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MiR-133b inhibits MPP+-induced apoptosis in Parkinson's disease model by inhibiting the ERK1/2 signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the effect of micro ribonucleic acid (miR)-133b on 1-methyl-4-phenylpyridinium ion (MPP^+)-induced apoptosis in the Parkinson's disease (PD) model.

MATERIALS AND METHODS: PC12 cells were induced by different concentrations of MPP^+ to establish the PD cell model. Subsequently, the survival rate of PC12 cells was detected using Cell Counting Kit-8 (CCK-8) assay. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression of miR-133b in the PD model induced by different concentrations of MPP^+. Next, PC12 cells were transfected with miR-133b mimic and miR-negative control (NC), and divided into MPP^+ group, MPP^+ + miR-NC group and MPP^+ + miR-133b mimic group. Transfection efficiency was verified using qRT-PCR. The apoptosis of cells was detected using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Moreover, the expressions of extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphorylated (p)-ERK1/2 were determined using Western blotting.

RESULTS: After MPP^+ treatment, the survival rate of PC12 cells significantly declined ($p<0.05$). MPP^+ exhibited toxicity against PC12 cells in a concentration-dependent manner. Meanwhile, cell survival rate decreased remarkably with the increase of MPP^+ concentration ($p<0.05$). With increased concentration of MPP^+, the expression of miR-133b in the PD cell model declined significantly ($p<0.05$). The apoptosis of PC12 cells was remarkably inhibited by overexpression of miR-133b in the PD cell model ($p<0.05$). In addition, the protein expression of p-ERK1/2 in PC12 cells was notably reduced after overexpression of miR-133b in the PD cell model ($p<0.05$).

CONCLUSIONS: MiR-133b is lowly expressed in the PD cell model. Furthermore, overexpression of miR-133b inhibits cell apoptosis in the PD cell model by regulating the ERK1/2 signaling pathway.

Key Words:
Parkinson's disease (PD), MiR-133b, Apoptosis, ERK1/2 signaling pathway.

Introduction

Parkinson's disease (PD), the most common neurodegenerative dyskinesia, is characterized by motor symptoms (such as resting tremor, stiffness, and postural instability), and non-motor symptoms (such as autonomic, mental and sensory cognitive disorders, and dementia)[1]. PD is the second major neurodegenerative disease after Alzheimer's disease and affects about 1% of the population aged above 60 years old[2], with more than 100,000 deaths every year worldwide[3]. In addition to lowering the life quality of patients, PD brings heavy economic burden on the society. It is estimated that the economic burden of PD may be up to 23 billion dollars annually in the USA[4]. The non-motor symptoms of PD, a chronic and progressive disease, usually occur many years before dyskinesia. The pathology of PD is the degeneration of nigrostriatal dopaminergic neurons and the accumulation of α-synuclein and other proteins. This may eventually lead to insufficient dopamine activity[5,6]. PD is the result of complex interaction among genetic and environmental factors[7]. In recent years, great progress has been made in the pathogenesis and molecular mechanism of PD. However, there are still no ef-

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fective molecular markers for early intervention of Parkinson's disease.

Micro ribonucleic acids (miRNAs) are a class of non-coding RNAs with about 22 bases in length. They can bind to the 3'UTR of messenger RNAs (mRNAs), ultimately participating in mRNA degradation or deadenylation and translation inhibition. MiRNAs have been observed involved in various physiological and pathological processes[8,9]. They can regulate protein expression involved in a variety of biological processes, such as cell proliferation, differentiation, cycle, stemness maintenance, and apoptosis[10]. Considering that one miRNA can control the expression of hundreds of transcripts, miRNAs possess huge regulatory potential. This is the reason why the entire phenotype of diseases can be actually affected by regulating a single miRNA. Therefore, there is a correlation between the miRNA network and the pathogenesis of neurodegenerative diseases, including PD[11]. In addition, there are also changes in the expression levels of several miRNAs in different PD models and the brain of PD patients[12,13]. MiR-133b is highly expressed in midbrain dopaminergic neurons, whereas is lowly expressed in the midbrain of PD patients[14]. In mouse embryonic stem (ES) cells, miR-133b negatively regulates the differentiation of ES cells into dopaminergic neurons. This indicates that miR-133b may play an important role in the pathogenesis of PD. Besides, miR-133b is a promoter of cervical carcinoma development through the activation of extracellular signal-regulated kinase (ERK) and AKT1 pathways[15]. Hence, it is speculated that miR-133b may exert a protective effect in the PD model *via* regulating the ERK1/2 signaling pathway.

1-methyl-4-phenylpyridinium ion (MPP^+) is the most relevant and commonly-used toxin for the establishment of PD model *in vitro*. Its resulting clinical, biochemical and neuropathological changes are similar to those of idiopathic PD. In the current study, the survival rate of primary PC12 cells treated with MPP^+ remarkably declined, which was accompanied by a significant decrease in the expression of miR-133b. The overexpression of miR-133b inhibited cell apoptosis in the PD cell model, accompanied by changes in the ERK1/2 signaling pathway. All these findings suggest that one of the potential mechanisms of reversion of MPP^+-induced axonal degeneration may be that miR-133b overexpression inhibits the activation of the ERK1/2 signaling pathway.

Materials and Methods

Cell Culture

PC12 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) under 5% CO_2 at 37°C. The culture medium was replaced every other day. Upon reaching 80% of confluence, the cells were digested with 0.25% trypsin (Thermo Fisher Scientific, Waltham, MA, USA) and passaged until the logarithmic growth phase.

Establishment of PD Cell Model

MPP^+ (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in deionized water and prepared into the stock solution (10 mmol/L). PC12 cells were seeded in a culture dish and treated with different concentrations of MPP^+ (0.5, 1 and 2 mmol/L) for 24 h upon reaching 80-90% confluence. Finally, the PD cell model was successfully established *in vitro*.

Cell Proliferation Assay

PC12 cells were first inoculated into 96-well plates, and the medium was discarded after adherence. Then, the cells were incubated with different concentrations of MPP^+ (0.5, 1 and 2 mmol/L) for 24 h, with 5 replicates at each concentration. According to the instructions of Cell Counting Kit-8 (CCK-8) assay, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added into each well, followed by incubation for 2 h in dark. Absorbance at 450 nm was finally measured using a micro-plate reader, based on which cell survival rate was calculated.

Cell Transfection

After treatment with 1 mmol/L MPP^+, PC12 cells were seeded into a 24-well plate. The cells were divided into four groups, including: blank control group (Control group, no treatment), MPP^+ group (treated with 1 mmol/L MPP^+ only), MPP^+ + miR-133b mimic group (transfected with 100 nM miR-133b mimic according to the instructions of the miRNA transfection reagent), and MPP^+ + miR-negative control (NC) group (transfected with 100 nM miR-NC). At 24 h

after transfection, the cells were collected, and total RNA was extracted. After 48 h, the protein was extracted from cells for Western Blotting analysis.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reversely transcribed into cDNA using PrimeScript™ RT-PCR kit (TaKaRa, Dalian, China), followed by qRT-PCR using LightCycler 480 II system (Roche, Basel, Switzerland). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references for gene and miR-133b, respectively. Relative expression levels of genes were measured using the $2^{-\Delta\Delta CT}$ method. Primers used in this study were shown in Table I.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Assay

PC12 cells were fixed with formaldehyde, washed with phosphate-buffered saline (PBS) for 3 times, and permeabilized with 1% Triton X-100. After reaction with TdT solution at room temperature, color development was performed. Finally, the cells were observed under a microscope, and $TUNEL^+$ cells were counted.

Western Blotting

Tissues were first lysed on ice using radioimmunoprecipitation assay (RIPA) lysis buffer (Roche, Basel, Switzerland) supplemented with protease inhibitor (PMSF). After centrifugation at 14,000 rpm for 20 min, the supernatant was collected. Protein concentration was quantified using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). After separation *via* 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), protein samples were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Next, the membranes were sealed with 5% skimmed milk powder for 1 h and incubated with specific primary antibodies of β-actin (1:2000, Abcam, Cambridge, MA, USA), ERK1/2 (1:1000, Abcam, Cambridge, MA, USA) and phosphorylated (p)-ERK1/2 (1:1000, Abcam, Cambridge, MA, USA) at 4°C overnight. On the next day, the membranes were incubated with corresponding secondary antibodies for 2 h at room temperature. Immuno-reactive bands were finally detected using the SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Numerical variables were expressed as mean ± standard deviation (SD). Independent-samples *t*-test was adopted for the comparison between two groups. $p<0.05$ was considered statistically significant.

Results

Effect of MPP^+ Treatment on PC12 Cell Viability

CCK-8 assay showed that the survival rate of PC12 cells declined significantly after MPP^+ treatment ($p<0.05$). MPP^+ exhibited toxicity against PC12 cells in a concentration-dependent manner, and cell survival rate decreased markedly with the increase of MPP^+ concentration ($p<0.05$) (Figure 1).

Effect of MPP^+ Treatment on Expression of MiR-133b in PC12 Cells

After MPP^+ treatment, the expression level of miR-133b in PC12 cells was significantly lower than that in the Control group ($p<0.05$). With the increase of MPP^+ concentration, the expression of miR-133b in PC12 cells declined remarkably ($p<0.05$) (Figure 2).

Table I. Primer sequences.

Index	F (5′-3′)	R (5′-3′)
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
miR-133b	GGGTTTGGTCCCCTTCA	TGGTGTCGTGGAGTCG
U6	GCTTCGGCACATATACTAAAAT	AACGCTTCACGAATTTGCGT
ERK1/2	ATATCCTTGGCTACTAAC	TATGGCTACAATGATTCTA

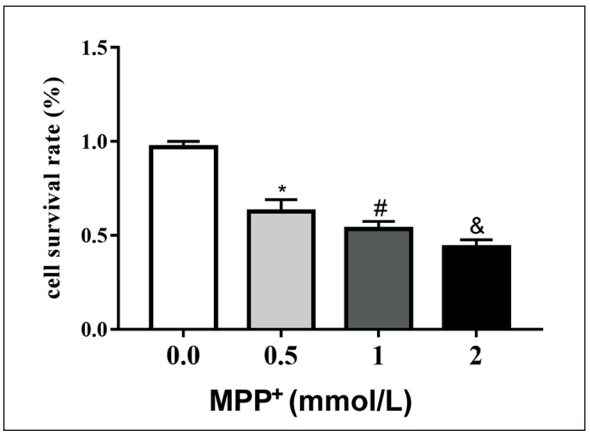

Figure 1. Effects of MPP^+ at different concentrations on survival rate of PC12 cells. Note: *p<0.05 *vs.* Control group (0.0), #p<0.05 *vs.* 0.5 mmol/L MPP^+, &p<0.05 *vs.* 1 mmol/L MPP^+.

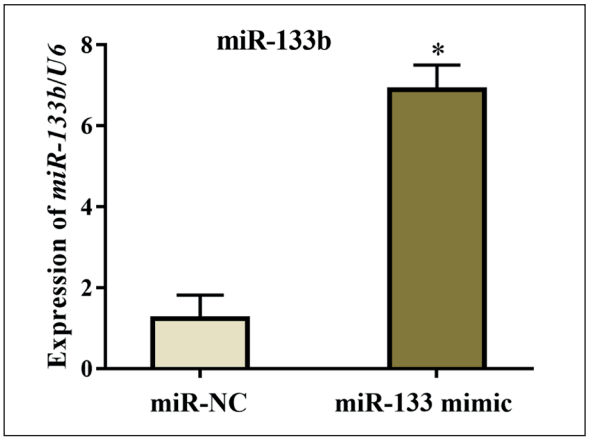

Figure 3. Expression of miR-133b in cells after transfection detected *via* qRT-PCR. Note: *p<0.05 *vs.* MPP^+ + miR-NC group.

Effect of MiR-133b Transfection on Expression of MiR-133b

QRT-PCR results showed that the expression of miR-133b was evidently higher in MPP^+ + miR-133b mimic group than MPP^+ + miR-NC group, and the difference was statistically significant (p<0.05). These findings indicated that the transfection efficiency of miR-133b in PC12 cells was higher after MPP^+ treatment (Figure 3).

Overexpression of MiR-133b Inhibited PC12 Cell Apoptosis in PD Model

No statistically significant difference was observed in the apoptosis rate between MPP^+ group and MPP^+ + miR-NC group (p>0.05). However, cell apoptosis in the two groups was markedly higher than the Control group (p<0.05). Meanwhile, cell apoptosis rate was notably lowered in MPP^+ + miR-133b mimic group compared with MPP^+ + miR-NC group (p<0.05) (Figure 4).

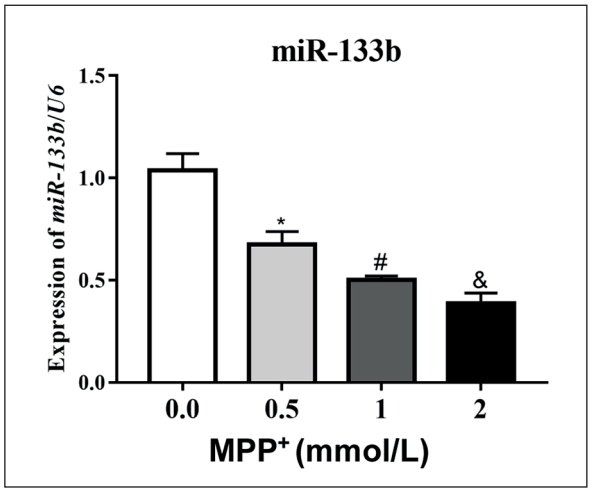

Figure 2. Effects of MPP^+ at different concentrations on expression of miR-133b in PC12 cells. Note: *p<0.05 *vs.* Control group (0.0), #p<0.05 *vs.* 0.5 mmol/L MPP^+, &p<0.05 *vs.* 1 mmol/L MPP^+.

Effect of MiR-133b Overexpression on ERK1/2 Signaling Pathway Protein Levels

QRT-PCR results showed that the mRNA expression of ERK1/2 displayed no significant difference between MPP^+ group and MPP^+ + miR-NC group (p>0.05). However, it was significantly upregulated in the two groups in comparison with Control group (p<0.05). Meanwhile, the mRNA expression of ERK1/2 remarkably declined in MPP^+ + miR-133b mimic group compared with MPP^+ + miR-NC group (p<0.05) (Figure 5). As shown in Figure 6, the results of Western blotting manifested that there was no apparent difference in the protein expression of p-ERK1/2 between MPP^+ group and MPP^+ + miR-NC group (p>0.05). However, the protein expression of p-ERK1/2 in the two groups was distinctly higher than that in Control group (p<0.05). In addition, the protein expression of p-ERK1/2 prominently declined in MPP^+ + miR-133b mimic group compared with MPP^+ + miR-NC group (p<0.05)

Discussion

Despite many studies on the pathogenesis of PD in recent years, PD remains a major challenge in the field of neurobiology due to no definite

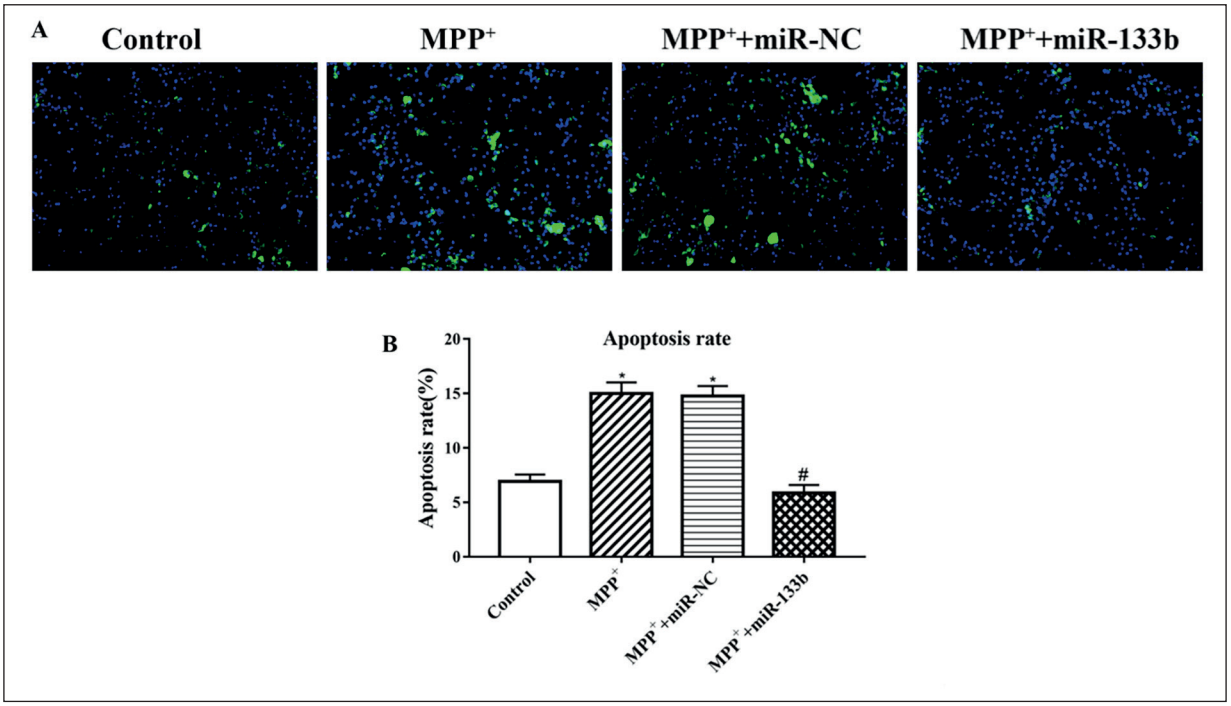

Figure 4. Comparison of apoptosis rate among groups after transfection (magnification: 400×) Note: *p<0.05 *vs.* Control group, #p<0.05 *vs.* MPP$^+$ + miR-NC group.

treatment methods[8,16]. The progress of researches on PD is limited by the low utilization of brain tissues and the dependence on cadaver samples. Therefore, several kinds of PD cell and animal models have been developed and widely used as alternatives for these clinical tissues that are hard to be obtained. In this investigation, the commonly-used toxic agent MPP$^+$ was used to induce the PD model in PC12 cells.

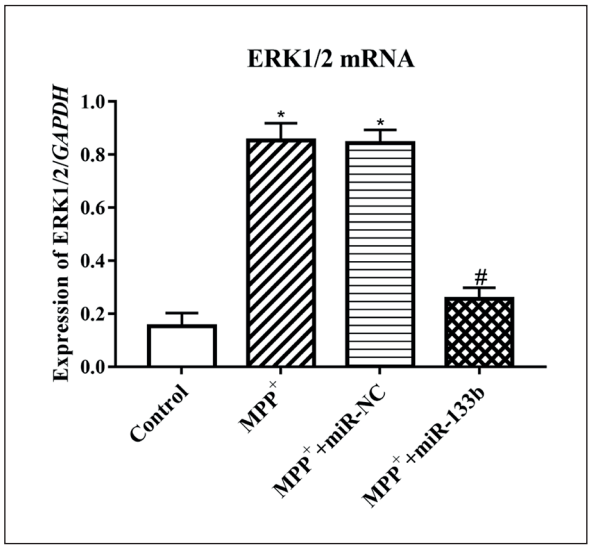

Figure 5. Effect of miR-133b overexpression on ERK1/2 mRNA expression determined *via* qRT-PCR. Note: *p<0.05 *vs.* Control group, #p<0.05 *vs.* MPP$^+$ + miR-NC group.

MiRNAs, a class of non-coding single-stranded RNA molecules, play key regulatory roles during biological development and mainly regulate the expression of target genes at the post-transcriptional level. Therefore, miRNAs exert important effects on tumorigenesis, biological development, organ regulation and metabolism[17,18]. It has been proved *in vitro* and *in vivo* that many miRNAs participate in the apoptosis, autophagy, inflammation, mitochondrial dysfunction and oxidative stress in PD. Consistently, miR-7, miR-153 and miR-221 exert regulatory roles in the PD cell model[19,20]. The expression of miR-133b is specifically lacked in brain tissues of PD patients and animal models[14]. Meanwhile, the expression of serum miR-133b also declines significantly in PD patients[21]. MiR-133b has multiple biological activities. In the present study, we manifested that the overexpression of miR-133b could prevent MPP$^+$-induced neuronal apoptosis. Besides, the balance between apoptosis and proliferation is critical to keeping normal cell activity. The disruption of such balance often results in disease development and progression. In mammalian cells, 5 mitogen-activated protein kinase

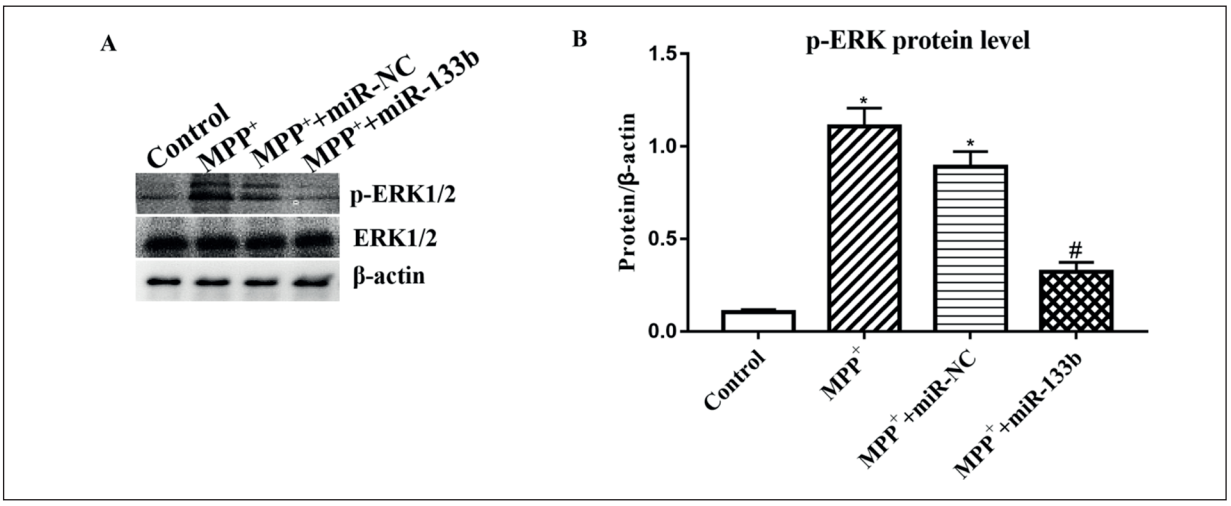

Figure 6. Effect of miR-133b overexpression on ERK1/2 protein expression determined *via* Western blotting. Note: *$p<0.05$ *vs.* Control group, $^{\#}p<0.05$ *vs.* MPP^+ + miR-NC group.

(MAPK) families have been determined, including ERK1 and ERK2, c-Jun N-terminal kinases (JNKs) (JNK1, JNK2 and JNK3); p38 kinase isozymes (p38α, p38β, p38γ and p38δ), ERK3/ERK4, and ERK5[22-24]. The MAPK pathway is an important regulator of cell proliferation and survival[25]. As key members of the MAPK family, ERK1 and ERK2 integrate extracellular signals to promote the proliferation and growth of most cells, as well as the learning and memory ability of nerve cells[26]. Phosphorylation of ERK1/2 in brain tissues is weakened with age. However, the exact role of ERK1/2 in brain aging remains unknown[27,28]. In this study, it was found that overexpression of miR-133b could suppress the activation of ERK1/2. Considering that the proteins or RNAs interacting with miR-133b in nerve cells may exert multiple functions, and that the ERK1/2 signaling pathway is complex, it is worth further study.

The differential expression of miR-133b in tissues and serum of PD patients has been reported previously. However, there is still no research indicating that miR-133b regulates the growth and apoptosis of nerve cells through the ERK1/2 signaling pathway. In this study, the results manifested that in the MPP^+-induced PD cell model, miR-133b expression was reduced remarkably, accompanied by increased protein expression level of p-ERK1/2. The above findings demonstrate for the first time that miR-133b can inhibit the activation of ERK1/2 and improve MPP^+-induced apoptosis in the PD cell model. Although the mechanism of action of the ERK1/2 signaling pathway remained to be deeply explored, our findings provided new ideas for such a mechanism in PD, which might have important significance for studying the role of miR-133b in PD pathogenesis.

Conclusions

To sum up, restoring the expression of miR-133b may be a new and attractive treatment method for Parkinson's disease.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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