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MicroRNA-599 regulates the development of Parkinson's disease through mediating LRRK2 expression

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Abstract. – OBJECTIVE: This study investigates whether microRNA-599 can inhibit the progression of Parkinson's disease (PD) by regulating the LRRK2 expression. We aim to search for a new therapeutic target for PD.

MATERIALS AND METHODS: A mouse model of PD was first established. A relative amount of TH+ neurons in the mouse brain was quantified by immunohistochemistry. The expression levels of microRNA-599 and LRRK2 in mouse brain tissues were determined by the quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot. Cell model of PD was constructed by MPP+ treatment in SH-SY5Y cells. The expression levels of microRNA-599 and LRRK2 in MPP+-induced SH-SY5Y cells were examined as well. We verified the binding condition between microRNA-599 and LRRK2 through dual-luciferase reporter gene assay. The viability and apoptosis in MPP+-induced SH-SY5Y cells overexpressing microRNA-599 were determined by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively.

RESULTS: Compared with normal mice, TH+ neurons were fewer in the brain tissue of PD mice. MicroRNA-599 expression was lower, while LRRK2 expression was higher in brain tissues of PD mice relative to controls. Meanwhile, *in vitro* expression of microRNA-599 was downregulated and LRRK2 expression was upregulated in MPP+-induced SH-SY5Y cells. Dual-luciferase reporter gene assay verified the binding condition between microRNA-599 and LRRK2. The microRNA-599 overexpression downregulated the LRRK2 expression in SH-SY5Y cells, and conversely, the microRNA-599 knockdown upregulated the LRRK2 expression. Of note, the microRNA-599 overexpression protected MPP+-induced viability decrease and apoptosis acceleration in SH-SY5Y cells.

CONCLUSIONS: MicroRNA-599 is lowly expressed in both *in vivo* and *in vitro* PD model. MicroRNA-599 inhibits the development of PD through regulating the LRRK2 expression.

Key Words:
MicroRNA-599, LRRK2, Apoptosis, PD.

Introduction

As a neurodegenerative disease, Parkinson's disease (PD) slowly impairs substantia nigra and nigrostriatal pathway in middle-aged and elderly people. Deficiency of melanin-containing dopamine (DA) neurons is pronounced in the substantia nigra pars compacta of PD patients[1,2]. Multiple factors are involved in the pathogenesis of PD, including environmental and genetic factors, and the latter greatly increases the susceptibility of PD. Massive degeneration of dopaminergic neurons induced by oxidative stress, mitochondrial dysfunction, neurotoxicity of excitatory amino acids, and neuronal apoptosis finally leads to the onset of PD[3,4].

MicroRNA is a single-strand, non-coding RNA of about 18 to 22 nucleotides in length. It regulates target gene expressions at the post-transcriptional level by complementary pairing with the target mRNA, thereby exerting its biological effects[5,6]. More than 2,000 microRNAs have been discovered in humans, which are vital in the normal physiological activities and pathological development of various diseases[7,8].

PD is a common neurodegenerative disease. In 2004, two independent researches identified that LRRK2 is a causative gene of autosomal dominant familial PD in multiracial families[9,10]. Risk variability of LRRK2 is only 0.1-4% in sporadic PD patients from Asia or Europe, but is strikingly up to 23% in familial PD of Asian patients. The LRRK2 mutation leads to the DA neuron deficiency in the substantia nigra pars compacta and Lewy body deposition in neurons[11,12]. Studies[13,14] have found that enhanced kinase activity caused

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by genetic mutations in the LRRK2 domain is an important mechanism leading to dopaminergic neuronal damage.

This work aims to elucidate the role of microRNA-599 in the development of PD and its underlying mechanism.

Materials and Methods

Establishment of In vivo PD Model in Mice

Twelve C57BL/6 mice were habituated for 1 week prior to the experiments, and they were given free access to drinking and food. Mice were randomly assigned into model group (n=6) and control group (n=6). After ether anesthesia, mouse neck was held to upward the nostrils. 1 g/L lipopolysaccharide (LPS) dissolved in 0.9% sodium chloride solution was slowly dropped into each side of nostril using a pipette and stand for 10 s to keep the drug solution fully infiltrated. LPS administration was daily given for consecutive 30 days. Mice in control group were administrated with the same volume (0.9% of sodium chloride solution) in the same way. Statistically significant differences in the signs of PD and behavioral scores in mice between model group and control group were considered as the successful construction of the PD model. This study was approved by the Animal Ethics Committee of Shanxi Provincial People's Hospital Animal Center.

Immunohistochemistry

After the injection with LPS or sodium chloride solution, the mice were anesthetized with 8% chloral hydrate. Next, the mice were perfused into the heart with paraformaldehyde. The brain around the coronary artery of the mice was subjected to sectioning on ice with the thickness of 1mm. And at last, the sections were stained to point out the TH-positive cells. Immunohistochemistry was quantified using Image-Pro Plus software (Version X; Media Cybernetics, Silver Springs, MD, USA).

Cell Culture

Human neuroblastoma cells SH-SY5Y were purchased from Conservation Genetics CAS Kunming Cell Bank (KCB2006107YJ) (Kunming, China). SH-SY5Y cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% streptomycin-penicillin. Cells were incubated at 37°C, 5% CO_2, and the medium was regularly changed.

SH-SY5Y cells in the exponential growth phase were digested, inoculated, and cultured until the cell density reached 60%. Cells were treated with 50 ng/mL MPP^+ for 24 hours and harvested for subsequent experiments.

Cell Transfection

SH-SY5Y cells were inoculated in a 6-well plate and cultured until cell density was up to 60%. Cells were transfected with microRNA-599 mimics, anti-microRNA-599 or NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After transfection for 6 h, fresh medium was replaced.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA). The purity of RNA sample was measured by an ultraviolet spectrophotometer and stored at -80°C until use. The complementary deoxyribose nucleic acid (cDNA) was reversely transcribed, and the SYBR Green method was used for PCR detection. Primer sequences were as follows: MicroRNA-599, F: 5′-UUGUGUCAGUUUVUCAAAC-3', R: 5′-UUGAUAAACUGACACAA-3'; U6, F: 5′-CTCGCTTCGGCAGCACA-3′, R: 5′-AACGCTTCACGAATTTGCGT-3′; LRRK2, F: 5′-TGGGTTGGTCACTTCTGC-3′, R: 5′-CATTGGCTGGAAATGAGTGC-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: 5′-ACCCACTCCTCCACCTTTGA-3′, R: 5′-CTGTTGCTGTAGCCAAATTCGT-3′.

Western Blot

Total protein was extracted for determining the protein expression. The protein sample was quantified by bicinchoninic acid (BCA; Pierce, Rockford, IL, USA), separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and blocked with 5% skim milk. Membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by enhanced chemiluminescence (ECL).

Dual-Luciferase Reporter Gene Assay

SH-SY5Y cells were inoculated in 24-well plates with 3×10^5 cells per well. Cells were co-transfected with wild-type or mutant-type psi-CHECK-2 LRRK2 vector with microRNA-599 mimics or miR-NC using Lipofectamine 2000. At 24 h later, luciferase activity was determined

using the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Cell Proliferation Assay

SH-SY5Y cells were cultured in a 96-well plate at a density of 5×10^4/mL, with 100 μL per well. After cell culture for 24 h, 10 μL of cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well. Absorbance at 450 nm wavelength was recorded after incubation for 1 h using a Microplate Reader (Bio-Tech Company, Hercules, CA, USA).

Cell Apoptosis Assay

SH-SY5Y cells were washed with PBS twice, digested and fixed in pre-cold 70% ethanol at 4°C for 30 min. Subsequently, cells were induced with 5 ml of Annexin V-FITC (fluorescein isothiocyanate) and 1 mL of Propidium Iodide (PI) (50 mg/mL) for 5 min. Apoptosis was determined using flow cytometry (Becton-Dickinson, Mountain View, CA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) was utilized for statistical analysis. Normally distributed measurement data were represented as mean ± standard deviation ($\bar{x}\pm s$). Differences between the two groups were analyzed by the *t*-test. $p<0.05$ was considered as statistically significant.

Results

MicroRNA-599 Was Lowly Expressed in the in vivo PD Model

The number of TH^+ cells in the ipsilateral brain tissues of PD mice was markedly reduced compared to normal mice (Figure 1A). Meanwhile, we found that the expression level of

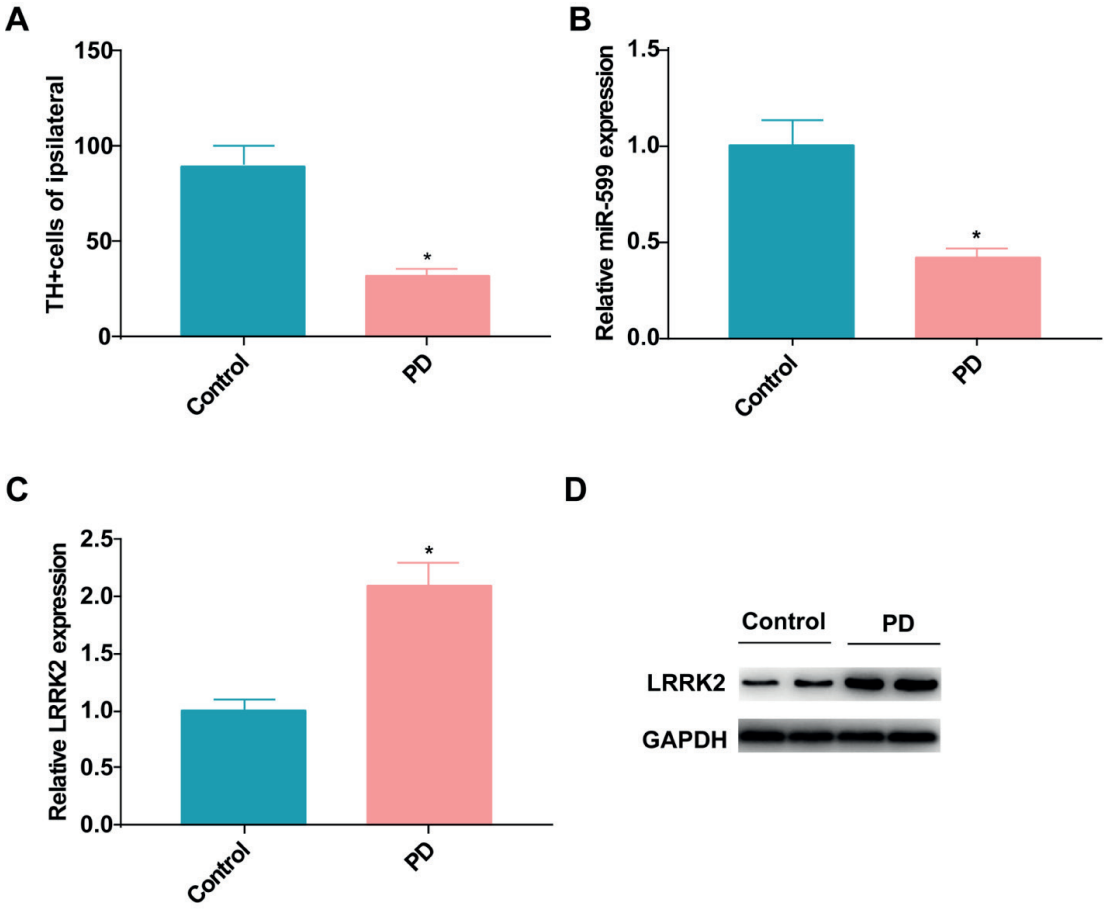

Figure 1. MiR-599 was lowly expressed in the *in vivo* PD model. ***A,*** The number of TH^+ cells in the ipsilateral brain tissues of PD mice was markedly reduced compared to normal mice. ***B,*** Expression level of miR-599 was lower in brain tissues of PD mice than controls. ***C, D,*** Both mRNA (***C***) and protein (***D***) expression levels of LRRK2 were significantly elevated in brain tissue of PD mice relative to controls. *$p<0.05$.

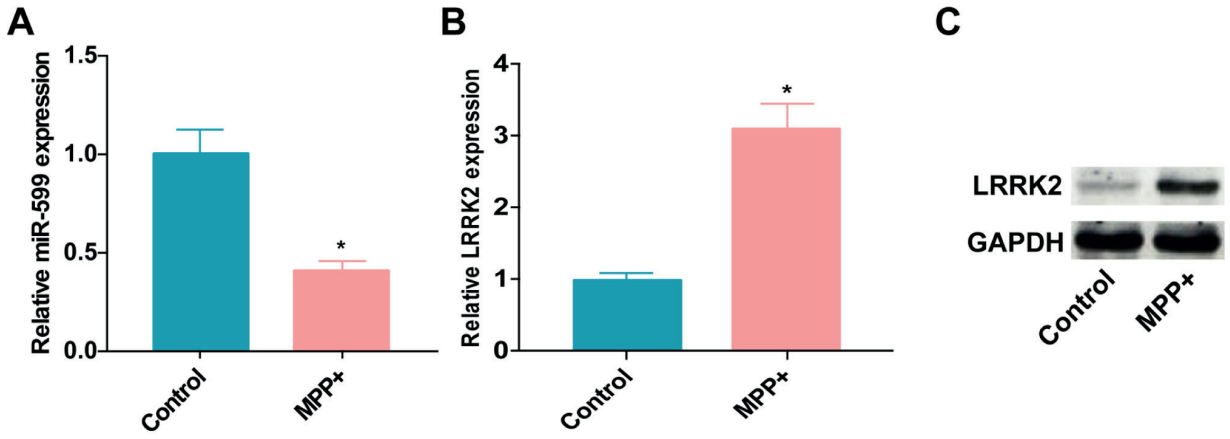

Figure 2. MiR-599 was lowly expressed in the *in vitro* PD model. ***A***, MPP^+ treatment in SH-SY5Y cells downregulated miR-599 expression at mRNA level. ***B***, MPP^+ treatment in SH-SY5Y cells upregulated LRRK2 expression at mRNA level. ***C***, MPP^+ treatment in SH-SY5Y cells upregulated LRRK2 expression at protein level. *$p<0.05$.

microRNA-599 was lower in brain tissues of PD mice than controls (Figure 1B). Both mRNA and protein expression levels of LRRK2 were significantly elevated in brain tissue of PD mice relative to controls (Figure 1C-1D). The above results indicated that microRNA-599 was lowly expressed and LRRK2 was highly expressed in PD mice.

MicroRNA-599 Was Lowly Expressed in the in vitro PD Model

After MPP^+ treatment in SH-SY5Y cells, microRNA-599 expression was markedly reduced, while LRRK2 expression was elevated at the mRNA level (Figure 2A-2B). Identically, the protein level of LRRK2 was upregulated in MPP^+-induced SH-SY5Y cells (Figure 2C). We confirmed that microRNA-599 was lowly expressed and LRRK2 was highly expressed in the *in vitro* PD model.

MicroRNA-599 Targeted on LRRK2 Expression

To explore further the interaction between microRNA-599 and LRRK2 in the development of PD, we predicted the binding site of microRNA-599 to LRRK2 (Figure 3A) and constructed wild-type (LRRK2 WT) and mutant-type (LRRK2 MT) LRRK2 luciferase reporter vectors. Luciferase activity was quenched in SH-SY5Y cells co-transfected with LRRK2 WT and microRNA-599 mimics, suggesting the binding of LRRK2 to microRNA-599 (Figure 3B). Subsequently, both mRNA and protein levels of LRRK2 were found to be upregulated by the microRNA-599 knockdown. Conversely, LRRK2 expression was downregulated by microRNA-599 overexpression (Figure 3C-3D). The above indicated that microRNA-599 could target LRRK2 expression in human neuroblastoma cells.

MicroRNA-599 Protected MPP+-Induced Viability Decrease Through Targeting LRRK2

Next, we focused on the roles of microRNA-599 and LRRK2 in MPP^+-induced neuronal injury. It was found that the microRNA-599 overexpression downregulated the increased mRNA and protein levels of LRRK2 in MPP^+-induced SH-SY5Y cells (Figure 4A-4B). Furthermore, CCK-8 results showed that the overexpression of microRNA-599 reversed the decrease in neuronal viability caused by MPP^+ treatment (Figure 4C). It was demonstrated that microRNA-599 reversed the decrease in cell viability caused by MPP^+ treatment in SH-SY5Y cells through inhibiting the LRRK2 expression.

MicroRNA-599 Protected MPP+-Induced Neuronal Apoptosis

MPP^+ treatment greatly induced apoptosis in SH-SY5Y cells as flow cytometry indicated, while microRNA-599 overexpression partially prevented against MPP^+-induced neuronal apoptosis (Figure 5A-5B). It was indicated that the microRNA-599 overexpression protected neuronal apoptosis in the *in vitro* PD model.

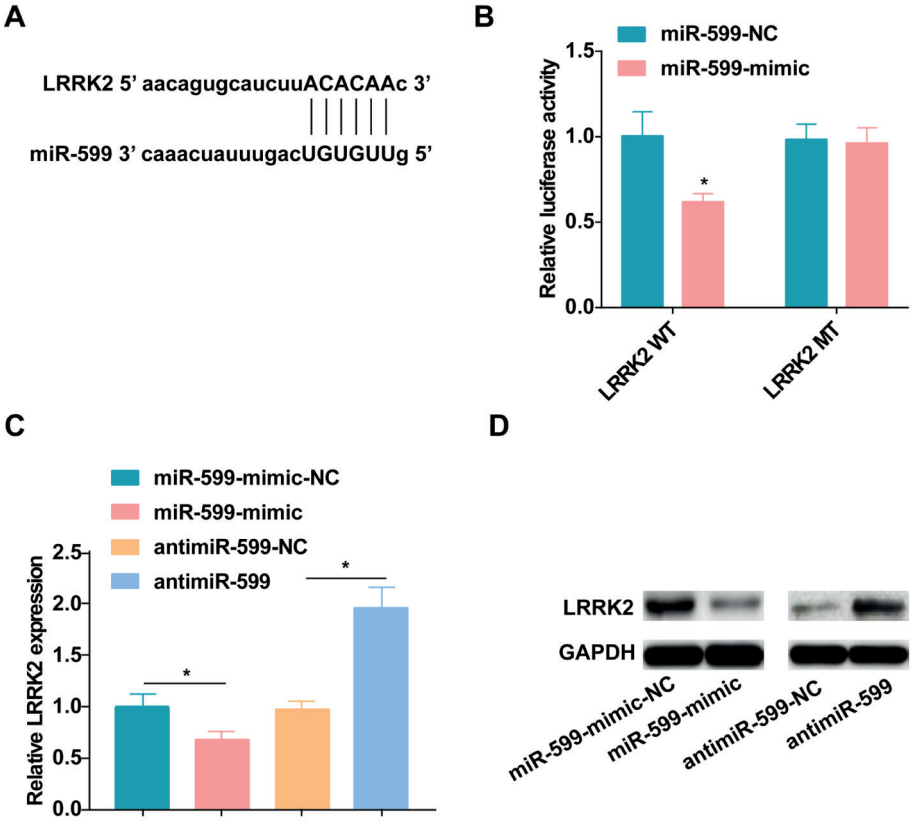

Figure 3. MiR-599 targeted on LRRK2 expression. ***A,*** The predicted binding site of miR-599 to LRRK2. ***B,*** Dual-luciferase reporter gene assay showed that luciferase activity was quenched in SH-SY5Y cells co-transfected with LRRK2 WT and miR-599 mimics. ***C-D,*** Both mRNA and protein levels of LRRK2 were found to be upregulated by miR-599 knockdown. Conversely, LRRK2 expression was downregulated by miR-599 overexpression. *$p<0.05$.

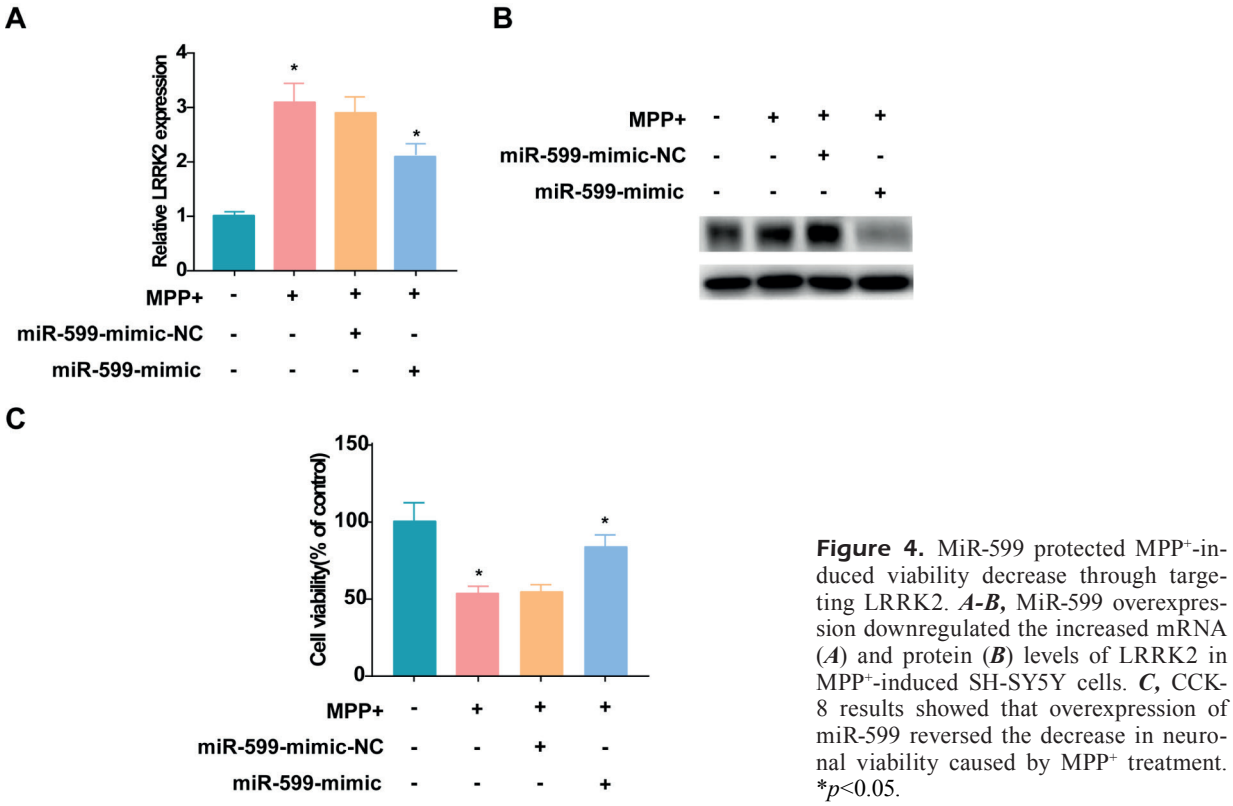

Figure 4. MiR-599 protected MPP^+-induced viability decrease through targeting LRRK2. ***A-B,*** MiR-599 overexpression downregulated the increased mRNA (***A***) and protein (***B***) levels of LRRK2 in MPP^+-induced SH-SY5Y cells. ***C,*** CCK-8 results showed that overexpression of miR-599 reversed the decrease in neuronal viability caused by MPP^+ treatment. *$p<0.05$.

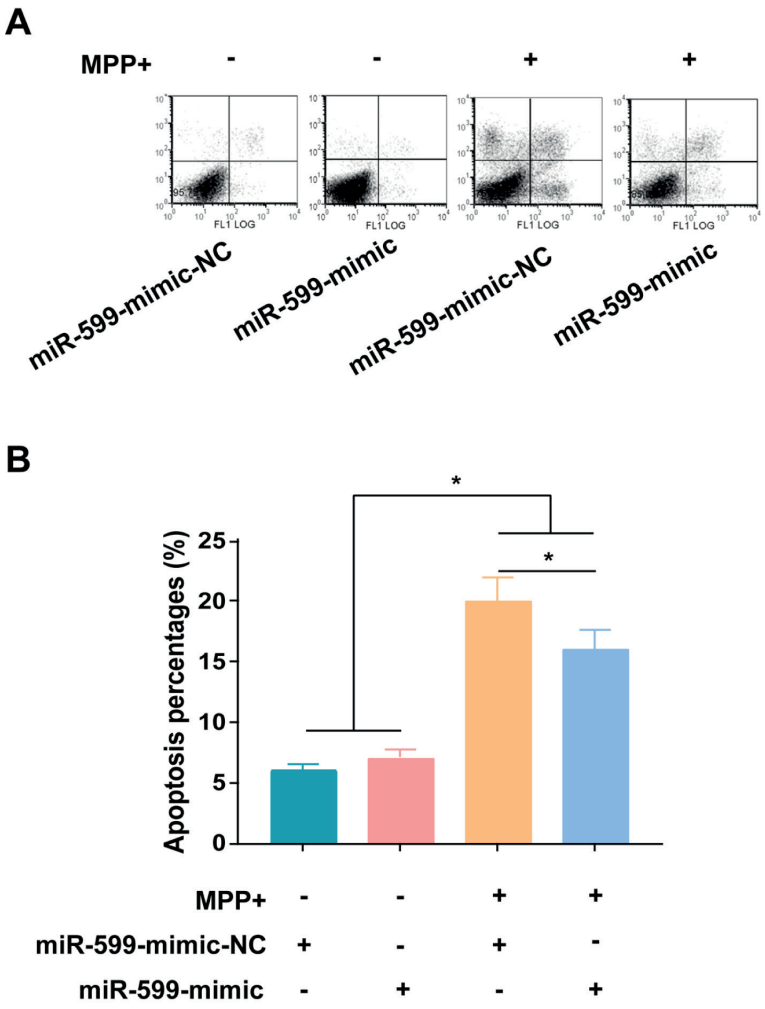

Figure 5. MiR-599 protected MPP^+-induced neuronal apoptosis. ***A-B,*** Flow cytometry indicated that MPP^+ treatment greatly induced apoptosis in SH-SY5Y cells, while miR-599 overexpression partially prevented against MPP^+-induced neuronal apoptosis. *$p<0.05$.

Discussion

PD is a neurodegenerative disease characterized by massive degeneration and progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* of the basal ganglia[15,16]. It is believed that aging, mitochondrial dysfunction, inflammation, and oxidative stress are responsible for the etiology of PD[17,18].

MicroRNAs are ubiquitous in organisms. Functionally, microRNAs regulate gene expressions by inhibiting mRNA translation *via* binding to 3′UTR of target mRNA[19,20]. Differentially expressed microRNAs have been identified in PD, which participate in neuronal cell proliferation, differentiation, apoptosis, and autophagy[4,21].

Apoptosis, also known as programmed cell death, triggers the process of natural cell death by activating endogenous DNA endonucleases. Apoptosis helps to maintain the homeostasis of tissues and cells[22,23]. However, excessive apoptosis is a pathological phenomenon. Massive neuronal apoptosis is found in neurodegenerative diseases[24,25]. In mammals, LRRK2 leads to apoptosis by activating p38 MAPK and MKK7 through the JNK pathway[26,27]. LRRK2 deficiency exerts a protective effect on DA neurons[28].

In this study, we found that microRNA-599 protected neuronal cells by regulating LRRK2 expression. MicroRNA-599 was lowly expressed, while LRRK2 was highly expressed in brain tissues of PD mice. To demonstrate that microRNA-599 could target and regulate LRRK2 expression, we co-transferred the LRRK2 lucife-

rase reporter vector and microRNA-599 mimics in SH-SY5Y cells. The dual-luciferase reporter gene assay verified our speculation that microRNA-599 directly bound to LRRK2. Moreover, microRNA-599 was capable of negatively regulating LRRK2 expression at both mRNA and protein levels. The overexpression of microRNA-599 protected the damage of SHP-SY5Y cells by MPP^+ treatment through downregulating LRRK2 expression.

Conclusions

We found that microRNA-599 is lowly expressed in both *in vivo* and *in vitro* PD model. MicroRNA-599 inhibits the development of PD through regulating LRRK2 expression.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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