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Original Article

MicroRNA-10a inhibits A30P α-synuclein aggregation and toxicity by targeting proapoptotic protein BCL2L11

Huimin Liang[1*], Bingqian Ding[1*], Junhui Liang[2], Xiaoyan Shi[3], Xin Jiang[1], Yang Gao[1]

[1]Department of Neurology, Huaihe Hospital of Henan University, Kaifeng, Henan, P. R. China; [2]Department of Laboratory Medicine, Wugang People's Hospital, Pingdingshan, Henan, P. R. China; [3]College of Pharmacy, Henan University, Kaifeng, Henan, P. R. China. [*]Equal contributors.

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Abstract: Parkinson's disease (PD) is the second most common neurodegenerative disorder around the world, and is characterized by progressive loss of nigrostriatal dopaminergic neurons. Certain microRNAs (miRNAs) are aberrantly expressed in the post-mortem brain tissues of patients with PD and *in vivo* PD model mice. However, the role of brain-enriched miRNA (miR)-10a in PD has not been studied. To investigate the regulatory role of miR-10a on α-synuclein (α-syn) in the pathology of PD, the present study aimed to examine whether upregulation of miR-10a attenuated A30P α-syn mutant aggregation and cellular toxicity. miRNA expression analysis by reverse transcription-quantitative polymerase chain reaction demonstrated that miR-10a expression was decreased in the midbrain of A30P α-syn transgenic mice and in SH-SY5Y human neuroblastoma cells transfected with A30P α-syn. In addition, miR-10a mimics were used to upregulate miR-10a expression. It was revealed that the upregulation of miR-10a suppressed α-syn intracellular accumulation and toxicity in α-syn-overexpressing SH-SY5Y cells. In addition, miR-10a overexpression resulted in a reversal of the A30P α-syn-induced upregulation of proapoptotic protein Bcl-2-associated X protein and cleaved caspase-3 expression and downregulation of antiapoptotic protein B-cell lymphoma-2 (BCL2) expression. A luciferase reporter assay demonstrated that BCL2-like 11 (BCL2L11), an apoptosis inducer, was a novel target gene of miR-10a. A30P α-syn aggregation and toxicity were alleviated by knocking down endogenous BCL2L11 in SH-SY5Y cells using a small interfering RNA specific for BCL2L11. In conclusion, these results demonstrate that miR-10a may serve a functional role in α-syn-induced neuronal pathology by inhibiting expression of BCL2L11 and that upregulation of miR-10a expression may be a useful therapeutic strategy for the treatment of PD.

Keywords: Parkinson's disease, microRNA-10a, α-synuclein, toxicity, B-cell lymphoma like 11

Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder around the world, which is characterized by a reduction of dopaminergic neurons in the substantia nigra pars compacta (SNpc), corpus striatum, and brain cortex [1]. α-synuclein (α-syn), an abundant presynaptic protein, has the capability to regulate synaptic vesicle mobilization in central neurons. α-syn has been demonstrated to be the primary component of Lewy bodies, which are the intracellular aggregates found in brains of PD patients [2]. The accumulation of misfolded and aggregated proteins is involved in the pathogenesis of PD [3]. Mutations in α-syn, in particular the dominant mutation A30P, induce aberrant aggregation of α-syn and results in neurotoxicity, thereby causing early-onset familial PD [4-6]. Therefore, strategies aimed at rescuing α-syn-mediated toxicity and modifying the inclusion morphology may provide an effective treatment option for PD patients.

B-cell lymphoma 2-like 11 (BCL2L11) is a proapoptotic BH3-only BCL2 family member that has been demonstrated to interact with BCL2, BCL2L1/BCL-extra large, and BCL2L3 (also known as MCL1), and acts as a vital apoptotic activator [7]. In healthy cells, BCL2L11 interacts with dynein light chain 1 (DYNL1, also known as LC8) and remains inactive. When cells are stimulated by a toxicant, BCL2L11 is released from DYNLL1 and induces apoptosis by inactivating BCL2 and activating BCL-2-

associated X protein (BAX)-BCL2 antagonist/killer 1 [8]. A previous study demonstrated that BCL2L11 expression is upregulated during neuronal apoptosis [9]. In addition, BCL2L11 expression was reported to be upregulated in the ventral midbrain of a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse model, and BCL2L11-deficient mice exhibited a protective effect against MPTP-induced SNpc dopaminergic neuronal death [10]. Conversely, another study revealed that BCL2L11 inhibited autophagy by mislocalization of Beclin 1 from the endoplasmic reticulum to the microtubules [11]. BCL2L11 was also demonstrated to be a dual agent that regulated autophagy in drug resistance [12]. Although proapoptotic effects of BCL2L11 have been reported, the underlying molecular mechanism of BCL2L11 in dopaminergic neuronal apoptosis during PD progression remains unknown.

microRNAs (miRNAs) are a class of endogenous, small noncoding RNAs that regulate of gene expression by targeting mRNAs for cleavage or translational repression [13]. A number of studies have demonstrated that miRNAs are involved in the pathology of several neurodegenerative diseases, including PD and Alzheimer's disease (AD); however, their functions remain poorly understood [14, 15]. In dopaminergic neurons, elevated miRNA (miR)-126 was previously revealed to impair insulin-like growth factor 1 (IGF-1) and enhance vulnerability to the neurotoxin 6-hydroxydopamine by regulating the IGF-1/phosphoinositide 3-kinase signaling pathway [16]. miR-10b was downregulated in acutely rejected kidney allografts (17), and *in vitro* experiments revealed that miR-10b mediated acute rejection of renal allografts by directly targeting BCL2L11 [17]. A miRNA expression profiling analysis revealed that miR-10a expression was significantly decreased in A30P α-syn transgenic mice, which suggested that miR-10a may be involved in the pathological process of PD [18]. Based on these previous data, the present study hypothesized that miR-10a may inhibit A30P α-syn aggregation and toxicity by regulating the expression of the pro-apoptotic protein BCL2L11.

In the present study, miR-10a was revealed to inhibit BCL2L11 expression by binding to its 3'-untranslated region (UTR). The reduced BCL2L11 expression led to suppression in the formation of α-syn aggregates and associated toxicity. These data suggested that miR-10a may serve as a therapeutic target for the treatment of PD.

Materials and methods

Animals

Ten wild type (WT) C57BL/6J mice and ten A30P α-syn transgenic C57BL/6J mice weighting about 20 g (male; age, 12 months; Jackson Laboratory, Bar Harbor, ME, USA) were housed under standard conditions at a temperature of 25°C, 50-60% humidity, 12-h light/dark cycles and were provided with standard laboratory feed and water *ad libitum*. The mice were sacrificed prior to analysis. The animal experiments were reviewed and approved by the Animal Care and Use Committee of Huaihe Hospital of Henan University (Henan, China) and were in compliance with the Chinese National Institute of Health Guidelines for Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used in the present study.

Cell culture

SH-SY5Y human neuroblastoma cells (American Type Culture Collection, Manassas, VA, USA) were used as an *in vitro* model for PD in the present study, as these cells exhibit many characteristics of dopaminergic neurons. For example, SH-SY5Y cells have a low degree of differentiation and are able to synthesize dopamine and dopamine transporter proteins. Therefore, the SH-SY5Y cell line has been used as an *in vitro* model of PD in previous years [19]. SH-SY5Y and 293T cells (American Type Culture Collection) were maintained in 25 cm^2 flasks (Corning Inc., Corning, NY, USA) with Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA, USA), penicillin (100 U/ml; Gibco; Thermo Fisher Scientific, Inc.), and streptomycin (100 μg/ml; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere with 5% CO_2. The growth medium was replaced every 2 or 3 days.

Cell transfection

SH-SY5Y cells (5×10^4/ml) cultured in 24-well plates were transfected at 50% confluence

using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Prior to transfection, 2 μl Lipofectamine 2000 and 1.5 μg plasmids, pre-diluted with 25 μl of serum-free DMEM (without antibiotics), were mixed gently to combine. Following incubation for 15 min at 25°C, the complete culture medium was replaced with 350 μl serum-free DMEM and the transfection mixture was added to the cells in a drop-wise manner. The culture medium was replaced with fresh complete culture medium 6 h post-transfection. After 48 h of culture, cells were harvested for subsequent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the midbrain of 12-month-old mice using TRIzol regent (Invitrogen; Thermo Fisher Scientific, Inc.), and RNA was extracted from cultured cells using a mirVana miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's protocol. The extracted RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) at wavelengths of 260 and 280 nm. Equal amounts of RNA (1.5 μg) were reverse transcribed to cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). cDNA for miRNAs was synthesized using a TaqMan miRNA reverse transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). cDNA was stored at -20°C until further use. mRNA and miRNA expression levels were detected by qPCR using Power SYBR Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression levels of the GAPDH housekeeping gene and U6 small nuclear RNA were used as internal standards for BCL2L11 and miR-10a expression, respectively. The data obtained were calculated by using the $2^{-\Delta\Delta Cq}$ method [20].

MTT assay

Cell proliferation was evaluated by the MTT assay. Briefly, cells (5×10^3) were seeded into each well of a 96-well flat-bottomed plate. Following 24 h incubation at 37°C, cells were transfected with A30P α-syn (4 μg/ml; Genewiz, Suzhou, China), miR-10a mimics (Genewiz; 50 nmol/l), miR-10a inhibitors (Genewiz; 50 nmol/l) or small interfering RNA (siRNA; Genewiz; 50 nmol/l) targeting BCL2L11 using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following incubation for 48 h, 50 μl MTT solution (2 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into each well and incubated for 4 h at 37°C in the dark. The MTT solution was removed and dimethyl-sulfoxide (150 μl) was added to each well to dissolve the formazan crystals. Finally, the absorbance was measured at a wavelength of 490 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Lactate dehydrogenase (LDH) release assay

LDH activity in the cell culture medium was detected using a Cytotoxicity Detection kit LDH (Roche Applied Science, Penzberg, Germany). One day prior to transfection, SH-SY5Y cells (5×10^3) were seeded and grown in a 96-well plate at 37°C. At 48 h post-transfection, the LDH assay was conducted according to the manufacturer's protocol. Cytotoxicity was calculated as follows: Cytotoxicity (%) = [(experimental value - low control)/(high control - low control)] ×100%. Low control was the LDH in culture supernatants of SH-SY5Y cells only; high control was the maximum releasable LDH in culture supernatants of SH-SY5Y cells following lysis with Triton X-100 at a final concentration of 0.5% for 4 h. Absorbance at a wavelength of 490 nm was detected using a microplate reader (Bio-Rad Laboratories, Inc.).

Dual-luciferase reporter assay

293T cells were plated in a 24-well plate 24 h prior to transfection. Following incubation, 0.3 μg luciferase reporter plasmid containing the WT BCL2L11 3'-UTR or a mutant (Mut) BCL2-L11 3'-UTR (Shanghai GenePharma Co., Ltd., Shanghai, China) and 50 nM miR-10a mimics or scramble mimics negative control (miR-NC; Shanghai GenePharma Co., Ltd.) were co-transfected into each well using Lipofectamine 2000 (Invitrogen). The luciferase reporter plasmids were constructed commercially and purchased from Shanghai GenePharma Co., Ltd. A 177-bp segment from the 3'-UTR of the BCL2L11 gene containing the miR-10a binding site was cloned into the pRL-SV40 vector (Promega Corporation, Madison, WI, USA). The sequence of this segment was as follows: 5'-ccacttataaatagcactgatctggctgtatactgatccatcactaacctgttttctaggacccagcgtatgtagcatttgtattgcagtttccctggctt-

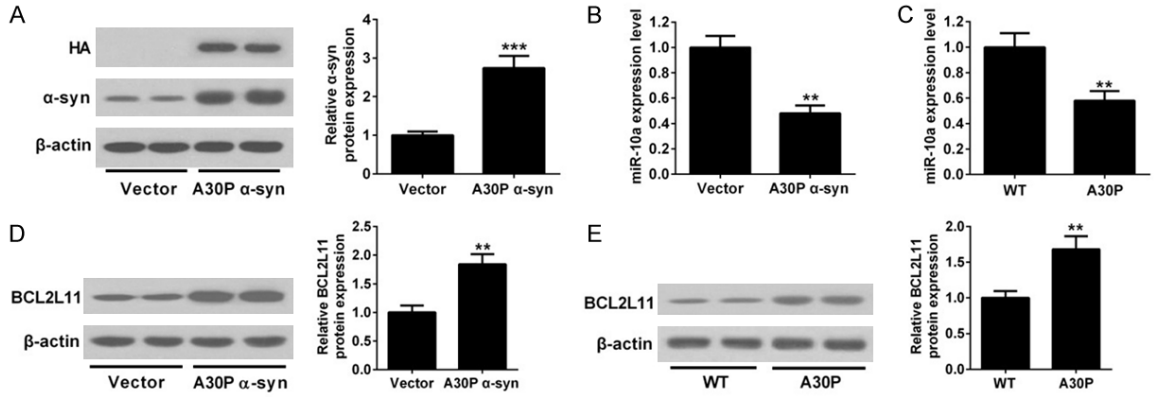

Figure 1. Expression of miR-10a in α-syn A30P transgenic mice and A30P α-syn-overexpressed SH-SY5Y cells. Protein expression was detected by Western blotting and microRNA levels were detected by reverse transcription-quantitative polymerase chain reaction. A: At 48 h post-transfection, cells were harvested and subjected to Western blotting using an anti-α-syn antibody. B: miR-10a expression levels in SH-SY5Y cells transfected with A30P α-syn. C: miR-10a expression levels in the midbrain of male A30P α-syn transgenic mice. D: BCL2L11 protein expression in SH-SY5Y cells transfected with A30P α-syn. E: BCL2L11 protein expression in the midbrain of male A30P α-syn transgenic mice. Data are presented as the mean ± standard deviation (n=4); $^{**}P<0.01$ and $^{***}P<0.001$ vs. vector or WT control. α-syn, α-synuclein; BCL2L11, B-cell lymphoma like 11; miR, microRNA; WT, wild-type.

acttgtgttttgcactgatgaattttgacagggtaattgccacttt acttgtgcaatactgctgtaaataactgca-3'. At 48 h post-transfection, cells were collected and then lysed with passive lysis buffer for 15 min at room temperature and the luciferase expression was determined by using the Dual-Luciferase Reporter 1000 System (Promega Corporation), according to the manufacturer's protocol.

Western blot analysis

Protein was extracted from midbrain tissues of A30P α-syn transgenic mice or SH-SY5Y cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). The homogenate was centrifuged at 12,000× g for 30 min at 4°C and the pellet was discarded. Total protein concentration was measured using the bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 20 μg protein was separated by SDS-PAGE. Following separation, proteins were transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% nonfat milk in TBS containing 0.1% Tween-20 at room temperature for 1 h and subsequently probed with primary antibodies overnight at 4°C. Antibodies against α-syn (1:500; catalog no. 2642), BCL2L11 (1:500; catalog no. 2819), and β-actin (1:2000; catalog no. 4967) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-BCL2 (1:1500; catalog no. sc-23960) and anti-BAX (1:1500; catalog no. sc-65532) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The anti-cleaved caspase-3 antibody (1:500; catalog no. ab49822) was obtained from Abcam (Cambridge, UK). The anti-HA-Tag antibody (1:1000; catalog no. CW0092) was obtained from CWbiotech (Beijing, China). Following primary antibody incubation, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000; catalog no. ab97051 and ab6789; Abcam) for 1 h at room temperature. Protein bands were visualized using an Enhanced Chemiluminescence detection system (EMD Millipore) and exposed to medical X-ray films.

Immunocytochemistry

For immunocytochemistry, SH-SY5Y cells were seeded onto coverslips (Thermo Fisher Scientific, Inc.) in a 24-well plate and cultured for 24 h prior to transfection. At 48 h post-transfection, cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, permeabilized with 0.3% Triton X-100 for 10 min and blocked with 5% goat serum for 45 min. Cells were incubated with an anti-α-syn antibody (1:300; catalog no. ab32127; Abcam) for 4 h at room temperature. Following three washes with PBS, cells were incubated with an Alexa Fluor 488-labeled secondary antibody (1:500; catalog no. A27034; Invitrogen, Thermo Fisher Sci-

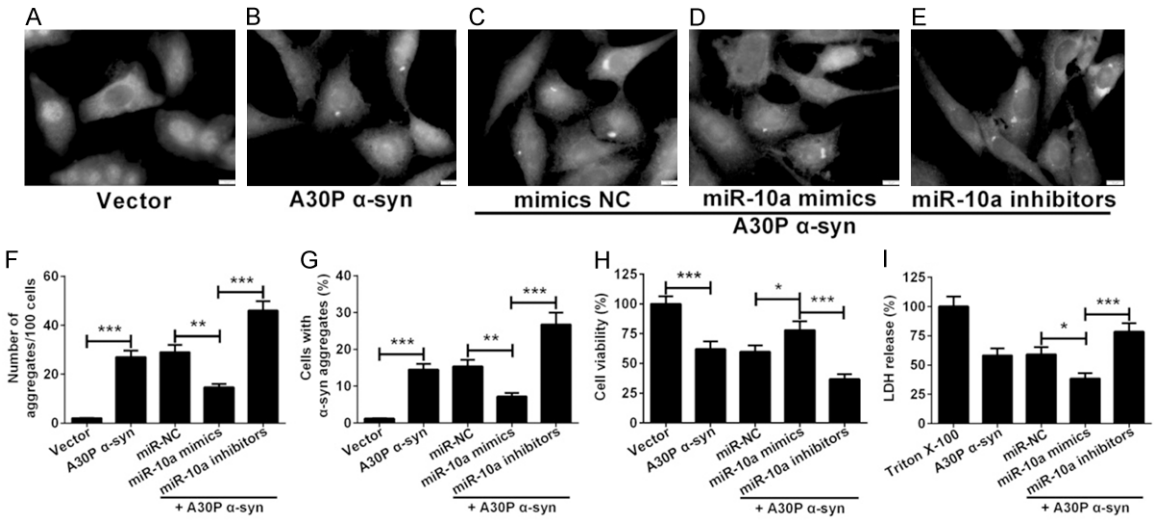

Figure 2. Effects of miR-10a on α-syn aggregation and toxicity. SH-SY5Y cells were transfected with (A) empty vector, (B) A30P α-syn, (C) A30P α-syn + miR-NC, (D) A30P α-syn + miR-10a mimics and (E) A30P α-syn + miR-10a inhibitor, followed by immunostaining with a human anti-α-syn antibody. (F) Quantification of the number of α-syn-positive aggregates in every 100 cells. (G) Percentage of all cells in which α-syn aggregates were observed. (H) Cell viability was assessed by MTT assay. (I) Cytotoxicity was assessed by LDH assays. Scale bar, 10 μm. Data are presented the mean ± standard deviation (n=3); $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. α-syn, α-synuclein; LDH, lactate dehydrogenase; miR, microRNA; NC, negative control.

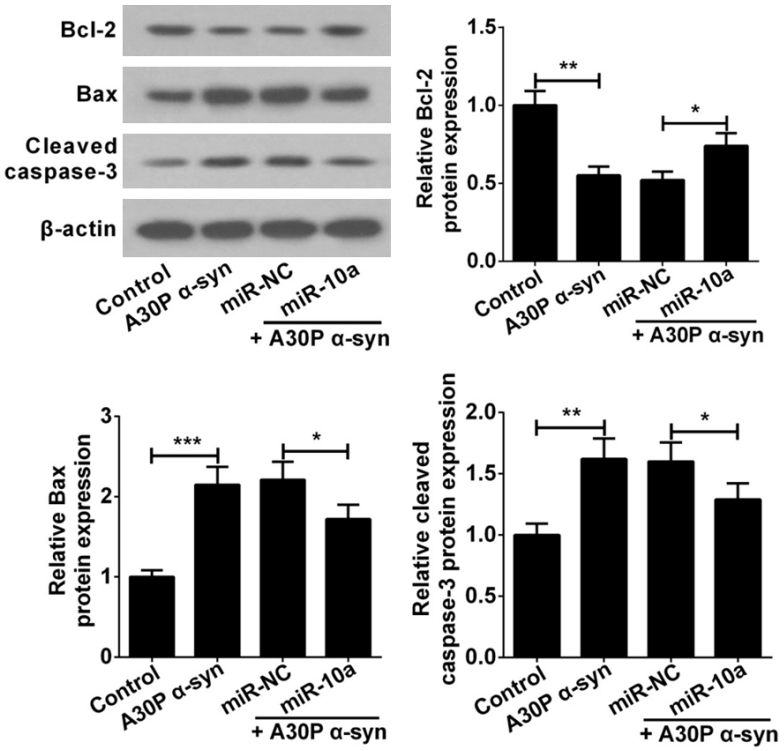

Figure 3. Effects of miR-10a on the intrinsic apoptosis pathway. SH-SY5Y cells were transfected with or without A30P α-syn and co-transfected with either miR-NC or miR-10a mimics. Protein expression levels of BCL2, BAX, and cleaved caspase-3 were detected by Western blotting. Data are presented as the mean ± standard deviation (n=3). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. α-syn, α-synuclein; BAX, BCL2-associated X protein; BCL2, B-cell lymphoma-2; miR, microRNA.

entific, Inc.) for 1 h at room temperature in the dark. Nuclei were stained with 0.5 μg/ml DAPI (Sigma-Aldrich; Merck KGaA) as a method to count the total number of cells per field. Cell fluorescence was visualized using a confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis

All experiments were repeated independently at least three times. Data were analyzed by Student's t-test or one-way analysis of variance followed by Tukey's post-hoc test. Results were expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference. All data analysis was performed using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA).

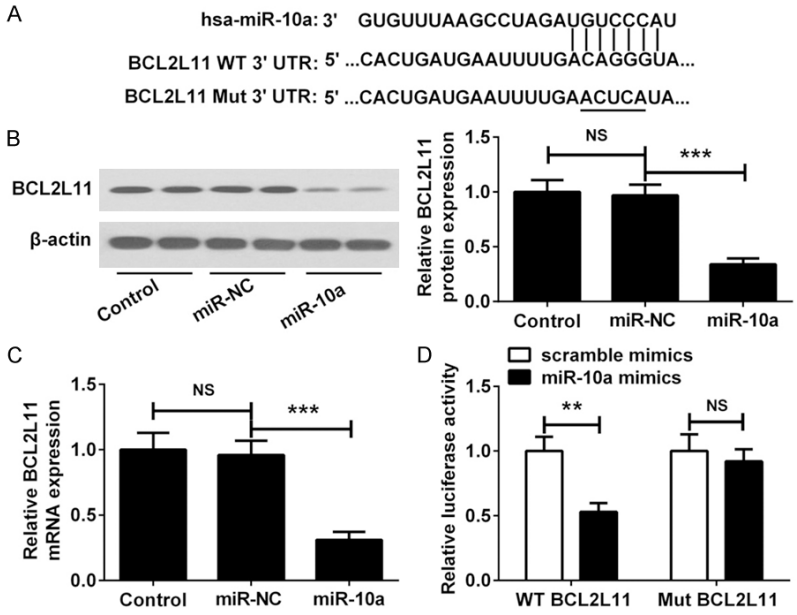

Figure 4. miR-10a inhibits the expression of BCL2L11 by directly binding to its 3'-UTR. (A) Putative binding sites for miR-10a in the 3'-UTR of BCL2L11 mRNA. The mutated region in BCL2L11 3'-UTR reporter constructs is labeled with a horizontal line. The expression of BCL2L11 at the (B) protein and (C) mRNA level was determined by Western blotting and reverse transcription-quantitative polymerase chain reaction, respectively. (D) SH-SY5Y cells were transfected with luciferase reporter vectors with either the BCL2L11-WT 3'-UTR or the BCL2L11-Mut 3'-UTR, along with miR-10a mimics or miR-NC. Cells were harvested 24 h post-transfection and then subjected to the luciferase activity assay. Data are presented as the mean ± standard deviation (n=3). $^{**}P<0.01$ and $^{***}P<0.001$. 3'-UTR, 3'-untranslated region; BCL2L11, B-cell lymphoma like 11; miR, microRNA; Mut, mutant; NC, negative control; NS, not significant; WT, wild-type.

Results

miR-10a is downregulated and BCL2L11 is upregulated in A30P α-syn transgenic mice and SH-SY5Y cells transfected with A30P α-syn

To study the effects of A30P α-syn on miR-10a and BCL2L11 expression, SH-SY5Y cells were transfected with a plasmid overexpressing A30P α-syn (pcDNA3.1-HA-A30P α-syn). Following transfection, the expression levels of total α-syn protein were elevated in the cells transfected with the A30P α-syn plasmid compared with cells transfected with the vector control (**Figure 1A**). RT-qPCR was performed to determine the expression levels of miR-10a in SH-SY5Y cells transfected with A30P α-syn and in male PD-associated A30P α-syn transgenic mice. A reduction of miR-10a expression was observed in SH-SY5Y cells transfected with A30P α-syn (**Figure 1B**). Consistent with a previous study [17], the present results demonstrated that the expression level of miR-10a was decreased in the midbrain of A30P α-syn transgenic mice compared with that in WT mice (**Figure 1C**). Western blotting revealed that the BCL2L11 protein was overexpressed in SH-SY5Y cells transfected with A30P α-syn and in the midbrain of A30P α-syn transgenic mice (**Figure 1D** and **1E**, respectively). This data suggested that downregulation of miR-10a and upregulated BCL2L11 may be implicated in the pathogenesis of PD.

miR-10a suppresses A30P α-syn aggregation and toxicity

To investigate whether transfection with miR-10a attenuated the formation of α-syn aggregates, immunocytochemistry was performed using an anti-α-syn antibody (**Figure 2A-E**). Upregulation of miR-10a expression in A30P α-syn-transfected cells inhibited the formation of α-syn aggregates; however, downregulation of miR-10a by inhibitor transfection enhanced the formation of α-syn aggregates (**Figure 2F** and **2G**). Cell proliferation was assessed by MTT assay. Transfection with A30P α-syn overexpression vector decreased cell viability by ~38% compared with the empty vector control group, whereas the cell viability was significantly increased in the A30P α-syn + miR-10a mimics group compared with the A30P α-syn + miR-NC group and the miR-10a inhibitors group (**Figure 2H**). In addition, LDH assays were conducted to examine the protective role of miR-10 against A30P α-syn-induced toxicity (**Figure 2I**). The release of LDH by SH-SY5Y cells co-transfected with A30P α-syn and miR-10a mimics was lower compared with the miR-NC group and the miR-10a inhibitors group. Both MTT and LDH assay results demonstrated that miR-10a overexpression reduced the toxicity of A30P α-syn in SH-SY5Y cells.

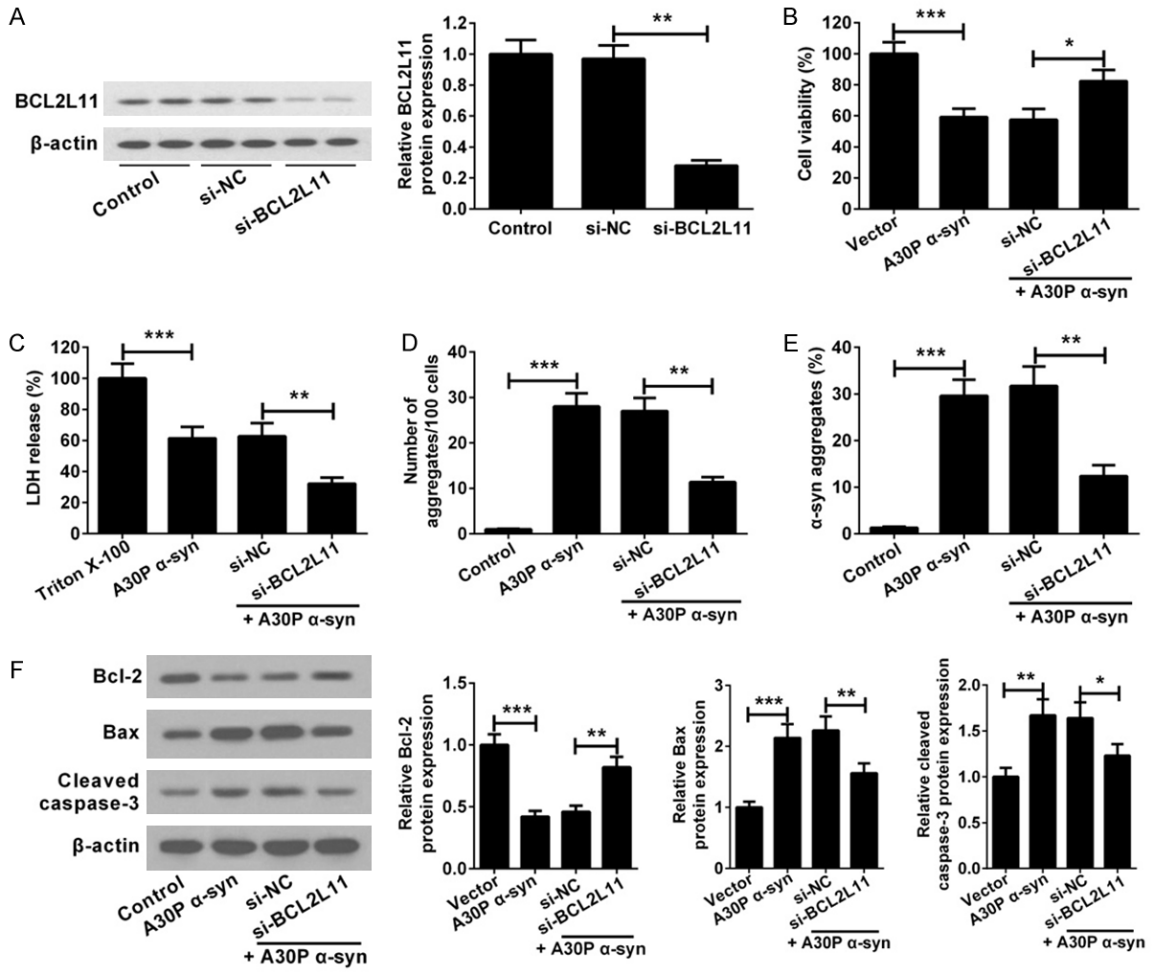

Figure 5. Effect of BCL2L11 knockdown on A30P α-syn aggregation and toxicity in SH-SY5Y cells. (A) The inhibitory effect of si-BCL2L11 on the expression of BCL2L11 proteins was confirmed by Western blotting. (B) Cell viability and (C) cytotoxicity were evaluated 48 h post-transfection with A30P α-syn and si-BCL2L11 by MTT and LDH assays, respectively. (D) α-syn-positive aggregates in every 100 cells were counted. (E) Percentage of cells with α-syn aggregates. (F) Western blotting was conducted to measure the expression levels of BCL2, BAX, and cleaved caspase-3 in SH-SY5Y cells co-transfected with A30P α-syn and si-BCL2L11. Data are presented as the mean ± standard deviation (n=3); $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. α-syn, α-synuclein; BAX, BCL-2-associated X protein; BCL2, B-cell lymphoma-2; BCL2L11, B-cell lymphoma like 11; LDH, lactate dehydrogenase; si, small interfering RNA.

miR-10a suppresses the intrinsic apoptosis pathway

To further investigate the roles of miR-10a in A30P α-syn-induced neural apoptosis, SH-SY5Y cells were co-transfected with A30P α-syn overexpression vector and miR-10a mimics. Western blotting was performed to determine the expression levels of apoptosis-associated proteins, including BAX, cleaved caspase-3, and BCL2 (**Figure 3**). BAX and cleaved caspase-3 expression were significantly reduced, whereas BCL-2 expression was significantly increased in the miR-10a + A30P α-syn group compared with the miR-NC + A30P α-syn group.

BCL2L11 is a direct target of miR-10a

By using TargetScan software (www.targetscan.org), BCL2L11 was predicted to be a putative target of miR-10a. The putative miR-10a target site in BCL2L11-WT 3'-UTR region and the modified binding sequence in BCL2L11-Mut 3'-UTR are provided in **Figure 4A**. Results obtained from Western blotting revealed that the protein expression levels of BCL2L11 were significantly decreased in cells transfected with miR-10a mimics compared with expression in the miR-NC group (**Figure 4B**). BCL2L11 mRNA expression was also significantly reduced by miR-10a mimics compared with the miR-NC group (**Fi-**

gure 4C). To test whether BCL2L11 was directly targeted and regulated by miR-10a, 293T cells were co-transfected with a luciferase reporter containing either the BCL2L11-WT 3'-UTR or the BCL2L11-Mut 3'-UTR and either miR-10a mimics or miR-NC. miR-10a overexpression reduced the luciferase activity of the reporter gene containing the BCL2L11-WT 3'-UTR, but no significant alterations were indicated in the BCL2L11-Mut group (**Figure 4D**). Therefore, miR-10a may directly target the 3'-UTR of BCL2L11 and may be associated with cell death induced by α-syn neurotoxicity.

Downregulation of BCL2L11 inhibits α-syn aggregation and toxicity

In order to test whether BCL2L11 was involved in the inhibitory effects of miR-10a on α-syn aggregation and cytotoxicity, siRNAs were used to knock down endogenous BCL2L11 expression in SH-SY5Y cells. The expression level of the BCL2L11 protein in the si-BCL2L11 group was reduced compared with that in the si-NC group (**Figure 5A**). Furthermore, the results of MTT and LDH assays suggested that knockdown of BCL2L11 attenuated α-syn toxicity (**Figure 5B** and **5C**). The number of α-syn aggregates per 100 cells and the percent of cells with α-syn aggregates were decreased in the si-BCL2L11 + A30P α-syn group compared with the si-NC + A30P α-syn group (**Figure 5D** and **5E**). A decrease in the expression levels of pro-apoptotic proteins BAX and cleaved caspase-3 and an increase in the expression level of anti-apoptotic protein BCL2 were observed in the si-BCL2L11 + A30P α-syn group compared with the si-NC + A30P α-syn group (**Figure 5F**). These results suggest that BCL2L11 may be a vital mediator in miR-10a-mediated suppression of α-syn aggregation and toxicity in SH-SY5Y cells.

Discussion

α-syn has been demonstrated to be involved in the pathogenesis of PD [2]. WT α-syn is selectively degraded by the chaperone-mediated autophagy pathway, but pathogenic mutants A53T α-syn and A30P α-syn block this degradation pathway by binding to lysosome-associated membrane protein type-2A, which results in excessive accumulation of toxic α-syn proteins [21]. A30P α-syn aggregation has been reported to impair the stability and reduce the survival rate of adult-born dopaminergic neurons in the olfactory bulb [22]. Results from the present study demonstrate that the expression of miR-10a is decreased in SH-SY5Y cells transfected with A30P α-syn overexpression vector and in the midbrain of A30P α-syn transgenic mice. It was also demonstrated that miR-10a inhibited the expression of BCL2L11 by binding to the 3'-UTR of BCL2L11 mRNA. In addition, the inhibition of BCL2L11 mRNA expression reduced A30P α-syn-induced toxicity in SH-SY5Y cells. To the best of our knowledge, the present study identified, for the first time, the potential neuroprotective role of the miR-10a/BCL2L11 signaling in an *in vitro* model of PD.

miRNAs are abundantly expressed in the central nervous system and serve a vital role in neuronal biology. To date, miRNAs have been demonstrated to be involved in neuronal development, memory, synaptic plasticity, neurogenesis, and neuronal degeneration [23, 24]. In MPTP-induced cells and mouse models of PD, the expression levels of miR-7 were reduced [25]. miR-7 represses α-syn expression by targeting the 3'-UTR of α-syn mRNA and thereby protects cells against oxidative stress [25]. In SH-SY5Y cells exposed to 1-methyl-4-phenylpyridinium, miR-7 exerts neuroprotective effects by suppressing mitochondrial fragmentation, mitochondrial depolarization, cytochrome c release, the generation of reactive oxygen species (ROS), and the release of mitochondrial calcium [26]. miR-34b and miR-34c, both of which have been reported to be downregulated in the brains of patients with of PD, repress α-syn expression, whereas the inhibition of miR-34b and miR-34c enhances α-syn expression and promotes the formation of α-syn aggregates [27]. A previous study revealed that miR-10a expression was upregulated in PD cerebrospinal fluid exosomes compared with the expression in healthy controls [28]. A previous study reported that miR-10a is downregulated in a mouse model of PD [18]. Therefore, the present study focused on the roles of miR-10a in the pathobiology of PD. RT-qPCR results revealed that the expression levels of miR-10a were decreased in the midbrain of A30P α-syn transgenic mice and in SH-SY5Y cells overexpressing A30P α-syn. In addition, it was demonstrated that miR-10a attenuated α-syn aggregation and toxicity through suppression of BCL2L11 expression in SH-SY5Y cells. In addition, the A30P α-syn-induced upregulation of

BAX, cleaved caspase-3 expression, and downregulation of BCL2 expression were reversed by miR-10a mimics *in vitro*.

The BCL2L11 protein is a key factor in activating the intrinsic apoptotic pathway in neurons under both physiological and pathophysiological conditions. Dominant-negative c-Jun contributes to neuronal survival by decreasing BCL2L11 expression [29]. BCL2L11 knockdown attenuated the forkhead box O3-induced overproduction of ROS and impaired mitochondrial respiration in neuronal cells, which led to apoptosis [30]. BCL2L11 was identified as the direct target of miR-9, and miR-9 was reported to mediate neuronal apoptosis by targeting BCL2L11 in ischemic brain injury [31]. High BCL2L11 expression in neurons leads to neurodegenerative disorders such as AD, PD, and Huntington's disease [32]. In the present study, to explore the underlying molecular mechanism of BCL2L11 in PD pathogenesis, a dual luciferase assay was used to test whether miR-10a recognized the 3'-UTR of BCL2L11. It was demonstrated that BCL2L11 may be a direct target gene of miR-10a. In addition, downregulation of BCL2L11 attenuated A30P α-syn aggregation and toxicity in SH-SY5Y cells, which was accompanied by decreased protein expression of BAX and cleaved caspase-3 and increased protein expression of BCl2.

In conclusion, the results of the present study suggest that BCL2L11 may serve a vital role in the control of α-syn aggregation and cytotoxicity *in vitro*. miR-10a overexpression reduced α-syn aggregation and cytotoxicity, at least partially, by inhibiting the expression of BCL2L11. Identification of BCL2L11 as a target gene of miR-10a in the present study may provide an improved understanding of underlying molecular mechanisms of miR-10a-mediated neuroprotective function, which may be a potential drug target for treating PD.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Huimin Liang, Department of Neurology, Huaihe Hospital of Henan University, 8 Baobei Road, Kaifeng 475000, Henan, P. R. China. Tel: +86-371-23906778; Fax: +86-371-23906778; E-mail: hmliang068@163.com

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