

# LncRNA NNT-AS1 is a major mediator of cisplatin chemoresistance in non-small cell lung cancer through MAPK/Slug pathway

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**Abstract.** – **OBJECTIVE:** To investigate the role of long non-coding ribonucleic acid (lncRNA) nicotinamide nucleotide transhydrogenase-antisense RNA1 (NNT-AS1) in cisplatin (DDP) resistance in non-small cell lung cancer (NSCLC) and its molecular mechanism.

**PATIENTS AND METHODS:** Fluorescence quantitative Real-time polymerase chain reaction (qRT-PCR) was used to detect the expression levels of lncRNA NNT-AS1 in NSCLC cell lines (A549 and SPCA-1) and DDP-resistant cell lines (A549/DDP and SPCA-1/DDP). Corresponding plasmids of si-NNT-AS1 and si-NC were conducted. Then, methyl thiazolyl tetrazolium (MTT) assay was applied to detect the changes in half inhibition concentration (IC<sub>50</sub>) values of DDP in A549/DDP and SPCA-1/DDP cells after interference in lncRNA NNT-AS1 expression. Clone formation assay and flow cytometry were employed to detect the changes in proliferation, cycle and apoptosis of A549/DDP and SPCA-1/DDP cells caused by si-NNT-AS1. Protein expressions of molecular markers in mitogen-activated protein kinase (MAPK)/Slug signaling pathway after interference in lncRNA NNT-AS1 expression was detected by Western blotting. The differential expressions of lncRNA NNT-AS1 in 10 pairs of drug-resistant and non-resistant tissues were detected by qRT-PCR.

**RESULTS:** QRT-PCR results showed that the expression of lncRNA NNT-AS1 in drug-resistant cells was higher than that in parental cells. The IC<sub>50</sub> value of DDP in drug-resistant cells was increased by interfering with the expression of lncRNA NNT-AS1. Clone formation assay and flow cytometry revealed that interfering in the expression of lncRNA NNT-AS1 could inhibit the proliferation, promote the apoptosis and cell cycle arrest of drug-resistant cells. Western blotting assay found that after interfering the expression of lncRNA NNT-AS1, the expressions of molecular markers in MAPK/Slug signaling pathway were downregulated. qRT-PCR assay indicated that there were 9 pairs of drug-resistant tissues with up-regulated lncRNA NNT-AS1 expression in a total of 10 pairs of drug-resistant tissues.

**CONCLUSIONS:** lncRNA NNT-AS1 is highly expressed in drug-resistant NSCLC tissues and cells, promoting the DDP resistance of NSCLC cells through the MAPK/Slug signaling pathway.

*Key Words:*

NSCLC, lncRNA NNT-AS1, Cisplatin resistance, MAPK/Slug signaling pathway.

## Introduction

Non-small cell lung cancer (NSCLC) manifests as faster growth and division, as well as earlier diffusion and metastasis. Most NSCLC patients are already in the advanced stage in the first diagnosis. Hence, the majority of NSCLC patients lost the opportunity for radical surgery. Currently, non-surgical treatment has become an important part of the treatment for NSCLC<sup>1</sup>. Non-surgical treatment mainly includes targeted therapy and chemotherapy. For NSCLC patients with positive mutation of epidermal growth factor receptor (EGFR), EGFR-tyrosine kinase inhibitor (TKI) targeted therapy is preferred, such as the use of gefitinib. However, EGFR-TKI has a limited effect on NSCLC patients with negative EGFR mutation. Therefore, chemotherapy is the best option for these patients<sup>2,3</sup>. Among chemotherapy schemes, the

combination of platinum-based chemotherapy is widely used. For example, the effect of cisplatin (DDP) has been clinically proven. Nonetheless, DDP resistance of NSCLC patients should not be ignored, and further exploration of the mechanism of DDP resistance in NSCLC is of great significance<sup>4</sup>.

Common mechanisms of DDP resistance in NSCLC include chromosome abnormality, reduced intracellular drug accumulation, enhanced deoxyribonucleic acid (DNA) damage repair function, increased cell detoxification and altered tumor microenvironment<sup>5,6</sup>. Recent studies have reported that more and more long non-coding ribonucleic acids (lncRNAs) affect the occurrence and development of tumors through regulating expressions of target genes. lncRNA is a class of non-coding RNA with a transcript length of over 200 nt, which was previously considered as a byproduct of the transcription of RNA polymerase II without specific biological functions<sup>7</sup>. Investigations have indicated that lncRNA can participate in DDP resistance in NSCLC as an important regulatory factor. Wang et al<sup>8</sup> found that lncRNA maternally expressed gene 3 (MEG3) can regulate the expression of sex-determining region Y-box 7 (Sox7) through the adsorption of microRNA (miRNA)-21-5P, thus increasing the sensitivity of NSCLC cells to DDP. Shi et al<sup>9</sup> confirmed by *in vitro* and *in vivo* experiments that interfering the expression of lncRNA retinoid-related orphan receptor (ROR) increases the sensitivity of NSCLC cells to DDP by inhibiting the phosphatidylinositol 3-kinase (PI3K)/serine/threonine kinase (AKT)/mammalian target of rapamycin (mTOR) signaling pathway.

lncRNA nicotinamide nucleotide transhydrogenase-antisense RNA1 (NNT-AS1) is located in the chromosome 5p12 region, with a full length of 3304 bp. At first, Wang et al<sup>10</sup> found that NNT-AS1 expression is up-regulated in colorectal cancer. It is proved through *in vitro* and *in vivo* experiments that lncRNA NNT-AS1 can promote the proliferation and metastasis of colon cancer cells. However, the relationship between lncRNA NNT-AS1 and DDP resistance in NSCLC has not been reported. We firstly investigated the differential expressions of lncRNA NNT-AS1 in NSCLC cells and drug resistant cells. Functional experiments further confirmed that highly expressed lncRNA NNT-AS1 was able to promote DDP resistance in NSCLC by regulating mitogen-activated protein kinase (MAPK)/Slug signaling pathway.

## Patients and Methods

### Patients

Lung cancer tissue samples were collected from inoperable advanced NSCLC patients with at least one measurable lesion in Respiratory Department and Oncology Department of our hospital. Samples were resected via percutaneous lung thick needle aspiration or bronchofiberscope lung biopsy, and placed in liquid nitrogen for preservation. All patients were treated with DDP-based first-line chemotherapy for 4-6 weeks. Therapeutic efficacy was evaluated every 2 weeks. Next, 10 cases of partial response (PR) and 10 cases of progressive disease (PD) were selected. This study was approved by the Ethics Committee of Shanghai Pulmonary Hospital. Patients signed the informed consent. The curative effect was evaluated according to the recent Response Evaluation Criteria in Solid Tumors stipulated by the World Health Organization, which was divided into complete response (CR), PR (partial response), stable disease (SD) and PD (progressive disease).

### Cell Culture

Human lung adenocarcinoma A549 and SPCA-1 cell lines were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Drug-resistant cells A549/DDP and SPCA-1/DDP were induced and preserved in our laboratory. A549 and drug-resistant cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI)-1640 (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). SPCA-1 and drug-resistant cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum. After 3 times of normal passages of drug-resistant cells, medium was added with 1 µg/mL DDP (purchased from our hospital) to maintain its drug resistance. Culture environment was 5% CO<sub>2</sub> and 37°C.

### Fluorescence Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from A549, SPCA-1, A549/DDP and SPCA-1/DDP cells and NSCLC tissues using TRIzol reagents (Invitrogen Corporation, Carlsbad, CA, USA). RNA was reversely transcribed into complementary DNA (cDNA) based on the instructions stated on AMV reverse transcription kits (TaKaRa Biotechnology Co., Ltd., Dalian, China). Fluorescence qRT-PCR was

performed using 2×SYBR Green PCR Master Mix (TaKaRa Biotechnology Co., Ltd., Dalian, China) with cDNA as a template. PCR conditions were as follows: 95°C for 10 min, and a total of 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. The expression level of lncRNA NNT-AS1 was expressed as  $2^{-\Delta\Delta Ct}$  value, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

### **Primers and siRNAs**

Primers required for qRT-PCR were: lncRNA NNT-AS1, 5'-TCTCCTAAGTCGAGGACTAGC-3' (forward) and 5'-AGGCACTCACTAGCATCACGCT-3' (reverse); GAPDH, 5'-CCCACTTGAAGGGTGGAGCCAA-3' (forward) and 5'-TGGCATGGACTGTGGTCATGA-3' (reverse). Interference sequences: si-NNT-AS1 #1, sense 5'-CGACAGUGCUIGUGAACUUTT-3', and antisense 5'-CCAUUCAGUACGAGAGUCCTT-3'; si-NNT-AS1 #2, sense 5'-ACUGACGUCGACCAUGUGATT-3', and antisense 5'-ACUGUCAUCAGUAUCCCAT-3'. si-NNT-AS1 #3, sense 5'-CCAAGGCUGGAACUGAUATT-3', and antisense 5'-GCAACGAAUCCGGAUCGCATT-3'. Primers and interference sequences were synthesized by TaKaRa Biotechnology (Dalian, China).

### **Detection of Half Inhibition Concentration (IC<sub>50</sub>) Value Through Methyl Thiazolyl Tetrazolium (MTT) Assay**

Cells in experimental group and control group were seeded into a 96-well plate at 4000/well, with 200  $\mu$ L in each well. Upon 12 h of cell attachment and cell synchronization, original medium was discarded. 5 replicate wells were set for each sample. After the cell attachment was completed, RPMI-1640 medium or DMEM containing different doses of DDP (1, 2, 4, 8, 16, 32 and 64  $\mu$ g/mL) was added. After that, cells in control group were added with DDP-free RPMI-1640 or DDP medium. After 48 h of culture, 10  $\mu$ L (5 mg/mL) MTT (Sigma-Aldrich, St. Louis, MO, USA) were added, a microplate reader was used to read the optical density (OD) value at a wavelength of 490 nm. The average OD value of five replicate wells was taken. Linear regression was used to calculate IC<sub>50</sub>.

### **Clone Formation Assay**

Cells in experimental and treatment groups were seeded in a 6-well plate at 1000/well. DDP was added in the 6-well plate at concentrations of

0.0  $\mu$ g/mL, 1.0  $\mu$ g/mL and 2.0  $\mu$ g/mL, respectively. Medium was replaced every 3 days. After 14 days, cells were subjected to formaldehyde fixation, crystal violet staining, photographing and counting.

### **Flow Cytometry**

Drug-resistant cells were transfected with si-NNT-AS1 or control sequences, and incubated with DDP at concentrations of 0  $\mu$ g/mL, 1  $\mu$ g/mL and 2  $\mu$ g/mL, respectively. After 48 h of culture, cells were harvested, re-suspended in precooled 75% alcohol and fixed at -20°C overnight. Then, the intracellular DNA content was detected according to the instructions of propidium iodide (PI) staining by flow cytometry (Roche Applied Science, Basel, Switzerland). The percentage in each phase was calculated by software. For cell apoptosis detection, the specific procedures were as the same as the above. After being treated for the required time in each group, cells were collected and re-suspended using the buffer in Annexin V-fluorescein isothiocyanate (FITC) kits. After 5  $\mu$ L AnnexinV-FITC and 10  $\mu$ L PI staining solution were added successively, the mixture was mixed evenly and incubated at room temperature in a dark place for 15 min, followed by detection on equipment.

### **Western Blotting**

Total protein was extracted from NSCLC cells and drug-resistant cells, electrophoresed on a 10% polyacrylamide gel, and transferred onto a membrane. After membrane transfer, 5% skimmed milk powder was used for blocking at room temperature for 2 h. Then, cells were incubated with mouse anti-human (MAPK1, p-MAPK1, Slug, GAPDH) antibody at 1:1500 and 4°C for 12 h, followed by three times of washing with phosphate-buffered solution and Tween-20 (PBST-20) to remove unbound primary antibody. After that, secondary antibody was diluted at 1:1000, added onto the membrane and incubated at room temperature for 1-2 h. Color development was performed with electrochemiluminescence (ECL) kits (Thermo Fisher Scientific, Waltham, MA, USA).

### **Statistical Analysis**

Statistical Product and Service Solutions (SPSS) 13.0 software (Chicago, IL, USA) were adopted for statistical analysis and data processing. The t-test was used for comparisons between two groups. One-way analysis of variance was employed for further comparisons among groups.

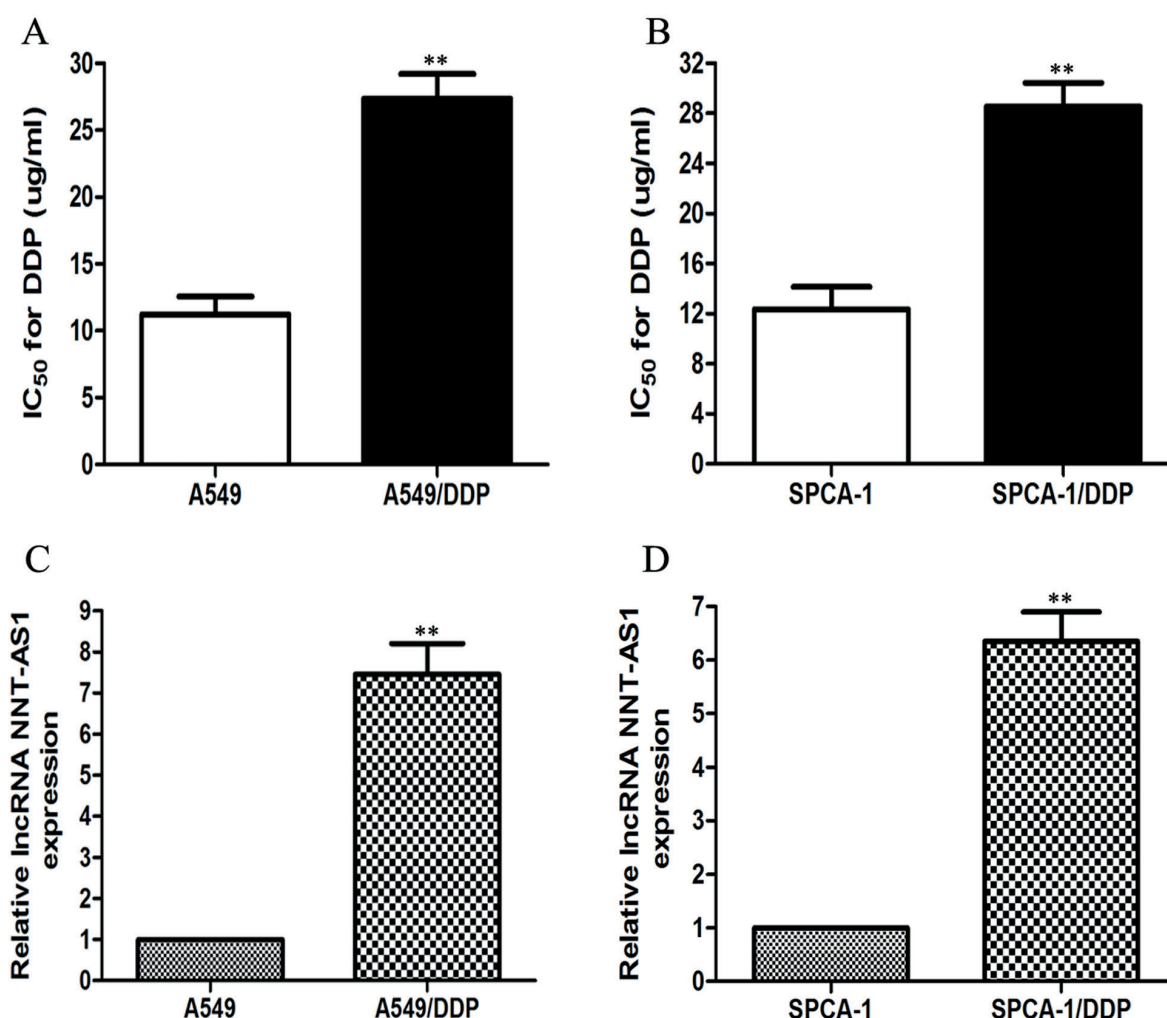
Least significant difference (LSD) test was applied for further pairwise comparisons among groups.  $p < 0.05$  suggested that the difference was statistically significant.

## Results

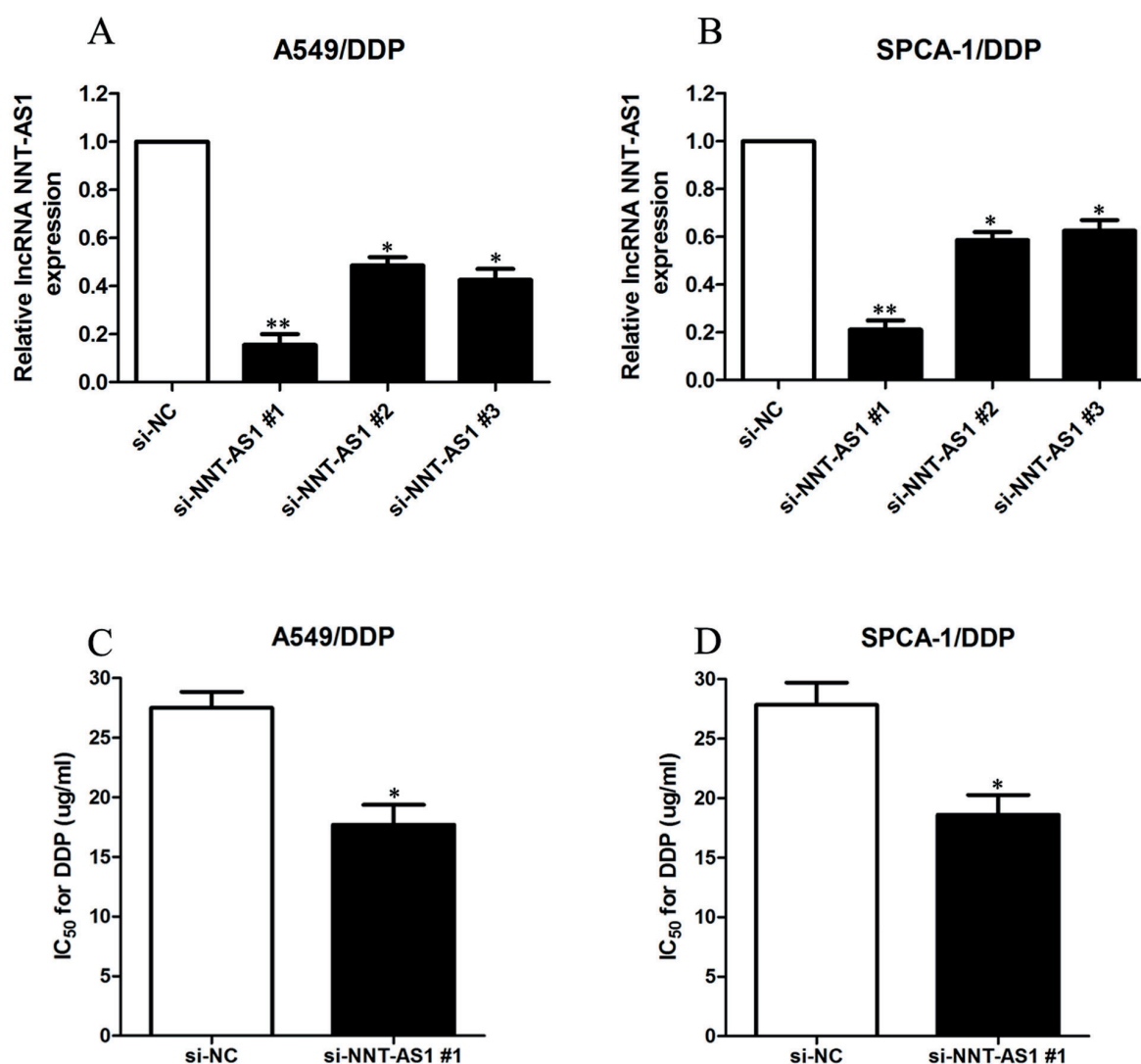
### *LncRNA NNT-AS1 was Highly Expressed in Drug-Resistant NSCLC Cells*

A549 and SPCA-1 cells were treated with different concentrations of DDP to induce drug-resistant cell lines. Then, MTT assay was performed to detect  $IC_{50}$  values in NSCLC cells and drug-resistant cells. The data showed that after treatment

with DDP for 24 h, the  $IC_{50}$  values of DDP in A549 and A549/DDP cells were  $(11.23 \pm 1.33) \mu\text{g/mL}$  and  $(27.37 \pm 1.83) \mu\text{g/mL}$ , respectively (Figure 1A). In SPCA1 and SPCA-1/DDP cells, the  $IC_{50}$  values were  $(12.37 \pm 1.89) \mu\text{g/mL}$  and  $(28.54 \pm 1.90) \mu\text{g/mL}$ , respectively (Figure 1B). Subsequently, the relative expression levels of lncRNA NNT-AS1 in A549/DDP cells and SPCA-1/DDP cells were measured via qRT-PCR. The results showed that compared with those in A549 and SPCA-1 cells, the relative expression levels of lncRNA NNT-AS1 were elevated in A549/DDP cells and SPCA-1/DDP cells (Figure 1C and 1D), suggesting that lncRNA NNT-AS1 is related to DDP resistance in NSCLC.



**Figure 1.** LncRNA NNT-AS1 expressions in drug resistant cells. **A**,  $IC_{50}$  values of DDP in A549 and A549/DDP cells detected by MTT. **B**,  $IC_{50}$  values of DDP in SPCA-1 and SPCA-1/DDP cells detected by MTT. **C**, and **D**, Relative expression levels of lncRNA NNT-AS1 in NSCLC resistant cells and parental cells measured via qRT-PCR, with GAPDH as the internal control. (\*\* $p < 0.01$ , \* $p < 0.05$ ).



**Figure 2.** Relationship between lncRNA NNT-AS1 expression and DDP resistance of NSCLC cells. **A**, and **B**, Efficiency detected after drug resistant NSCLC cells are transfected with si-NNT-AS1 and si-NC for 48 h, with GAPDH as the internal reference. **C**, IC<sub>50</sub> value of DDP in A549/DDP/si-NNT-AS1 or A549/DDP/si-NC cells detected by MTT assay. **D**, IC<sub>50</sub> value of DDP in SPCA-1/DDP/si-NNT-AS1 or SPCA-1/DDP/si-NC cells. (\*\* $p < 0.01$ , \* $p < 0.05$ ).

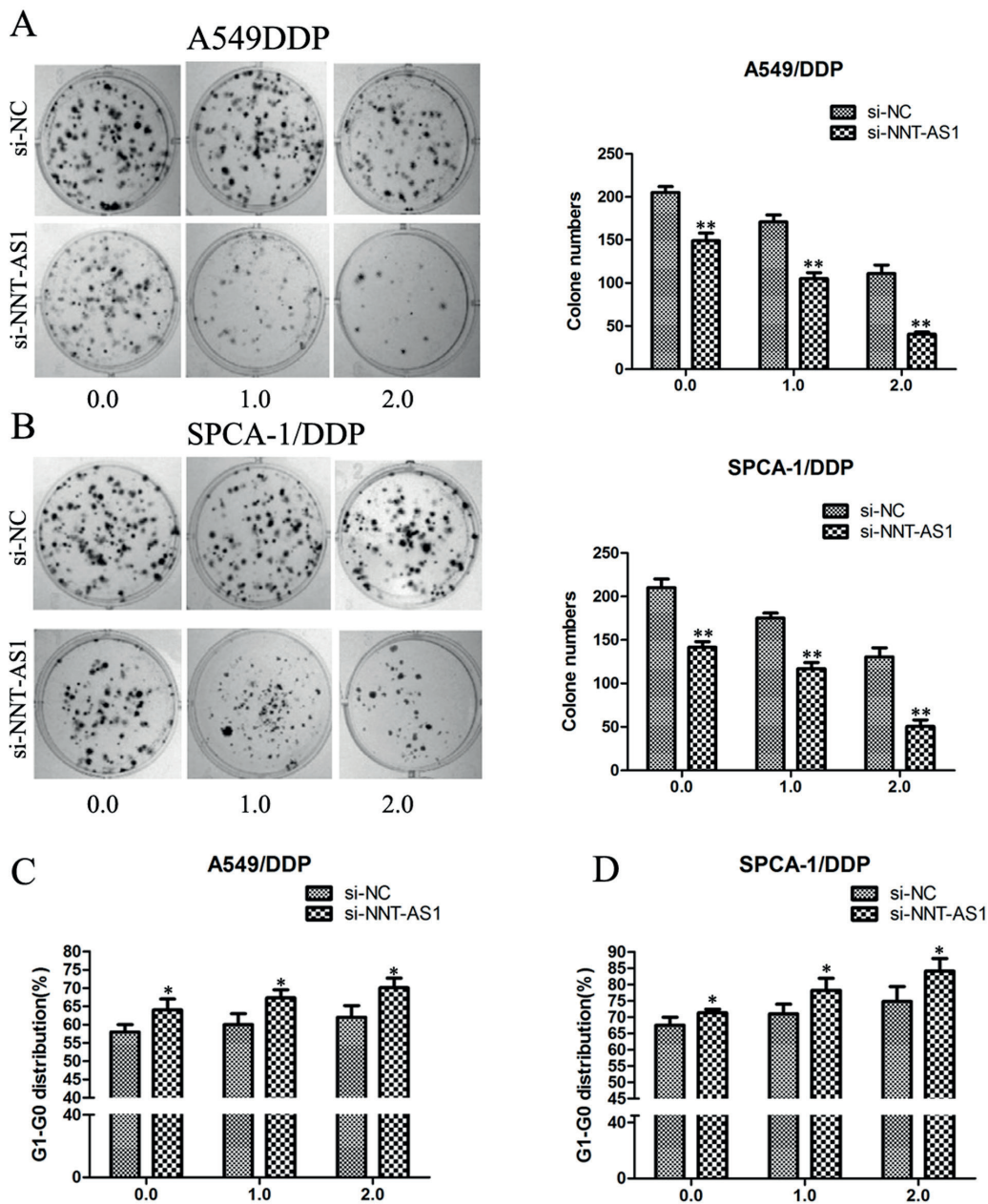
### Effect of si-NNT-AS1 on DDP Sensitivity of Drug Resistant NSCLC Cells

In order to investigate the role of lncRNA NNT-AS1 in the formation of drug resistance, lncRNA NNT-AS1 interference sequences were designed, synthesized and transfected into drug resistant cells. The interference efficiency was tested by qRT-PCR (Figure 2A and 2B). Next, MTT assay was used to detect the IC<sub>50</sub> of DDP in A549/DDP and SPCA-1/DDP cells transfected with si-NC or si-NNT-AS1. The results indicated that the IC<sub>50</sub> values of DDP in A549/DDP and SPCA-1/DDP cells after knockdown of lncRNA NNT-AS1 expression were (27.51±1.34) and (27.85±1.87) μg/

mL, respectively. Besides, IC<sub>50</sub> values in control group were (17.71±1.67) and (18.57±1.62) μg/mL, respectively (Figure 2C and 2D).

### Impact of si-NNT-AS1 on Proliferation of Drug Resistant NSCLC Cells

The results of clone formation assay revealed that after interference in the expression of lncRNA NNT-AS1, the proliferation ability of drug resistant cells was significantly inhibited, and the number of colony was decreased with the increase of DDP concentration (Figure 3A and 3B). Flow cytometry was used to detect the role of si-NNT-AS1 in cell cycle distribution,



**Figure 3.** Effect of si-NNT-AS1 combined with DDP on the proliferation ability of drug resistant NSCLC cells. **A**, and **B**, Proliferation ability of drug resistant NSCLC cells detected via clone formation assay after cells were transfected with si-NNT-AS1 or si-NC and treated with different concentrations of DDP (0, 1.0 and 2.0 μg/mL). **C**, and **D**, Cell cycle distribution of drug resistant NSCLC cells detected by flow cytometry after cells were transfected with si-NNT-AS1 or si-NC and treated with different concentrations of DDP (0, 1.0 and 2.0 μg/mL). (\*\* $p < 0.01$ , \* $p < 0.05$ ).

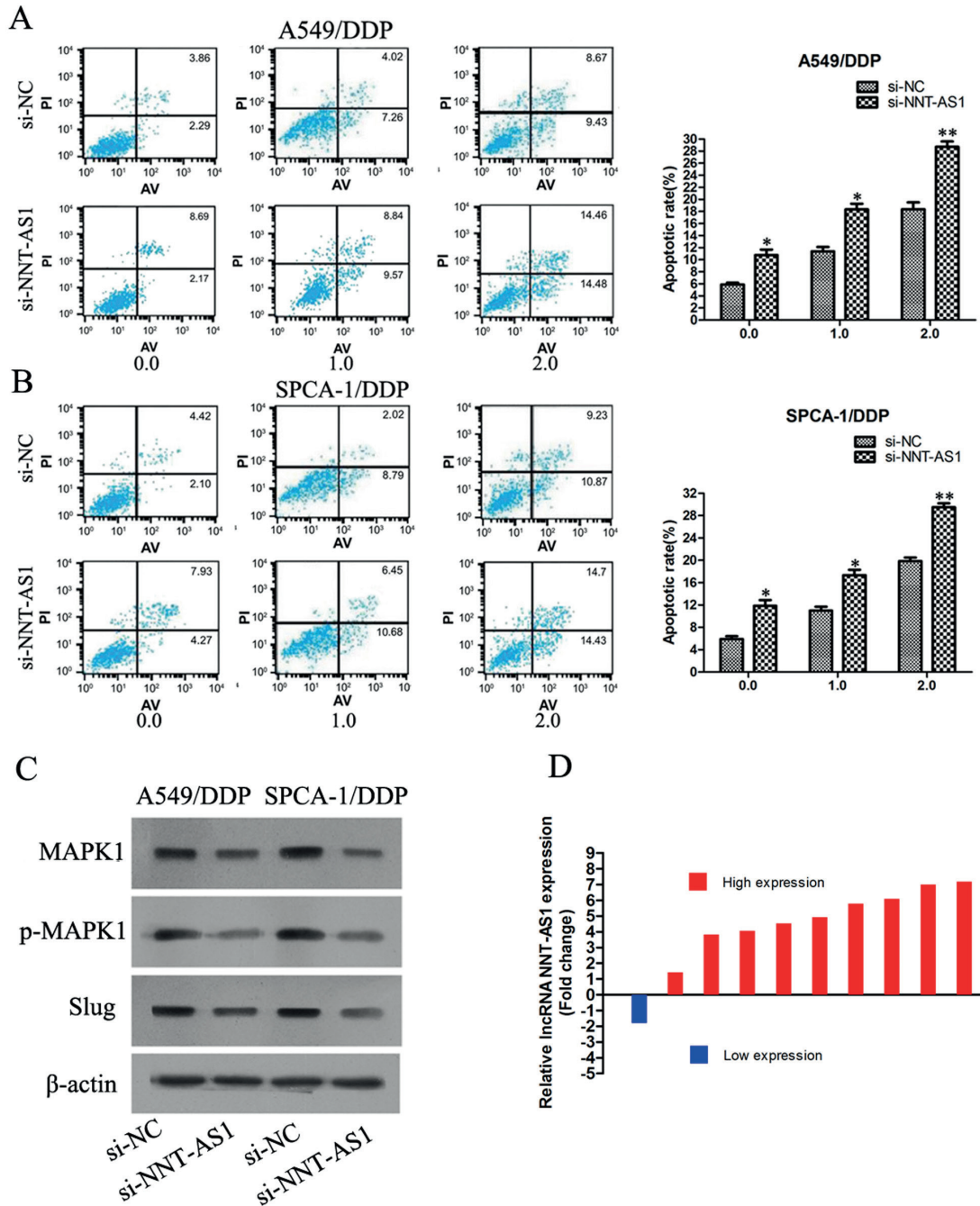
and the results showed that compared with that in control group, cell cycle of drug-resistant cells in si-NNT-AS1 group was arrested in G1/G0 phase. The above experiments confirmed that interfering lncRNA NNT-AS1 expression can significantly reduce the proliferation ability of DDP-treated A549/DDP and SPCA-1/DDP cells (Figure 3C and 3D).

### ***Influence of si-NNT-AS1 on Apoptosis of Drug-Resistant NSCLC Cells***

Flow cytometry was employed to detect the effect of lncRNA NNT-AS1 on the apoptosis of drug-resistant cells. The results indicated that the apoptosis rate of cells in experimental group was significantly higher than that in control group after DDP treatment (Figure 4A). Besides, the apopto-

sis rate in experimental group was increased in a dose-dependent manner (Figure 4B). Through literature review, it was found that the MAPK/Slug signaling pathway was closely related to the oc-

currence and development of tumors. It has been reported<sup>11</sup> that lncRNA PTENP1 can promote the proliferation and metastasis of breast cancer cells through activation of MAPK signaling pa-



**Figure 4.** LncRNA NNT-AS1 regulates the MAPK/Slug pathway. *A*, and *B*, Changes in apoptosis of drug resistant NSCLC cells detected via flow cytometry after cells were transfected with si-NNT-AS1 or si-NC and treated with different concentrations of DDP (0, 1.0 and 2.0 µg/mL). *C*, Protein expressions of MAPK/Slug pathway molecular markers were detected by Western blotting assay after cells were transfected with si-NNT-AS1 or si-NC. *D*, Relative expression levels of lncRNA NNT-AS1 in 10 cases of DDP resistant and non-resistant NSCLC tissues measured by qRT-PCR, with GAPDH as the internal reference. (\*\* $p < 0.01$ , \* $p < 0.05$ ).

thway. In hepatocellular carcinoma, lncRNA cancer susceptibility candidate 2 (CASC2) regulates the changes in biological behaviors of liver cells and hepatocellular carcinoma through the MAPK signaling pathway<sup>12</sup>. In our work, lncRNA NNT-AS1 could regulate expressions of molecular markers in MAPK/Slug signaling pathway (Figure 4C). Lastly, qRT-PCR was used to detect the relative expression level of lncRNA NNT-AS1 in 10 cases of drug-resistant NSCLC tissues. The results showed that the relative expression level of lncRNA NNT-AS1 was up-regulated compared with that in non-resistant tissues (Figure 4D).

## Discussion

For the majority of patients with advanced NSCLC, platinum-based chemotherapy regimens are still the first-line therapeutic schedules for advanced NSCLC, especially DDP<sup>13</sup>. However, DDP-based chemotherapy regimens are not always effective in the treatment of advanced NSCLC. A study found that patients undergoing preoperative DDP-based chemotherapy regimen present a survival hazard ratio (HR) of 0.87 and a reduction of 13% in mortality risk. However, the 5-year survival rate is increased by only about 5%<sup>14</sup>, indicating that acquired DDP resistance of lung cancer cells is an important cause hindering the long-term survival of patients.

DDP was first discovered by Michele Peyrone in 1844 (*Ann Chemie Pharm* 1844; 51: 1-29). DDP exerts its anticancer mechanism by inducing DNA damage and interfering with the process of DNA damage repair. It has a broad spectrum of anti-tumor effects and is clinically used in the treatment of tumors, such as lung cancer, ovarian cancer and gastric cancer<sup>15</sup>. Recent studies have shown that abnormal expressions of lncRNAs are closely correlated with DDP resistance. Liu et al<sup>16</sup> found that lncRNA homeobox transcript antisense intergenic RNA (HOTAIR) expression in DDP resistant A549/DDP cell line is significantly higher than that in A549 cell line. lncRNA HOTAIR knockdown can reverse the sensitivity of drug resistant cells to DDP by up-regulating p21 to arrest cells in the G0/G1 phase. Xia et al<sup>17</sup> discovered that lncRNA MEG3 is also involved in DDP resistance in NSCLC, suggesting that down-regulated MEG3 increases the DDP resistance of NSCLC cells via Wnt/ $\beta$ -catenin signaling pathway. This research group first discovered that lncRNA NNT-AS1 participates in the DDP

resistance of NSCLC cells, and preliminarily studied its underlying molecular mechanism. MAPK is a group of serine/threonine kinases that can be activated by various signals<sup>18</sup>. MAPK signal transduction pathway exists in most cells with multiple levels of cellular regulation. MAPK pathway participates in the regulations of cell proliferation, differentiation, transformation, apoptosis and other biological reactions<sup>19-21</sup>. MAPK is a bridge between extracellular stimuli and intracellular gene expressions, which plays an important role in the chemoresistance of tumors.

## Conclusions

We showed that lncRNA NNT-AS1 can promote the DDP resistance of NSCLC cells by regulating the MAPK/Slug signaling pathway. New targeted drugs against lncRNA NNT-AS1 may provide a theoretical basis for clinical reversal of DDP-resistance in NSCLC.

## Conflict of Interest

The Authors declare that they have no conflict of interest.

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