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Original Article

The role and mechanism of microRNA184 in MPTP-induced nerve damage

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Abstract: Parkinson's disease (PD) is a degenerative disease of the central nervous system which causes aged dementia. MPTP is known to trigger neural injury for PD-like syndromes. MicroRNAs (miRs) can facilitate neural development and synaptic plasticity. Previous studies have shown the association between abnormal expression of mirR-184 and neural disease including glioma. However, the role and mechanism of miR-184 in MPTP-induced neural injury remains to be illustrated. Neuro-2a cells were divided into six groups: control group; MPTP group; miR-184 mimics NC group; miR-184 inhibitor NC group; miR-184 mimics group; and miR-184 inhibitor group. Real-time PCR was used to detect miR-184 expression, while MTT was used to measure cell proliferation. Caspase 3 activity and expression of transcriptional factor Nrf2, Bcl-2, and Bax were also measured. miR-184 was up-regulated in Neuro-2a cells after MPTP treatment ($P<0.05$ compared to control group). Transfection of miR-184 mimic inhibited Neuro-2a cell proliferation, increased caspase 3 activity, and Bax expression, as well as decreased Bcl-2 and Nrf2 expression ($P<0.05$ compared to MPTP group). Transfection of miR-184 inhibitor facilitated Neuro-2a cell proliferation, reduced caspase 3 activity or Bax expression, together with increased Bcl-2 and Nrf2 expression ($P<0.05$ compared to MPTP group). miR-184 is up-regulated in MPTP-induced neural injury. Silencing of miR-184 expression alleviates MPTP-induced injury possibly through facilitating the neuroprotective factor Nrf2 expression, modulating apoptosis/anti-apoptosis balance, as well as inhibiting cell apoptosis.

Keywords: MicroRNA-184, Parkinson's disease, MPTP, cell apoptosis, Nrf2

Introduction

Parkinson's disease (PD) is a degenerative disease of the central nervous system. It is mainly featured with substantia nigra-striatum circuit degeneration, progressively decrease or denature of dopaminergic neurons in substantia nigra pars compacta, including increased acidophilic inclusion body, α-synuclein, and aggregation of inclusion bodies, leading to decreased dopamine synthesis and deficiency at striatum neurons, eventually resulting in the development of neural disorders [1, 2]. Progression of PD eventually causes aged dementia [3]. PD is commonly observed in aged people over 65 years old, with an incidence rate being only lower than Alzheimer's disease (AD). The PD incidence is increasing over the world, which is a major health concern next to cardiovascular disease [4, 5]. A complicated mechanism and risk factors underlie PD pathogenesis, including hereditary, aging, environment, infection, excitotoxicity, oxidative stress, mitochondrial disorder and immune dysfunction, among which genetic and environmental factors were believed to be important, although the exact molecular mechanism remains poorly understood [6, 7]. Exposure or inhalation of 1-mehtyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been known to induce a PD-like syndrome in rodents which is used to mimic PD related neuronal damage [8, 9]. Due to complicated PD pathogenesis, the identification of effector molecules and target sites are research focuses to improve the treatment efficacy [10].

MicroRNAs (miRs) are small molecule RNAs consisting of 19~25 nucleic acids with similar molecular biological features, that participate in regulating various body biological functions such as growth/development and environmental acclimation. Due to its various forms, miRs

can be modulated by physiological and developmental signals [11, 12]. Each miR can regulate more than 200 target genes, suggesting that at least 1/3 of functional coding genes in human are modulated by miRs [13]. Recent studies have shown the involvement of miRs in various patho-physiological processes, as it can facilitate neurodevelopment and synaptic plasticity formation [14]. miRs can modulate proliferation, apoptosis, and differentiation of neurons, and are associated with PD pathogenesis and progression [15]. Previous studies have shown that abnormal expression of miR-184 was related with neurological diseases such as glioma [16]. Its role in PD, however, has not been illustrated. This study thus investigated the functional role of miR-184 in MPTP-induced neural damage and related mechanisms, in an attempt to determine the molecular mechanism of PD pathogenesis, and to provide evidence for PD diagnosis and treatment.

Materials and methods

Major equipment and reagent

Human neural crest stem cell Neuro-2a cell line was purchased from ATCC cell bank (US). DMEM medium, fetal bovine serum (FBS) and streptomycin-penicillin were purchased from Hyclone (US). DMSO and MTT powders were purchased from Gibco (US). Trypsin-EDTA lysis buffer was purchased from Sigma (US). Caspase 3 activity assay kit and PVDF membrane were purchased from Pall Life Sciences (US). EDTA was purchased from Hyclone (US). Western blotting reagent was purchased from Beyotime (China). ECL reagent was purchased from Amersham Biosciences (US). Rabbit anti-human Bcl-2 monoclonal antibody, anti-human Bax monoclonal antibody, anti-human Nrf2 monoclonal antibody, and mouse anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibody were all purchased from Cell Signaling (US). miR-184 mimic and inhibitor were purchased from Jikai Gene Chem (China). RNA extraction kit, reverse transcription kit were purchased from Axygen (US). Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad (US). Other common reagents were purchased from Sangon (China).

Neuro-2a cell culture and grouping

Neuro-2 cells stored in liquid nitrogen were resuscitated in 37°C water-bath until fully thawing. Cells were centrifuged at 1000 rpm for 3 minutes, and re-suspended in 3 ml fresh medium and were removed to 5 ml culture flask which contained 3 ml fresh culture medium. Cells were kept in a humidified chamber at 37°C with 5% CO_2 for 24~48 hours. Neuro-2a cells were seeded into 6-well plates at 1×10^5 per cm^2 containing 10% FBS and 90% high-glucose DMEM medium (with 100 U/ml penicillin and 100 μg streptomycin). Cells were kept in a humidified chamber at 37°C with 5% CO_2. Cells at log-phase with 2nd to 8th generation were randomly divided into the control group; the MPTP group, which received 300 μM MPTP [8]; the miR-184 mimics NC group; the miR-184 inhibitor NC group; the miR-184 mimics group; and the miR-184 inhibitor group.

Liposome transfection of miR-184 mimics and inhibitors

miR-184 mimics (5'-UGU UAC AAC AGU GUC GUG GA-3'), miR-184 inhibitor (5'-ACC GGU CUG UUU GAG AGA-3') or negative controlled oligonucleotides (miR-184 mimics NC: 5'-AUG CAU UAG GCC CGG AUU G-3'; and miR-184 inhibitor NC: 5'-ACA AGU AGG CGC GUA UG-3') were transfected into Neuro-21 cells after MPTP treatment. In brief, cells were cultured in 6-well plate until reaching 70%~80% confluence. miR-184 mimics/inhibitor or NC sequences were mixed with liposome in 200 μl serum-free medium for 15 minutes at room temperature incubation. Lipo2000 reagent was then mixed with miR-184 mimics/inhibitor or controlled dilutions for a 30 minute room temperature incubation. Serum was removed from cells, followed by PBS rinsing and addition of 1.6 ml serum-free culture medium. Cells were then kept in a humidified chamber at 37°C with 5% CO_2 for 6 hours, followed by addition of serum-containing medium in 48 hour continuous incubation for further experiments.

Real-time PCR for miR-184, Bcl-2, Bax and Nrf2 expression

Trizol reagent was used to extract RNA from all groups of Neuro-2a cells. Reverse transcription was performed according to the manual instruction, using primers designed by Primer6.0 and synthesized by Invitrogen (China) (**Table 1**). Real-time PCR was performed on target genes under the following conditions: 56°C for 1 minute, followed by 35 cycles each containing 92°C for 30 seconds, 58°C for 45 seconds and 72°C for 35 seconds. Data were collected and calculated for CT values of all samples and

Table 1. Primer sequences

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
miR-184	CTTAGTGGTCTCTACTTGTT	TCACCCTCTCACAGCTTG
Bcl-2	CCCACCTCTTCTAGAATCT	TATTGGACCTCGCGGTAATT
Bax	AGTCTCCTCTAGATTCATCT	GACCGACCTCGGTCGAATT
Nrf2	AGTGGGGTCTCTAGCTTGTT	CTCCCAACACAGCTTG

standards based on fluorescent quantification using GAPDH as the internal control. Standard curve was plotted using CT values of standards, followed by semi-quantitative analysis using $2^{-\Delta Ct}$ method.

MTT assay for cell proliferation in all groups

Neuro-2a cells at log-phase were digested, counted, and seeded into 96-well plate at 5×10^3 cells per well density. After 24-hour incubation, supernatants were discarded. Cells were randomly divided into the control group, the MPTP group, the miR-184 mimics NC group, the miR-184 inhibitor NC group, the miR-184 mimics group, and the miR-184 inhibitor group treated as above-mentioned. After 48 hours, 20 μl sterile MTT solution was then added into each test well in triplicates. With 4 hours of continuous culture, the supernatant was completely removed, with the addition of 150 μl DMSO for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values was measured at 570 nm in a microplate reader. The proliferation rate was calculated in each group. Each experiment was repeated in triplicates for statistical analysis.

Caspase 3 activity assay

Caspase 3 activity in myocardial tissues was evaluated using test kit. In brief, cells were digested by trypsin, and centrifuged at 600 g for 5 minutes at 4°C. The supernatant was discarded, followed by addition of cell lysis buffer and iced incubation for 15 minutes. The mixture was then centrifuged at 20,000 g for 5 minutes at 4°C, followed by the addition of 2 mM Ac-DECD-pNA. OD values at 450 nm wavelength were measured to reflect caspase 3 activity.

Western blotting for Bcl-2, Bax, and Nrf2 protein expression level

Neuro-2a cell proteins were firstly extracted. In brief, RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 2 μg/ml Aprotinin, 2 μg/ml Leupeptin, 1 mM PMSF, 1.5 mM EDTA and 1 mM NaVandade) (Thermo Fisher Scientific) was used to lyse cells on ice for 15~30 minutes, followed by ultrasound rupture (5 s×4) and centrifugation (4°C, 10,000 g, 15 minutes). Supernatants were saved and quantified for protein contents, and were stored at -20°C for further Western blotting. Proteins were then separated on 10% SDS-PAGE gel, transferred to PVDF membrane (Thermo Fisher Scientific) using semi-dry method. Non-specific background was removed using 5% defatted milk powder at room temperature for 2 hours, followed by addition of anti-Bcl-2 monoclonal antibody (1:1000 dilution) (Cell Signaling Technology), anti-Bax monoclonal antibody (1:2000) (Cell Signaling Technology), anti-Nrf2 monoclonal antibody (1:1500) (Cell Signaling Technology) and incubation at 4°C overnight. On the next day, the membrane was rinsed in PBST, and incubated with goat anti-rabbit secondary antibody (1:2000) for 30 minutes incubation in the dark. After PBST rinsing, ECL reagent (Thermo Fisher Scientific) was used to develop the membrane for 1 min, followed by exposure under X-ray for observing results. Protein imaging analysis system and Quantity One software were used to scan X-ray films for observing band density. Each experiment was repeated four times (N=4) for further analysis.

Statistical analysis

All data were presented as mean ± standard deviation (SD). Student t-test was used to compare means between two groups. SPSS11.5 software was used for data analysis. ANOVA was employed for comparing difference among multiple groups. A statistical significance was defined when $P<0.05$.

Results

Increased miR-184 expression in MPTP-treated neural cells

Real-time PCR was used to measure miR-184 expression in MPTP-treated Neuro-2a cells 48 hours after transfection. Results showed significantly elevated miR-184 in MPTP-treated group ($P<0.05$ compared to control group, **Fi-**

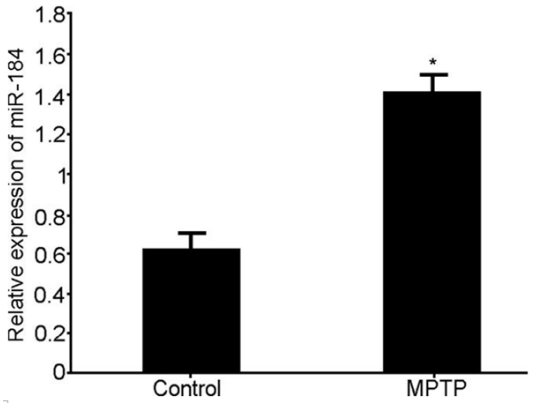

Figure 1. miR-184 expression in MPTP-treated neurons. *, P<0.05 compared to the control group.

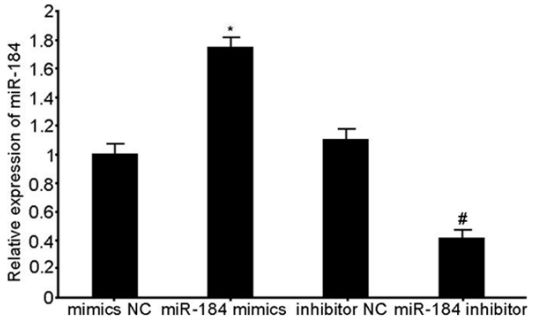

Figure 2. miR-184 expression after transfecting mimics or inhibitor. *, P<0.05 compared to the mimics NC group; #, P<0.05 compared to the inhibitor NC group.

gure 1), indicating that MPTP-induced neural injury increased miR-184 expression.

Manipulation of MiR-184 expression using mimics or inhibitor transfection

Real-time PCR was employed to detect the regulation of miR-184 in MPTP-treated Neuro-2a cells after transfection with miR-184 mimics or inhibitors. Results showed that the transfection of miR-184 mimics significantly facilitated its expression in MPTP-treated cells (P<0.05 compare to mimics NC group). Transfection of miR-184 inhibitor, however, effectively inhibited its expression in MPTP-treated cells (P<0.05 compared to inhibitor NC group, **Figure 2**).

Decreased/increased neuron proliferation after miR-184 mimics/inhibitor transfection

MTT assay was employed to test the effect of miR-184 mimics or inhibitor transfection on

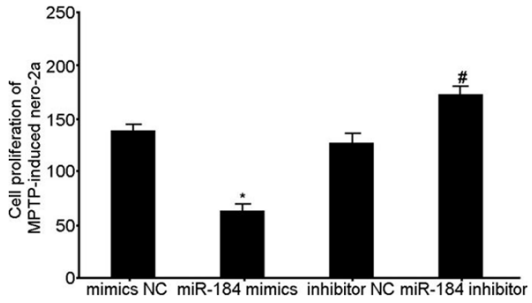

Figure 3. MPTP-treated Neuro-2a cell proliferation by miR-184 manipulation. *, P<0.05 compared to the mimics NC group; #, P<0.05 compared to the inhibitor NC group.

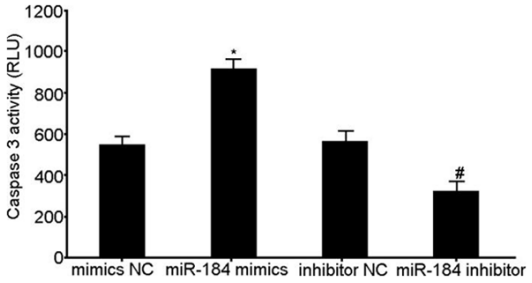

Figure 4. Caspase 3 activity and miR-184. *, P<0.05 compared to the mimics NC group; #, P<0.05 compared to the inhibitor NC group.

MPTP-treated neuron proliferation. Results showed significant inhibition on Neuro-2a cell proliferation by miR-184 mimics transfection and hence force up-regulation (P<0.05 compared to mimics NC group). In contrast, transfection of miR-184 inhibitor decreased its expression and facilitated MPTP-treated cell proliferation (P<0.05 compared to inhibitor NC group, **Figure 3**). These results suggest that down-regulation of miR-184 could facilitate neuron proliferation *in vitro* after MPTP-induction.

Caspase 3 activity in MPTP-treated neurons

Caspase 3 activity kit was used to analyze the effect of miR-184 on MPTP-treated Neuro-2a cells. Results revealed significantly elevated caspase 3 activity after miR-184 up-regulation by mimics transfection (P<0.05 compared to mimics NC group). In contrast, transfection of miR-184 inhibitor down-regulated its expression and inhibited caspase 3 activity (P<0.05 compared to inhibitor NC group (P<0.05 compared to inhibitor NC group, P<0.05, **Figure 4**).

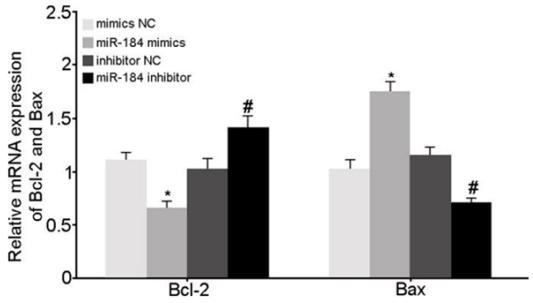

Figure 5. Effect of miR-184 manipulation on Bcl-2 and Bax mRNA levels in MPTP-treated Neuro-2a cells. *, P<0.05 compared to the mimics NC group; #, P<0.05 compared to the inhibitor NC group.

These results suggest that over-expression of miR-184 induces neuron apoptosis via enhancing caspase 3 activity.

The effect of miR-184 on Bcl-2 and Bax mRNA levels in Neuro-2a cells after MPTP induction

Real-time PCR was further employed to test the effect of miR-184 on Bcl-2 and Bax mRNA level in MPTP-treated Neuro-2a cells. Results show that up-regulation of miR-184 by mimics transfection significantly down-regulated Bcl-2 mRNA while up-regulated Bax mRNA expression (P<0.05 compared to mimics NC group). In contrast, transfection of miR-184 inhibitor elevated Bcl-2 and decreased Bax mRNA levels (P<0.05 compared to inhibitor NC group, P< 0.05, **Figure 5**).

Regulation of miR-184 and Bcl-2/Bax protein levels in neurons

Western blotting was used to test the effect of miR-184 on Bcl-2 and Bax protein levels in MPTP-treated Neuro-2a cells. Results found significantly down-regulated Bcl-2 and up-regulated Bax proteins after miR-184 mimics transfection (P<0.05 compared to mimics NC group). Whereas, transfection of miR-184 inhibitor on MPTP-treated cells decreased miR-184 expression, elevated Bcl-2, and suppressed Bax protein expressions (P<0.05 compared to the inhibitor NC group, **Figure 6**).

Nfr2 expression in MPTP-treated neural cells after miR-184 mimics/inhibitor transfection

Real-time PCR and Western blotting were employed to test the effect of miR-184 on Nrf2

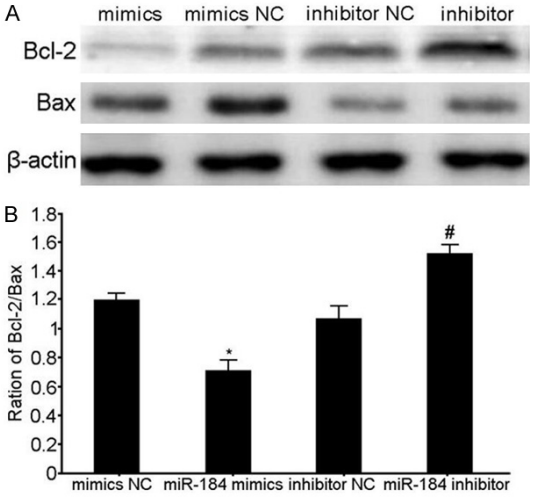

Figure 6. Effect of miR-184 on Bcl-2 and Bax protein in MPTP-treated Neuro-2a cells. A: Western blot analysis of Bcl-2 and Bax protein expression. B: Quantification of the expression of Bcl-2 and Bax. *, P<0.05 compared to the mimics NC group; #, P<0.05 compared to the inhibitor NC group.

expression. Results showed that the up-regulation of miR-184 by mimics transfection significantly suppressed Nrf2 mRNA and protein expressions (P<0.05 compared to mimics NC group). In contrast, miR-184 inhibitor transfection into MPTP-treated Neuro-2a cells elevated Nrf2 mRNA/protein expression levels (P<0.05 compared to inhibitor NC group, **Figure 7**).

Discussion

miRs have now become an important research hotspot due to their involvement in various biological and pathological processes via manipulating post-transcriptional levels of several genes. Target genes of miRs include cell growth factors, transcriptional factors, cell death molecules, and signal molecules, all of which contribute to cell proliferation, differentiation, apoptosis or even cell death [17]. miRs can modulate development and metabolism at post-transcriptional level [18]. Although MPTP does exist in natural environment, it still has similar chemical structure with paraquat, which is an agricultural drug. Both paraquat and MPTP induce neurons for apoptosis, and cause oxidative stress, which further leads to decreased dopamine in substantia nigra, plus neuronal damage, both of which leads to PD-like syndrome [19, 20]. Previous studies have shown the correlation between miR expression alternation

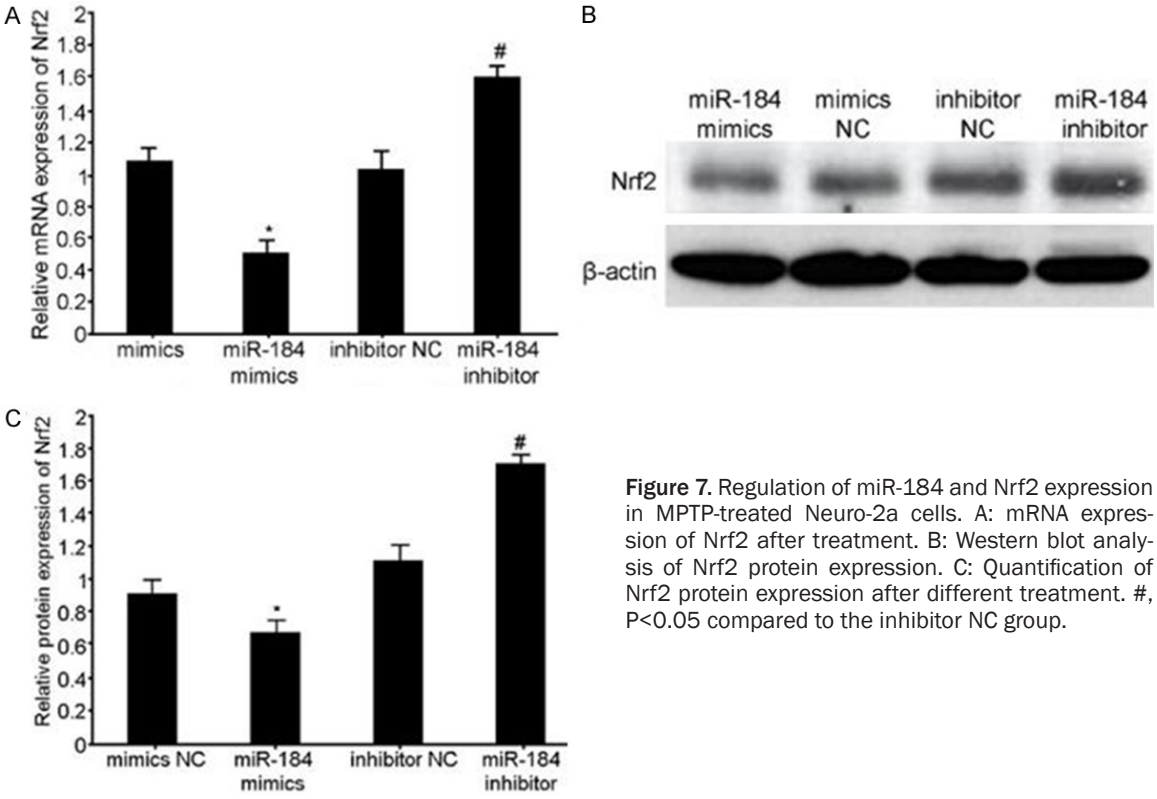

Figure 7. Regulation of miR-184 and Nrf2 expression in MPTP-treated Neuro-2a cells. A: mRNA expression of Nrf2 after treatment. B: Western blot analysis of Nrf2 protein expression. C: Quantification of Nrf2 protein expression after different treatment. #, $P<0.05$ compared to the inhibitor NC group.

and PD risk. As miR-184 is known to be related with certain neurological diseases [16], its role in PD, however, remains poorly understood. This study thus treated Neuro-2a cells with MPTP, and demonstrated up-regulation of miR-184 in MPTP-induced cells.

Transfection of miR-184 mimics increased its expression in MPTP-treated Neuro-2a cells and inhibited its proliferation. Transfection of miR-184 inhibitor obtained opposite effects. These results collectively indicate that miR-184 might be a target for MPTP-induced neurological injury. Therefore, inhibition of miR-184 promotes neuronal proliferation. Further studies analyzed the related mechanism of miR-184 in MPTP-induced neural injury. Apoptosis is a regulatory mechanism for body homeostasis. Up-regulation of apoptosis inhibits neural cell proliferation. The imbalance of anti-apoptotic gene Bcl-2 and pro-apoptotic gene Bax leads to higher levels of neural cells and neural injury, including PD [21]. Initiation of cell apoptotic program activates apoptotic molecules, in which caspase 3 is the most potent factor which can induce apoptosis of tumor cells [22]. This study showed that transfection of miR-184 mimic facilitated caspase 3 activity, down-regulated Bcl-2, and up-regulated Bax, thus suppressing Bcl-2/Bax ratio. However, inhibition of miR-184 expression had opposite effects. The transcriptional factor for cellular oxidative stress Nrf2 can protect neural toxicity induced by MPTP or paraquat [23]. However, whether miR-184 can affect Nrf2 still requires investigation. This study showed decreased Nrf2 expression after over-expression of miR-184 in MPTP-induced injured Neuro-2a cells. However, inhibition of miR-184 expression facilitated Nrf2 expression, thus exerting protective roles.

Conclusion

miR-184 is up-regulated in MPTP-induced injured neural cells. Down-regulation of miR-184 can alleviate MPTP-caused neural injury possibly through facilitating neuroprotective factor Nrf2 expression, modulating apoptosis-anti-apoptosis balance, inhibiting cell death, suggesting miR-184 might be a novel target for the treatment of PD.

Disclosure of conflict of interest

None.

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