

Original Research

MiRNA-1976 Regulates the Apoptosis of Dopaminergic Neurons by Targeting the *PINK1* Gene

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Abstract

Introduction: Parkinson's disease (PD), which is a neurodegenerative disease, requires urgently needed biomarkers to explore its mechanism. We screened for differences in the expression of microRNAs (miRNAs) and identified miR-1976 as a possible biomarker. **Methods:** Twenty-three patients and 30 controls were included in this study. Dopaminergic neurons from C57/BL mice were cultured. The miRNA expression profiles were analyzed using an miRNA microarray. MiR-1976 was identified as an miRNA that was differentially expressed between PD patients and age-matched controls. Lentiviral vectors were constructed, then apoptosis in dopaminergic neurons was analyzed using MTS (multicellular tumor spheroids) and flow cytometry. Transfection of miR-1976 mimics into MES23.5 cells was performed, and target genes and biological effects were analyzed. **Results:** Overexpression of miR-1976 increased apoptosis and mitochondrial damage in dopaminergic neurons. *PINK1* (*PINK1*-induced kinase 1) was the most common target protein of miR-1976, and silencing of *PINK1* caused mitochondrial damage and increased apoptosis of MES23.5 cells. **Conclusions:** MiR-1976 is a newly discovered miRNA that exhibits a high degree of differential expression with respect to the apoptosis of dopaminergic neurons. Given these results, increased expression of miR-1976 may increase the risk of PD by targeting *PINK1* and may therefore be a useful biomarker for PD.

Keywords: Parkinson's disease; miR-1976; *PINK1*; biomarker; target protein; dopaminergic neuron; apoptosis

1. Introduction

Parkinson's disease (PD) is a multifarious neurodegenerative disease that affects the neurons in the substantia nigra pars compacta. The incidence of PD in those aged 65 years is 1.7%, but this prevalence increases to 4%–5% for those aged ≥85 years old [1]. A report issued by the Central Committee and the State Council of China indicated that in late 2019, there were 2.54 billion people aged 60 years or older in the Chinese population [2]. Because older individuals are at a higher risk of developing PD, and given that China's elderly population is increasing, the prevalence of this condition is likely to increase. People diagnosed with PD experience significant movement difficulties and may present with a range of non-motor symptoms including sleep disorders, cognitive deficits, and mental health issues, that also stand to reduce the individual's quality of life. However, despite the significant implications of this burden on both the individual and the health care sector, the cause or trigger of these neurodegenerative changes remains unknown. One of the primary challenges of studying the potentially relevant neurophysiological and environmental factors that contribute to the onset of PD is that current diagnostic methods rely on detecting motor symptoms, which only appear after significant dopaminergic neuronal

loss. By identifying biomarkers that can be used to predict which people have a higher risk of developing PD, it could be feasible to diagnose the condition earlier and obtain a better understanding of the pathology.

It is widely speculated that complex interactions between genetic and environmental factors may play a part in triggering the neurodegenerative changes that lead to the symptoms of PD [3–6]. Most dopaminergic neurons are located on the dense part of the substantia nigra and are extremely vulnerable to oxidative stress, a commonly reported risk factor for PD [7]. By studying the biological regulatory mechanisms of the dopaminergic neurons, it may prove possible to gain important insights into the pathogenesis of PD. For example, some research has shown that excessive apoptosis of dopaminergic neurons that lead to the changes underpinning PD was associated with specific dysfunction of the neurons themselves [8,9]. To evaluate the key factors that regulate the apoptosis of dopaminergic neurons, we utilized dopaminergic neurons as experimental cells in the hope of better understanding how PD develops. In many instances, neuronal cell death is modulated by changes in gene expression. The myocyte enhancer factor, encoded by the myocyte enhancer factor-2 gene, is expressed in neurons and plays a key role in the nitrosative



stress-induced dysfunction in the isogenic human induced pluripotent stem cell Parkinson's model [10]. Both glycogen synthase kinase-3 β and caspase-3 have turned out to be involved in the apoptosis of dopaminergic neurons in PD; however, the therapeutic effects of targeting these genes are still controversial [11].

MicroRNAs (miRNAs) are small RNA molecules that perform non-coding functions in both plants and animals. According to previous studies, dysregulation of miRNAs and their associated biological processes is a possible cause of common pathophysiological conditions such as PD [12, 13]. Some miRNAs have been identified to be dysregulated in PD in three areas: brain/neuronal models, cerebrospinal fluid, and blood [14]. Among these miRNAs, some play potential roles in interactions with PD risk genes, mitochondrial function, and immune pathways. To date, research has identified several different functions of miRNAs in humans, but the potential function of miRNAs on dopaminergic neurons is still unknown. It was observed in our previous pilot study [15] that miR-1976 showed high differential expression concerning apoptosis of dopaminergic neuronal cells; therefore, it may be involved in the development of PD and has potential as a new biomarker.

MiR-1976 is an miRNA molecule that is 20 nucleotides in length and is located on chromosome 1p36.11. A high-through sequence study has demonstrated that compared with healthy lung tissue, this miRNA was downregulated in non-small cell lung cancer tissue [16]. Similarly, low expression of miR-1976 has been implicated in the biological function of breast cancer [17]. Although several studies have reported an association between miR-1976 and certain cancer types, it remains unclear whether this miRNA plays a role in the development of PD.

In our study, we profiled differentially expressed miRNAs in PD patients and non-PD control subjects. To ascertain the biological functions and the predicted target genes of miR-1976, we cultured and analyzed dopaminergic neurons (MES23.5) in this study.

2. Materials and Methods

2.1 Participants

Twenty-three PD patients (male: 12; female: 11) with a mean age of 63.5 ± 1.0 years and on anti-parkinsonian medications were recruited from the Brain Hospital affiliated with the Nanjing Medical University and included in this study (Table 1). The participants with PD were diagnosed according to PD diagnostic criteria released by the Movement Disorders and PD Group within the Chinese Neurology Association. Participants diagnosed with other comorbid conditions, such as diabetes, arthritis, or heart disease were excluded from the study. In addition to the PD cohort, 30 age- and gender-matched healthy volunteers (male: 15, female: 15) with a mean age of 65.0 ± 0.8 years were contacted via the physical examination center of our hospital and invited to participate in this study as members

of the control group. To be eligible for inclusion, all controls were required to be free of neurological conditions and have no history of diabetes, arthritis, or heart disease. The protocols for this research were approved by the appropriate Ethics Committees of the Nanjing Brain Hospital (Ethics Reference No: 2017-KY010), and all participants involved gave their written informed consent to participate.

Table 1. Demographic characteristics of PD and control groups.

	Control (n = 30)	PD (n = 23)	p value
Age (y)	65.5 \pm 0.8	63.5 \pm 1.0	0.078
Gender (Male%)	50%	52%	
Height (cm)	166.4 \pm 10.3	166.1 \pm 8.9	0.722
Weight (kg)	67.6 \pm 15.3	63.4 \pm 12.7	0.091
BMI (kg/m ²)	24.4 \pm 3.9	23.0 \pm 4.1	0.073

Data are mean \pm SD values. BMI, body mass index.

The PD participants were predominantly in the early stages of the disease (Hoehn & Yahr score 1.4 ± 0.9) and had an average unified Parkinson's Disease rating scale (UPDRS) total score of 40.9 ± 17.9 . For the three UPDRS sub-scales, UPDRS I, II, and III, the mean scores were 2.8 ± 2.2 , 11.5 ± 7.4 , and 26.6 ± 10.8 , respectively (Table 2).

Table 2. Disease characteristics of PD participants.

UPDRS I	2.8 (2.2)
UPDRS II	11.5 (7.4)
UPDRS III	26.6 (10.8)
UPDRS Total	40.9 (17.9)

Abbreviations: UPDRS, Unified Parkinson's Disease Rating Scale. Data are mean (SD).

2.2 Animals and Treatments

Eight-week-old C57/BL mice were provided by Nanjing Medical University (Nanjing, Jiangsu, China). Forty C57/BL mice (20 males and 20 females) were acclimatized and kept at a temperature of 22 ± 2 °C and a humidity of $60\% \pm 2\%$ with ad libitum feeding. All experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of our hospital (Ethics Reference No: 2017-KY010). Twenty of the C57/BL mice received the miR-1976 mimic lentiviral vector injected into neurons of the substantia nigra and were euthanized 24 h after receiving treatment. Meanwhile, the remaining 20 mice received only saline injections into the neurons of the substantia nigra and served as controls.

2.3 Cell Culture and Transfection

The dopaminergic neuronal cell line (MES23.5) used in this study was cultured in Gibco Dulbecco's Modi-

fied Eagle Medium (DMEM) supplemented with 15% fetal bovine serum at 37 °C and 5% CO₂ before being prepared for transfection.

To facilitate analysis, HSA-miR-1976 mimics (and negative controls), *PINK1 shRNA*, and *PINK1* inhibitors were purchased from Shengbo Biotechnology Co., Ltd (Shanghai, China). Once the MES23.5 cells that were cultured in DMEM had reached approximately 50% confluence, cells (1×10^6) were seeded into each well of a 6-well plate and transfected with the above mimics or inhibitors using Lipofectamine™ 2000 (11668, Invitrogen, CA, USA) according to the manufacturer's protocol. These cells were subsequently collected after 12 h, 24 h, and 36 h and used in the subsequent experiment.

2.4 Pretreatment of Human Blood Samples

Blood samples (3 mL) were drawn from each subject and were centrifuged at 3500 rpm for 5 min. Plasma samples were then stored at –80 °C before analysis.

2.5 Total RNA Isolation

Total RNA was isolated using the TRIzol protocol. In brief, TRIzol (15596, Ambion, Carlsbad, CA, USA) was added to the plasma samples. Following a 10 min incubation at ambient temperature, 200 µL of chloroform was added and mixed vigorously. The mixture was then centrifuged at 14,000 rpm for 15 min at a temperature of ~4 °C. The upper aqueous phase was then transferred into a new tube and combined with 500 µL of ethanol to facilitate RNA extraction. The RNA precipitant obtained by centrifugation was washed with 75% ethyl alcohol, and the integrity of extracted total RNA was determined using a microspectrophotometer (MSP100, Angstrom Sun Technologies Inc, New York, NY, USA).

2.6 MiRNA Isolation

Total RNA (100 µg) was added to 5 times the volume of lysis buffer, then combined with 1/10th the volume of miRNA Homogenate Additive and 1/3rd the volume of ethyl alcohol. The mixture was subsequently centrifuged at 5000 rpm for 1 min. Following centrifugation, the precipitate was combined with 700 µL of miRNA Washing Solution 1 and 500 µL of miRNA Washing Solution 2 and recentrifuged an additional two times. Finally, 50 µL of Elution Solution was added to the column and left for 2 min prior to centrifuging the samples for 1 min at 10,000 rpm. The flowthrough containing the miRNA was stored at –80 °C until used.

2.7 MiRNA Microarray Experiments

Microarray data was normalized using the R package *frma* from Bioconductor and analyzed with the R *limma* package. Unless otherwise indicated, data were considered significant with a *p*-value lower than 0.05 and absolute fold change higher than 1. First, the solution used for microarray

hybridization was prepared by combining 2.4 µL of 15% formamide, 3.2 µL of 0.2% sodium dodecyl sulfate, 4 µL of $3 \times \text{SSC}$, 1.6 µL of $50 \times$ Denhardt's solution, and 6.4 µL of diethyl pyrocarbonate. Following this process, the RNA described above was added to this solution and incubated for 3 min at 95 °C. Hybridization of the miRNAs was performed on the mammalian miRNA microarray chip (V3.0), after which the hybridized chips were washed and scanned on a microarray scanner (G2565CA, Agilent, Santa Clara, CA, USA).

2.8 Real-time Polymerase Chain Reaction (PCR)

Total RNA was extracted using the procedures described above for microarray hybridization. Both primers for each miRNA (miR-1976, miR-153, miR-103a, miR-29a, miR-210, miR-375, miR-146a, and miR-101a) and the internal reference U6 reverse transcription primers were provided by Ribobio (Guangzhou, Guangdong, China). For real-time PCR, the cDNA from individual samples of total RNA was synthesized using the FastQuant RT kit (KR106, TIANGEN, Beijing, China). The real-time PCR was performed in a 20 µL reaction system comprising 10 µL SYBR Green (163795-75-3, TaKaRa, Kyoto, Japan), 1 µL upstream/downstream primers, 1 µL deoxynucleotide solution, 2 µL Taq polymerase, and 5 µL cDNA. Cycling conditions included 10 min at 95 °C pre-denaturation, followed by 40 cycles of 5 sec denaturation at 95 °C, 2 min annealing at 60 °C, and 30 sec elongation at 72 °C. All reactions were performed on a Prime Quantitative PCR detector (7300, ABI, Arlington, VA, USA).

2.9 Luciferase Assay

The 3'UTR fragment of *PINK1*, which contains binding sites for miR-1976 (WT) and a 3'UTR fragment with a corresponding mutation (MUT), were designed based on the results of information prediction. The primers used in this assay were miR-1976-F (CTC CTG CCC TCC TTG CTG T) and U6-F (CCT AGC ACC ATG AAG ATC AAG AT). They were then plated into a 24-well plate and co-transfected with 40 nM of either an miR-1976 mimic or a negative control and 100 ng of either the *PINK* 3'UTR WT or MUT reporter using the Lipofectamine™ (3000, Invitrogen, CA, USA). Forty-eight h later the cells were collected, and the luciferase activity was measured using the dual luciferase assay system (E2920, Promega, Madison, WI, USA).

2.10 Electron Microscopy

The MES23.5 cells were first cultured and then digested using pancreatin. After washing with phosphate-buffered saline, cells were centrifuged for 10 min at 800–1000 rpm, after which the supernatant was discarded, and the remaining precipitate was resuspended at 1500 rpm for 10 min. The compacted cell mass was then fixed with 2.5% glutaraldehyde and 1% osmic acid. After embed-

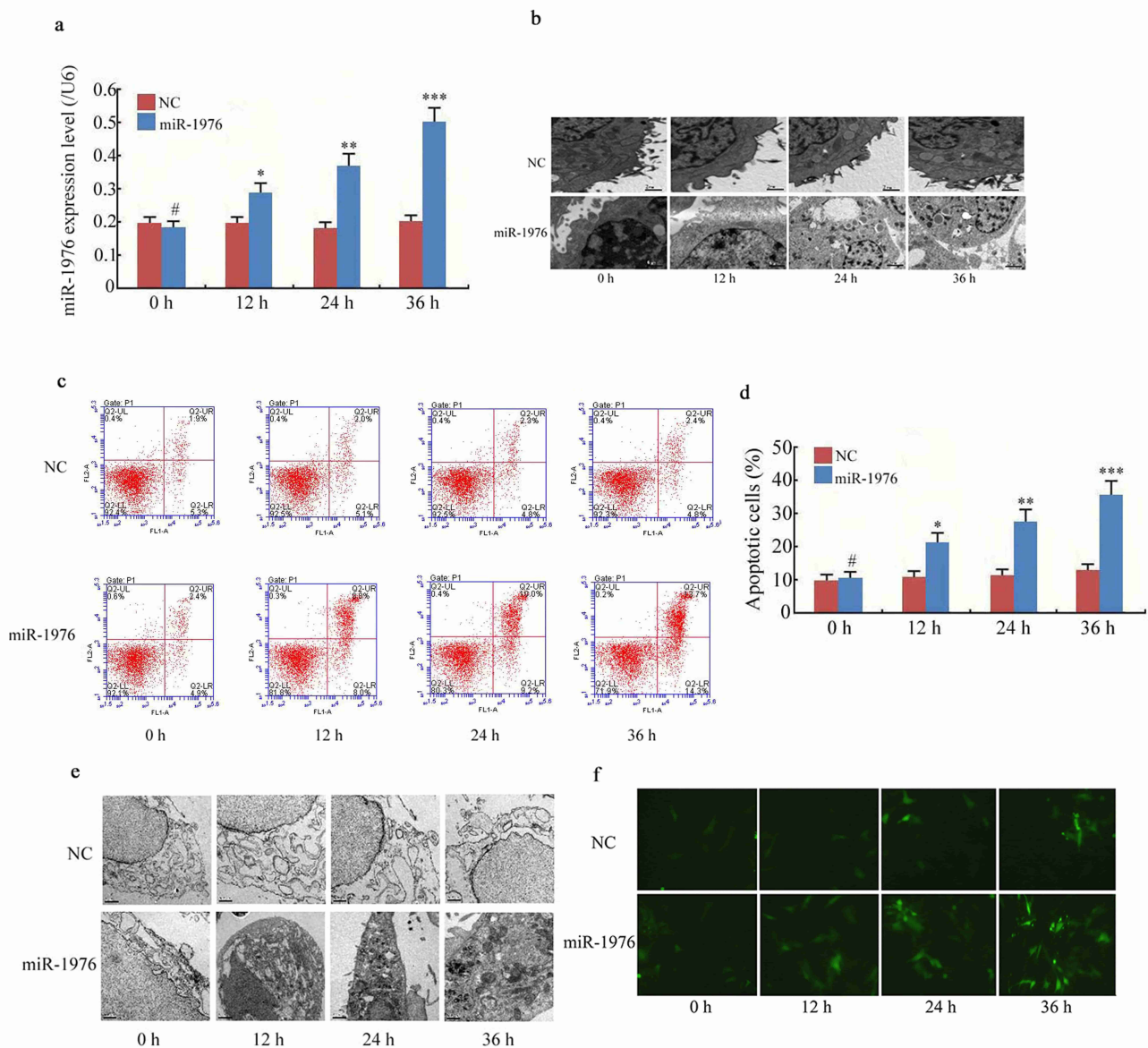


Fig. 2. Overexpression of miR-1976 increased apoptosis of dopaminergic neurons. (a) Overexpression of miR-1976 was verified using real-time PCR. (b) Apoptotic bodies stained with FITC observed under the electron microscope. (c,d) Cell apoptosis rate reflected using a flow cytometry chart. (e) MiR-1976 induced greater cell apoptotic bodies in C57/BL mice. (f) MiR-1976 induced more cell apoptosis shown via the TUNEL assay. NC, normal control. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; #, $p > 0.05$.

ance (ANOVA) when there were more than two groups. The nonparametric Mann-Whitney U test was performed to compare the miRNA expression between patients and normal controls, and the Kruskal-Wallis test was used when there were more than two groups. The Mann-Whitney U test and the Kruskal-Wallis test were used to evaluate the correlations between the results of the miRNA expression and other parameters. A two-tailed p -value of < 0.05 was considered statistically significant. Categorical variables were evaluated using the Chi-square test. For comparison of differences, $p < 0.05$ was considered statistically significant.

3. Results

3.1 MiRNA Microarray in PD Patients

To assess differences in the expression of miRNAs between the healthy volunteers and the PD cohort, total RNA from plasma was collected from the 13 participants with PD and the 11 age-matched controls to facilitate miRNA microarray expression profiling. Based on the bioinformatics evaluation, 18 miRNAs that were differentially regulated between the two study cohorts ($p < 0.05$) were detected. Among these miRNAs, 11 (miR-29a, 103a, 1976, 153, 30b, 103a, 7, 9, 129, 132, and 105) were shown to be upregu-

Table 3. The sequence conservation of miR-1976 among different species detected via the miRBase searching method.

Species	miRNA	Conserved sequence
Human	has-miR-1976	33-CCUCCUGCCCUCUUGCUGU-52
Macaca mulatta	mml-miR-1976	33-CCUCCUGCCCUCUUGCUGU-52
Horse	eca-miR-1976	31-CCUCCUGCCCUCUUGCUGU-50
House mouse	mmu-miR-1976	37-CCUCCUGCCCUCUUGCUGU-55
Zebrafish	dre-miR-1976	53-CCUCCUGCCCUCUUGCUGU-72
Rattus norvegicus	rno-miR-1976	33-CCUCCUGCCCUCUUGCUGU-51
Tropical clawed frog	xtr-miR-1976	34-CCUCCUGCCCUCUUGCUGU-56
Pongo pygmaeus	ppy-miR-1976	35-CCUCCUGCCCUCUUGCUGU-56
Ornithorhynchus anatinus	oan-miR-1976	38-CCUCCUGCCCUCUUGCUGU-57

lated in participants with PD compared with controls, while seven (miR-210, 375, 146a, 101a, 34b, 34c, and 56) were downregulated (Fig. 1a).

To examine whether these miRNAs were related to PD progression, plasma RNA was collected again from the same cohorts, PD patients and the healthy controls, and the expression of these miRNAs were detected using real-time PCR. The results confirmed that the expression of miR-1976, miR-153, miR-103a, and miR-29q was significantly upregulated in PD patients, while the expression of miR-210, miR-375, miR-146a, and miR-101a was significantly downregulated in PD patients compared with controls. Of these miRNAs, the greatest difference was observed in the expression of miR-1976, with the PD cohort having a $5.64 \times$ higher expression than the control group ($p < 0.01$) (Fig. 1b). Given the large difference observed for miR-1976 expression between the PD patient and control cohorts, we hypothesized that a higher level of miR-1976 might be relevant to the development of PD.

The expression level of miR-1976 was determined in PD patients and normal controls using real-time PCR. Results showed that miR-1976 was significantly upregulated in the serum of PD participants compared with that of matched non-PD controls (Fig. 1c). We then examined the sequence conservation of miR-1976 among different species via miRBase searching. Alignment results showed that the sequence “CCUCCUGCCCUCUUGCUGU” had high conservation among many species, including humans, Macaca mulatta, horses, house mice, zebrafish, Rattus norvegicus, tropical clawed frogs, Pongo pygmaeus, and ornithorhynchus anatinus (Table 3).

3.2 Overexpression of MiR-1976 Increased Dopamine Neuronal Cell Apoptosis

To evaluate the overexpression of miR-1976 on apoptosis of MES23.5 cells, the miR-1976 mimics or mock control mimics were transfected using liposomes into MES23.5 cells. Real-time PCR confirmed the high overexpression at 12 h, 24 h, and 36 h post-transfection (Fig. 2a). The effects of miR-1976 overexpression on cell apoptosis were subsequently further evaluated. The FITC staining results showed more apoptotic bodies observed under the electron

microscope when cells were transfected with miR-1976 mimics (Fig. 2b). Similarly, the overexpression of miR-1976 showed a strong cell apoptotic rate as reflected using a flow cytometry chart (Fig. 2c,d).

For the *in-vivo* study, the effects of overexpression of miR-1976 on the dopaminergic neuronal cells of mice were assessed by constructing an overexpressing miR-1976 mouse model that involved injecting miR-1976 mimics via a lentiviral vector into the substantia nigra of C57/BL mice. As a result, miR-1976 induced more cell apoptotic bodies with relatively integrated cell cytomembranes and organelles (Fig. 3e). These results were supported by the TUNEL assay, which showed cell apoptosis in the group treated with the miR-1976 mimics (Fig. 3f). Collectively, these data suggest that the miR-1976-induced nerve cell apoptosis might be crucial in the development of PD.

3.3 Prediction and Analysis of MiR-1976 Target Genes

Using the GO and pathway programs, as well as the microarray that we had performed in our preliminary study, we identified that phosphatidylinositol 3-kinase (*PI3K*) was a predicted target of miR-1976. Furthermore, bioinformatics analysis identified two pathways (*PINK1* and *MAPK*-mitogen-activated protein kinase) that were significantly correlated with miR-1976 ($p < 0.0001$).

To detect the effects of miR-1976 on the *PINK* or *MAPK* signaling pathways, the mRNAs of the proteins *PINK1*, *MAPK4*, 6, 16, and *BPI* (binding protein1) were evaluated via real-time PCR in miR-1976 mimic treated MES23.5 cells or empty vector treated cells (NC). Results showed that the high expression of miR-1976 significantly inhibited the expression of *PINK1* ($p < 0.05$) but showed no significant effect on expression of proteins of the *MAPK* signaling pathway. These results indicated that the *PINK1* signaling pathway might be a target pathway of miR-1976 (Fig. 3a).

We subsequently explored whether the target sequence of miR-1976 was in *PINK1*. We found that *PINK1* harbors one conserved miR-1976 cognate site named 89-91 of *PINK1* 3'-UTR (Fig. 3b), which is a predicted target of miR-1976. To identify whether *PINK1* expression was indeed regulated by miR-1976, the *PINK1* 3'-UTR

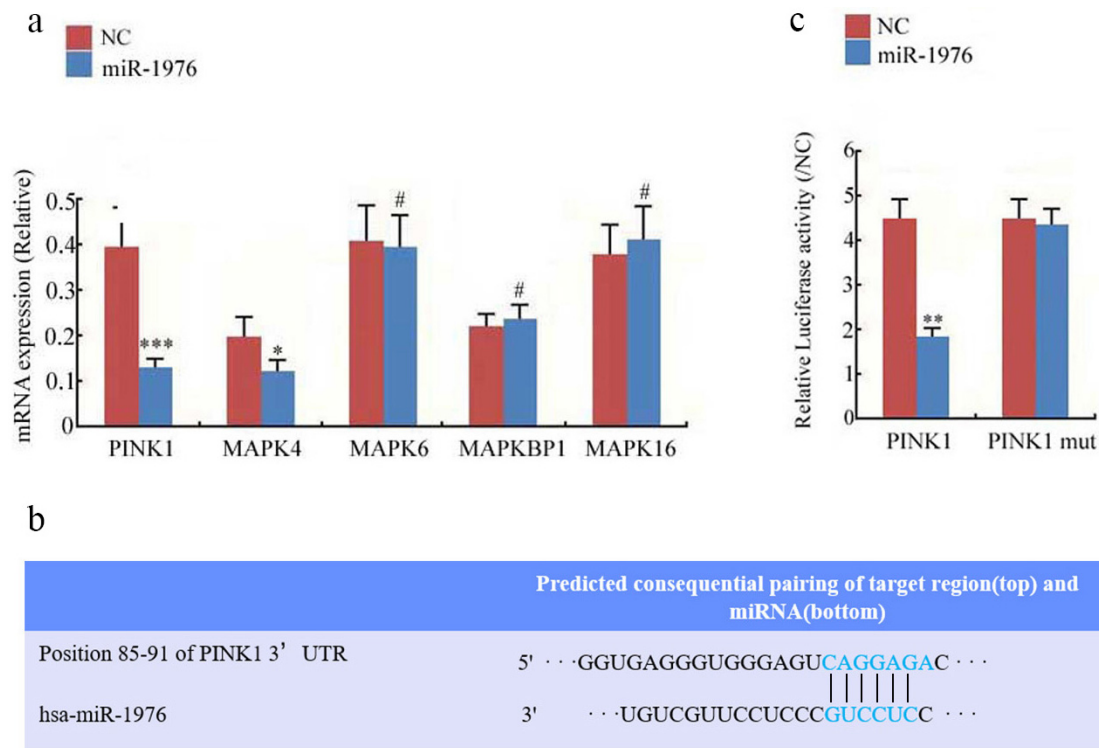


Fig. 3. The prediction and analysis of miR-1976 target genes. (a) The effect of miR-1976 on the *PINK* or *MAPK* signaling pathways. (b) The target sequence of miR-1976 in *PINK1* was detected using TargetScan. (c) Dual-luciferase assay demonstrated that the *PINK1* expression was regulated by miR-1976. NC, normal control. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; #, $p > 0.05$.

was cloned into a luciferase reporter plasmid (Fig. 3c) that was used to quantify the ability of miR-1976 to inhibit expression of the *PINK1* coding region. The luciferase assay showed that miR-1976 suppressed luciferase activity when the reporter plasmid carried the wild type *PINK1* 3'-UTR. However, no significant suppression was observed when the reporter plasmid carried a mutant *PINK1* 3'-UTR. These results demonstrated that miR-1976 directly binds to the predicted binding site in the *PINK1* 3'-UTR.

3.4 Silencing of *PINK1* Expression Increases the Apoptosis of MES23.5 Cells and the Number of and Changes in the Morphology of Mitochondria

To detect the effect of *PINK1* on dopamine neuronal cells, we constructed the *PINK1* shRNA lentivirus vector to infect MES23.5 cells. We first observed cell apoptosis using flow cytometry at 0-, 12-, 24-, and 36-h time points after transfection. As shown in Fig. 4a, low expression of *PINK1* induced more cell apoptosis in a time-dependent fashion, indicating that the inhibition of *PINK1* regulated cell apoptosis. This result was supported by the observed changes in mitochondrial morphology, which showed that the number of mitochondria was reduced with increased time. Furthermore, relative to the 0-h time point, a greater number of phagosomes and fewer mitochondrial cristae were found at the 36-h mark (Fig. 4b).

4. Discussion

In the present research, we first identified several differences with respect to the expression of miRNAs in people with PD. High expression of miR-1976 was found in these individuals and was prominent in dopamine neurons (MES23.5 cells). To assess the functional results of miR-1976 upregulation *in-vitro* and *in-vivo*, we examined cell apoptosis in both the dopamine neuronal cells and the neurons in the substantia nigra of mice. We found that the introduction of the miR-1976 molecule into the substantia nigra neurons of mice induced neuronal apoptosis both *in vitro* and *in vivo*. This suggests that miR-1976 can trigger neuronal apoptosis, leading to the underlying mechanism of PD. Via bioinformatics analysis, we identified *PINK1* as the target gene of miR-1976, and these results were confirmed via dual-luciferase assay. Furthermore, lower expression of *PINK1* turned out to contribute to higher neuronal apoptosis. Thus, the target gene, *PINK1*, was shown to be involved in the apoptosis of dopaminergic neurons. Collectively, these results indicated that miR-1976 might play a crucial role in the development of PD by targeting the *PINK* signaling pathway.

It has been noted that miRNAs were stable in plasma and serum samples and can be useful for detecting disease conditions [18,19]. In the circulatory system, miRNAs exist in the form of high-density lipoproteins (HDLs) and are mainly derived from the budding exocytosis or active secre-

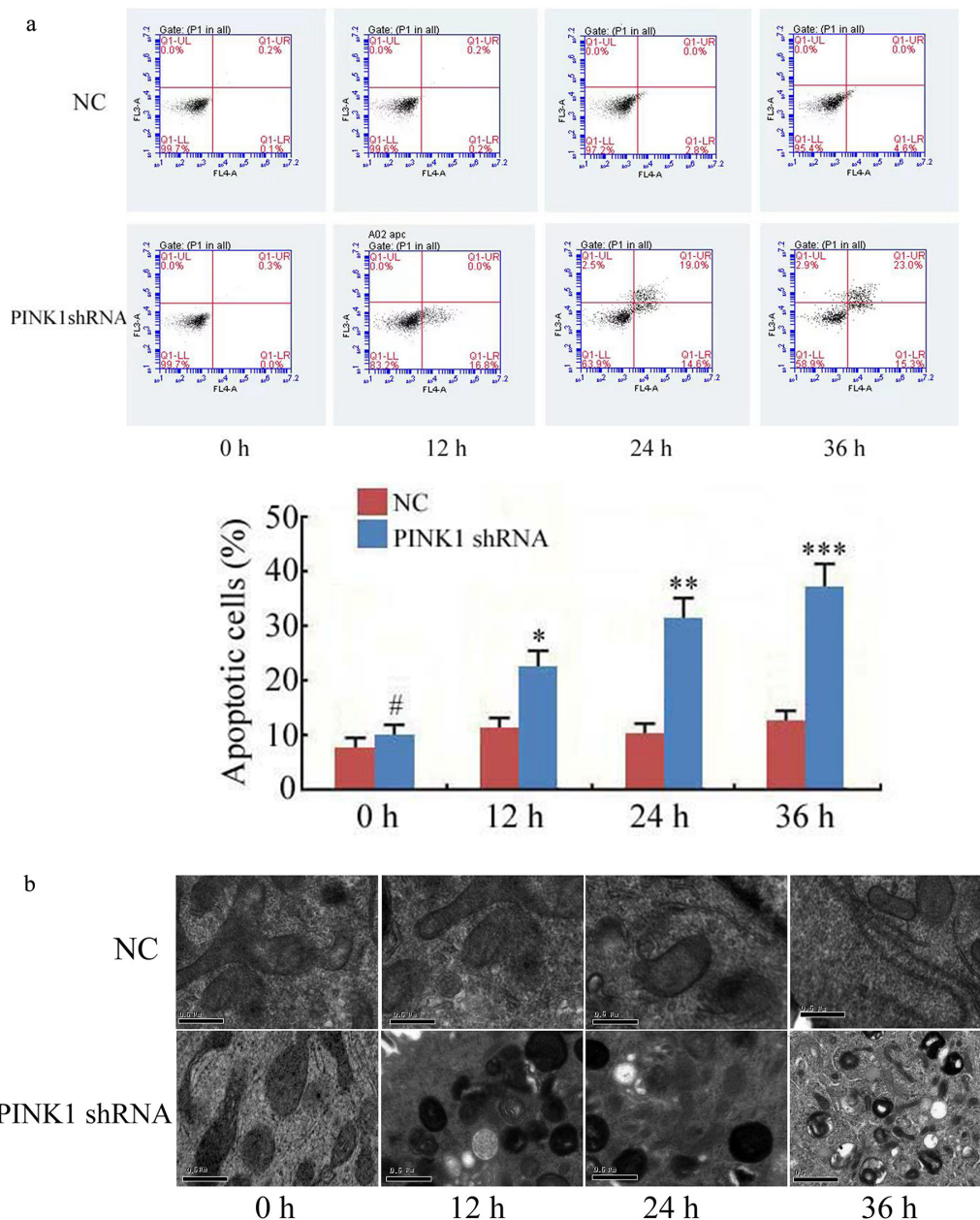


Fig. 4. Silencing of *PINK1* expression increases the apoptosis of MES23.5 cells and affects the morphology of mitochondria. (a) Cell apoptosis was observed via flow cytometry at 0-, 12-, 24-, and 36-h time points after transfection. (b) Electron microscopy showed more phagosomes and fewer mitochondrial cristae. NC, Normal control. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; #, $p > 0.05$.

tion of various cells [20]. Conversely, the plasma miRNA can re-enter the cell and function as specific protein binders and in endocytosis [21,22]. Therefore, miR-176 present in the blood can reflect its expression levels in brain tissue. In this study, we found that the plasma miR-176 level was higher in people with PD compared to controls, indicating that miR-176 might be associated with PD progression. Based on this hypothesis, we subsequently identified the target gene of miR-176 and investigated the biological function of miR-176 in dopaminergic cells via transfection involving miR-176 mimics. Results showed that miR-

176 induced dopaminergic neuronal apoptosis. Bioinformatics were used to screen for possible targets of miR-176 using KEGG analysis, and *PINK*, *MAPK*, transmembrane protein, and *PI3-K/AKT* signaling pathways were identified as potentially being associated with miR-176. Among these pathways, *PINK* proved to be the most plausible protein that was targeted by miR-176. Therefore, it was hypothesized that the risk of developing PD might be because of the higher levels of miR-176 in PD serum targeting *PINK1*. Results from the dual-luciferase assay provided support for this hypothesis.

Subsequently, in this study we addressed why the downregulation of *PINK1* significantly reduced the proliferation of dopaminergic neurons. Previous research has shown that knockout/knockdown of *PINK1* was associated with the increased susceptibility of several types of cancer cells to apoptosis by decreasing mitochondrial respiration, ATP generation, and mitochondria membrane potential, or by increasing reactive oxygen species [23,24]. Therefore, while *PINK1* has been observed to be related to the cancer process, it has also been linked with the presence of PD. The *PINK1* protein synthesis process is well known. It is first transcribed in the nucleus and then translated in the cytoplasm before being transported into the mitochondria. It promotes mitochondrial health and protects the cell against mitochondrial-mediated apoptosis caused by cell death [25]. For instance, a previous study has shown that silencing of the *PINK1* gene resulted in inactivity of the myocyte enhancer factor-2 protein, which induced tumor cell apoptosis [26]. Furthermore, in a PD mouse model, mutation of the *PINK1* gene resulted in mitochondrial dysfunction and neuronal apoptosis, which ultimately resulted in the development of PD [27]. To determine the effect of *PINK1* on cell death, we silenced the *PINK1* gene by using a shRNA lentiviral vector. We demonstrated that silencing the *PINK1* gene induced apoptosis and mitochondrial damage in the dopaminergic neurons, which was similar to what was found in the PD mouse model.

In the dopaminergic neurons, *PINK1* downregulation reduced mitochondrial defects and dysfunction, as demonstrated by the reduced mitochondrial mass, increased physalides, and fewer mitochondrial cristae. Dysfunction of the mitochondria could affect the signaling pathways that mediate apoptosis of dopaminergic neurons. Therefore, we hypothesized that the upregulation of miR-1976 might be associated with apoptosis in dopaminergic neurons and the development of PD by targeting *PINK1*. It is interesting to note that up to now, more than 100 homozygous mutations in the *PINK1* gene have been reported to be associated with PD [28].

5. Conclusions

In conclusion, we screened for differences in the expression of miRNAs between people with PD and healthy age- and gender-matched controls, and via our miRNA expression profiling study, we identified miR-1976 as a possible biomarker of PD. To further investigate the relationship between miR-1976 and PD, we performed a bioinformatics analysis and showed that *PINK1* may be a target of miR-1976 and hence, may play a key role in PD progression.

Our study had several limitations. One major problem involves the delivery of candidate miRNA-1976 to specific sites in the brain by getting it across the blood–brain barrier, which will be required if miRNA-1976 is to be used as a therapeutic. Stabilizing and extending the life of delivered miRNAs could be difficult to accomplish because

miRNAs are degraded easily. Furthermore, utilizing miRNAs as a treatment may be a double-edged sword. While it is advantageous that miRNAs as powerful modulators of gene expression have the ability to alter several signaling pathways at once to switch the cellular physiology from an apoptotic state to one that favors survival, it also means that side effects in unspecific sites could be problematic. Hence, specific and effective delivery systems for miRNA-based therapy are vital. These challenges could slow down the progress of miRNA research. Furthermore, given the small population, it is recommended that this work be replicated on a larger sample size to further develop our understanding of the possible pathways involved in PD progression. Nonetheless, we believe that the present study provides data that may be useful for furthering our understanding of the mechanism of PD.

Availability of Data and Material

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Author Contributions

FQ—Conceptualization; FQ, YW—Data curation; FQ, YW—Formal analysis; FQ, YW, GX—Investigation; FQ, YW, GX—Methodology; FQ—Supervision; FQ, YW, GX, HC, MD, HJ—Writing and original draft; FQ, YW, GX, HC, MD, HJ—Writing, review and editing.

Ethics Approval and Consent to Participate

All experimental procedures were approved by the Human Research Ethics Committee of Nanjing Brain Hospital (Ethics Reference No: 2017-KY010). All experimental procedures complied with the guidelines for human studies and were conducted ethically in accordance with the World Medical Association Declaration of Helsinki. All subjects (or their parents or guardians) have given their written informed consent.

All animals were kept in a pathogen-free environment and fed ad lib. The procedures for care and use of animals were approved by the Ethics Committee of the Nanjing Brain Hospital and all applicable institutional and governmental regulations concerning the ethical use of animals were followed (Ethics Reference No: 2017-KY010).

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Conflict of Interest

The authors declare no conflict of interest.

References

- [1] Vincenza F, Andrea S, Binit C. Parkinson's Disease: New Insights into Pathophysiology and Rehabilitative Approaches. Parkinson's Disease. 2016; 2016: 3121727.
- [2] Fang Q. CPC Central Committee and State Council Issue Outline of Digital Village Development Strategy. China City Planning Review. 2019. Available at: http://www.gov.cn/zhengce/2019-04/16/content_5383610.htm (Accessed: 16 April 2019).
- [3] Wang T, Bruggeman KF, Kauhausen JA, Rodriguez AL, Nisbet DR, Parish CL. Functionalized composite scaffolds improve the engraftment of transplanted dopaminergic progenitors in a mouse model of Parkinson's disease. Biomaterials. 2016; 74: 89–98.
- [4] Fahmy AM, Boulais J, Desjardins M, Matheoud D. Mitochondrial antigen presentation: a mechanism linking Parkinson's disease to autoimmunity. Current Opinion in Immunology. 2019; 58: 31–37.
- [5] Geibl F, Henrich MT, Oertel WH. Mesencephalic and extramesencephalic dopaminergic systems in Parkinson's disease. Journal of Neural Transmission. 2019; 126: 377–396.
- [6] Villar-Cheda B, Dominguez-Mejide A, Valenzuela R, Granado N, Moratalla R, Labandeira-Garcia J. Aging-related dysregulation of dopamine and angiotensin receptor interaction. Neurobiology of Aging. 2014; 35: 1726–1738.
- [7] Singh S, Jamwal S, Kumar P. Neuroprotective potential of Quercetin in combination with piperine against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. Neural Regeneration Research. 2017; 12: 1137–1144.
- [8] Barallobre MJ, Perier C, Bové J, Laguna A, Delabar JM, Vila M, et al. DYRK1a promotes dopaminergic neuron survival in the developing brain and in a mouse model of Parkinson's disease. Cell Death and Disease. 2014; 5: e1289.
- [9] Ghosh A, Saminathan H, Kanthasamy A, Anantharam V, Jin H, Sondarva G, et al. The Peptidyl-prolyl Isomerase Pin1 up-regulation and Proapoptotic Function in Dopaminergic Neurons. Journal of Biological Chemistry. 2013; 288: 21955–21971.
- [10] Ryan S, Dolatabadi N, Chan S, Zhang X, Akhtar M, Parker J, et al. Isogenic Human iPSC Parkinson's Model Shows Nitrosative Stress-Induced Dysfunction in MEF2-PGC1 α Transcription. Cell. 2013; 155: 1351–1364.
- [11] Hernandez-Baltazar D, Mendoza-Garrido M, Martinez-Fong D. Activation of GSK-3 β and caspase-3 occurs in Nigral dopamine neurons during the development of apoptosis activated by a striatal injection of 6-hydroxydopamine. PLOS One. 2013; 8: e70951.
- [12] Li H, Yu L, Li M, Chen X, Tian Q, Jiang Y, et al. MicroRNA-150 serves as a diagnostic biomarker and is involved in the inflammatory pathogenesis of Parkinson's disease. Molecular Genetics and Genomic Medicine. 2020; 8: e1189.
- [13] Uwatoko H, Hama Y, Iwata IT, Shirai S, Matsushima M, Yabe I, et al. Identification of plasma microRNA expression changes in multiple system atrophy and Parkinson's disease. Molecular Brain. 2019; 12: 49.
- [14] Goh SY, Chao YX, Dheen ST, Tan EK, Tay SS. Role of MicroRNAs in Parkinson's Disease. International Journal of Molecular Sciences. 2019; 20: 5649.
- [15] Qiu F, Wu Y, Cao H, Hua P, Zhang Y, Du MY, et al. Study of aberrantly expressed microRNAs involved in Parkinson's disease and effective mechanism of miRNA-1976. Journal of Clinical Neurology. 2017; 30: 171–174.
- [16] Yang C, Sun C, Liang X, Xie S, Huang J, Li D. Integrative analysis of microRNA and mRNA expression profiles in non-small-cell lung cancer. Cancer Gene Therapy. 2016; 23: 90–97.
- [17] Wang J, Ma G, Han X, Liang M, Wang X, Xia T, et al. The low expression of miR-1976 in plasma samples indicating its biological functions in the progression of breast cancer. Clinical and Translational Oncology. 2020; 22: 2111–2120.
- [18] Bagheri Khoulenjani N, Saniee Abadeh M, Sarbazi-Azad S, Jaddi NS. Cancer miRNA biomarkers classification using a new representation algorithm and evolutionary deep learning. Soft Computing. 2021; 25: 3113–3129.
- [19] Palanikumar L, Al-Hosani S, Kalmouni M, Saleh HO, Magzoub M. Hexokinase II-Derived Cell-Penetrating Peptide Mediates Delivery of MicroRNA Mimic for Cancer-Selective Cytotoxicity. Biochemistry. 2020; 59: 2259–2273.
- [20] Mo M, Chen L, Fu Y, Wang W, Fu SW. Cell-free Circulating miRNA Biomarkers in Cancer. Journal of Cancer. 2012; 3: 432–448.
- [21] Duttgupta R, Jiang R, Gollub J, Getts R, Jones K. Impact of cellular miRNAs on circulating miRNA biomarker signatures. PLOS One. 2011; 6: e20769.
- [22] Vickers KC, Remaley AT. Lipid-based carriers of microRNAs and intercellular communication. Current Opinion in Lipidology. 2012; 23: 91–97.
- [23] Boonying W, Joselin A, Huang E, Qu D, Safarpour F, Iyirhiaro G, et al. Pink1 regulates FKBP5 interaction with AKT/PHLPP and protects neurons from neurotoxin stress induced by MPP. Journal of Neurochemistry. 2019; 150: 312–329.
- [24] Dai X, Xu W, Li T, Lu J, Yang Y, Li Q, et al. Involvement of phosphatase and tensin homolog-induced putative kinase 1–Parkin-mediated mitophagy in septic acute kidney injury. Chinese Medical Journal. 2019; 132: 2340–2347.
- [25] Steer EK, Dail MK, Chu CT. Beyond Mitophagy: Cytosolic PINK1 as a Messenger of Mitochondrial Health. Antioxidants and Redox Signaling. 2015; 22: 1047–1059.
- [26] Grenier K, Kontogiannia M, Fon EA. Short Mitochondrial ARF Triggers Parkin/PINK1-dependent Mitophagy. Journal of Biological Chemistry. 2014; 289: 29519–29530.
- [27] Hertz N, Berthet A, Sos M, Thorn K, Burlingame A, Nakamura K, et al. A Neo-Substrate that Amplifies Catalytic Activity of Parkinson's-Disease-Related Kinase PINK1. Cell. 2013; 154: 737–747.
- [28] Nybø C, Gustavsson E, Farrer M, Aasly JO. Neuropathological findings in PINK1-associated Parkinson's disease. Parkinsonism & Related Disorders. 2020; 78: 105–108.