

LncRNA NEAT1 promoted MPP⁺-induced ferroptosis *via* regulating miR-150-5p/BAP1 pathway in SK-N-SH cells

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As widely reported, dysregulated ferroptosis is closely associated with Parkinson's disease (PD) progression. The goal of the present study was to probe the roles of long non-coding RNA (lncRNA) nuclear enriched assembly transcript 1 (NEAT1) in regulating ferroptosis in PD. PD cell model was constructed by subjecting SK-N-SH cells to 1-methyl-4-phenylpyridinium (MPP⁺) for 24 h. The RNA levels of NEAT1, miRNA (miR)-150-5p and BRCA1-associated protein 1 (BAP1) were evaluated using qRT-PCR. The protein levels of glutathione peroxidase 4 (GPX4), BAP1 and solute carrier family 7 member 11 (SLC7A11) were determined using western blot. Cell viability was assessed using 3-(4,5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) assay. In addition, fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was employed to determine the ROS level. Moreover, the levels of GSH, MDA and Fe²⁺ were also measured. Finally, the interactions among NEAT1, miR-150-5p and BAP1 were identified by dual luciferase reporter gene assay, and/or RIP assay. Upregulated NEAT1 was observed in PD cell model. Knockdown of NEAT1 elevated viability and GSH level in PD cell model, and reduced ROS, MDA and Fe²⁺ levels. Moreover, NEAT1 functioned as a sponge to suppress miR-150-5p expression. Moreover, miR-150-5p overexpression suppressed ferroptosis in PD cell model. We subsequently found that miR-150-5p regulated SLC7A11 expression by directly binding to BAP1. miR-150-5p inhibition or BAP1 overexpression mitigated the anti-ferroptosis effect mediated by sh-NEAT1. Taken together, knockdown of NEAT1 mitigated MPP⁺-induced ferroptosis through regulating BAP1/SLC7A11 axis by sponging miR-150-5p, indicating the potential of NEAT1 as a promising therapeutic target for PD.

Key words: lncRNA NEAT1, miR-150-5p, ferroptosis, BAP1, Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is a common type of neurodegenerative disease (Wen et al., 2020). As the second most common neurological disease, about 6 million people worldwide suffer from PD, and the number of PD patients is still growing rapidly (Marras et al., 2018). However, the entire and precise etiology of PD still needs to be illuminated. There is currently no cure for PD, only some drugs can improve the symptoms of PD (Connolly et al., 2014; Erkinen et al., 2018). Therefore, it's urgent and necessary to find new therapeutic targets to improve the treatment and diagnosis approaches of PD.

Ferroptosis is a form of iron-dependent cell death, and is characterized by consumption of glutathione (GSH), accumulation of lipid peroxidation products and lethal reactive oxygen species (ROS) (Dixon et al., 2012; Xie et al., 2016). Accumulated evidence indicates that ferroptosis plays a vital role in the pathogenesis and development of PD (Guiney et al., 2017; Mahoney-Sánchez et al., 2021). The accumulation of iron is observed in PD (Masaldan et al., 2019). Do Van et al. (2016) confirmed that ferroptosis was the main cause of dopaminergic neuron death in PD. In addition, suppression of ferroptosis could inhibit MPTP-induced dopaminergic cell death in mice. All these evidence suggested that

ferroptosis was closely related to neuronal damage in PD. However, the role of ferroptosis in PD and the specific molecular mechanism in regulating ferroptosis in PD remain unknown.

Non-coding RNAs (ncRNAs) composed of long non-coding RNA (lncRNA) and microRNA (miRNA) are considered to be the research focus of “next generation” biology (Tao et al., 2015). lncRNA refers to a single stranded RNA (ssRNA) with the transcripts more than 200 nts and function in many biological processes (Iyer et al., 2015). As well known, lncRNAs were involved in PD development (Wan et al., 2017). lncRNA HAGLROS was reported to enhance apoptosis in PD (Peng et al., 2019). Besides, lncRNA UCA1 was markedly upregulated in brains of PD mice and MPP⁺-treated SH-SY5Y cells, and UCA1 silence could suppress cell apoptosis of PD cell model (Lu et al., 2018). lncRNA nuclear enriched assembly transcript 1 (NEAT1), as a generally highly expressed in various tissues, has been confirmed to be closely related to the development of PD (Xie et al., 2019; Liu et al., 2021). lncRNA NEAT1 was highly expressed in in MPTP-induced PD mouse model and MPP⁺-induced PD cell model, and NEAT1 silence enhanced cell viability, inhibited apoptosis in PD cell model (Liu et al., 2021). Although the role of NEAT1 in PD progression has been widely confirmed, it is unknown whether NEAT1 is involved in the regulation of ferroptosis in PD and what its regulatory pathway is. MiRNAs refer to non-coding single-stranded RNA molecules with a length of approximately 22 nts, which participate in post-transcriptional regulation of gene expressions (Bartel, 2004). It's widely reported that lncRNAs play the role as ceRNAs via acting as endogenous decoys for miRNAs, in turn affecting the binding of miRNAs to their targets (Salmena et al., 2011), which is considered as one of the important way to clarify the molecular mechanism of lncRNAs. Previous study demonstrated that miR-150 was closely related to nerve function, specifically miR-150 overexpression alleviated the isoflurane-induced cognitive dysfunction and neuroinflammation (Cui et al., 2020). Besides, Li et al. (2020) revealed that miR-150 was markedly down-regulated in PD patients. Therefore, the study of miR-150-5p might help us better understand the pathogenesis of PD. In the current study, we predicted that miR-150-5p had a binding site to NEAT1 through the starBase database. However, little is known about the regulatory relationship between miR-150-5p and NEAT1 in regulating ferroptosis in PD, which is worthy of further research.

BRCA1-associated protein 1 (BAP1), as a histone 2A deubiquitinating enzyme, was confirmed as a risk factor for PD (Keo et al., 2020). However, the function of BAP1 in regulating PD progression remains unknown. Previous study identified solute carrier family 7 mem-

ber 11 (SLC7A11) as a target of BAP1 (Zhang et al., 2019). SLC7A11, as an amino acid transporter, has the ability to protect cells from accumulated lipid hydroperoxide and from ferroptosis via mediation of cystine import and glutathione biosynthesis (Dixon et al., 2012; Stockwell et al., 2017). We found that BAP1 was the target of miR-150-5p through Starbase database prediction, therefore we speculated that BAP1/SLC7A11 axis was the downstream target of miR-150-5p in regulating ferroptosis in PD.

Here we probed the function of lncRNA NEAT1/miR-150-5p/BAP1/SLC7A11 axis on regulating ferroptosis in PD. NEAT1 upregulated BAP1 by targeting miR-150-5p, thereby inhibiting SLC7A11 expression. Our research provided a new treatment strategy and potential therapeutic targets for PD treatment.

METHODS

Cell culture and treatment

SK-N-SH cells were obtained from ATCC (VA, USA). All cells were cultured in DMEM (Gibco, MD, USA) containing 10% FBS (Gibco) in a humidified atmosphere of 5% CO₂ at 37°C. SK-N-SH cells were treated with 0.25, 0.5 or 1 mM MPP⁺ (Sigma-Aldrich, MO, USA) for 24 h to construct PD cell model.

Cell transfection

The overexpression plasmid of NEAT1 (oe-NEAT1), the overexpression plasmid of BAP1 (oe-BAP1), the short hairpin RNA of NEAT1 (sh-NEAT1) and mimics/inhibitor of miR-150-5p as well as their negative controls (vector, sh-NC, mimics NC and inhibitor NC) were obtained from GenePharma (Shanghai, China). For *in vitro* transfection, cells were transfected with oe-NEAT1, oe-BAP1, sh-NEAT1, miR-150-5p inhibitor, miR-150-5p mimics or their negative controls using Lipofectamine™ 3000 (Invitrogen, CA, USA).

3-(4,5-dimethylthiazolyl)2-5-diphenyltetrazolium bromide (MTT) assay

Cells were suspended in complete DMEM medium (Gibco) mixed with 5 mg/mL MTT (Sangon, Shanghai, China). Cells were incubated in MTT solution for 4 h at 37°C. After incubation, all the medium was removed. Then dimethyl sulfoxide (DMSO) (Sigma-Aldrich, MO, USA) was added and the absorbance at 490 nm was detected by a microplate reader (Bioteke, Beijing, China).

Quantitative real-time polymerase chain reaction (QRT-PCR)

Total RNA was isolated from cells by using TRIzol reagent (Thermo Fisher Scientific, MA, USA). cDNA was synthesized with HiFiScript cDNA synthesis kit (Life Technologies, CA, USA). Then, the cDNA was used for qRT-PCR assay conducted on an Eppendorf MasterCycler RealPlex4 (Eppendorf, Wesseling-Berzdorf, Germany) using an Ultra SYBR Mixture kit (Thermo Fisher Scientific). To detect the expression of miRNA-150-5p, PCR reaction systems were prepared using miScript SYBR Green PCR Kit (Qiagen, CA, USA). The relative expressions of miRNA and mRNA were respectively normalized by U6 and GAPDH and calculated by $2^{-\Delta\Delta CT}$ method. The primers were listed as follows (5'-3'):

NEAT1 (F): GTGGCTGTTGGAGTCGGTAT
 NEAT1 (R): TAACAAACCACGGTCCATGA
 miR-150-5p (F): CGGCGGCAAAGTGCTTACAG
 miR-150-5p (R): GGGCATAATCGGCTAATACA
 BAP1 (F): CCTGAGGAGTCCAAGTCAGC
 BAP1 (R): CTGGAGGCTTCACCACTAGC
 GAPDH (F): CTGACTTCAACAGCGACACC
 GAPDH (R): GTGGTCCAGGGGTCTTACTC
 U6 (F): CTCGCTTCGGCAGCACA
 U6 (R): AACGCTTCACGAATTTGCGT

Western blot

The proteins were isolated from cells by using RIPA (Thermo Fisher Scientific) mixed with 1% protease inhibitor and phosphatase inhibitor (Thermo Fisher Scientific), and concentrations of protein were assessed using a BCA Kit (Beyotime, Shanghai, China). Lysate samples were separated using SDS-PAGE, and then transferred to a PVDF membrane (Millipore, MA, USA). Then, membranes were incubated with primary antibodies including glutathione peroxidase 4 (GPX4) (Abcam, Cambridge, UK), BAP1 (Abcam) and SLC7A11 (Abcam). Anti-GAPDH antibody (Abcam) was served as a loading control. After washed with PBS-T, membranes were then incubated with the corresponding secondary antibody labeled with HRP (Abcam) for 60 min. The membranes were covered with ECL reagents (Beyotime) and the images were performed by GEL imaging system (Bio-Rad, CA, USA). The quantification of proteins was analyzed by the software ImageJ (National Institutes of Health, MD, USA).

Measurement of Fe²⁺

Cells were harvested and homogenized with PBS. The supernatant was used to performed Fe²⁺ level

detection by using iron assay kit (Abcam). Briefly, samples were added to wells and incubated with assay buffer for 30 min at 37°C. Then iron probe was added and incubated for 60 min at 37°C. And the absorbance was detected by a microplate reader (Bioteke).

Measurement of GSH

The level of GSH was determined using GSH content detection kit (Solarbio, Beijing, China, BC1170). Briefly, cells were resuspended in reagent I and frozen and thawed for 2-3 times. The supernatant was collected by centrifugation. 140 µL of reagent II and 40 µL of reagent III were subsequently added into 20 µL of supernatant, and the absorbance at 412 nm was detected by a microplate reader (Bioteke).

Measurement of ROS

For ROS detection, cells were incubated with 10 µM DCFH-DA (Beyotime) for 25 min. Cells were subsequently washed with PBS, and fluorescence was detected using a fluorescence microplate reader at the excitation/emission 488/525 nm.

Measurement of malondialdehyde (MDA)

The level of MDA in cell lysates was detected using MDA assay kit (Abcam, ab118970) in accordance to the manufacturer's instruction. Briefly, the MDA in the sample reacted with thiobarbituric acid (TBA) to generate a MDA-TBA adduct. The MDA-TBA adduct was quantified colorimetrically (optical density (OD)=532 nm).

Dual luciferase reporter gene assay

We predicted the target of gene binding site between NEAT1 and miR-150-5p using a common online tool, starBase (<http://starbase.sysu.edu.cn/index.php>). The sequence of NEAT1 containing the potential binding site of miR-150-5p was amplified by PCR. Site-directed mutagenesis of the miR-150-5p binding site in NEAT1 sequence was performed using a quick change site-directed mutagenesis kit (Stratagene, CA, USA). Wild-type (wt) and mutant-type (mut) reporter plasmids of NEAT1 sequences were cloned into PGL3 vector (GenePharma). Then, cells were plated onto 24-well plate and were co-transfected with NEAT1-wt or NEAT1-mut plasmids and miR-150-5p mimics or mim-

ics NC by Lipofectamine™ 3000 (Invitrogen). The Luciferase activity was examined using a dual-luciferase reporter assay system (Promega, WI, USA). The same method was used to verify the binding relationship between miR-150-5p and BAP1.

RNA immunoprecipitation (RIP)

RIP assay was conducted by using RIP kit (Millipore). Briefly, cells were lysed with a complete RIP lysis buffer. Cell extract was incubated with immunoglobulin G (IgG) (Abcam, 1:50, ab172730) and Ago2 (Abcam, 1:50, ab186733) antibody at 4°C overnight to precipitate the complex. RNA was purified from the complex. cDNA was synthesized with HiFiScript cDNA synthesis kit according to manufacturer's instruction (Life Technologies). Then the cDNA was used for qRT-PCR assay. GAPDH was served as a house-keeping control. The fold enrichment of NEAT1 and miR-150-5p were determined as described by the manufacturer.

Data analysis

All data was obtained from at least three replicate experiments. Results were expressed as mean \pm standard deviation (SD). Statistical analysis was performed using GraphPad Prism 6.0. Between-group differences and multi-group comparisons were determined using Student's *t* test and one-way Analysis of Variance (ANOVA) followed by Tukey's *post hoc* test, respectively. The *p* values less than 0.05 were considered significant.

RESULTS

LncRNA NEAT1 enhanced MPP⁺-induced ferroptosis in SK-N-SH cells

To explore the expression level of LncRNA NEAT1 in PD *in vitro* model, we subjected SK-N-SH cells to MPP⁺ and the result of qRT-PCR displayed that the RNA level of NEAT1 was upregulated in a dose-dependent manner ($P < 0.05$; Fig. 1A). Previous study revealed that ferroptosis participates in PD progression (Mahoney-Sánchez et al., 2021), so we sought to probe whether NEAT1 could regulate ferroptosis in PD. As shown in Fig. 1B, the RNA level of NEAT1 in PD cell model was reduced following sh-NEAT1 transfection, suggesting the transfection was successful. Result of MTT assay subsequently demonstrated that cell viability

was obviously reduced following MPP⁺ treatment, while NEAT1 knockdown partially rescued this effect ($P < 0.05$; Fig. 1C). Besides, MPP⁺ treatment induced elevated levels of MDA, ROS and Fe²⁺, and reduced level of GSH, however, NEAT1 knockdown eliminated these effects ($P < 0.05$; Fig. 1D-G). Finally, the protein levels of GPX4 and SLC7A11 was reduced by two-thirds following MPP⁺ treatment, while NEAT1 silence partially abolished the effects ($P < 0.05$; Fig. 1H-J). In sum, NEAT1 silence could suppress MPP⁺-induced ferroptosis in SK-N-SH cells.

miR-150-5p was a target of NEAT1

Bioinformatics software was employed to predict the target of NEAT1, and we found that NEAT1 had a binding site to miR-150-5p (Fig. 2A). Dual luciferase reporter gene assay and RIP assay were employed to further verify this binding relationship between NEAT1 and miR-150-5p. The results revealed that NEAT1 directly bound to miR-150-5p ($P < 0.05$; Fig. 2B-C). We found that the level of miR-150-5p in SK-N-SH cells was gradually reduced with the increase of MPP treatment concentration ($P < 0.05$; Fig. 2D). Results of qRT-PCR subsequently demonstrated that the level of miR-150-5p in PD *in vitro* model was significantly downregulated following transfection with oe-NEAT1, while the level was markedly increased following NEAT1 knockdown ($P < 0.05$; Fig. 2E-F). In total, miR-150-5p acted as a role as the target of NEAT1.

miR-150-5p suppressed MPP⁺-induced ferroptosis in SK-N-SH cells

Then we overexpressed miR-150-5p in SK-N-SH cells and treated cells with 1 mM MPP⁺ for 24 h. the level of miR-150-5p in PD *in vitro* model was markedly upregulated by about 2-fold following miR-150-5p mimics transfection, suggesting the transfection was successful ($P < 0.05$; Fig. 3A). Cell viability was obviously reduced following MPP⁺ treatment, while miR-150-5p overexpression partially rescued this effect ($P < 0.05$; Fig. 3B). In addition, decreased levels of MDA, ROS and Fe²⁺, and increased level of GSH were observed in MPP⁺ + miR-150-5p mimics group compared with MPP⁺ + mimics NC ($P < 0.05$; Fig. 3C-G). Finally, the protein levels of GPX4 and SLC7A11 in the MPP⁺ + miR-150-5p mimics group were upregulated by about 2-fold in comparison with the MPP⁺ + mimics NC ($P < 0.05$; Fig. 3H-I). Taken together, miR-150-5p overexpression could inhibit ferroptosis in PD *in vitro* model.

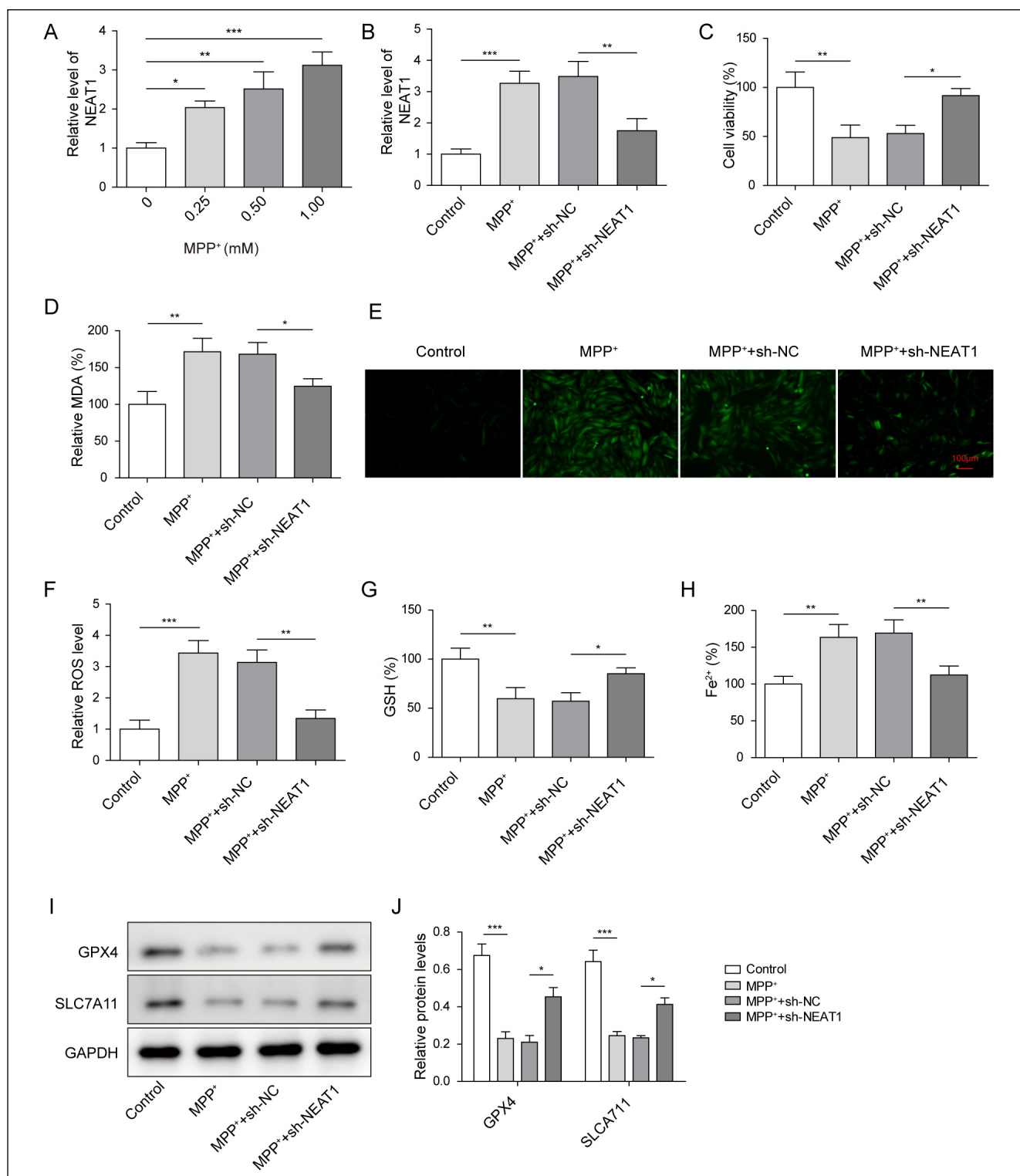


Fig. 1. LncRNA NEAT1 enhanced MPP⁺-induced ferroptosis in SK-N-SH cells. (A) SK-N-SH cells were subjected to different concentrations of MPP⁺ for 24 h, then the RNA level of NEAT1 in cells was determined using qRT-PCR. Then we knocked down NEAT1 in SK-N-SH cells and treated cells with 1 mM MPP⁺ for 24 h. (B) qRT-PCR was employed to assess the RNA level of NEAT1. (C) Cell viability was determined using MTT assay. (D) MDA level was evaluated using MDA assay kit. (E) Fluorescent probe DCFH-DA was employed to determine ROS level. (F) The quantitative analysis of ROS in (E). (G) GSH level was detected using GSH content detection kit. (H) Fe²⁺ level was detected by using iron assay kit. (I) The protein levels of GPX4 and SLC7A11 were assessed using western blot. (J) The quantitative analysis of proteins in (I). The data were expressed as mean ± SD. All data was obtained from at least three replicate experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

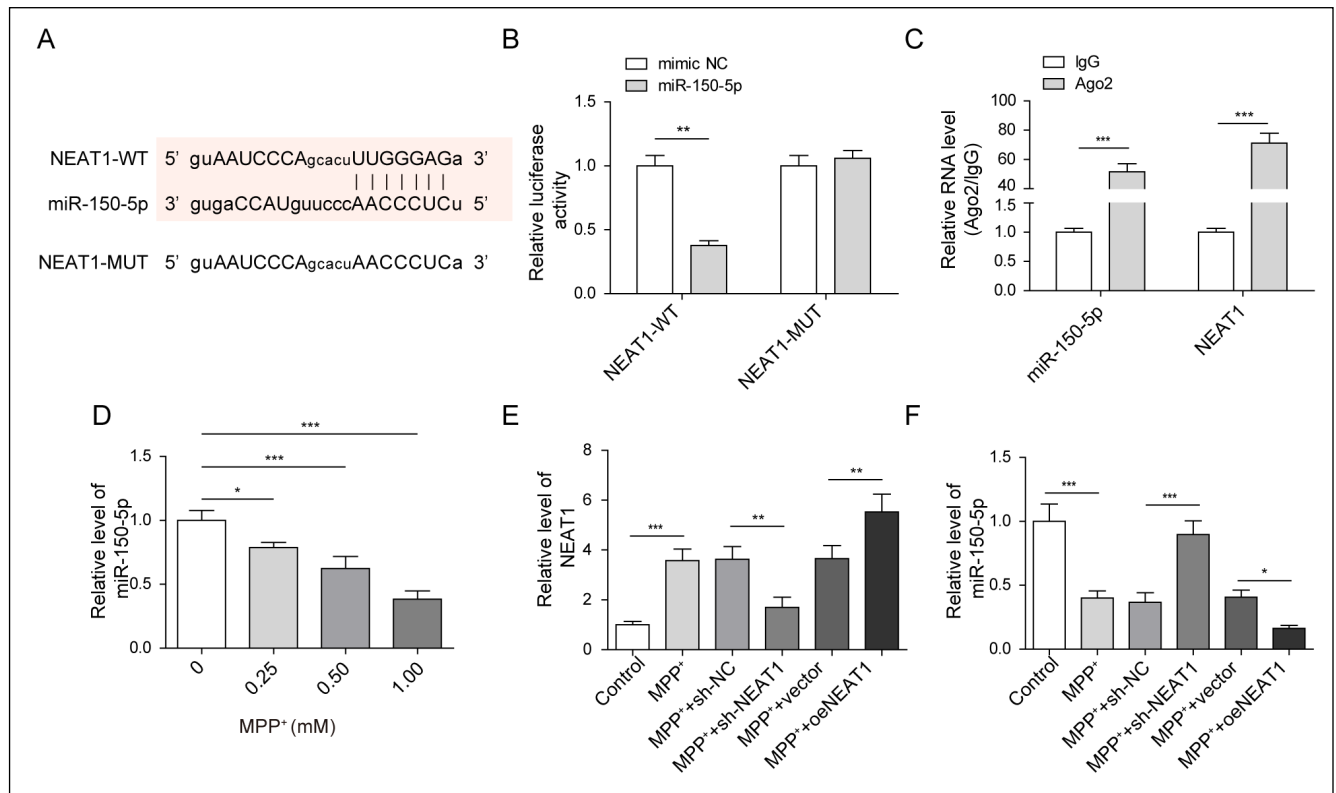


Fig. 2. miR-150-5p was a target of NEAT1. (A) Bioinformatics software starBase was applied to the binding site between miR-150-5p and NEAT1. (B) Dual luciferase reporter gene assay was performed to verify the binding relationship between miR-150-5p and NEAT1. (C) Interaction between miR-150-5p and NEAT1 was verified using RIP assay. (D) Cells were subjected to MPP⁺ for 24 h, then the level of miR-150-5p was assessed using qRT-PCR. (E-F) The levels of miR-150-5p and NEAT1 in cells following NEAT1 inhibition and NEAT1 overexpression were detected using qRT-PCR. The data were expressed as mean \pm SD. All data was obtained from at least three replicate experiments. * P <0.05, ** P <0.01, *** P <0.001.

miR-150-5p regulated SLC7A11 expression by directly binding to BAP1

Further research was performed to explore the downstream target of miR-150-5p. Bioinformatics software was conducted to predict that miR-150-5p had a binding site to BAP1 (Fig. 4A). Then dual luciferase reporter gene assay displayed that miR-150-5p directly bound to BAP1 mRNA 3'UTR (P <0.05; Fig. 4B). We found that both the mRNA and protein levels of BAP1 in SK-N-SH cells were gradually increased with the increase of MPP⁺ treatment concentration (P <0.05; Fig. 4C-E). Results of qRT-PCR and Western blot subsequently demonstrated that both mRNA level and protein level of BAP1 in PD cell model were significantly downregulated following transfection with miR-150-5p mimics, while the expressions were markedly increased following miR-150-5p inhibition (P <0.05; Fig. 4F-I). Finally, the protein level of SLC7A11 was upregulated, and both the mRNA and protein levels of BAP1 were reduced in PD cell model following miR-150-5p mimics transfection, while oe-BAP1 reversed the effect of miR-150-5p (P <0.05; Fig. 4J-L). In

summary, miR-150-5p regulated SLC7A11 expression via directly binding to BAP1.

NEAT1 enhanced MPP⁺-induced ferroptosis in SK-N-SH cells via regulating miR-150-5p/BAP1 axis

Next, in order to study whether NEAT1 acted as a role in regulating MPP⁺-induced ferroptosis in SK-N-SH cells by regulation of miR-150-5p/BAP1 axis, we knocked down NEAT1 in PD cell model, and set up a functional rescue experiment: MPP⁺ + sh-NEAT1 + miR-150-5p inhibitor group and MPP⁺ + sh-NEAT1 + oe-BAP1 group. Results of qRT-PCR displayed that the level of miR-150-5p was significantly elevated and BAP1 mRNA level was reduced in MPP⁺-treated SK-N-SH cells following NEAT1 knockdown. The level of miR-150-5p was markedly reduced and BAP1 mRNA level was obviously elevated in MPP⁺ + sh-NEAT1 + miR-150-5p inhibitor group compared to MPP⁺ + sh-NEAT1 group; miR-150-5p expression unchanged and BAP1 expression was obviously elevated in MPP⁺ + sh-NEAT1 +

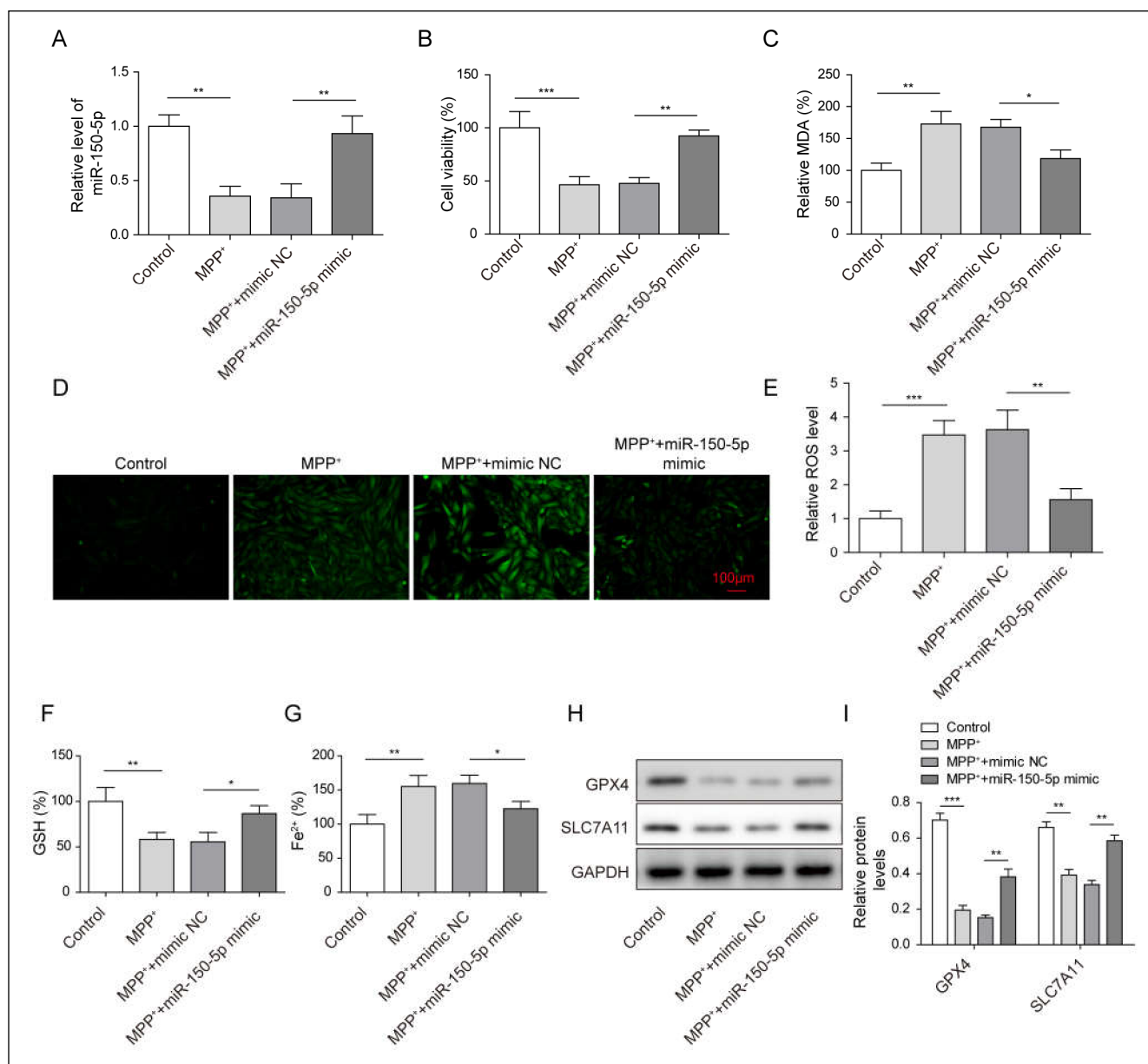


Fig. 3. miR-150-5p suppressed MPP⁺-induced ferroptosis in SK-N-SH cells. We overexpressed miR-150-5p in SK-N-SH cells and treated cells with 1 mM MPP⁺ for 24 h. (A) qRT-PCR was employed to assess the level of miR-150-5p. (B) MTT assay was performed to evaluate cell viability. (C) MDA level was evaluated using MDA assay kit. (D) Fluorescent probe DCFH-DA was employed to determine ROS level. (E) The quantitative analysis of ROS in (D). (F) GSH level was detected using GSH content detection kit. (G) Fe²⁺ level was detected by using iron assay kit. (H) Western blot was employed to determine the protein levels of GPX4 and SLC7A11. (I) The quantitative analysis of proteins in (H). The data were expressed as mean \pm SD. All data was obtained from at least three replicate experiments. * P <0.05, ** P <0.01, *** P <0.001.

oe-BAP1 group compared to MPP⁺ + sh-NEAT1 group (P <0.05; Fig. 5A). As shown in Fig. 5B, cell viability of MPP⁺-treated SK-N-SH cells was markedly elevated by NEAT1 knockdown, while it was abolished following miR-150-5p inhibition or BAP1 overexpression. What's more, levels of MDA, ROS and Fe²⁺ was significantly reduced, and level of GSH was markedly elevated in MPP⁺-treated SK-N-SH cells after sh-NEAT1 transfection, while miR-150-5p inhibition or BAP1 over-

expression reversed the effect of sh-NEAT1 (P <0.05; Fig. 5C-5G). Besides, co-transfection with sh-NEAT1 and miR-150-5p inhibitor or co-transfection with sh-NEAT1 and oe-BAP1 resulted in increased protein level of BAP1 and decreased protein levels of GPX4 and SLC7A11 in MPP⁺-treated SK-N-SH cells compared with MPP⁺ + sh-NEAT1 group (P <0.05; Fig. 5H-I). In conclusion, NEAT1 enhanced ferroptosis in PD *in vitro* model via regulation of miR-150-5p/BAP1 axis.

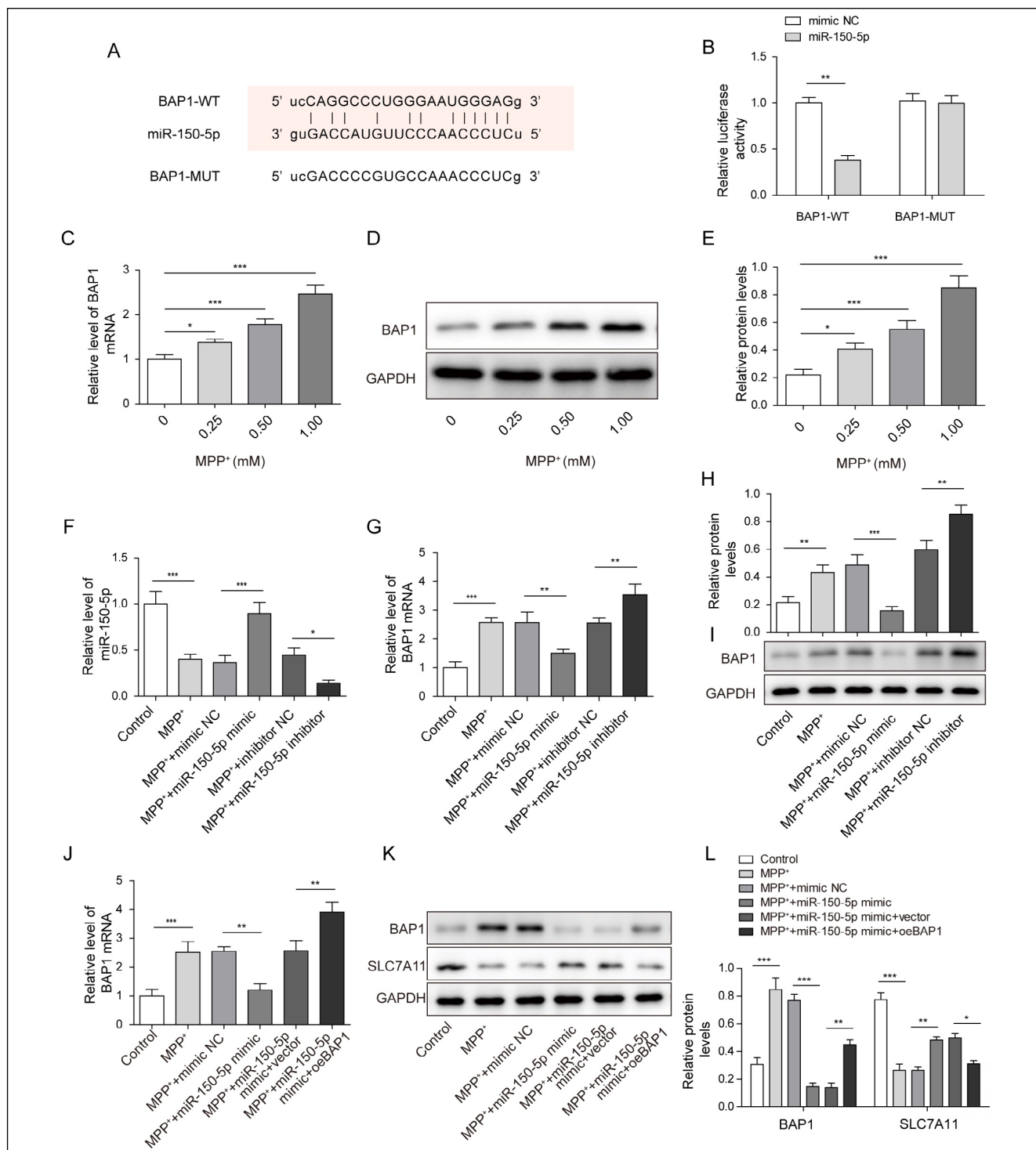


Fig. 4. miR-150-5p regulated SLC7A11 expression by directly binding to BAP1. (A) Bioinformatics software starBase was applied to the binding site between miR-150-5p and BAP1. (B) Dual luciferase reporter gene assay was performed to verify the binding relationship between miR-150-5p and BAP1. (C-E) SK-N-SH cells were subjected to MPP⁺ for 24 h, then the mRNA and protein levels of BAP1 expression were assessed by qRT-PCR and western blot, respectively. (F) miR-150-5p expression in cells following miR-150-5p inhibition and miR-150-5p overexpression was detected using qRT-PCR. (G-I) The mRNA and protein levels of BAP1 in SK-N-SH cells following miR-150-5p inhibition and miR-150-5p overexpression were detected using qRT-PCR and western blot, respectively. Cells were divided into six groups: control, MPP⁺, MPP⁺ + mimics NC, MPP⁺ + miR-150-5p mimics, MPP⁺ + miR-150-5p mimics + vector and MPP⁺ + miR-150-5p mimics + BAP1. (J) The mRNA level of BAP1 was assessed using qRT-PCR. (K-L) The protein levels of BAP1 and SLC7A11 were evaluated using western blot. The data were expressed as mean \pm SD. All data was obtained from at least three replicate experiments. * P <0.05, ** P <0.01, *** P <0.001.

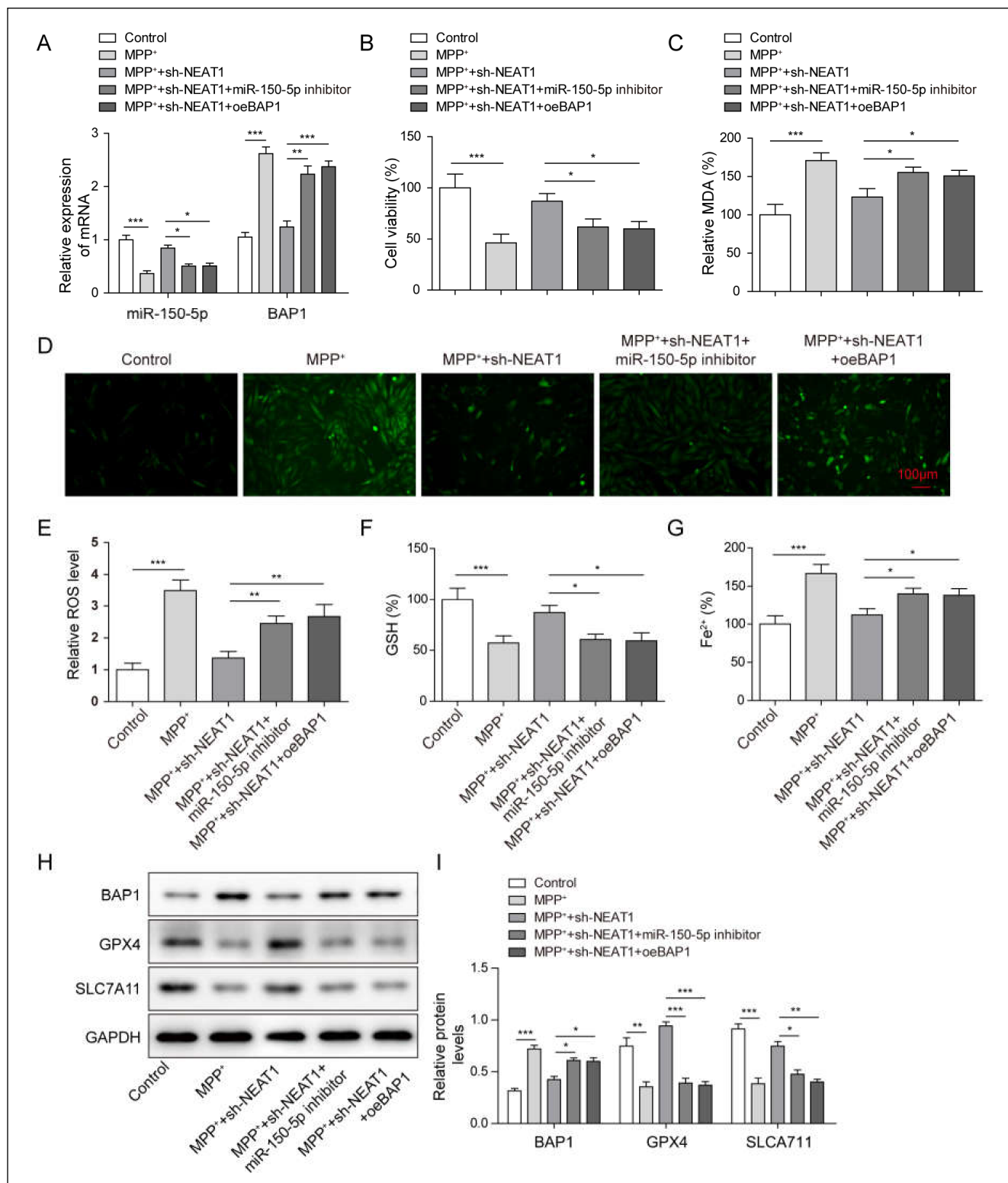


Fig. 5. NEAT1 enhanced MPP⁺-induced ferroptosis in SK-N-SH cells *via* regulating miR-150-5p/BAP1 axis. SK-N-SH cells were divided into 5 groups: control, MPP⁺, MPP⁺ + sh-NEAT1, MPP⁺ + sh-NEAT1 + miR-150-5p inhibitor and MPP⁺ + sh-NEAT1 + oeBAP1 group. (A) The RNA levels of miR-150-5p and BAP1 were assessed by qRT-PCR. (B) Cell viability was determined using MTT assay. (C) MDA level was evaluated using MDA assay kit. (D) Fluorescent probe DCFH-DA was employed to determine ROS level. (E) The quantitative analysis of ROS in (D). (F) GSH level was detected using GSH content detection kit. (G) Fe²⁺ level was detected by using iron assay kit. (H-I) The protein levels of BAP1, GPX4 and SLC7A11 were assessed by western blot. The data were expressed as mean \pm SD. All data was obtained from at least three replicate experiments. * P <0.05, ** P <0.01, *** P <0.001.

DISCUSSION

PD is one of the most common neurodegenerative diseases. The number of PD patients is increasing at a terrible rate, and it is estimated that there will be 9 million PD patients in 2030 (Dorsey et al., 2007; Wirdeltd et al., 2011). Due to the slow progress of PD and the lack of effective treatment for PD, PD has caused a huge burden on society. Ferroptosis is a new cell death pathway that has not been studied in PD until recently, but there are strong reasons to believe that this pathway is involved in PD progression (Guiney et al., 2017). Therefore, inhibition of ferroptosis might have broad-acting benefits in PD where there are currently no disease modifying therapies. However, little is known about the molecular mechanism of ferroptosis in PD.

Recently, much evidence has suggested that lncRNAs are closely related to PD progression (Wang et al., 2018). For example, lncRNA SNHG1 enhanced MPP⁺-induced neurotoxicity *via* regulation of AKT pathway through sponging miR-153-3p (Zhao et al., 2020). NEAT1 was previously reported to facilitate MPP⁺-induced cell autophagy in PD *via* interacting with PINK1 (Yan et al., 2018). Here we found that the RNA level of NEAT1 in SK-N-SH cells was elevated with the dose of MPP⁺ treatment, which was totally consistent with a previous study (Xie et al., 2019). Additionally, NEAT1 knockdown dramatically suppressed ferroptosis in MPP⁺-treated SK-N-SH cells.

The ceRNA hypothesis has been widely recognized as a novel regulatory mechanism pertinent to lncRNAs (Denzler et al., 2014). Therefore, we further clarified the possible regulatory axis of NEAT1 in PD. By using the bioinformatics software starBase, we found that NEAT1 had a binding site to miR-150-5p. MiR-150-5p was identified as a diagnostic biomarker of PD and was involved in the inflammatory pathogenesis of PD (Li et al., 2020). Here, dual luciferase reporter gene assay and RIP assay displayed that NEAT1 directly bound to miR-150-5p. The RNA level of miR-150-5p in SK-N-SH cells was reduced by MPP⁺ treatment. Additionally, miR-150-5p expression was increased by NEAT1 silence, while was reduced following NEAT1 overexpression in MPP⁺-treated SK-N-SH cells. Above all, we came to the conclusion that NEAT1 acted as an endogenous sponge to suppress miR-150-5p expression. Having identified the direct relationship between NEAT1 and miR-150-5p, we assumed that NEAT1 might regulate ferroptosis in PD *via* its negative regulatory effects on miR-150-5p. Function assays displayed that miR-150-5p overexpression obviously inhibited ferroptosis in PD *in vitro* model. Moreover, miR-150-5p inhibition mitigated the anti-ferroptosis effect med-

itated by sh-NEAT1. In summary, NEAT1 mediated the pro-ferroptosis effect in PD *via* directly interacting with miR-150-5p.

Subsequent starBase prediction and dual luciferase reporter gene assay verified that BAP1 was the target of miR-150-5p. BAP1 was a type of ubiquitin C-terminal hydrolases (Louie et al., 2020). Previous study demonstrated that PD displayed a significant imbalance in the ubiquitination/deubiquitination processes (Sharma et al., 2020). More importantly, BAP1 was reported as a genetic risk factor for PD (Keo et al., 2020). In the present study, both the mRNA and protein levels of BAP1 in SK-N-SH cells were elevated by MPP⁺ treatment. Additionally, we found that miR-150-5p could negatively regulate BAP1 expression. By analyzing the functions in depth, it's evident that BAP1 overexpression mitigated the anti-ferroptosis effect mediated by sh-NEAT1 or miR-150-5p mimics. Therefore, we came to the conclusion that NEAT1 acted as the ceRNA to sponge miR-150-5p in SK-N-SH cells to positively regulate BAP1 expression. As previously described, BAP1 enhanced ferroptosis *via* reducing H2A ubiquitination in the promoter region of SLC7A11 (Zhang et al., 2019). Here, we proved that NEAT1 activated BAP1/SLC7A11 axis in PD by targeting miR-150-5p.

CONCLUSION

In conclusion, our research proved that lncRNA NEAT1 promoted MPP⁺-induced ferroptosis *via* regulating miR-150-5p/BAP1 axis in SK-N-SH cells. Considering that there might be multiple targets or pathways for NEAT1, it's essential to deeply explore the other possible functional mechanisms and interactions.

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