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Original Article

Silencing of SIAH1 in SH-SY5Y affects α-synuclein degradation pathway

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Abstract: Seven in absentia homolog (SIAH) is a ubiquitin ligase that monoubiquitinates α-synuclein. Lewy bodies are characteristically rich in monoubiquitinated α-synuclein. We aimed to determine the effect of siRNA-SIAH1 on α-synuclein autophagy and UPS degradation in SH-SY5Y. SIAH1 expression was measured with real-time quantitative PCR and Western Blot. Cell proliferation was measured by CCK-8 assay; cell apoptosis assayed by flow cytometry. Relative protein expressions were measured by Western Blot. mRNA levels of relative protein were measured by real-time quantitative PCR. The expression of α-synuclein, LC3-II and SIAH1 were observed by confocal microscopy. We found: (1) Transfection efficiency of SIAH1-siRNA into SH-SY5 measured approximately 89% by flow cytometry. (2) siRNA silencing of SIAH1 promoted cellular proliferation and suppressed apoptosis. (3) Protein and mRNA expression of α-synuclein, LC3-II and p53 decreased after SIAH1 knockdown. E1 protein and mRNA levels increased after SIAH1 siRNA. These data show silencing SIAH1 increased cell proliferation and inhibited apoptosis in SH-SY5Y neuroblastoma cells. SIAH1 knockdown enhanced the clearance of non-aggregated α-synuclein by UPS. SIAH1 is a potential target for treatment of Parkinson's disease.

Keywords: Seven in absentia homolog (SIAH), α-synuclein, Parkinson's disease, autophagy, ubiquitin-proteasome system (UPS)

Introduction

Parkinson's disease (PD) affects approximately 1% of the population over the age of 65, and is the second most common progressive neurodegenerative disorder after Alzheimer's disease [1]. PD is clinically characterized primarily by resting tremors, bradykinesia, rigidity, postural instability, and other motor and non-motor symptoms. The pathology of PD is characterized by selective loss of dopamine neurons (DA), and accumulation of α-synuclein in neurons of substantia nigra (SN) and other brainstem regions [2, 3]. The neuropathological hallmark of PD is the presence of intracytoplasmic Lewy bodies (LBs), composed primarily of mono- and polyubiquitinated α-synuclein protein [4]. E3 ubiquitin ligase-SIAH (seven in absentia homolog) plays an important role in α-synuclein monoubiquitylation and in modulating α-synuclein aggregation, and may contribute to the formation of Lewy bodies [5]. The main degradation pathway of intracellular α-synuclein aggregates is via autophagy by the lysosomal degradation pathway [6, 7].

E3 ubiquitin ligase-SIAH supports α-synuclein aggregation and monoubiquition, but the mechanism of SIAH1 in autophagy through the lysomomal pathway and the upbiquitin proteasome system (UPS) pathway remains unknown. SIAH1 may activate or down-regulate autophagy via p53, thus promoting or inhibiting the degradation of monoubiquitylated α-synuclein. In order to clarify this, we silenced SIAH1 and analyzed the effects on UPS degradation pathway and autophagy-lysosomal degradation pathway.

Results

SIAH1 gene silencing by predesigned siRNAs

To silence SIAH1 expression, we designed three pairs of siRNAs. After 6 h transfection, the siR-

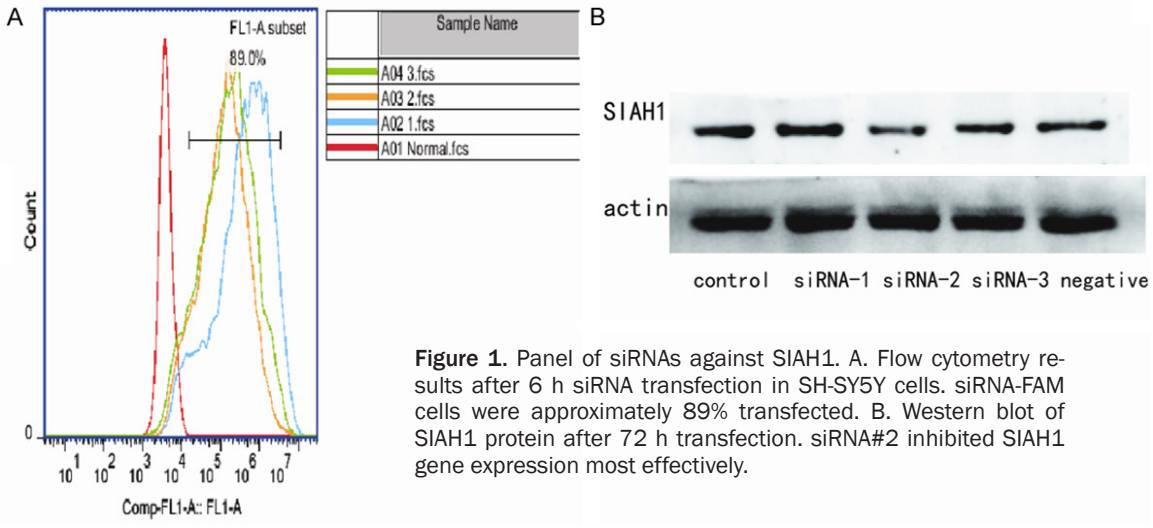

Figure 1. Panel of siRNAs against SIAH1. A. Flow cytometry results after 6 h siRNA transfection in SH-SY5Y cells. siRNA-FAM cells were approximately 89% transfected. B. Western blot of SIAH1 protein after 72 h transfection. siRNA#2 inhibited SIAH1 gene expression most effectively.

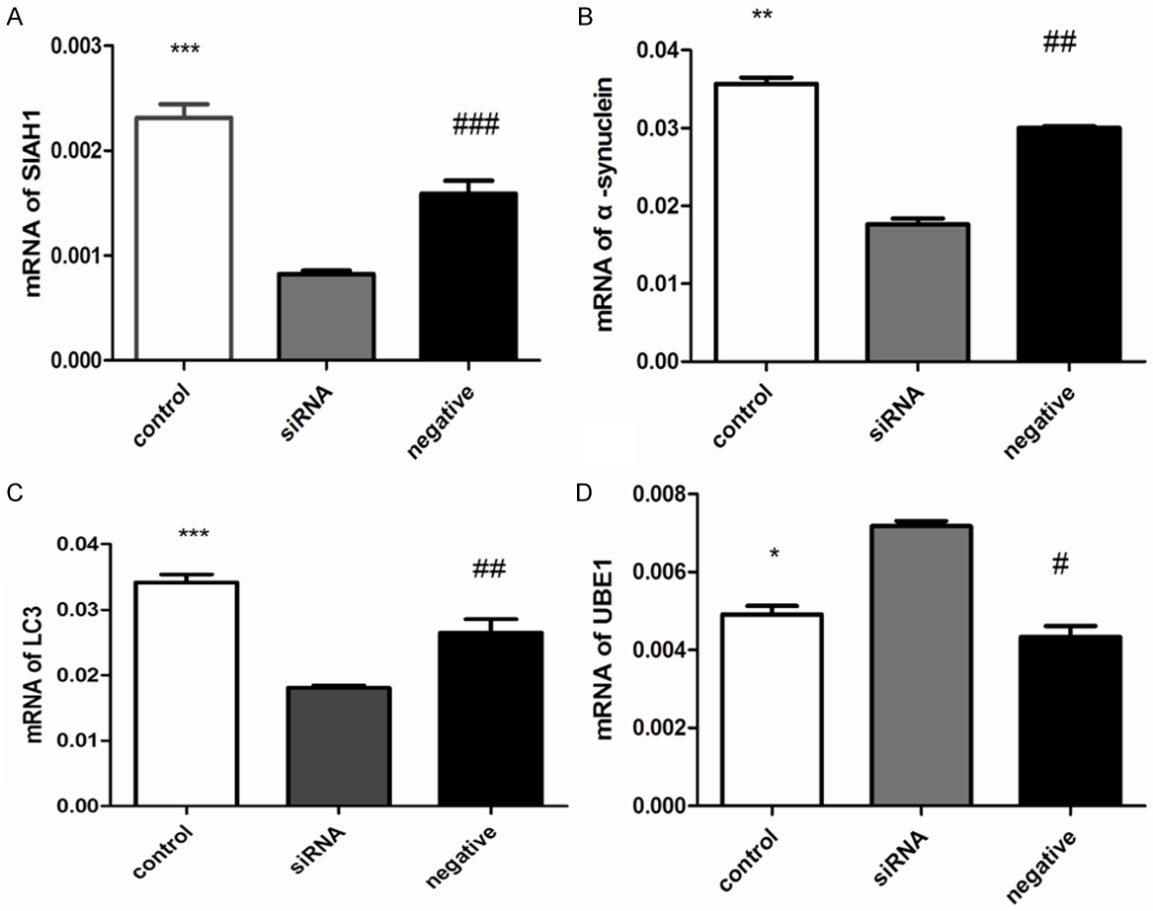

Figure 2. Real time PCR analysis of mRNA levels after siSIAH1 treatment in SH-SY5Y cells. A. SIAH1. B. α-synuclein level reduced significantly (**P<0.01) (##P<0.01). C. LC3-II level reduced significantly (***P<0.001) (##P<0.01). D. UBE1 mRNA increased significantly (*P<0.05, #P<0.05).

NA-FAM cells were approximately 89% transfected in SH-SY5Y cells measured by flow cytometry. After 72 h transfection, we found siRNA #2 showed the greatest suppression of SIAH1 and was selected for subsequent assays (**Figure 1**).

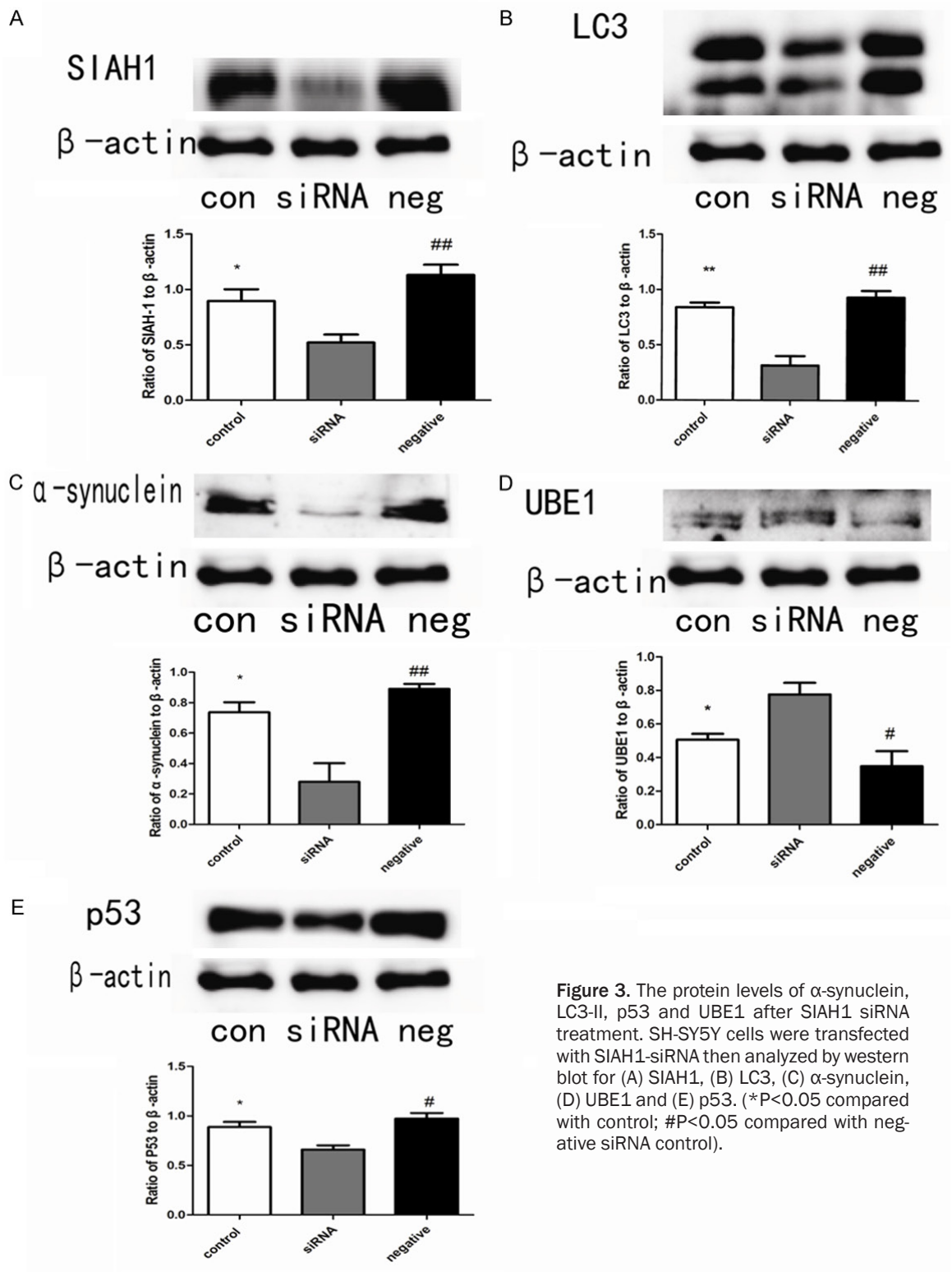

Figure 3. The protein levels of α-synuclein, LC3-II, p53 and UBE1 after SIAH1 siRNA treatment. SH-SY5Y cells were transfected with SIAH1-siRNA then analyzed by western blot for (A) SIAH1, (B) LC3, (C) α-synuclein, (D) UBE1 and (E) p53. (*P<0.05 compared with control; #P<0.05 compared with negative siRNA control).

SIAH1 silencing increased cell proliferation in SH-SY5Y cells

To examine the effect of SIAH1 siRNA on cell proliferation, we measured cell proliferation with a CCK-8 assay. We found that SIAH1 knockdown significantly increased SH-SY5Y cell proliferation compared with control and negative siRNA groups. This indicates that silencing SIAH1 may be beneficial to H-SY5Y cells.

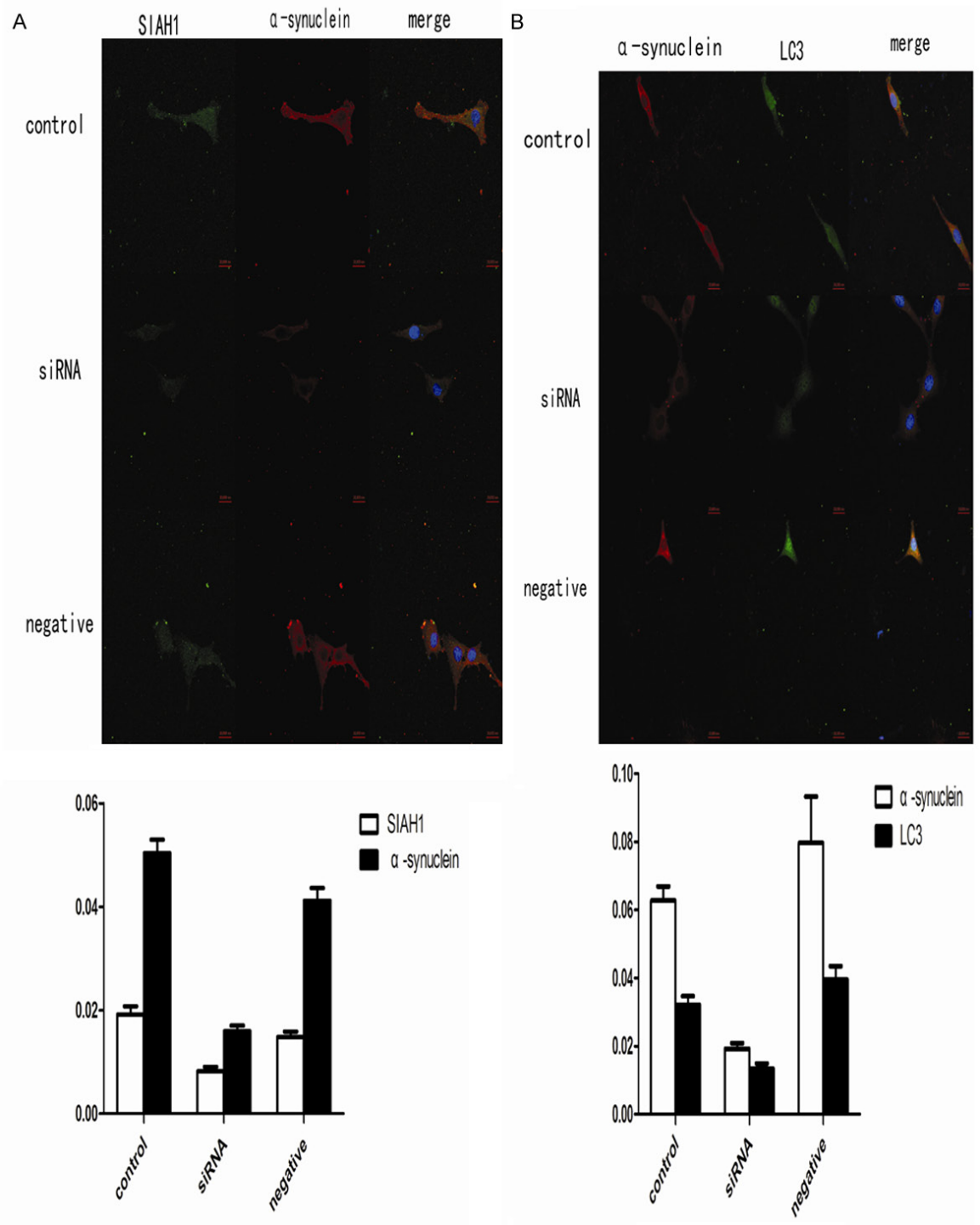

Figure 4. Colocalizatoin of α-synuclein, SIAH-1 and LC3 in SH-SY5Y cells. The treated cells were immuostained with anti-α-synuclein (red), anti-LC3 (green) antibodies and anti-SIAH1 (green) antibodies nuclei were further stained with DAPI (blue). Immunoreactivity was visualized under a confocal laser scanning microscope. Scale bars = 20 μm.

SIAH1 silencing affected cell apoptosis

We measured apoptosis of SH-SY5Y cells after SIAH1 siRNA using flow cytometry. We found the mean percent of apoptosis in control cells, siRNA-SIAH1 transfected cells and negative siRNA transfected cells was 5.01%, 3.53% and 5.36% respectively. Apoptosis in siRNA treated

cells was significantly lower compared with control cells. Our results show a non-significant decrease compared with negative siRNA group.

SIAH1 silencing suppressed α-synuclein expression and the autophagy pathway

Our results show SIAH1 siRNA suppressed α-synuclein in both mRNA and protein levels in SH-SY5Y cells compared with negative siRNA and control (**Figures 2B**, **3C**). To further examine the role of SIAH1 in autophagy, we analyzed expression of pathway proteins. Microtubule-associated protein 1 light chain 3 (LC3) is cleaved by Atg4 into LC3-I and LC3-II, LC3-II associated with both outer and inner membranes of autophagosome, and the amount of LC3-II correlates with the extent of autophagasome [8]. We found LC3-II mRNA and protein reduced after siRNA treatment (**Figures 2C**, **3B**).

SIAH1 silencing the UPS pathway

The UPS pathway plays a critical role in α-synuclein degradation. To investigate the effect of SIAH1 on the UPS pathway, we measured the protein level of UBE1, that important for the UPS degradation. We found UBE1 protein elevated in SH-SY5Y cells after SIAH1 siRNA treatment compared with mock cells ($t = 3.480$, $P<0.05$) or negative siRNA ($t = 3.765$, $P<0.05$) (**Figure 3D**). Consistent with our western blot results, UBE1 mRNA increased in SH-SY5Y cells after SIAH1 siRNA transfection compared with mock cells ($t = 8.739$, $P<0.05$) or negative siRNAs ($t = 9.170$, $P<0.05$) (**Figure 2D**).

Crosstalk between UPS pathway and autophagy pathway

UPS pathway and ALP pathway are two important protein degradation systems, and growing evidence suggests that there is a crosstalk between UPS pathway and autophagy pathway. The previous study has shown that p53 has an important in mediating UPS inhibition induced autophagy activation [9]. We measured the protein level of p53. We found p53 protein elevated in SH-SY5Y cells after SIAH1 siRNA treatment compared with mock cells ($t = 3.326$, $P<0.05$) or negative siRNA (4.400, $P<0.05$) (**Figure 3E**).

SIAH-1, α-synuclein and LC3 colocalization in SH-SY5Y cell

We investigated the autophagic degradation effect of SIAH1 siRNA using confocal microscopy. Double immunofluorescence staining with anti-SIAH and anti-α-synuclein antibodies shows that α-synuclein aggregations decreased after SIAH1-siRNA treatment (**Figure 4A**). In SH-SY5Y cells, the LC3 was significantly reduced following SIAH1-siRNA treatment (**Figure 4B**). SIAH-1, α-synuclein and LC3 lost its colocalization in SH-SY5Y cells.

Discussion

PD pathology is characterized by the accumulation of α-synuclein aggregates, known as Lewy bodies, in the substantia nigra (SN) of the midbrain [10]. The UPS and autophagy-lysosome pathway (ALP) are the two major degradation pathways that maintain protein homeostasis in cells. While the UPS clears most short-lived and soluble proteins [11], the ALP degrades longer-lived macromolecules and dysfunctional organelles [12]. α-synuclein exists in three forms: monomers, dimers and protofibrils [13]. However, the mechanism of α-synuclein degradation by the UPS or the autophagy pathway remains unknown. UPS pathway degrades monomeric α-synuclein [14]. Only a small proportion of soluble α-synuclein oligomers, not including monomeric α-synuclein, are degraded by the 26S proteasome [15]. α-synuclein monoubiquitylation by E3 ubiquitin ligase-SIAH plays a critical role in α-synuclein aggregation and contributes to Lewy body formation [16]. Monoubiquitination of α-synuclein by SIAH1 is cleared solely by the 26S proteasome, while deubiquitinated α-synuclein is degraded mainly by autophagy [17]. Our study supports the hypothesis that SIAH1 knockdown decreases α-synuclein monoubiquitination and aggregation, contributing to increasing monomers of α-synuclein.

The E3 ubiquitin ligases-SIAH are RING finger ubiquitin ligases composed of a catalytic RING domain, two zinc finger domains, and a substrate binding domain [18-20]. Mice express Siah1a, Siah1b, and Siah2 gene; humans express Siah1 and Siah2 [21]. We inhibited SIAH1 expression with designed siRNAs transfected into SH-SY5Y cells. Our data show SIAH1 knockdown lead to decreased p53, increased

cell proliferation and apoptosis suppression. Our results are consistent with previous studies showing inhibition of SIAH1 reduced apoptosis [22].

In our study, SIAH1 knockdown significantly inhibited α-synuclein expression, suggesting a key role in α-synuclein monoubiquitylation and aggregation. This may provide a therapeutic opportunity in treating PD. Our results are consistent with previous research showing SIAH1 monoubiquitylates α-synuclein and leads to aggregation, a toxic environment in cells [16].

In our experiments, RNAi-mediated downregulation of SIAH1 effectively decreased protein and mRNA of autophagic vacuoles marker (LC3-II). We can infer autophagy pathway decreased after SIAH1 siRNA treatment.

In addition, we also found that UBE1 protein and mRNA expression levels increased. α-synuclein degradation through the UPS consists of two consecutive steps. First, conjugation of multiple ubiquitin moieties to the substrate occur, then 26S proteasome complex degrades it [23]. Ubiquitylation occurs via a three-step cascade catalyzed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligase enzyme (E3). After the initial ubiquitin is attached, polyubiquitylation of the substrate adds ubiquitin molecules, forming ubiquitin chains [24, 25]. In our study, we found the expression of E1 increased, indicating the UPS degradation pathway was activated after SIAH1 siRNAs transfection.

In conclusion, our results show SIAH1 knockdown inhibited cellular apoptosis and increased cell proliferation. In addition, knockdown of SIAH1 decreased α-synuclein monoubiquitination and aggregation. These findings argue that the UPS degradation pathway was activated upon SIAH1 inhibition. Our data suggest SIAH1 may be a promising candidate for PD treatment.

Materials and methods

Cell culture

SH-SY5Y neuroblastoma cells were a kind gift from the Institute of Neuroscience. SH-SY5Y cells were cultured at 37°C and 5% CO_2 in DMEM/F12 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, USA). The culture medium was changed every two days and the cells were passaged when they reached 80 to 90% confluency. SH-SY5Y cells were divided into three groups as follows: i) a blank control group; ii) an experimental group transfected with siRNA-SIAH1; iii) a negative control group transfected with a null siRNA.

CCK-8 assay

Cell viability was assayed using the Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). After the control siRNA and SIAH1 specific siRNAs were transfected into SH-SY5Y cells in 96-well-plate 48 h, 10 μL of the CCK-8 reagent was added and incubated at 37°C for 2 h. The optical density (OD) was measured at 450 nm. Cell viability was calculated using the data and presented relative to controls. Results of transfection were read relative to controls, assuming the absorbance of controls was 100%. All values were calculated using mean ± S.D.

Apoptosis assay

Measured SH-SY5Y cells apoptosis after transfection control siRNA and SIAH1-siRNA for 72 h using Annexin V/FITC kit (BD Pharmingen, USA) in accordance with the manufacturer's instructions and detected by flow cytometry.

siRNA design and transfection

siRNAs targeting SIAH1 were designed and synthesized by Suzhou GenePharma. 1#siRNA Sense: 5'-GCUCACAUGUUGUCCAACUTT-3', Anti-sense: 5'-AGUUGGACAACAUGUGAGCTT-3'; 2#siRNA2 Sense: 5'-CCUGGUGCUUCCUGUAAAUTT-3', Antisense: 5'-AUUUACAGGAAGCACCAGGTT-3'; 3#siRNA3 Sense: 5'-GCGACUGUCUAGUCUUUGATT-3', Anti-sense: 5'-UCAAAGACUAGACAGUCGCTT-3'. Cells were transfected with siRNA and Lipofectamine-2000 (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded in a 6 or 24-well-plate at a confluence of 60-70%. 100 pmol siRNA (20 μM) was mixed with 5 μl Lipofectamine-2000 in 250 μl serum-free OPI-MEM medium (Gibco, USA) and incubated at room temperature for 30 minutes to form a complex. After washing cells with PBS, the 500 μl transfection mixtures were added to each well. 24 h after the transfection, the medium was replaced with fresh

500 μl DMEM/F12 medium containing 10% FBS. 48 h after transfection, cells were collected for Real-time PCR and immunofluorescence microscopy. 72 h after transfection, proteins were collected for Western Blot.

Western blotting

72 h after transfection control siRNA and SIAH1-siRNA into SH-SY5Y cells in 6-well-plate, protein was isolated according to instructions of the protein extraction kits (Beyotime Biotechnology, China). Protein concentrations determine using BCA protein assay kits (Bioworld, USA). Equal quantities of protein extracts were loaded onto 12% SDS-PAGE gels and wet-transferred to PVDF membranes. After blocking with 5% powdered skim milk for 1 h, the membranes were incubated overnight at 4°C with anti-SIAH (1:100 SantaCruz, America), anti-α-synuclein (1:500, Abcam, USA), anti-LC3 (1:2000, Abcam, USA), UBE1 (1:1000, Cell Signaling, USA), p53 (1:1000 Abcam USA). β-actin (1:1000 Abcam USA). The membranes were incubated with HRP-conjugated secondary antibodies for 2 h at room temperature, and visualized using ECL kits (Bioworld, USA). Determined the band intensities with Quantity One Software (Tanon 5200, China).

Real time quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated from cells using Trizol reagent (Life Invitrogen) according to the manufacture's protocol and was converted to cDNA using PrimeScript RT Master Mix (Perfect Real Time) (Takara, Japan). cDNA were amplified by real-time PCR using SYBR 7500 Fast Real-time PCR System (Applied Biosystems). The following primers were used: SIAH sense primer 5'CTGTCGCCCCAAACTTACAT-3' and antisense primer 5'-CCACATGGATACCATCAAACTG-3'. α-synuclein sense primer 5'-CCTCAGCCCAGAGCCTTTC-3' and antisense primer 5'-CCTCTGCCACACCCTGCTT-3'. LC3 sense primer 5'-GAGTGGAAGATGTCCGGCTC-3', antisense primer: 5'-CCAGGAGGAAGAAGGCTTGG-3'; UBE1 sense primer 5'-CCCTACATGACCAAGGCACT-3', antisense primer 5'-CCAGGAGGAAGAAGGCTTGG-3'. β-actin sense primer: 5'-TCAGGTCATCACTATCGGCAAT-3'; antisense primer: 5'-AAAGAAAGGGTGTAAAACGCA-3'. PCR products were subjected to a melting-curve analysis to confirm the PCR specificity.

Immunofluorescence microscopy

After 24 h transfection SH-SY5Y cells with SIAH1-siRNA, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.25% triton X-100 for 5 min. Cells were washed with PBS three times every 15 min. After blocking for 1 h in BSA (2% goat serum+0.2% BSA+0.2% triton), cells were incubated with primary anti-SIAH-1 (1:100 dilution), anti-LC3 (1:250 dilution) and anti-α-synuclein (1:100 dilution) overnight at 4°C. Cells were treated with the appropriate secondary antibody treatment for 1 h and DAPI (1:100 dilution) was added for 5 min to stain nucleus. Cells evaluated under a Confocal microscope (ZEISS, LSM710, Germany).

Statistical analysis

Data are presented as the mean ± standard deviation. Two group comparisons were performed using Student's t-test. Multiple group comparisons were performed using one-way analysis of variance and Fisher's least significant difference. Values of $P < 0.05$ were set as statistically significant.

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Disclosure of conflict of interest

None.

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