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Effects of miR-153 on angiogenesis in MCAO rats through Shh signaling pathway

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Abstract. – OBJECTIVE: Cerebrovascular disease is a disease which has the highest mortality in China. Angiogenesis in the ischemic region after cerebral infarction is closely related to its prognosis. Recent studies found that microRNAs (miRNAs) are involved in the regulation of neovascularization. MicroRNA-153 (MiR-153) has protective effects on the ischemic injury, but its relationship with the Sonic Hedgehog (Shh) signaling pathway is still unclear. This work aimed to investigate the role of miR-153 in angiogenesis of middle cerebral artery occlusion (MCAO) rats through the Shh signaling pathway.

MATERIALS AND METHODS: The rat cerebral ischemic injury (MCAO) model was established by thread embolism and treated by Agomir-153 and 5-EI. MiR-153 expression was detected using Real Time-Polymerase Chain Reaction (RT-PCR). The neurological function was assessed. The infarct area of the brain and the capillary density were determined using 2,3,5-triphenyl tetrazolium chloride (TTC) method. The Shh signaling pathway and angiogenesis-related factors were tested by Western blot assay.

RESULTS: Agomir-153 or Agomir-153 combined with 5-EI significantly increased miR-153 expression, reduced the infarct area, and promoted the generation of cerebral capillaries in the MCAO model. 5-EI partially blocked the protective effects of Agomir-153 and angiogenesis. The up-regulation of miR-153 markedly inhibited patched (PTC) expression and activated the Shh signaling pathway.

CONCLUSIONS: The up-regulation of miR-153 rats activated the Shh signaling pathway to promote angiogenesis and improve prognosis through lipid-coated Patch (PTC) in MCAO. MiR-153 was considered to be a new therapeutic target for promoting angiogenesis after MCAO.

Key Words:

MiRNA, Shh signaling pathway, MCAO rat, Angiogenesis.

Introduction

Cerebrovascular disease is a disease which has the highest mortality and morbidity in China[1]. Mortality and prognosis of patients with cerebral infarction are closely related to neovascularization in the ischemic region. Effectively promoting angiogenesis after cerebral infarction can directly restore the supply of oxygen and nutrients in damaged brain tissues. Therefore, facilitating angiogenesis in the ischemic region after cerebral infarction has been considered one of the most promising ways to treat cerebral infarction[2]. Previous studies found that after the establishment of a cerebral infarction model (MCAO) in rats via thread embolism method, there is spontaneous angiogenesis in the cerebral infarct area. The lumen forms on 3 days and up to 7-14 days[3]. Recent studies showed that the synergistic effects of angiogenin (ANG) and vascular endothelial growth factor (VEGF) are important in vascular angiogenesis and maturation. Among them, ANG-2 and VEGF act synergistically to promote angiogenesis, while ANG-1 significantly increases endothelial cell survival, enhances neovascular closure and stability, and promotes neovascularization[4]. Shh is a secreted protein that is a member of the hedgehog family. By binding to the transmembrane protein lipid-coated patch (PTC), Shh promotes the release and activation of Smoothened (Smo), and stimulates the downstream zinc finger family transcription factor Gli transfer from outside to the inside of the nucleus. Internally, it binds with DNA and induces the transcription of the target gene. In the neurogenic tumor, the Shh signaling pathway plays an important role in promoting stromal and angiogenesis, and promotes vascular proliferation in developing embryos, ischemic corneas and limbs of aged

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rats[5]. As an extremely potent angiogenesis factor, the Shh signaling regulates VEGF signaling molecules and ANG-1/ANG-2 levels[6]. At present, the regulation of the Shh signaling pathway is still unclear. MicroRNAs (miRNAs) are a class of non-coding RNAs with regulatory functions that bind specifically to the 3'-UTR of mRNA to form silencing complexes, therefore inhibiting mRNA translation or promoting mRNA degradation. It was found that circulating miRNAs can be stably presented in cerebrospinal fluid or blood and affects angiogenesis in physiological or pathological states through the regulation of angiogenic key signaling molecules[7]. Among them, we found that miR-153 may target the Shh signaling pathway to affect angiogenesis in MCAO rats through miRanda, TargetScan and PicTar database matching. This work aimed to investigate the role of miR-153 in angiogenesis of MCAO rats through the Shh signaling pathway.

Materials and Methods

Main Materials and Reagents

Rabbit anti-rat PTC, Shh, VEGF, ANG-1, and ANG-2 polyclonal antibodies were purchased from Abcam (Cambridge, MA, USA). TaKaRa TRIzol total RNA extract was purchased from Sigma-Aldrich (St. Louis, MO, USA). Agomir-miRNA-153 was purchased from Bio-Engineering Co., Ltd. (Dalian, China). Chloroform, isopropyl alcohol, and anhydrous alcohol were purchased from Shanghai Reagent No. 1 Plant (Shanghai, China). Phosphate buffer was purchased from Boster (Wuhan, China). Enhanced chemiluminescence (ECL) luminescent kit was purchased from Beyotime Biotechnology (Shanghai, China). Bicinchoninic acid (BCA) protein kit, radioimmunoprecipitation assay (RIPA) total protein lysate, and cocktail protease inhibitor were purchased from KGI Biosciences Inc. (Shanghai, China).

Main Instruments

The CO_2 incubator was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The clean bench was purchased from Suzhou Purification Equipment Factory (Suzhou, China). Real Time-Polymerase Chain Reaction instrument was purchased from Applied Biosystems (Foster City, CA, USA). Vertical plate electrophoresis and electrophoresis apparatus were purchased from Bio-Rad (Hercules, CA, USA). The brain stereotaxic apparatus was supplied by Shenzhen Ruiwoode Life Co., Ltd. (Shenzhen, China). Ultra-low temperature refrigerator was purchased from NuAire (Plymouth, MN, USA). Fluorescence microscopy was purchased from Leica (Frankfurt, Germany).

Experimental Animals

6-8 week old female Sprague Dawley (SD) rats weighed between 180 and 220 g were purchased from the Experimental Animal Center of the Zhejiang University. The rats were housed in a clean animal room with room temperature at 24°C, the relative humidity at 60%, and 12 h day/night cycle. The litter was changed every day to avoid infection. Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of The First Affiliated Hospital of the Jiaxing University (Zhejiang China) Jiaxing, China.

Grouping

The rats were divided into four groups, including the sham group, MCAO model group, Agomir-153 group, and Agomir-153 and Shh signaling pathway inhibitor 5-EI combination group, with 10 rats in each group.

MCAO Model Establishment

The rat permanent middle cerebral artery occlusion model was prepared using the Longa line plug method[8]. After inhalational anesthesia, the rat was fixed on the operating table in a supine position. The soft tissue was bluntly dissected through the midline neck incision. The common carotid artery and vagus nerve were carefully separated and the common carotid artery was ligated. A small opening of 1/3 of the diameter was punctured at distance of 2.5-3 mm from the bifurcation of the common carotid artery. Polythiolysine-coated tether was inserted into the internal carotid artery. Next, the incision was stitched and sterilized. Postoperative rectal temperature, respiration and heart rate were monitored. Rats after MCAO were placed in a separate squirrel cage. After MCAO rats were awake, neurological evaluation was performed. In the sham group, the insertion depth of the sling line was slightly shallower than 8-10 mm. The Agomir-153 group and the Agomir-153 and Shh signaling pathway inhibitor 5-EI combination group were administered with Agomir-153 or Shh signaling pathway inhibitor 5-EI by tail vein injection at a concentration of 5 μg/ml/100 g, respectively.

Rat Neurological Function Score

According to Longa's 5-point scoring method[9], scoring was started from the time when the MCAO rat first recovered completely. 0 point, normal without neurological deficit; 1 point, unilateral forelimb cannot be straightened after rising; 2 points, the body tilted to one side when the rat was crawling forward; 3 points, the rat's crawling body fell to the side; 4 points, coma or cannot crawl spontaneously.

Cerebral Infarction Area Determined by TTC Method

The MCAO rats were anesthetized at 24 h after the successful administration and the brains were decapitated. Next, the sample was continuously coronally cut to 2 mm thick slices and stained with 2% 2,3,5-Triphenyltetrazolium chloride (TTC) in the dark for 30 min at 37°C. After being fixed in 4% paraformaldehyde solution at 4°C for 2 h, the section was photographed. The ischemic region of the cerebral infarction was white, while the normal brain tissue was red or purple. Therefore, the cerebral infarct area can be visually observed.

Capillary Density Assessment

A total of 0.2 ml of FITC-labeled dextran was injected intravenously 10 min before the rats were sacrificed; then, the brain tissue was used to prepare frozen brain section. At last, the sample was observed and photographed under a fluorescence microscope.

Total Protein Extraction

The brain tissue of each group was washed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA). Next, the lysate was added with 10 μl of 100 mM phenyl methane sulfonyl fluoride (PMSF). After mechanical homogenization, the sample was lysed on ice for 5-10 min and centrifuged at 4°C and 12000 r/min for 5 min.

Western Blot

The Western blot method was based on literature[10]. The extracted total protein solution was quantified by bicinchoninic acid (BCA) and then calibrated to a uniform concentration. After adding the buffer, the sample was placed in boiling water for 5 min to denature. Samples were separated by using electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane at 300 mA for 1 h. Next, the membrane was incubated in rabbit anti-rat PTC, Shh, VEGF, ANG-1, and ANG-2 polyclonal antibodies (1:1000) overnight at 4°C. Then, the membrane was incubated with the corresponding secondary antibody (1:1000) at 37°C for 2 h. The bands were visualized by chemiluminescence.

Real Time-Polymerase Chain Reaction

The brain tissue was added with TRIzol, homogenized, and placed on ice for 5 min. Then the sample was moved into a 1.5 ml Eppendorf (EP; Eppendorf, Hamburg, Germany) tube and added with 200 μl of chloroform. After shaken vigorously for 15 s, the sample was set at room temperature for 3 min. After centrifuged at 4°C and 12000 ×g for 15 min, the upper aqueous phase was carefully pipetted into a new Eppendorf tube. Next, the sample was added with 500 μl of isopropanol and set at room temperature for 10 min. After being centrifuged at 4°C and 12000×g for 10 min, the supernatant was removed and the sample was washed three times with 1 ml of ethanol. Finally, the supernatant was carefully removed and added with 20 μl of diethyl pyrocarbonate (DEPC) water to obtain mRNA. The primers for miR-53 were designed and synthesized by Sigma-Aldrich (St. Louis, MO, USA). The primer sequence was: forward 5'-TTGAATTCTAACACCTTCGTGGCTACAGAG-3', reverse 5'-TTAGATCTCATTTATCGAGGGAAGGATTG-3'. U6 was selected as the loading control, forward, 5'-CTCGCTTCGGCAGCACA-3', reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The PCR reaction was performed in a 50 μl system according to manual. The reaction procedure contained 50°C for 30 min, 95°C 5 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C 50 s. The Real Time-Polymerase Chain Reaction amplification curve and dissolution curve were confirmed to calculate the relative expression level by comparing the cycle threshold (Ct) value of the target gene with the Ct value of the reference gene through the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

SPSS 19.0 software (SPSS, Armonk, NY, USA) was used to perform the data analysis. Measurement data were presented as mean ± standard deviation (SD) and tested for normality and homogeneity of variance. For the variable with a normal distribution, the Student's t-test was used to compare the differences between the two groups

and the Tukey's Post-Hoc test was used to validate the one-way analysis of variance (ANOVA) for comparing measurement data among groups. $p<0.05$ was considered statistically significant.

Results

MiR-153 Expression Changes

First, we used Real Time-Polymerase Chain Reaction to detect miR-153 expression in brain tissue (Figure 1). Compared with the sham group, miR-153 expression was significantly lower in the model group ($p<0.05$). MiR-153 agomir or combining with 5-EI treatment markedly elevated miR-153 expression ($p<0.05$).

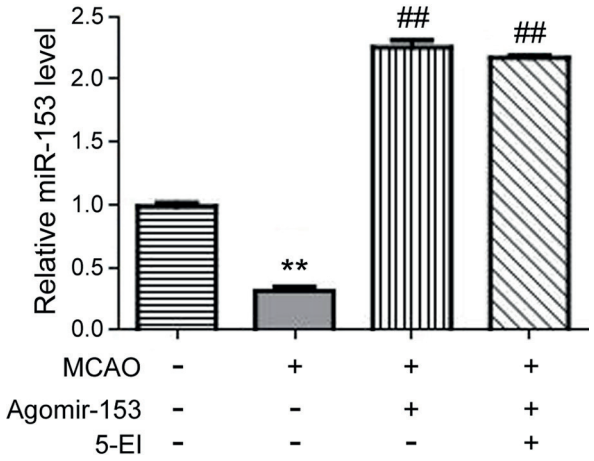

Figure 1. MiR-153 expression changes. $^{**}p<0.05$, compared with the sham group. $^{\#\#}p<0.05$, compared with the model group.

Shh Signaling Pathway Protein Expression

MiRanda, TargetScan, and PicTar prediction found that PTC may be the target of miR-153. Thus, we examined the expression levels of PTC and Shh (Figure 2). PTC level was significantly increased in the model group ($p<0.05$). MiR-153 agomir or combining with 5-EI treatment markedly inhibited the expression level of PTC compared with the model group ($p<0.05$). However, the expression level of Shh showed a completely opposite change from the PTC expression. The results suggested that the up-regulation of miR-153 expression remarkably suppressed PTC expression and activated the Shh signaling pathway.

Cerebral Infarction Area

TTC staining was adopted to observe the cerebral infarct area (Figure 3). The white area in the figure exhibited the infarcted brain area. It was observed that in the sham group, there was almost no infarct. However, in the model group, a large number of infarctions appeared in the left hemisphere. MiR-153 agomir or combining with 5-EI treatment apparently reduced the area of cerebral infarction. In addition, 5-EI can block the protective effect of agomiR-153 on cerebral infarction.

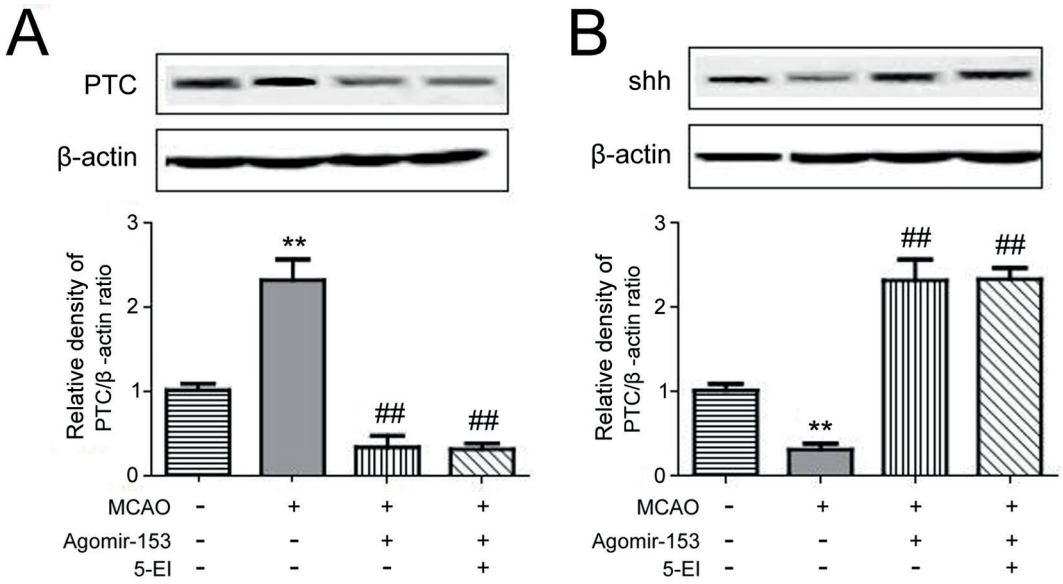

Figure 2. PTC and Shh signaling pathway protein expression. $^{**}p<0.05$, compared with the sham group. $^{\#\#}p<0.05$, compared with the model group.

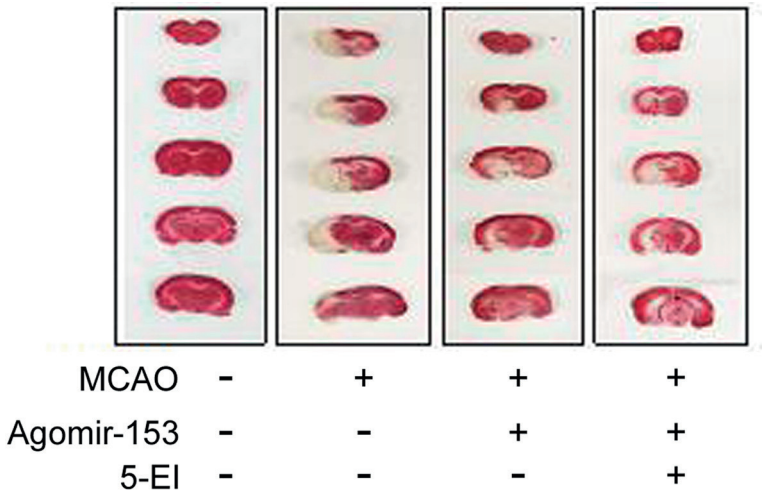

Figure 3. Cerebral infarction area.

Neurological Score

The neurological function scores for each group of rats were shown in Figure 4. In the model group, the neurological scores were significantly increased compared with the sham group. MiR-153 agomir or combining with 5-EI treatment markedly reduced the neurological score compared with the model group.

Capillary Density Evaluation

Capillary density in rat brain was examined by injection of FITC-labeled dextran (Figure 5). The number of neovascularization in the MCAO model group was remarkably decreased compared with the sham group. AgomiR-153 intervention significantly elevated the number of neovascularization, indicating that the up-regulation of miR-153 level can promote angiogenesis. The combined application of agomir-153 and 5-EI apparently restrained the number of capillaries compared to the agomiR-153 group, suggesting that the promotion role of miR-153 on angiogenesis was achieved through the activation of the Shh signaling pathway.

Shh Signaling Pathway Related Protein Expression

VEGF, ANG-1, and ANG-2 were important proangiogenic factors regulated by the Shh signaling pathway. Compared with the sham group, the expression levels of VEGF, ANG-1, and ANG-2 in the model group were significantly lower. MiR-153 agomir or combining with 5-EI treatment up-regulated VEGF, ANG-1, and ANG-2 levels in the brain (Figure 6).

Discussion

The death of patients caused by cerebrovascular disease exceeds that of the other diseases. Even if the patients survive, they often lose the ability to work, leading to a heavy burden on families and society[11]. When the supply of cerebral blood flow is disturbed, the affected neurons will die quickly. However, due to the clear division of neurons in the brain, surviving neurons cannot replace the function of dead neurons. Therefore, a deep understanding of the regulatory mechanism of angiogenesis after cerebral infarction and restoration of cerebral blood flow as soon as possible is of great significance for the protection and rescue of damaged neurons in cerebrova-

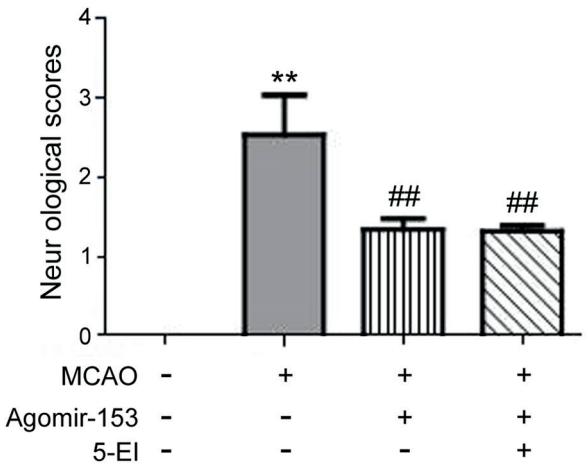

Figure 4. Neurological score. $^{**}p<0.05$, compared with the sham group. $^{\#\#}p<0.05$, compared with the model group.

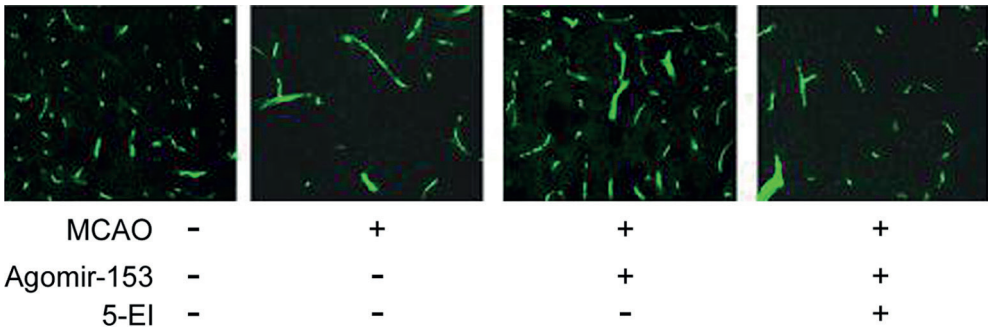

Figure 5. Capillary density evaluation.

scular diseases[12]. In this work, we investigated the role of miR-153 in angiogenesis through the Shh signaling pathway in MCAO rats. It is found that miR-153 may promote the activation of the Shh signaling by targeting PTC, which facilitates neovascularization in MCAO rats. Secretory protein Sonic Hedgehog (Shh), a member of the Hedgehog family, promotes the proliferation of stroma and blood vessels in tumors, accelerates vascular proliferation in ischemic corneas, and develops embryos and limbs in aged rats[13]. It was considered that Shh-induced limb and corneal neovascularization may not only rely on VEGF, but also include ANG-1 and ANG-2[14,15]. During angiogenesis, the regulation of angiogenic factors, such as VEGF and ANG-1 secretion by astrocytes, is an essential factor. In this study, we observed that in the MCAO rat model, Shh signal and the expression levels of VEGF, ANG-1 and ANG-2 were significantly inhibited. At the same time, there was a positive correlation between the activation of Shh signal and the density of microvessels generated in the brain, suggesting that Shh signaling pathway has an important role in angiogenesis. MiRNAs are a class of non-coding RNAs with regulatory functions. Recent studies showed that changes in miRNA expression levels are involved in the pathogenesis of cerebral ischemia[16]. In cerebral ischemic disease or experimental cerebral ischemia model, a large number of miRNAs have been reported to be differentially expressed[17]. For example, it was reported[18] that miR-424 protects against microglial activation in a permanent ischemic injury mouse model. MiR-376 was found to promote angiogenesis in rat cerebral ischemic injury induced by ischemia-reperfusion[19]. It was also suggested that miRNAs can affect neural production in cerebral ischemic tissue, which provides a sufficient theoretical basis for miRNA regulation in cerebral ischemia[20]. In ischemic heart disease, the role of miR153 was found in screening for miRNAs illustrating the diagnostic value[21]. In the cultured hippocampal neurons, the protective effects of the up-regulation of miR-153 on hypoxia were also found[22,23]. According to targetscan databases, it was found that miR-153 may effectively regulate the PTC, which is a transmembrane protein that

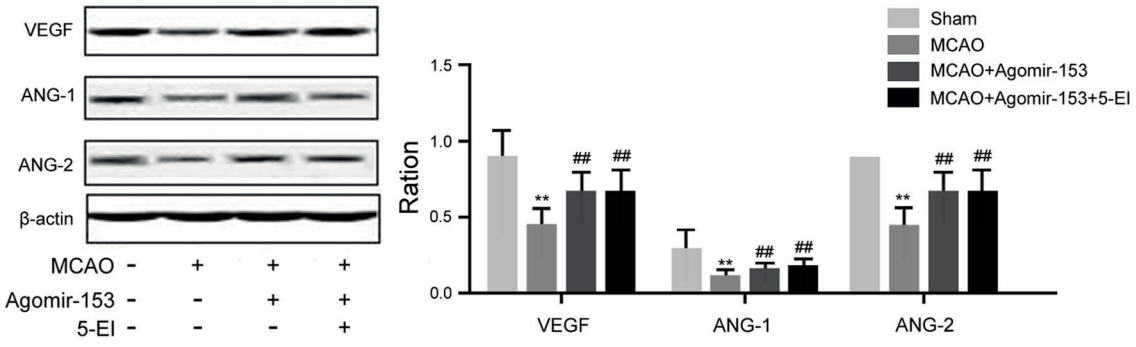

Figure 6. VEGF, ANG-1, and ANG-2 expression changes. $^{**}p<0.05$, compared with the sham group. $^{\#\#}p<0.05$, compared with the model group.

participates in the Shh signaling pathway. When Shh binds with PTC, it can relieve Smo and activate Shh signal. In this work, the miR-153 level in the MCAO model was found to be markedly reduced. The application of agomir to up-regulate the level of miR-153 can activate the Shh signaling pathway and promote angiogenesis to play a protective role. We observed significant down-regulation of miR-153 and up-regulation of PTC in the MCAO model. The use of agomiR-153 up-regulated miR-153 levels and inhibited PTC expression. It suggested that miR-153 up-regulation may activate the Shh signaling pathway by inhibiting PTC expression. For the first time, it indicated the protective role of miR-153 in ischemic brain diseases and possible targets, which provided new ideas and theoretical basis for the research on the role of miRNA in cerebral ischemic diseases. However, this study also has a few limitations, such as miR-153 targeting PTC should be verified at the cellular level using the Luciferase reporter gene method. We showed the role of miR-153 in regulating Shh signaling and angiogenesis, provided a theoretical basis for the role of miRNA in cerebral ischemic injury. Therefore, miRNA may be used as a potential treatment target for ischemic brain injury.

Conclusions

We found that the up-regulation of miR-153 rats activated the Shh signaling pathway to promote angiogenesis and improve prognosis through lipid-coated PTC in MCAO. MiR-153 was considered to be a new therapeutic target for promoting angiogenesis after MCAO.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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