induces Cyr61 protein expression and suppresses Bax protein expression in SAH mice. The present study aimed to determine whether p53 regulates the Cyr61 intrinsic pathway in SAH mice. As shown in Fig. 7, suppression of p53 significantly enhanced Cyr61 protein expression and suppressed Bax protein expression in SAH mice compared with in the control group.

p53 inhibitor suppresses caspase-3 activity in SAH mice. To explore whether p53/microRNA-22 affects the apoptotic mechanism in SAH mice, caspase-3 activity was measured using a commercial kit. As shown in Fig. 8, caspase-3 activity was significantly reduced by suppression of p53 expression in SAH mice compared with in the control group.

Knockdown of p53 suppresses p53 protein expression and increases IL-6 mRNA expression in HEB cells. In an in vitro model, si-p53 was transfected into LPS-treated HEB cells to suppress p53 protein expression. As shown in Fig. 9A, si-p53 significantly suppressed p53 protein expression in HEB cells treated with LPS compared with in the negative control group. Furthermore, knockdown of p53 significantly increased IL-6 mRNA expression in HEB cells treated with LPS compared with in the negative control group (Fig. 9B).

Knockdown of p53 suppresses microRNA-22 expression in HEB cells. Using an in vitro model, the present study explored whether p53 knockdown affects microRNA-22 expression. Knockdown of p53 significantly inhibited microRNA-22 expression in HEB cells treated with LPS compared with in the negative control group (Fig. 10).
Knockdown of p53 increases Cyr61 protein expression and suppresses Bax expression in HEB cells. The present study evaluated whether p53 knockdown affects Cyr61 protein expression in HEB cells treated with LPS. As presented in Fig. 11, p53 knockdown significantly increased Cyr61 protein expression and suppressed Bax expression in LPS-treated HEB cells compared with the negative control group.

**Discussion**

SAH is characterized by bleeding into the subarachnoid space following the rupture of cerebral vessels or superficial cerebral vessels (17). The occurrence rate of SAH is low; however, it is associated with severe symptoms. According to statistics, ~12.4% patients directly succumb to SAH (2). In addition, 40-60% patients succumb within 48 h as a result of a second hemorrhage (18). Patients with SAH are often accompanied with severe neurological symptoms and cognitive impairment. Through examining the brain tissue of patients following SAH-induced mortality, severe ischemic injuries are commonly detected (2). The occurrence of these symptoms is associated with EBI and CVS following SAH. EBI not only induces fatal injury to patients, including hydrocephalus, but is also closely associated with sequelae (19). The present study indicated that microRNA-22 expression was...
upregulated in SAH mice. Therefore, targeting microRNA-22 expression may be considered a therapeutic approach for SAH.

It is well known that numerous proteins, including p53, Bcl-2 and caspases, are involved in vascular endothelial cell apoptosis in CVS; all of these proteins serve important roles in vascular endothelial cell and neuron apoptosis post-SAH (20). The present study demonstrated that downregulation of microRNA-22 in HEB cells increased IL-6 mRNA expression, induced Cyr61 expression, and suppressed Bax protein expression and caspase-3 activity. Zhang et al reported that microRNA-22 suppressed the growth of renal cell carcinoma cells via p53 (21). Therefore, the present study hypothesized that microRNA-22-mediated apoptosis may be mediated by targeting proapoptotic genes, including p53. It was hypothesized in the present study that microRNA-22 induced apoptosis through p53 in subarachnoid hemorrhage.

Following SAH, intracellular p53 is activated under the action of numerous factors, including hypoxia. Activated p53 is able to upregulate the target gene Bax, inhibit the expression of Bcl-2, and thus promote cell apoptosis (9). In a dog model of cerebellomedullary cistern CVS, CVS is induced by introducing blood twice into the cerebellomedullary cistern; the expression levels of p53 and cytochrome c (cyto C) are markedly increased in rat basilar artery endothelial cells following SAH, and apoptosis can be detected (22). p53 is an important tumor inhibiting factor that serves numerous roles in inhibiting cell cycle progression, promoting genome repair and inducing cell apoptosis (23). Following SAH, p53 is activated under the action of several factors, including hypoxia, and thus exerts its proapoptotic function on vascular endothelial cells and neurons in the brain through its target Bcl-2 and caspase family proteins, this results in the generation of EBI and vasospasm post-SAH (22). In the present study, the p53 inhibitor, pifithrin-α, suppressed p53 protein expression and increased IL-6 mRNA expression, decreased microRNA-22 expression and suppressed Bax protein expression in SAH mice.
Caspase-3 has been verified to serve an essential role in p53-mediated endothelial cell apoptosis after SAH (24). In bovine cerebral microvascular endothelial cells, oxyhemoglobin increased the expression of caspase-8, caspase-9, caspase-2 and caspase-3 in endothelial cells, and apoptotic cells could be detected (25). In addition, it has been reported that a caspase-3 inhibitor can effectively prevent CVS (26). Taken together, the present study indicated that the p53 inhibitor, pifithrin-α, suppressed caspase-3 activity and induced Cyr61 expression in SAH mice.

The Bcl-2 family is particularly important for p53-mediated endothelial cell apoptosis and the generation of CVS after SAH. p53 acts on the mitochondria via the Bcl-2 family proteins, thus leading to apoptosis. The Bcl-2 family is well known to participate in cell apoptosis (20). The Bcl-2 family comprises four homologous peptides, which are known as Bcl-2 homeodomains (BHs). All of the family members possess one of the four BHs (BH1-BH4) as the homologous region. The Bcl-2 protein family can be divided into two categories: i) The anti-apoptotic family, which consists of Bcl-2, Bcl-extra large and Bcl-w; and ii) the proapoptotic family, which can be further divided into two types dependent on structure: The first type is the Bax family, which is composed of Bax and BOK, Bcl-2 family apoptosis regulator; the other type is the BH3 protein family, which includes p53 upregulated modulator of apoptosis, NOXA, Bcl-2 interacting killer, BLK proto-oncogene, Src family tyrosine kinase, Bcl-2-associated agonist of cell death, Bcl-2 antagonist/killer 1 and BH3 interacting domain death agonist (8,20). High expression of intracellular Bax induces the release of cyto C by the mitochondria, which gives rise to the activation of caspase-9 and -3 in succession, thus inducing apoptosis. In addition, activated BH3-type proteins can induce or promote the activation of Bax, thus indirectly inducing the release of cyto C (24). Among the Bcl-2 family, the relative Bax/Bcl-2 ratio may serve a key role in determining cell survival or death (24). In the present study,
p53 expression was knocked down using si-p53; transfection with si-p53 suppressed microRNA-22 expression and Bax protein expression in HEB cells. These results suggest that p53/microRNA-22 may regulate inflammation and apoptosis and may be considered a therapeutic target for the treatment of SAH.

In recent years, increasing importance has been attached to the role of the immunoinflammatory response in the pathological mechanism underlying vasospasm. Clinical research has provided a large amount of evidence to demonstrate that a series of inflammatory factors, including adhesion molecules, cytokines, granulocytes, immunoglobulin and complement, which may serve important roles in the pathogenesis of CVS (27). Inflammatory factors, including adhesion molecules such as intercellular adhesion molecule-1 and nuclear factor-kB, and cytokines such as IL-1β, IL-6 and IL-8, have been reported to be associated with the pathogenesis of vasospasm; IL-6 in particular is markedly increased in the cerebrospinal fluid following SAH, thus indicating its important role in CVS (28). The results of the present study indicated that downregulated p53/microRNA-22 expression increased IL-6 mRNA expression, inhibited caspase-3 activity and induced Cyrf6 expression in HEB cells. Lin et al. revealed that the downregulation of microRNA-22 increased inflammation in rheumatoid arthritis (29). The results of the present study indicated that microRNA-22 promoted IL-6 mRNA expression and Cyrf6 protein expression to induce HEB cell apoptosis. Therefore, it may be hypothesized that p53/microRNA-22 regulates Cyrf6 and affects SAH-induced inflammation and apoptosis.

In conclusion, the present study revealed that the neuroprotective effects of p53/microRNA-22 are associated with regulation of IL-6 mRNA expression and the caspase-3/Bax signaling pathway in SAH. These results support the perspective that p53/microRNA-22 may be a rational therapeutic strategy for the clinical treatment of SAH.

Competing interests

The authors declare that they have no competing interests.

References


