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Original Article

MiR-205 Regulates *LRRK2* Expression in Dopamine Neurons in Parkinson's Disease through Methylation Modification

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Abstract

Background: We explored the methylation modification in miR-205 promoter during the pathological changes of Parkinson's disease (PD) and its regulation on *Leucine-Rich Repeat Kinase 2* (*LRRK2*), clarified the important role of methylation in miR-205 promoter region in PD, explained the role of miR-205 methylation in the pathological changes of PD, and looked for new targets for PD.

Methods: Methylation of miR-205 promoter regions was determined by cell genomic DNA, with model bisulfite treatment, and the transcription of miR-205 and *LRRK2* in PD model cells was determined by qPCR, and *LRRK2* expression was determined by Western blot. The binding sites of miRNAs in the non-coding region of *LRRK2* were analyzed by the targetscan database, and miR-205 expression in 293T cells was controlled. The correlation between miR-205 expression and *LRRK2* was determined to clarify the regulation mode of miR-205 on *LRRK2*.

Results: The level of miR-205 were reduced in the SH-SY5Y Parkinson model cells, and its promoter region was highly methylated, while *LRRK2* expression decreased in the model cells after 5-Azacytidine inhibition of methylation in miR-205 promoter region. According to the target scan database analysis, *LRRK2* non-coding region is a miR-205-specific binding site. After further miR-205 overexpression in 293T cells, the transcription and translation of *LRRK2* decreased in cells, which increased after the treatment of miR-205 inhibitor on *LRRK2*.

Conclusion: The methylation modification of miR-205 promoter region could regulate the transcription and translation of *LRRK2* in dopaminergic neurons, so miR-205 methylation regulation can serve as a new potential target for the treatment of PD.

Keywords: Parkinson's disease; miR-205; RNA interference

Introduction

Parkinson's disease (PD), also known as paralysis agitans syndrome, is an age-dependent neurodegenerative disease characterized by increased muscle tone and decreased movement, with an average age of onset of around 60, and the incidence increases significantly with age. Currently,

the incidence of PD in the total population is 0.3%, with 1% over 60 and 3% over 80, lacking effective control or reverse treatment (1, 2). In recent years, new therapeutic targets developed for *Leucine-Rich Repeat Kinase 2* (*LRRK2)* have been a hot spot in the whole field and a major breakthrough direction in the field, but drugs, such as *LRRK2* kinase inhibitors and glucocerebrosidase (GBA) regulators, are all in the early stage of clinical application development (2, 3). Therefore, PD has become a globally important issue in public health, bringing a heavy burden on society and families (4, 5).

The pathogenesis of PD is still in the exploring stage. It is widely believed that PD is the result acted with various factors, such as genetics, environment, aging, oxidative stress and so on (4, 6). As the continuous progress and development of biotechnology and the deepening of research, scientists found that *LRRK2* was detected in both familial and sporadic patients with PD, expressed in both early and late PD (7), with main pathogenic mechanism that may be related to pathological processes, such as mitochondrial dysfunction, oxidative stress response, overexpression of cysteine thiester, and inflammatory response (8). Current studies on *LRRK2* were all targeting *LRRK2* itself (7,9). If we focus on the regulation of *LRRK2* expression, the binding substrates, and the proteins or small molecular substances interacting with them, some previously unknown pathogenic mechanisms may be uncovered.

A growing number of studies have shown that certain miRNA have altered the expression when regulated by DNA abnormal methylation, which plays an important role in the development of disease, for example, hypermethylation in the promoter regions of miR-34b/c and miR-124a genes may play an important role in the development of cancer (10, 11). Bnormal expression of miR-205 is related to the occurrence of PD and is involved in biological processes, such as metabolism and neuromacronutrient signaling (12). MiR-205 could modulate the expression of LDL-associated receptor protein 1 in the brain and thus participate in pathological alterations in Alzheimer's disease (13). Recently, miR-205 has been able to regulate *LRRK2* expression associated with PD (14), but the specific mechanism of miR-205 promoter region methylation in regulating *LRRK2* expression in PD has not been reported and should be further explored.

In this project, we planned to investigate the mechanism of methylation modification in miR-205 promoter region that regulates *LRRK2* expression and explore precise medical ways that regulate miR-205 transcription.

Materials and Methods

Prediction of miR-205 binding sites

The binding sites of miRNAs in *LRRK2* noncoding region were analyzed with the targetscan database and screened for specific regulatory.

pGl3-*LRRK2* luciferase plasmid was purchased from BGI, which was performed with plasmid transfection 293T cell assay. The assay was divided into blank (NC) group (transfection reagent + cells), plasmid group, plasmid + miR-205 NC group and plasmid + miR-205 mimics group, and three complex holes were set for each sample. After the cells were transfected for 6h, the medium was replaced with fresh and complete medium. After 36-48 h of transfection, the luciferase assay was prepared, with the corresponding methods by the instructions of the luciferase assay kit.

Preparation of PD model cells

Human neuroblastoma cells (SH-SY5Y cells) were purchased from the Cell Resource Center of Peking Union Medical College and cultured with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin in high glucose DMEM (15), with the medium changed every 24 h. After the cells covered the bottom of the plate, Accutase enzyme was adopted to digest the cells into single cells, which were then seeded into the 12-well plate coated with gelatin at a density of 1*10^ 4/well. 10 uM of vitamin A was added to the culture medium, which was replaced after 48 h, and then cultured for another 24 h. Subsequently, vitamin A in the culture medium was replaced

with 80 nM of TPA (phorbol ester 12-O-tetradecanoylphorbol-13-acetate) and continued for 3 days (16).

Six days after inducing differentiation, SH-SY5Y cells had the biological characteristics of dopamine neurons, high expressed dopamine. SH-SY5Y cells were divided into two groups. The first group was cultured with DMEM medium containing 5% FBS as the control group. The other group was cultured with high glucose DMEM medium containing 1 mM MPP+ (1-methyl-4-phenyl-Pyri -dinium ion) and 5% FBS to induce PD model cells (17). After 48 h of culture, dopaminergic neurons were induced as PD model cells.

Inhibition assay of methylation in miR-205 promoter region

After inducing differentiation of SH-SY5Y, dopamine neurons cells were divided into four groups: dopamine nerve cell group, dopamine nerve cell 5-Azacytidine treatment group, dopamine nerve cell MPP+ treatment group, dopamine nerve cell MPP+ and 5-Azacytidine treatment group.

After induction, dopaminergic neurons were treated with 5-Azacytidine at 10 umol/L for 48 h. In the dopaminergic neurons MPP+ and 5-Azacytidine treatment group, the cells were treated with MPP+ and 5-Azacytidine for 48 h at the same time. All samples were collected for follow-up trials.

Detection of miR-205 methylation in PD model cells

SH-SY5Y cells were extracted and then induced into dopamine neurons. Genomic DNA was treated with bisulfite, all unmethylated cytosines were converted to uracils while the methylated cytosines remained unchanged. Subsequently, the primers for methylated and unmethylated sequences were designed for PCR. MSP amplification products were detected by electrophoresis to determine the methylation of miR-205 promoter region, and the transcription of miR-205 was further detected to determine the expression of *LRRK2*. *LRRK2* primer: upstream primer, 5'AGCAGGACAAAGCCAGCCTCA-3'; downstream primer, 5'-GATGGCAGCATTGGGATACAG-3'. miR-205 methylation primers (M): upstream primer 5'-TTGTTTTGATGATTATGAAGGAATG-3'; downstream primer 5'-ACCCCTAAACTAACTAAACCCAAAC-3'. miR-205 unmethylated primers (U): upstream primer 5'-TCTGCCCTGATGATCATGAAGG-3'; downstream primer 5'-CCTGGGCTGACTGAACCCAAGCC-3' (18, 19).

miR-205 interference assay

miR-205 precursors, miR-205 inhibitors, and miR-205-negative plasmids were purchased from Ambion (Austin, TX, USA). A 12-well plate was prepared for resuscitating 293T cells, which were seeded at a density of 1*10^ 5/well, and then cultured with DMEM medium containing 10% FBS. After 24 h of cultivation, the 293 T cells were divided into four groups: blank plasmid control group, nucleotide-negative control group, miR-205 precursor group, and miR-205 inhibitor group. After 48 h of transfection, the cells were collected and mRNA and protein were extracted for detection, to determine the relationship between miR-205 level and *LRRK2* expression.

qPCR assay

The cells were collected and mRNA was extracted with a kit. Total mRNA in tissues was extracted with the miRNA kit, as well as Qiagen miScript RT kit for reverse. Taking cDNA as template, miR-205 and *LRRK2* fragments were amplified and synthesized by DNA polymerase. *LRRK2* was transcripted with SYBR Green Master mix, with GAPDH as endogenous control by the instruction of the kit. U6 primer sequences, F: CTCGCTTCGGCAGCACA; R: AACGCTTCACGAAATTTGCGT. miR-205 primer sequences, F: CGTCCAACATTCCACCG; R: GTGCAGGGTCCGAGGT. miR-205 transcription was analyzed by All-in-One™ miRNA qRT-PCR detection kit, with U6 as an endogenous control (14). PCR conditions: heating at

95℃ for 40s, at 95℃ for 10s, at 60℃ for 30s, for 40 cycles in total, with the last cycle of heating at 72℃ for 5 min. Expression was calculated by 2-ΔΔCt, ΔΔCt = [Ct (target gene) - Ct (internal reference)] group 1 - [Ct (target gene) -Ct (internal reference)] group 2.

Western Blot

SH-SY5Y cells with inducing differentiation (MPP+ treatment group and untreatment group) and HEK293T cells after miR-205 interference were collected, and the protein was extracted with the protein extraction kit. The protein samples were separated by molecular weight by polyacrylamide electrophoresis and transferred to the hybrid membrane (blot), then the specificity of *LRRK2* was detected by *LRRK2* antibody (Novus, NB300-268), and finally the target protein was detected by qualitative or semi-quantitative detection. In the assay, protein extraction was performed by Western blot to determine the expression level of *LRRK2* in PD model cells and after RNA interference and regulation.

Results

High methylation of miR-205 promoter region in SH-SY5Y-induced PD model cells

To investigate the role of miR-205 methylation modification in PD, we first differentiated SH-SY5Y cells into dopaminergic neurons and showed decreased proliferation capacity and increased dopamine secretion after differentiation (Fig. 1 A, B). Differentiated cells were induced in vitro into PD model cells, and DNA and total RNA of non-Parkinson and Parkinson SH-SY5Y cells were extracted for methylation and transcription of miR-205, respectively. The results of qRT-PCR assay for PD model cells confirmed a significant decrease in miR-205 level (Fig. 1 C, $P<0.001$) and an increased methylation in the miR-205 promoter region, indicating that the methylation modification of miR-205 in neural cells during PD was involved in the regulation of pathological changes (Fig. 1D).

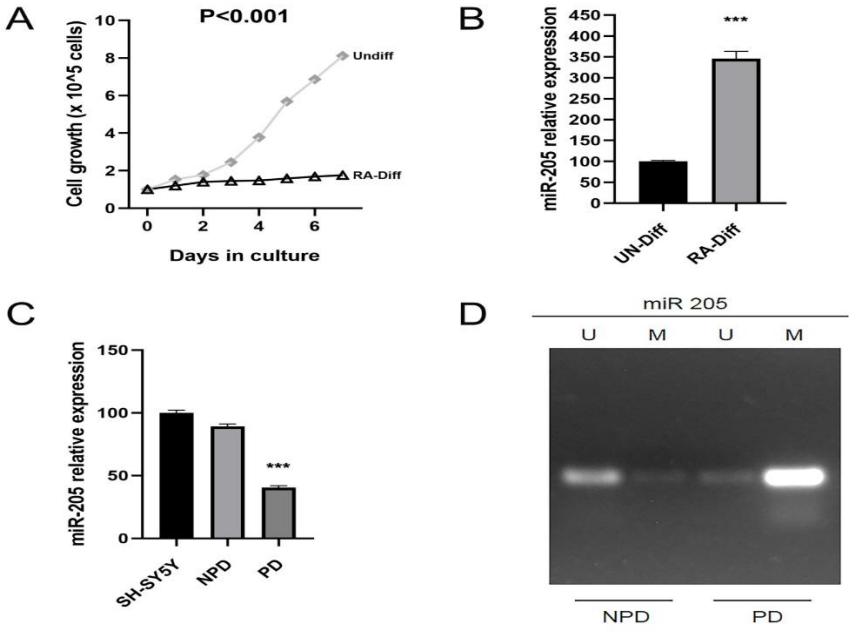

Fig. 1: Increased miR-205 methylation in SH-SY5Y cells of the PD model

Note: A, Cell proliferation decreased after SH-SY5Y induced differentiation into dopaminergic neurons; B, PCR showed increased dopamine transcription after differentiation of SH-SY5Y cells; C, miR-205 transcription significantly decreased in PD model cells; D, miR-205 methylation in PD model cells significantly increased after SH-SY5Y was induced to differentiate into dopaminergic neurons. ***, $P<0.001$. NPD, non-PD model cell group; PD, PD model cell group; U, unmethylation; M, methylation

High expression of LRRK2 in SH-SY5Y cells in the PD model

To detect the regulation of miR-205 methylation modification on *LRRK2*, we extracted RNA and proteins of non-Parkinson and Parkinson SH-SY5Y cells to further examined *LRRK2* expression in the PD model cells. The results showed that *LRRK2* transcription increased in the PD model cells (Fig. 2A, $P<0.001$), and WB assay also indicated increased *LRRK2* protein expression (Fig. 2B).

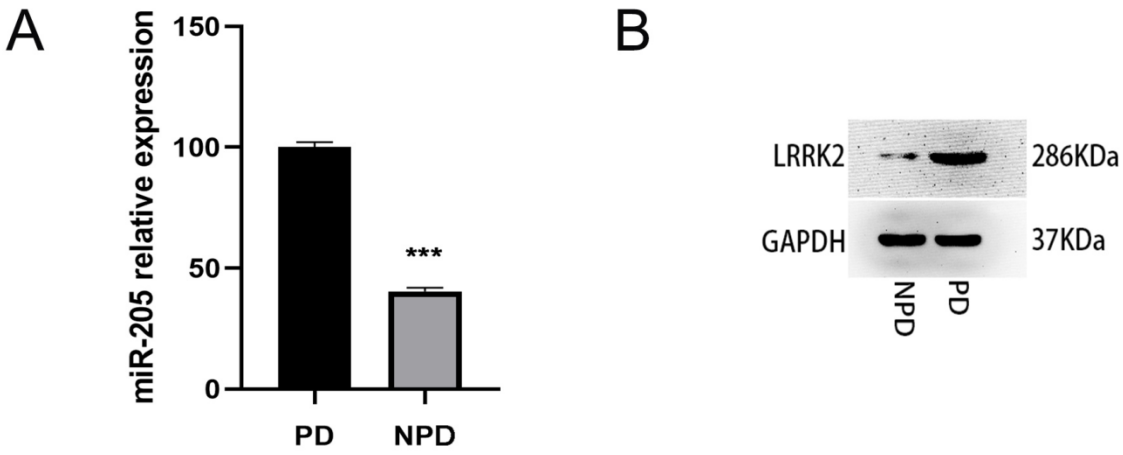

Fig. 2: Increased *LRRK2* expression in SH-SY5Y cells in the PD model
Note: A, *LRRK2* transcription in PD model cells significantly increased after SH-SY5Y was induced to differentiate into dopaminergic neurons. B, *LRRK2* protein expression significantly increased in the PD model cells. ***, $P<0.001$. NPD, non-PD model cell group; PD, PD model cell group

Specific binding of Mir-205 to LRRK2

Online analysis of the targetscan database revealed a specific binding region AUGAAGGA between sequences 116-123 of the 3' non-coding region of *LRRK2* gene (Table 1), and *LRRK2* is the target gene of miR-205.

Table 1: Specific binding sites of miR-205 to L RRK2

	Binding sequences	***Site Category***	***Context + score***
3 ' UTR 5'116-123 site of L RRK2	5' ...AAAUAGCUCGUGUGUAUGAAGG A...	8 mer	-0.41
Has-miR-205	3' GUCUGAGGCCACCUUACUUCCU		

After 293T cells were transfected with dual-luciferase for 36 h, the transfected cells were collected, and the fluorescence intensity was determined by the luciferase assay kit. The results showed that the luciferase activity of 293T cells decreased after transfection of pGl3-*LRRK2* luciferase plasmid and miR-205 mimics (Fig. 3, $P<0.0001$), suggesting the involvement of miR-205 in inhibiting the expression of target genes.

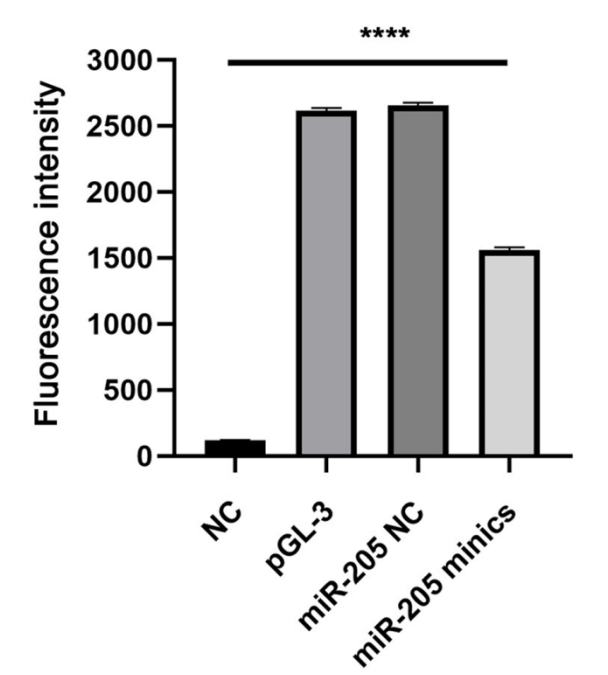

Fig. 3: Luciferase activity detection in 293T cells after being transfected with Pgl-3 plasmid
Note: NC, blank control; pGL-3, pGl3-*LRRK2* luciferase plasmid group; miR-205 NC, pGl3-*LRRK2* luciferase plasmid and miR-205 negative mimic group; miR-205 mimics, pGl3-*LRRK2* luciferase plasmid and miR-205 positive mimic group. miR-205 can specifically bind to *LRRK2* promoter region and inhibit its expression. ****, $P<0.0001$

The 5-Azacytidine inhibits LRRK2 expression in PD model cells

To validate that methylation in miR-205 promoter region regulated *LRRK2* expression during PD, we inhibited methylation in PD model cells with 5-Azacytidine. RNA and proteins were then extracted from each group to further examine the level of miR-205 and methylation of their promoter region in cells from different treatment groups, while the expression level of the corresponding *LRRK2* were examined. The results showed no significant change in miR-205 expression in the MPP+/5-ZacC group, but miR-205 expression in dopamine neurons in MPP+ group significantly decreased (Fig. 4 A, $P<0.0001$); and 5-Azacytidine inhibited methylation in miR-205 promoter region in PD model cells (Fig. 4B) and the level of mRNA in *LRRK2* decreased (MPP+/5-ZacC group), further inhibiting *LRRK2* expression (Fig. 4 C, D; $P<0.0001$).

miR-205 inhibits LRRK2 expression

To further determine that the changes in *LRRK2* expression were caused by transcriptional changes of miR-205, we transfected 293T cells into miR-205 and collected them after 48 h for mRNA and protein extraction. The level of miR-205 significantly increased in 293T cells after overexpression (Fig.5A, $P<0.0001$), while miR-205 significantly inhibited RNA transcription of *LRRK2* (Fig. 5B) and protein expression (Fig. 5C).

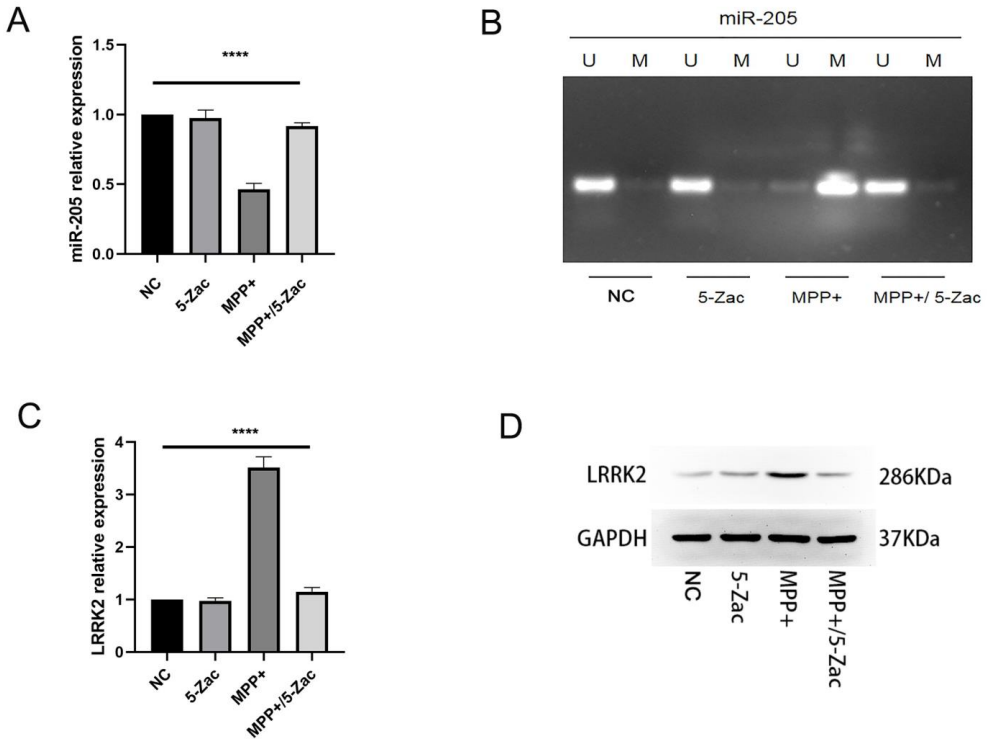

Fig. 4: The 5-Azacytidine inhibition assay

Note: A, 5-Azacytidine effectively promoted miR-205 transcription in cells after induction with MPP+; B, 5-Azacytidine effectively inhibited the methylation of miR-205 promoter region in cells after induction with MPP+; C and D, 5-Azacytidine inhibited *LRRK2* transcription and expression in PD model cells with induction with MPP+. ****, P<0.0001. NPD, non-PD model cell group; PD, PD model cell group; U, unmethylation; M, methylation; 5-Zac, 5-Azacytidine

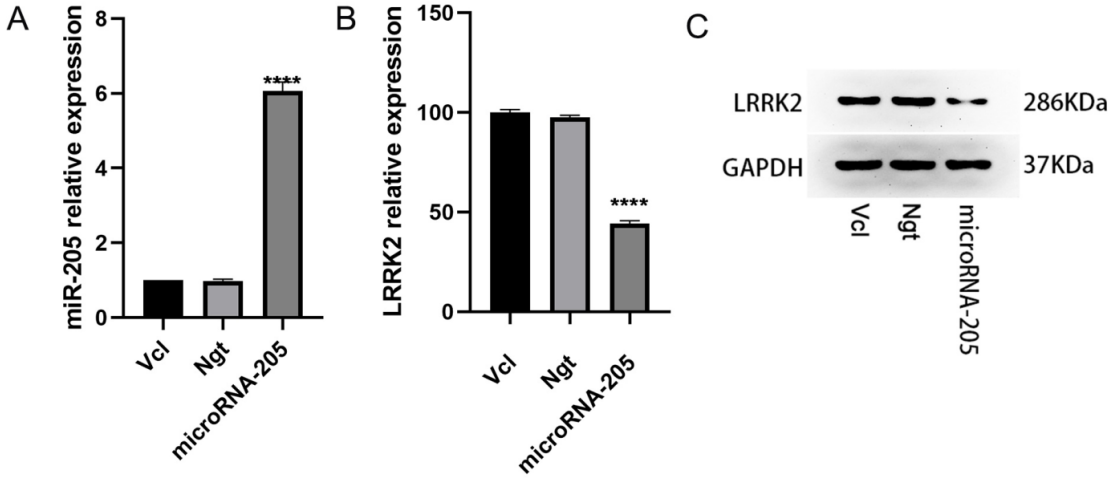

Fig. 5: miR-205 inhibits the transcription and expression of *LRRK2* in vitro

Note: A, the level of miR-205 significantly increased in 293T cells after 293T cell overexpression; B, the level of mRNA in *LRRK2* significantly decreased after miR-205 overexpression; C, *LRRK2* protein in 293T cells significantly decreased after overexpression of 293T cells on miR-205. ****, *P*<0.0001

miR-205 inhibitors promote LRRK2 expression

To further demonstrate the regulatory effect of miR-205 on *LRRK2*, we had 293T cells from the plates treated with the miR-205 inhibitor, and after 48 h, the cells were collected and mRNA and protein were extracted. Endogenous miR-205 transcription in 293T cells was inhibited by miR-205 inhibitors (Fig. 6A) and after inhibition of miR-205, the transcription (Fig. 6B) and translation (Fig. 6C) of *LRRK2* in 293T cells increased.

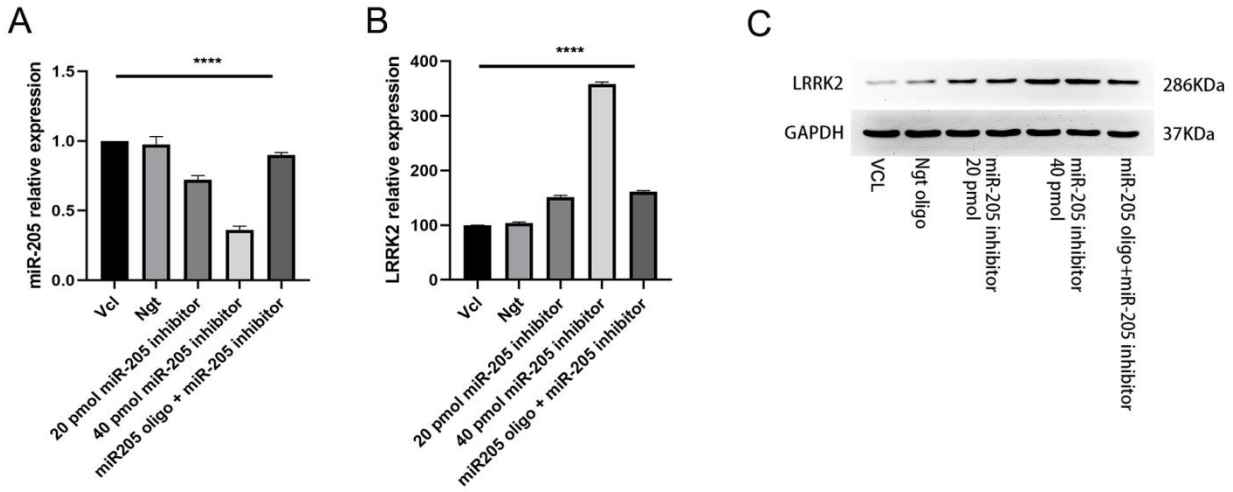

Fig. 6: miR-205 inhibitor up-regulated *LRRK2* expression in 293T cells
Note: A, After treatment on 293T cells with the miR-205 inhibitor, the mRNA level of *LRRK2* in the cells significantly increased; B and C, miR-205 inhibitors can promote the high expression of *LRRK2* in 293T cells. ****, P<0.0001. Vcl, blank plasmid control group; Ngt, nucleotide-negative control

Discussion

microRNAs (miRNAs) are a class of endogenous noncoding single-stranded small molecule RNA, which are highly conserved, with the size of 19-25bp. They can bind to base complementary pairing of target gene 3'-untranslated region (3'-UTR), to degrade or inhibit the translation of target gene mRNA, and to regulate the post-transcriptional activity of target gene. MiRNAs can be considered as the important regulatory mechanism in eukaryotes in the post-transcriptional process, which is closely related to malignancies, cardiovascular diseases, and neurodegenerative diseases, and plays a key role in the development and development of PD pathology (20). Thus, the relationship between miRNAs and PD has received widespread clinical attention. MiR-205 belongs to the microRNA family, and abnormal miR-205 expression suggested by PATIL et al. is related to the occurrence of PD and is involved in biological processes, such as metabolism and neurronutrient signaling regulation (12). This study found that miR-205 transcription significantly decreased in PD model cells, indicating an important regulatory role of miR-205 in the alteration of PD pathology, which is consistent with those reported in previous studies (14).

Methylation is an important epigenetic modification of protein and nucleic acids, including DNA methylation and histone methylation, involving gene expression regulation, protein function regulation and ribonucleic acid processing, which is closely associated with various diseases, such as cancer, aging and degenerative lesions. DNA

methylation is mainly used to regulate the stability, chromatin structure, conformation, protein and other related gene expression of genomic DNA (21, 22). Recently, during the development and progression of diseases, some miRNAs are regulated by abnormal DNA methylation, especially related to the CpG island methylation of tumor-related miR. For example, hypermethylation of miR-34B/C and miR-124A gene promoter regions plays an important role in the development and progression of cancer (10, 11). Hypermethylation of miR-34B/C gene promoter is mainly involved in the regulation of tumor lymph node metastasis, while hypermethylation of miR-124a gene promoter region is mainly related to the degree of tissue differentiation. These studies suggest that methylation modification of miRNA promoter region plays an important regulatory role in the pathological processes of the disease (23). We therefore further examined the methylation in miR-205 promoter region, showing a significant increase in methylation in miR-205 promoter region, based on which we further verified that *LRRK2* expression in PD model cells changed with miR-205 methylation, which was important for PD studies and subsequent treatment.

miRNAs regulates relevant biological functions by targeting relevant functional genes, mainly by complementing the 3' non-coding sequence of mRNA with the 2-8 bit base at its 5'-end. In the early stage of this study, we found that after dopaminergic neurons decreased in cells, PD model cells increased. To clarify whether there is a regulatory relationship between them, we performed miR-205 target gene prediction with the TargetScan database. TargetScan is mainly used to predict the complementarity of miRNA target genes and binding sites with target genes by computing the complementarity between the 2-8 nucleotides of the 5' -end of miRNA and the 3' -end untranslated region of the target gene (24, 25). miRNA target gene analysis based on the TargetScan database enables predictions for specific functions based on existing miRNA studies. Predicted by the TargetScan database, we found that *LRRK2* is a miRNA-205 regulatory target gene and highly specific, and the subsequent dual-luciferase reporter system assay further validated that miRNA-205 is able to negatively regulate *LRRK2* expression.

LRRK2 gene, containing 51 exons (26), is a complex multifunctional protein belonging to the Roco family (27), with a highly conserved Ras-like GTP enzyme domain ROC that is usually organized in tandem with COR (C-terminal of Roc). The ROC domain of *LRRK2* has similar effects to Ras, and the kinase of *LRRK2* regulates apoptosis (9). *LRRK2* is expressed in multiple types of neurons in cerebral cortex, dense nigra, and striatum, which is consistent with the motor disorder symptoms of PD (28). The results of our trial showed that both the transcription and translation of *LRRK2* increased in SH-SY5Y cells of the PD model. Combined with previous results, we found a negative correlation between miR-205 transcription and *LRRK2* expression in SH-SY5Y cells of the PD model, which is consistent with the results reported in the literature (14). Therefore, we further confirmed whether miR-205 could inhibit *LRRK2* expression in subsequent trials.

RNA interference refers to that the expression of a specific gene is inhibited by double-stranded RNA, simply RNAi (27). Several years after RNAi was discovered, specific gene silencing could be induced in mammalian cells, thus the application of RNAi technology has begun in mammalian cells (29). RNAi can effectively inhibit the post-transcriptional expression of genes as an important technical means in the field of gene functions and gene therapy research (30). With the extensive research and application of RNAi technology, miRNA can regulate *LRRK2* expression in animals of the PD model (31). Mir-205 could regulate *LRRK2* expression associated with PD (12, 14). Therefore, in this study, we used RNA interference to detect the regulation of Mir-205 on *LRRK2* gene expression. The test results showed that when miR-205 was overexpressed in cells, *LRRK2* expression in cells decreased. After treatment with the miR-205 inhibitor, intracellular *LRRK2* expression increased,

which further confirmed that miR-205 can inhibit *LRRK2* expression.

Conclusion

The methylation of miR-205 promoter region increased in cells of the PD model, and the results of the methylation inhibition assay indicated that the hypomethylation of miR-205 promoter region effectively inhibited *LRRK2* expression. These results suggest that methylation modification of miR-205 is the key to regulating *LRRK2* expression during altered PD pathology and a potential new target for the treatment of PD, bringing new hope for diagnosis and treatment of PD, and an important breakthrough direction for the treatment of PD.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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