The role of miR-146a in MPTP treated mice with Parkinson’s disease

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Abstract: Objective: To explore the expression and role of miR-146a in PD and its possible mechanisms. Methods: PD mice were constructed by intraperitoneal injection of MPTP, and the expressions of miR-146a in the midbrain at different time points were measured by RT-PCR and in situ hybridization combined with immunofluorescence. The expressions of miR-146a in BV2 cells were detected by RT-PCR. A luciferase reporter assay was used to verify the target gene of miR-146a, and the regulation of target gene IRAK1 by miR-146a was detected by RT-PCR and western blot. After the lateral ventricle administration: the inhibitor or negative control of miR-146a and the expression of IRAK1 were measured by RT-PCR and western blot. ELISA was used to measure the levels of the pro-inflammatory cytokines TNF-α, IL-1, and IL-6, and the apoptosis of neurons was detected by TUNEL combined with immunofluorescence. Results: Compared with the control group, the microglial cells were activated and the expression of miR-146a was significantly up-regulated in the MPTP treated mice. The expressions of miR-146a in BV2 cells treated with LPS were also increased. IRAK1 was a target gene of miR-146a and the expression of IRAK1 was regulated by miR-146a. In the MPTP treated mice, the expressions of IRAK1 were decreased, and the level of TNF-α, IL-1, IL-6, and neuron apoptosis were increased. And the expressions of IRAK1 and the levels of the pro-inflammatory factors were up-regulated by the miR-146a inhibitor, but there was no significant effect of the miR-146a inhibitor on neuron apoptosis. Conclusion: In MPTP treated mice, miR-146a was up-regulated, and it negatively regulated the expression of IRAK1 to involve it in the regulation of PD neuroinflammation. miR-146a might be a new biomarker for the early diagnosis of PD.

Keywords: Parkinson’s disease, apoptosis, neuroinflammation, miR-146a, IRAK1

Introduction

Parkinson’s disease (PD) is a common movement disorder disease, and its pathological features include the loss of dopaminergic (DA) neurons within the substantia nigra pars compacta (SNpc) and the abnormal accumulation of α-synuclein, resulting in the deposition of Lewy bodies [1, 2]. Epidemiological investigations show that age, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and heavy metals can induce PD [3], and the pathophysiological mechanisms underlying DA neuron loss are related to mitochondrial dysfunction, oxidative stress, and neuroinflammation, which can cause apoptosis or impair autophagy of the DA neurons [4-7]. Due to its irreversible pathology and complex pathogenesis, there are no effective methods to cure PD. Therefore, the study of early diagnosis indicators and new therapeutic targets have become hot topics in PD research.

Extensive evidence demonstrates that many miRNAs play important roles in diseases of neurodegeneration, including PD [8]. For example, miR-7 and miR-153 negatively regulate α-synuclein, a key gene related to inherited PD, to inhibit the progression of PD [9], and miR-34b/c is involved in the pathogenesis of PD by regulating mitochondrial function and oxidative stress [10]. miR-146a was the first miRNA found to regulate the immune inflammatory system and is more highly expressed in the central nervous system than in other organs [11]. Research demonstrates that miR-146a has an abnormal expression in nervous system tu-
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In addition, studies have revealed that miR-146a negatively regulates the expression of the main proteins in the toll-like receptor subtype TLR4 signaling pathway, such as the tumor necrosis factor receptor related factor 6 (TRAF6) and the interleukin 1 receptor kinase 1 (IRAK1), and then regulates the nuclear factor kappa B (NF-κB) and the downstream inflammatory factors IL-6, IL-1, and TNF-α and is involved in the inflammation process in the nervous system [14], so it has been speculated that miR-146a might be related to PD. However, there are few studies on the expression and function of miR-146a in PD. In this study, the expression of miR-146a, its target gene IRAK1, and the related inflammatory factors were measured for the first time, in an effort to explore the role and possible mechanisms of miR-146a in PD, finding a new biomarker for the early diagnosis and clinical treatment of PD.

Materials and methods

Experimental animals and treatment

Male C57BL/6 mice (SPF grade) about 8 weeks old and weighing (22 ± 2) g, were provided by the Experimental Animal Center of Zhengzhou University.

40 mice in the MPTP group were treated with MPTP to build a PD model: The mice were intraperitoneally injected with MPTP at 30 mg/kg/time/day for 5 consecutive days. The control mice (8 mice) were injected with saline every day. The mice in the MPTP group were killed at different time-points: 0, 1, 5, 10, and 20 days after the last MPTP injection (8 mice every time), to remove the brains, and, the ventral midbrains were dissected and stored at -80°C for further experiment.

32 mice were divided into 4 groups, the normal miR-146a inhibitor group and the normal negative control (NC) group, the MPTP miR-146a inhibitor group and the MPTP NC group, 8 mice in each group. The mice in the two normal groups had drugs administered into the right lateral ventricles only, and the mice in the MPTP groups had drugs administered into the right lateral ventricles and then had the MPTP injected intraperitoneally. The miR-146a inhibitor groups were given the miR-146a inhibitor, and the NC groups were given a miR-146a negative control. The lateral ventricle administration was conducted as follows: the mice were fixed to the stereotactic apparatus, the stereotactic intraventricular injection site was chosen according to stereotactic map of each mouse. Then the drill hole was created using a 0.8 mm diameter drill, and a PE catheter was inserted and then fixed. After 1 week, the mice were administered the miR-146a inhibitor or the miR-146a negative control (with a concentration of 20 nM of ribonucleotide, for a total volume of 10 µl) through the catheter, for 5 consecutive days. The treatment with the miR-146a inhibitor or the NC was performed 2 days prior to the injection of MPTP.

Cell culture and transfection

BV2 cells were provided from the Cell Culture Center, Institute of Basic Medicine, Chinese Academy of Medical Sciences. BV2 cells were maintained in DMEM (HyClone, USA) containing 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 µg/ml streptomycin and cultured at 37°C in a 5% CO₂ incubator. After three passes, the cells were inoculated into 24-well plates at 1 × 10⁵/well and divided into 5 groups. The saline, 1 µg/ml, 5 µg/ml, 10 µg/ml, and 100 µg/ml (final concentration) and the LPS were added into the cells respectively, and the cells were collected after 24 h.

The cells were seeded into 12 well plates and divided into 4 groups: the LPS group, the miR-146a mimic group (Mimic group), the negative control group (NC group), and the miR-146a inhibitor group (Inhibitor group). After being cultured for 24 h, the mimics, the NC, and the inhibitor of miR-146a were transfected with BV2 cells using lipofectamine 2000 according to the manufacturer's protocol. LPS with a final concentration of 5 µg/ml was added after transfection 48 h, and the cells were collected after 24 h.

Luciferase reporter assay

Based on bioinformatical predictions, a wild type fragment and a mutant fragment from the 3'UTR of the IRAK1 gene containing the miR-146a binding sites were produced by PCR with the primers, and then cloned into XhoI/NotI sites of psiCHECK™2 vector (Promega, USA). All of the recombinant plasmids were confirmed by sequencing. The psiCHECK2 recombi-
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nant vector (containing the 3'UTR of IRAK1 with the wild type or mutant type miR-146a binding site) and the miR-146a mimics or the negative control were cotransfected into the HKE293T cells according to the manufacturer's instructions. After 48 h, the luciferase activity was detected using the dual-luciferase reporter assay system detection kit (Promega, USA). The ratio of Rluc to Fluc was the relative activity of the luciferase.

Real time PCR (RT-PCR)

Total RNA was extracted from the mice midbrain tissues and cells in each group using Trizol, and cDNA was obtained by reverse transcription according to the kit's instruction. The expressions of miR-146a and IRAK1 were measured by RT-PCR, and the measurements were repeated three times. The relative expressions in each group were analyzed using the 2^{ΔΔCt} method. The primer sequences are shown in Table 1.

Western blot

Total proteins were extracted from the brain tissues of the mice, and the protein concentrations were determined using the BCA method. Then, SDS-PAGE electrophoresis was performed and blocked into 5% milk for 1 h after being transferred. The primary antibodies used were the following: IRAK1 and β-actin (1:1000, Abcam, British). After being washed, the secondary antibodies (1:2000, Abcam, U.K.) were incubated for 1 h at room temperature. BeyoECL Plus was added into the membrane to detect the protein bands, and the image analysis was performed using Image J to calculate the relative expression of the proteins with β-actin as the internal reference.

In situ hybridization combined with immunofluorescence

Paraffin embedding and sectioning (8 µm) was performed on the fixed brain tissues of the mice. The sections were put into dimethyl benzene for dewaxing and treated by 0.01% TritonX-100 for 2 min, then digested in trypsin K (1 µg/ml) at 37°C for 20 min. Pre-hybridization was performed at 42°C for 30 min. The Dig-miR-146a probe was obtained from the Abace Biology Company. Then a preheated hybridization solution containing the probe was added into the sections for hybridization at 42°C overnight. The sections were washed in a 2 × SSC solution containing 50% formamide, then a 1 × SSC, 0.5 × SSC solution in turn at 37°C. After being washed by Buffer I (1 mol/L NaCl, 0.1 mol/L Tris, pH = 7.5), buffer II (Buffer I contained 0.1 g/ml BSA) was used to block for 30 min at room temperature. The probe target complex was seen under the action of alkaline phosphatase-digoxin antibodies and Nitro-Blue-Tetrazolium/5-Bromo-4-Chloro-3-Indolyl-Phosphate (NBT/BCIP). After in situ hybridization for miR-146a, the sections were analyzed for immunofluorescence using the OX42 antibody (1:500, 4°C overnight) and a secondary antibody for the microglial cells.

Enzyme-linked immunosorbent assay (ELISA)

The midbrain tissues in mice were ground to prepare a tissue homogenate, and the supernatant was prepared using a conventional centrifuge (3000 g for 10 min) at 4°C. Then, the expressions of IL-6, IL-1, and TNF-α in the midbrain tissues were detected using ELISA kits.

TUNEL combined with immunofluorescence histochemistry

0.3% H₂O₂ was incubated for 10 min after the tissue sections were dewaxed. And after being washed with PBS, a TUNEL staining solution was incubated at 37°C for 2 h. Then the sections were blocked by BSA for 30 min. The primary antibody: tyrosine hydroxylase (TH) antibody (1:500) was incubated at 4°C overnight. A second antibody was labeled with Cy3 and incubated for 1 h. After washing, DAPI was used to stain the nuclei. The apoptosis of the neurons in the midbrain was observed under a fluorescence microscope.

Statistical analysis

All data were analyzed by SPSS 19.0. The data were represented as the mean ± standard deviation (X ± s). The comparisons of the miR-146a expressions in the MPTP treated mice and the BV2 cells were performed using a single factor analysis of variance, and the comparisons between two groups were analyzed by an LSD t test. The relative activity of luciferase in the different cells was compared using a t test. And the expressions of IRAK1, IL-6, IL-1, TNF-α, and the rates of apoptosis in the cells in the
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Table 1. The primer sequences in RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>miR-146a</td>
<td>5'-GGGTGAGAACTGAATTCCA-3'</td>
<td>5'-CAGTGCGTGTCGGAGAT-3'</td>
</tr>
<tr>
<td>U6 snRNA</td>
<td>5'-GCTTCCGCGCAGCATATACTAAAT-3'</td>
<td>5'-GCTTGAGAACTGAATTCCA-3'</td>
</tr>
<tr>
<td>IRAK1</td>
<td>5'-CCTGGATCAACCGCAACG-3'</td>
<td>5'-GTCTGGGAGCCTGGAAA-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-CGGCGCCCTATAAACCAG-3'</td>
<td>5'-CGCGGCGATATCATCATCCA-3'</td>
</tr>
</tbody>
</table>

Figure 1. The expression of miR-146a: A: The expression of miR-146a in the midbrains of the MPTP-treated mice; *Compared with the control group, P < 0.05; #Compared with the 0d group, P < 0.05. B: In situ hybridization combined with immuno-fluorescence to detect the expression of miR-146a in the microglial cells of MPTP-treated mice. C: The expression of miR-146a in BV2 cells with the treatment of LPS at different concentrations; *Compared with the control group, P < 0.05; #Compared with the 1 ug/ml group, P < 0.05.

Results

The expression of miR-146a in the midbrains of MPTP mice and LPS induced BV2 cells

The results of miR-146a expression in the midbrain of the MPTP mice were detected by RT-PCR, as shown in Figure 1A. Compared with the control group, the expression of miR-146a in the MPTP mice was gradually increased with the time of MPTP administration, and the difference was significant (P < 0.05). Inflammation is involved in PD, and miR-146a is associated with microglial activation. The changes of microglial cells and the intensity of miR-146a were measured by in situ hybridization combined with immunofluorescence. The results showed that the microglial cells were activated and the expression of miR-146a was up-regulated in the midbrains of MPTP mice. Additionally, the expressions of miR-146a in BV2 cells treated with 1 µg/ml LPS was not significantly different from the control group, but the expression of miR-146a in cells treated with other concentrations of LPS was significantly higher than in the control group (P < 0.05), but there were no differences among the groups.
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miR-146a targets to IRAK1

It was predicted that IRAK1 is a target gene of miR-146a by biological software. And the result was verified by the double luciferase reporter assay. The relative luciferase is shown in Figure 2A. And the results indicate that when transfected with IRAK1-WT, the luciferase activity in the miR-146a mimics group was significantly lower than it was in the NC group, which suggests that miR-146a could bind to 3′UTR of IRAK1 mRNA to inhibit the luciferase activity.

Regulation of endogenous IRAK1 by miR-146a

The mimics, inhibitor, and negative control of miR-146a were transfected into LPS-induced cells to detect the expression of IRAK1. The results showed that compared with the LPS group, the expression of IRAK1 mRNA in the mimics group decreased, while the expression in the inhibitor group increased, and the differences were significant. The expression of IRAK1 protein in each group was consistent with the results of the mRNA (Figure 2B and 2D). The results indicated that miR-146a could directly regulate the expression of IRAK1 in cells.

The effect of the miR-146a inhibitor on the IRAK1 and inflammatory factors in the midbrain of MPTP treated mice

In order to explore whether miR-146a could play a role in PD by regulating the expression of IRAK1, the expressions of IRAK1 mRNA, and protein were detected after exogenously inhibiting miR-146a in the right lateral ventricles of mice, and the results are shown in Figure 3. Compared with the normal mice, the expression of IRAK1 in the MPTP treated mice was decreased. There was no significant difference in the expression of IRAK1 in the miR-146a inhibitor group or in the NC group in the normal mice. And in the MPTP treated mice, the expression of IRAK1 in the miR-146a inhibitor group was higher than it was in the NC group, which was similar to the IRAK1 protein results. The results indicated that miR-146a negatively regulates the expression of IRAK1 in MPTP treated mice.

Figure 2. MiR-146a targets to IRAK1. A: Co-transfected psiCHECK2 recombinant vector and miR-146a mimics or NC in HEK293T cells, the relative activity of luciferase. C: The effect of exogenous miR-146a on the expression of IRAK1 mRNA; B and D: The effect of exogenous miR-146a on the expression of IRAK1 protein (* compared with the NC group in MPTP treated mice, P < 0.05).

Figure 3. The expression of IRAK1 mRNA and protein in the midbrain of mice after the inhibitor or NC of miR-146a was administered into the right lateral ventricles, (compared with the NC group in MPTP treated mice, P < 0.05).
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Table 2. The levels of TNF-α, IL-1 and IL-6 in midbrain (µg/L)

<table>
<thead>
<tr>
<th>Group</th>
<th>TNF-α</th>
<th>IL-1</th>
<th>IL-6</th>
</tr>
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<tbody>
<tr>
<td>Normal mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC group</td>
<td>2.631 ± 0.041</td>
<td>0.306 ± 0.012</td>
<td>1.285 ± 0.020</td>
</tr>
<tr>
<td>Inhibitor group</td>
<td>2.679 ± 0.038</td>
<td>0.308 ± 0.011</td>
<td>1.294 ± 0.019</td>
</tr>
<tr>
<td>MPTP treated mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC group</td>
<td>3.037 ± 0.050*</td>
<td>0.353 ± 0.011*</td>
<td>1.345 ± 0.024*</td>
</tr>
<tr>
<td>Inhibitor group</td>
<td>3.293 ± 0.046*</td>
<td>0.408 ± 0.013*</td>
<td>1.468 ± 0.025*</td>
</tr>
</tbody>
</table>

*Compared with the NC group in normal mice, P < 0.05; #Compared with the miR-146a inhibitor group in normal mice, P < 0.05; ΔCompared with the NC group in MPTP treated mice, P < 0.05.

The levels of TNF-α, IL-1, and IL-6 in the midbrains of the mice in each group were measured by ELISA, as shown in Table 2: in the MPTP treated mice, the levels of TNF-α, IL-1, and IL-6 were increased, and the levels of TNF-α, IL-1 and IL-6, in the miR-146a inhibitor group were higher than they were in the NC group, with a significant difference. The result demonstrated that the expression of miR-146a was up-regulated as the expression of IRAK1 decreased and the pro-inflammatory cytokines TNF-α, IL-1, and IL-6 increased in the MPTP treated mice, while the miR-146a inhibitor could up-regulate the expression of IRAK1 and the levels of the pro-inflammatory cytokines.

The effect of miR-146a inhibitor on the apoptosis of neurons in the midbrain of the MPTP treated mice

The apoptotic neurons were observed by TUNEL combined with TH immunohistochemistry, as shown in Figure 4. And the statistical results of the apoptotic neurons are shown in Figure 5. The percentage of apoptotic neurons in the MPTP treated mice was significantly higher than it was in the normal mice, but there were no significant differences in the apoptotic neurons between the miR-146a inhibitor group and the NC group. The results indicate that the miR-146a inhibitor had no significant effect on the apoptotic neurons in the midbrain.
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Discussion

The pathogenesis of PD is a complex and lengthy process that is the result of a combination of the environment and genetics. In recent years, more and more evidence indicates that in a variety of degenerative diseases, the hyper-activated microglial cells lead to neuroinflammation and oxidative stress, which results in damage to dopaminergic neurons. So, we speculated that neuroinflammation plays an important role in PD [15]. As one of the miRNAs involved in the inflammatory reaction process, miR-146a could activate microglial cells in the central nervous through NF-κB/JUK-STAT or other pathways to involve in the immune inflammatory response of nervous system diseases [16]. And it was reported that miR-146a is abundant in brain tissue and up-regulated in the brains of neuroinflammatory patients [17]. For example, miR-146a, which was found to be up-regulated in pilocytic astrocytomas, is predicted to target the ERK/MAPK and NF-κB signaling pathways, as well as regulate genes involved in senescence-associated inflammation and cell cycle control [18]; Wu [19] showed that miR-146a was up-regulated in the microvessels of multiple sclerosis-active lesions and the spinal cords of mice with experimental autoimmune encephalomyelitis, and brain endothelial miR-146a modulated NF-κB activity upon cytokine activation by targeting two novel signaling transducers, RhoA and NFA55, as well as molecules previously identified, IRAK1 and TRAF6, which are associated with decreased leukocyte adhesion during neuroinflammation. Lukiw et al. [20] indicated that the up-regulation of miR-146a coupled with the down regulation of CFH was observed in the AD brain and in interleukin-1β and/or oxidatively stressed HN cells in primary culture, while Muller [21] found that the levels of miR-146a were significantly decreased in CSF of AD patients. Dong et al. [22] observed that miR-146a was markedly decreased in AD patients’ serum, but miR-146a is significantly elevated in mild cognitive impairment, so miR-146a is dynamic in AD. However, there are few studies on the expression of miR-146a in the brain tissues of PD. In this study, the results showed that microglial cells were activated in the midbrains of the MPTP treated mice, and the expression of miR-146a was significantly higher than it was in the control group, and the expression of miR-146a was increased with MPTP treatment time; in vitro, miR-146a was also up-regulated in the LPS treated BV2 cells and the expression of miR-146a was increased with the increase of LPS concentration. The results indicated that miR-146a was up-regulated in the midbrain tissue of the MPTP treated mice, which might play a certain role in PD.

It had been verified by many studies that IRAK1 was a target gene of miR-146a, and miR-146a could negatively regulate the expression of IRAK1 [23]. This study also confirmed, through a luciferase report assay, that miR-146a targets IRAK1, and miR-146a can negatively regulate the expression of endogenous IRAK1. IRAK1 is a key intermediate adaptor protein in the TLR signaling pathway, and it can regulate downstream signaling processes, and a series of events that can lead to the induction of pro-inflammatory transcription factors, such as NF-κB, and it can eventually regulate the inflammatory gene expression and innate immune response, which play a certain role in driving neuropathology, stimulating apoptotic brain cell death and neural tissue degeneration [24, 25]. In the present study, the results also showed that, compared with normal mice, the expression of miR-146a and the levels of the inflammatory cytokines TNF-α, IL-1, and IL-6 were increased, but the expression of IRAK1 was decreased in the MPTP treated mice. And the miR-146a inhibitor could up-regulate the expression of IRAK1, but the levels of inflammatory cytokines were also increased. Many

Figure 5. The percentage of apoptotic neurons in the midbrains of the mice in each group.
studies found that the promoter regions of miR-146a contained the binding site of NF-κB, and activated NF-κB could activate the transcription of miR-146a to down-regulated the expression of IRAK1, meanwhile the expression of IRAK2 was compensatory up-regulated, so when the TLR signaling pathway was activated, miR-146a was up-regulated with the enhancement of the inflammatory response [23, 25]. Cui et al. [25] also found that there was a significantly independent regulation of IRAK1 and IRAK2, and miR-146a mediated the down-regulation of IRAK1 coupled to an NF-κB induced up-regulation of IRAK2 expression, which drives an extensively sustained inflammatory response. According to the existing studies, it was speculated that, in MPTP treated mice, due to neuroinflammation, the levels of the pro-inflammatory cytokines TNF-α and IL-1 were increased, which further up-regulate the expression of miR-146a. The up-regulation of miR-146a is coupled to the down-regulation of IRAK1, and a compensatory up-regulation of IRAK2, maintaining or driving the sustained inflammatory response. So, the results indicate that miR-146a negatively regulated IRAK1 but did not inhibit the inflammatory response. In addition, the study also found that the miR-146a inhibitor had no significant effect on the apoptosis of neurons in the midbrain. One reason for this result was that the miR-146a inhibitor could not affect IRAK2, so the regulatory effect of the miR-146a inhibitor on neuroinflammation was not sufficient to have a significant effect on the apoptosis of the neurons.

In conclusion, miR-146a was up-regulated in the MPTP treated mice, and it negatively regulated the expression of IRAK1's involvement in the regulation of the neuroinflammation of PD. The study firstly revealed that miR-146a might be associated with the progression of PD, and it might be a new biomarker for the early diagnosis of PD. However, the miR-146a inhibitor could not be used to inhibit the inflammatory response, and it has not yet been investigated whether miR-146a could mediate the down-regulation of IRAK1 coupled to an NF-κB induced up-regulation of IRAK2 expression in neuroinflammation during the progression of PD. So the role of miR-146a in PD still needs to be further comprehensively analyzed in subsequent studies.

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Disclosure of conflict of interest

None.

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