MICRORNA DYSREGULATION CONTRIBUTES TO BRAIN MICROVASCULAR INJURY, VASCULAR REGENERATION, AND INFLAMMATORY RESPONSE BY TARGETING P38/MAPK

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ABSTRACT

Objective: *To investigate how microRNA dysregulation contributes to brain microvascular injury, vascular regeneration, and inflammatory response by targeting p38/MAPK.*

Methods: *Brain microvascular endothelial Bend3 cells were selected for cell culture and various treatments. We obtained miRNA-451-overexpressing Bend3 cells (the miRNA-451 overexpression group), miRNA-451 control cells (the miRNA-451 control group), stable knockout miRNA-451 Bend3 cells (the miRNA-451 interference group), and miRNA-451 interference control cells (the miRNA-451 interference control group). The changes in cell proliferation, migration, and angiogenesis were compared between groups. The expression levels of PI3K, Akt, Erk, p38/MAPK, and MIF in each group of cells were compared. We randomly selected 60 SPF male C57BL/6 mice; these were divided into a sham operation group (stereotactic injection of 2 μL phosphate buffer into the brain), an miRNA-451 group (3μL miRNA-451 lentiviral solution injected into the brain stereotactically), and an miRNA-451 interference group (stereotactic injection of 2 μL miRNA-451 interference lentiviral fluid into the brain), with 20 mice in each group. We observed for neuron damage in the mouse hippocampus, the expression of blood-brain barrier-related protein ZO-1, and changes in inflammatory factors in the hippocampus of mice in each group. The expression changes of p38/mitogen-activated protein kinase (p38/MAPK) in each group of mice were compared.*

Results: *Compared with the miRNA-451 control group and the miRNA-451 interference control group, there was no significant difference in the cell proliferation capacity of the miRNA-451 overexpression group and the miRNA-451 interference group at 12 hours. At 24h and 36h, the cell proliferation ability of the miRNA-451 overexpression group was significantly lower, while the cell proliferation ability of the miRNA-451 interference group was significantly increased ($P<0.05$). Compared with the miRNA-451 control group, the miRNA-451 overexpression group showed significantly reduced cell clone formation ability, cell migration ability, angiogenesis ability, p38/MAPK expression levels, and MIF expression levels ($P<0.05$). Compared with the miRNA-451 interference control group, the miRNA-451 interference group showed significantly higher cell formation ability, cell migration, angiogenesis, p38/MAPK expression levels, and MIF expression ($P<0.05$). In the sham operation group, the neuron cells were closely packed and uniformly distributed; the intercellular space was normal, with obvious Nissl bodies. Neuronal cells in the miRNA-451 overexpression group were similar to those in the sham operation group. In the miRNA-451 interference group, the neuronal cells were arranged loosely and in a disorderly manner; the neuron cell body showed irregularity, and the intercellular space increased significantly. Compared with the sham operation group and the miRNA-451 overexpression group, the expression levels of the blood-brain barrier-related protein ZO-1, inflammatory factors, and p38/MAPK in the miRNA-451 interference group were significantly higher ($P<0.05$).*

Conclusions: *MicroRNA may contribute to brain microvascular injury, vascular regeneration, and the inflammatory response by targeting p38/MAPK.*

Keywords: *miRNA-451/MIF, p38/MAPK, brain microvascular injury, vascular regeneration, inflammatory response.*

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Introduction

Cerebrovascular disease refers to various diseases of the blood vessels in the brain; these can cause transient or long-lasting local or diffuse brain damage and ultimately lead to disability or death in patients. Cerebrovascular disease manifests primarily as headache, dizziness, disturbance of consciousness, aphasia, and hemiplegia. The integrity of the structure and function of cerebral blood vessels plays an important role in normal brain function. The impairment of the structure and function of the cerebral microvasculature may be involved in the occurrence of degenerative lesions[1].

Studies have found that cerebrovascular disease is a major risk factor for Parkinson's disease[2]. According to relevant statistics, 20–50% of Parkinson's patients have microvascular lesions, obvious microvascular structure destruction, thickening of the basement membrane, and keratin deposition[3]. Among early-stage Parkinson's patients, the normal structure between neurons and capillaries in the brain is reportedly damaged, and the structure and function of the blood-brain barrier is damaged.

This allows immune cells in peripheral blood to cross the blood-brain barrier and further destroy it, causing changes in the cerebrospinal fluid and in the central nervous system's inflammatory response[4]. MicroRNA (miRNA) is an endogenous, non-coding, small single-stranded RNA that is widely found in eukaryotic cells. It is abundantly expressed in the nervous system, demonstrates specific, time-sensitive, and regional characteristics, and has important relations with brain tissue development and neuronal differentiation[5]. Macrophage migration inhibitory factor (MIF) is an endocrine immune substance that can limit the activity of macrophages in the body and promote the formation of collateral circulation. Some studies have reported a negative regulatory relationship between miRNA-451 and MIF. miRNA-451/MIF is involved in the occurrence of microvascular injury, vascular regeneration, and inflammatory response, although its mechanism of action is not clear[6]. In this paper, we explore the role of miRNA-451/MIF in microvascular injury, vascular regeneration, and inflammation.

Materials and methods

Experimental animals and experimental cells

Experimental cells

brain microvascular endothelial cells (Bend3).

Experimental animals

80 SPF male C57BL/6 mice were randomly selected, aged 9 weeks and weighing (23.45±2.16) g. They were purchased from the Guangdong Medical Experimental Animal Center (production license: SCXK [Guangdong] 2017-0029).

Laboratory instruments and reagents

DMEM medium was provided by Shanghai Limin Industrial Co., Ltd. Fetal bovine serum was purchased from Shanghai Laichuang Biotechnology Co., Ltd. The thermostatic water bath was obtained from the Changzhou Jintan Youlian Instrument Research Institute (model: HH-600). The flow cytometer was purchased from Beckman Coulter Co., Ltd. (Model: CytoFLEX). The low-temperature, high-speed centrifuge was provided by Shanghai Shun Instrument Manufacturing Co., Ltd. (model: TG20KR-D). The ultra-low temperature refrigerator was procured from Beijing Shengke Xinde Technology Co., Ltd. (model: DW-86L728J). The biological microscope was purchased from Shenzhen Honghui Network Technology Co., Ltd. (model: LIOO S600T). The miniature handheld cranial drill was obtained from Shenzhen Ruiwode Life Technology Co., Ltd. (model: 78001).

Experimental methods and observation indicators

Cell culture

Bend3 cells were cultured in DMEM cell culture medium containing 5% fetal bovine serum and 5% CO_2 at 37°C. When the cells were cultured to about 85% culture medium saturation, they were subcultured. During the cell culture process, we were attentive to changing the cell culture medium and set up three groups in each group.

Lentivirus infection

5 μL of miRNA-451 lentivirus solution, miRNA-451 interfering lentivirus solution, and 5 μg/mL polybrene were added to 400 μL of serum-free culture solution. We aspirated the cell culture medium, added the diluted virus solution, and added 72 μg/mL puromycin after lentivirus infection. miRNA-451-overexpressing Bend3 cells (the miRNA-451 overexpression group), miRNA-451 control cells (the miRNA-451 control group), stable knockout miRNA-451 Bend3 cells (the miRNA-451 interference group), and miRNA-451 interference control cells (the miRNA-451 interference control group) were obtained after approximately 2 weeks.

Cell proliferation ability

The CCK-8 method was used to determine the cell proliferation ability of each group. The cultured cells were placed in a 24-well plate at 10,000 cells/well and cultured for 12h, 24h, and 36h, respectively; 10 μL CCK-8 solution was added to each well. The absorbance at 470 nm was measured.

Clone formation experiments

Bend3 cells subjected to the various treatments

were adjusted for cell concentration. They were inoculated in 6-well plates and cultured in an incubator. After 2 weeks of normal culture, they were stained with crystal violet solution, washed, and dried; the number of cell clones was observed with a microscope.

Transwell chamber experiments were used to determine cell migration capacity

Bend3 cells subjected to various treatments were made into cell suspensions. The upper chamber of the Transwell chamber was inoculated with 300 μL of resuspended cell culture medium, while 600 μL of serum-containing DMEM medium was added to the lower chamber.

These were cultured at room temperature for 24 h and stained with a crystal violet staining solution; five fields were randomly selected for observation under a microscope.

Lumen formation experiment

50 μl of Matrigel stock solution was drawn into a 96-well plate to avoid air bubbles. Bend3 cells were developed into a cell suspension and counted. The cells were cultured at 37°C for 24 h, and the angiogenesis ability of each group of cells was observed. The expression levels of PI3K, Akt, Erk, p38/MAPK, and MIF in each group of cells were determined by Western blotting.

Establishment of animal models

After anesthetizing the mice, their heads were fixed and the skin on their heads was cleaned, prepared, and disinfected, exposing their anterior ridges. The mouse hippocampus was positioned, and a positioning needle was used 2 mm posterior to the anterior condyle and 2.5 mm adjacent to the sagittal suture. At this point, a skull drill was used to drill the skull. A 5-μL syringe was used to inhale the lentiviral solution into the mouse hippocampus; the bone window was then closed and the skin was sutured. Mice were divided into the sham operation group (stereotactic injection of 2 μL phosphate buffer into the brain), the miRNA-451 group (3μL miRNA-451 lentiviral solution injected into the brain stereotactically), and the miRNA-451 interference group (stereotactic injection of 2 μL miRNA-451 interference lentiviral fluid into the brain), with 20 mice in each group.

Brain tissue sections were prepared and stained via Nissl staining; neuron damage in the hippocampus of each group of mice was observed under a microscope. The expression of the blood-brain barrier-related protein ZO-1 in the hippocampus of each group was determined by immunohistochemistry.

The changes of expression level in tumor necrosis factor-α (TNF-α), Cyclooxygenase-2 (COX-2), and Nitric oxide synthetase (iNOS) in the hippocampus of each group were measured by real-time quantitative PCR.Western blot was used to determine the expression level of p38/Mitogen-activated protein kinase (p38/MAPK) in each group of mice.

Statistical methods

The comparison of count data in this group of studies was performed using χ^2 comparison. Count data were compared using independent sample t-tests between the two groups, while single-factor multi-sample means were used for comparisons between multiple groups. The CCK-8 method and clone formation experiments were used to determine changes in cell proliferation ability.

Transwell chamber experiments were used to determine changes in cell migration capacity. Lumen formation experiments were used to determine the angiogenic capacity of the cells in each group. The expression levels of PI3K, Akt, Erk, p38/MAPK, and MIF in each group of cells were determined by Western blotting.

The changes in inflammatory factors TNF-α, COX-2, and iNOS in hippocampus of each group of mice were measured by real-time quantitative PCR. In this group of studies, we used SPSS 18.0 software for statistical data analysis and considered a statistical result of $P<0.05$ to be a statistically significant difference.

Results

Comparison of cell proliferation in each group

Compared with the miRNA-451 control group and the miRNA-451 interference control group, there was no significant difference in the cell proliferation capacity of the miRNA-451 overexpression group or the miRNA-451 interference group at 12 hours. At 24h and 36h, the cell proliferation ability of the miRNA-451 overexpression group was significantly reduced, while the cell proliferation ability of the miRNA-451 interference group was significantly increased ($P<0.05$). The results are shown in Table 1.

group	Cell proliferation capacity		
	12h	24h	36h
miRNA-451 overexpression group	0.47±0.09	1.12±0.12	2.26±0.25
miRNA-451 control group	0.51±0.11	2.07±0.15	3.73±0.36
t	0.488	8.566	5.809
P	0.651	0.001	0.004

group	Cell proliferation capacity		
	12h	24h	36h
miRNA-451 interference group	0.49±0.07	1.31±0.12	3.19±0.21
miRNA-451 interference control group	0.45±0.04	1.04±0.09	2.73±0.14
t	0.859	3.118	3.157
P	0.439	0.036	0.034

Table 1: Comparison of cell proliferation in each group ($\bar{x}\pm s$).

Comparison of results from the clone formation experiments

Compared with the miRNA-451 control group, the cell cloning ability of the miRNA-451 overexpression group was significantly reduced (P<0.05); compared with the miRNA-451 interference control group, the cell clone formation ability of the miRNA-451 interference group was significantly enhanced (P<0.05). The results are shown in Figure 1.

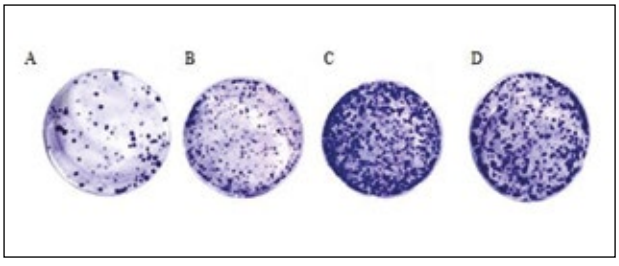

Figure 1: Comparison of results of clone formation experiments.
A: miRNA-451 overexpression group; B: miRNA-451 control group; C: miRNA-451 interference group; D: miRNA-451 interference control group.

Comparison of the cell migration ability of each group

Compared with the miRNA-451 control group, the miRNA-451 overexpression group showed significantly reduced cell migration ability (P<0.05); compared with the miRNA-451 interference control group, the miRNA-451 interference group had significantly enhanced cell migration ability (P<0.05). The results are shown in Figure 2.

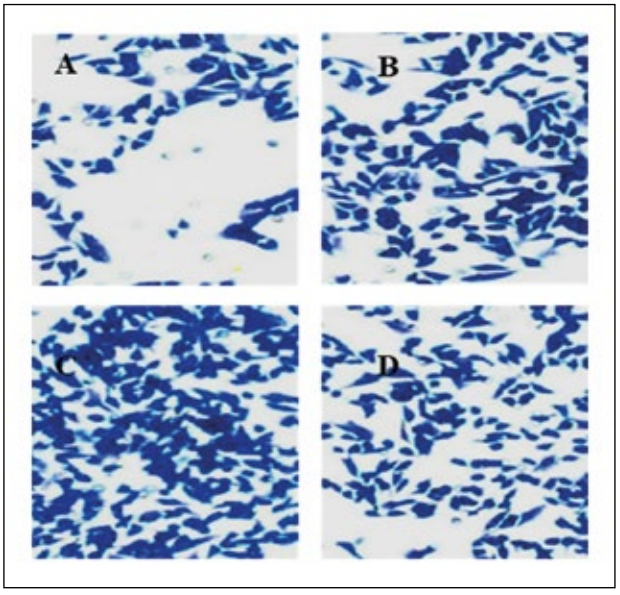

Figure 2: Comparison of the cell migration ability of each group.
A: miRNA-451 overexpression group; B: miRNA-451 control group; C: miRNA-451 interference group; D: miRNA-451 interference control group.

Comparison of the angiogenesis ability of each group of cells

Compared with the miRNA-451 control group, the angiogenesis ability of the miRNA-451 overexpression group was significantly reduced (P<0.05). Compared with the miRNA-451 interference control group, the angiogenesis ability of the miRNA-451 interference group was significantly enhanced (P<0.05). The results are shown in Figure 3.

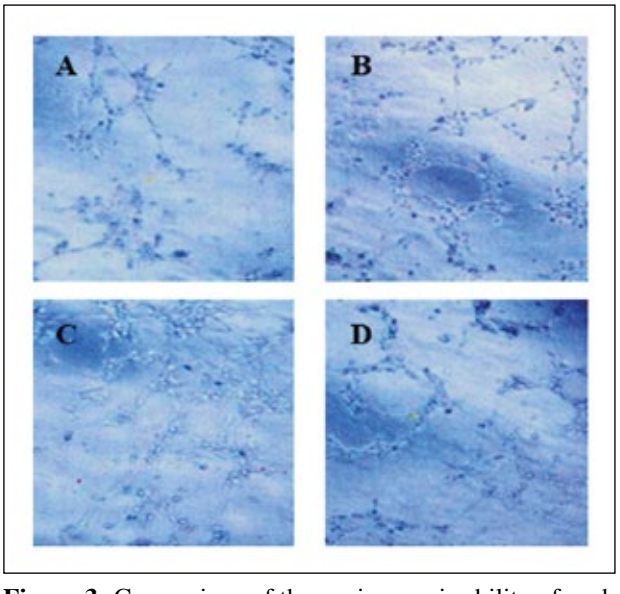

Figure 3: Comparison of the angiogenesis ability of each group of cells.
A: miRNA-451 overexpression group; B: miRNA-451 control group; C: miRNA-451 interference group; D: miRNA-451 interference control group.

Comparison of PI3K, Akt, Erk, and p38/MAPK expression levels in each group of cells

The results of Western blotting show that-compared to the miRNA-451 control group-the expression levels of p38/MAPK and MIF in the miRNA-451 overexpression group were significantly reduced (P<0.05). Compared with the miRNA-451 interference control group, the expression levels of p38/MAPK and MIF in the miRNA-451 interference group were significantly increased (P<0.05). There were no significant differences in the expression levels of PI3K, Akt, and Erk across the groups (P> 0.05). The results are shown in Figure 4.

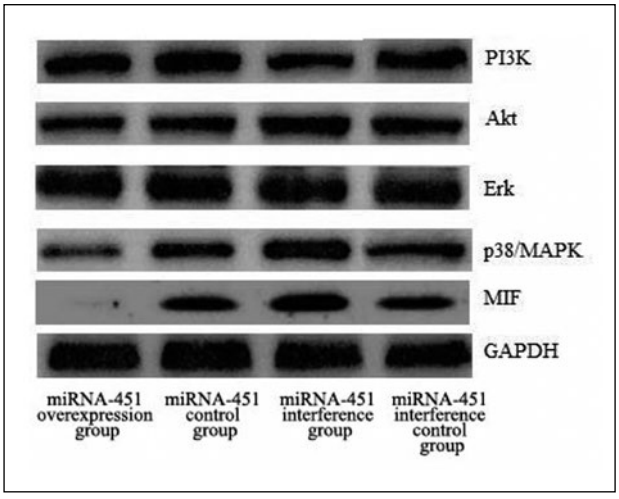

Figure 4: Comparison of PI3K, Akt, Erk, and p38/MAPK expression levels in each group of cells.

Comparison of neuron damage in the hippocampus of mice in each group

In the sham operation group, the neuron cells were closely packed and uniformly distributed, while the intercellular space was normal, with obvious Nissl bodies. Neurons in the miRNA-451 overexpression group were similar to those in the sham operation group. Neurons in the miRNA-451 interference group were disordered and loosely arranged; the neuron cell bodies showed irregularities, and the intercellular space was significantly increased. The results are shown in Figure 5.

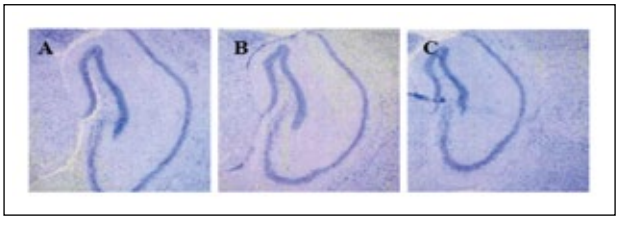

Figure 5: Comparison of neuron damage in hippocampus of mice in each group.
A: sham operation group; B: miRNA-451 overexpression group; C: miRNA-451 interference group.

Expression of blood-brain barrier-related protein ZO-1 in the hippocampus of each group of mice

Compared to the sham operation group and the miRNA-451 overexpression group, the expression level of blood-brain barrier-related protein ZO-1 in the miRNA-451 interference group was significantly increased (P<0.05). There was no significant difference in the expression levels of blood-brain barrier-related protein ZO-1 between the sham operation group and the miRNA-451 overexpression group (P>0.05). The results are shown in Figure 6.

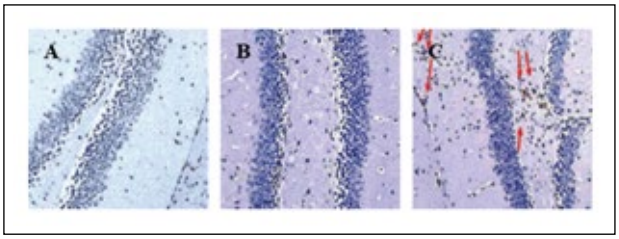

Figure 6: Neuron damage in hippocampus of mice in each group.
A: sham operation group; B: miRNA-451 overexpression group; FC: miRNA-451 interference group.

Comparison of inflammatory factors in hippocampus of different groups of mice

Compared with the sham operation group and the miRNA-451 overexpression group, the expression levels of TNF-α, COX-2, and iNOS in the miRNA-451 interference group were significantly higher (P<0.05). The results are shown in Table 2.

Group	TNF-α	COX-2	iNOS
Sham Operation Group	1.02±0.03	1.03±0.04	0.97±0.04
miRNA-451 Overexpression Group	2.74±0.96	1.25±0.23	1.15±0.23
miRNA-451 Interference Group	50.39±32.58^{ab}	57.52±94.63^{ab}	61.85±88.39^{ab}
F	6.65	5.25	7.52
P	0.030	0.048	0.023

Table 2: Comparison of Inflammatory Factors in Hippocampus of Different Groups of Mice ($\bar{x}\pm s$, %).
Note: a denotes $^{a}P<0.05$ compared with the sham operation group; b denotes $^{b}P<0.05$ compared with the miRNA-451 overexpression group.

Changes in p38/MAPK expression in mice of each group

Compared to the sham operation group and the miRNA-451 overexpression group, the expression level of p38/MAPK in the miRNA-451 interference group was significantly higher (P<0.05). The results are shown in Figure 7.

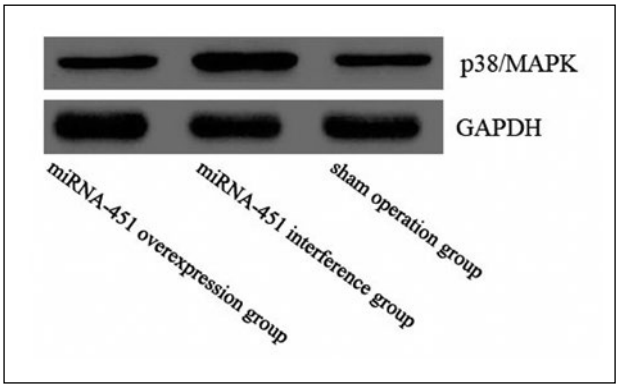

Figure 7: Changes in p38/MAPK expression in mice of each group

Discussion

The brain microenvironment provides the basis for maintaining the normal structure and functional activities of neurons. Brain microvascular disease and damage to the blood-brain barrier may cause neurons to be damaged or die. It has been reported that cerebral microvascular damage and vascular regeneration are important to the occurrence and development of Parkinson's disease and Parkinson's cognitive impairment[7]. For this paper, we selected Bend3 cells for cultivation and established a miRNA-451 lentivirus stereotactic injection model for mouse hippocampus, in order to investigate the mechanism of miRNA-451/MIF on Bend3 cell proliferation, migration, and vascular regeneration and to elucidate the role of miRNA-451 in hippocampal neuron damage and the inflammatory response in mice.

Angiogenesis refers to new blood vessels formed from existing capillaries or after the meridian development of capillaries; it is regulated by numerous proteins throughout the process. Some studies have suggested that the improvement of motor symptoms in Parkinson's patients with deep brain stimulation may be related to the improvement in microvascular structure, increased expression of proteins such as ZO-1, and decreased permeation of IgG from blood vessels[8].

Some studies have found that cerebral microvascular disease and injury may be involved in damage to the white matter. Furthermore, the occurrence and development of white matter are closely related to the destruction of cerebral vascular integrity[9]. The integrity of brain microvascular structure and function is important for normal brain function. Hinman et al.[10] report finding obvious microvascular structural damage and microvascular lesions in Parkinson's patients or animals. Microvascular injury stimulates a large influx of albumin, which can cause edema around blood vessels, obstruct microcirculation and blood flow in the brain, and eventually cause degeneration and neurodegeneration of neurons. Currently, many studies have found that deep brain stimulation can reduce clinical symptoms in patients with Parkinson's disease, which may stem from the improvement in microvascular structure, including increased capillary length and density or increased endothelial cell thickness[11]. Therefore, investigating miRNAs that can regulate angiogenesis for the treatment of Parkinson's disease has become a focus of scholarly attention.

miRNAs are endogenous, non-coding, single-stranded, small-molecule RNAs. Studies have confirmed that numerous miRNAs have been found in mammalian brain tissues, related to brain tissue development, neuronal differentiation, advanced neural functions, neurological diseases, mental illness, and brain tumors[12]. In recent years, it has been discovered that miRNAs are also involved in the regulation of neuronal apoptosis and cell cycle machines in Parkinson's disease. miRNA-451 is a multifunctional miRNA that can not only inhibit the proliferation of a variety of tumor cells, but also inhibit the differentiation of tumor cells, thereby promoting apoptosis of tumor cells. MIF is an endocrine immune substance that participates in the pathological processes of a variety of autoimmune cells and inflammatory diseases. Vincent et al.[13] have found that MIF is involved in processes such as macroangiopathy, diabetic microangiopathy, and angiogenesis.

A growing body of research confirms that the pathogenesis of Parkinson's is closely related to the level of inflammatory factors. MiRNA-451 is now known to be a miRNA associated with immune response and inflammation in higher vertebrates[14]. TNF-α, COX-2, and iNOS are all inflammation-related factors. According to reports, TNF-α may not only participate in inducing nerve injury by activating the pro-apoptotic pathway of the receptor street, but can also further cause nerve injury by inducing the expression of iNOS and COX-2[15].

MAPK is one of many fascinating signaling pathways, among others such as extracellular signaling-regulated kinase (Erk), c-Jun amino terminal kinase (JNK), Erk/macrotide-activated protein kinase 1 (ERK/mrap-1, BMK1), and p38/MAPK. Among these, the p38/MAPK signaling

pathway plays an important role in cytokine production, apoptosis, and other mechanisms. Zhou et al. (16) suggest that miRNA-451/MIF inhibits the p38/MAPK signaling pathway and exerts anti-inflammatory effects. The results of our paper indicate that miRNA-451 could regulate MIF expression. Interfering with miRNA-451 expression may significantly inhibit cell proliferation, promote cell migration and angiogenesis, and activate inflammatory reactions in the brain. The P38/MAPK signaling pathway may be crucial in influencing these processes. In summary, miRNA-451/MIF may contribute to cerebral microvascular injury, vascular regeneration, and the inflammatory response by regulating p38/MAPK.

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