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Downregulated long non-coding RNA TRPM2-AS inhibits cisplatin resistance of non-small cell lung cancer cells via activation of p53-p66^{shc} pathway

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Abstract. – OBJECTIVE: Non-small cell lung cancer (NSCLC), as an ordinary malignant tumor, presents with high death rate and poor prognosis. Few literatures have explored the association between NSCLC development and lncRNAs expression. This study focuses on the important role of a novel lncRNA TRPM2-AS in the development of chemo-resistance in NSCLC.

MATERIALS AND METHODS: The expression level of lncRNA TRPM2-AS was identified by using qRT-PCR assay. The apoptosis rate and the alteration of the cell cycle were detected by the flow cytometric analysis. Cell Counting Kit-8 assay (CCK8) was utilized for detecting chemo-sensitivity of the cisplatin-resistant A549/DDP cells. The p53 and p66^{shc} protein levels were detected by Western blotting assay.

RESULTS: A549/DDP cells presented remarkably higher expression of lncRNA TRPM2-AS than paired A549 cells. Moreover, re-sensitization to cisplatin was seen in A549/DDP cells after lncRNA TRPM2-AS knockdown. On the contrary, the sensitivity of lncRNA TRPM2-AS-overexpressed A549 cells to cisplatin decreased obviously when compared with the control. Furthermore, downregulated lncRNA TRPM2-AS induced cell apoptosis and altered cell cycle distribution through activating the p53-p66^{shc} pathway.

CONCLUSIONS: We suggest that lncRNA TRPM2-AS participates in the resistance of NSCLC cells to cisplatin, which may provide a new therapeutic target of NSCLC.

Key Words
LncRNAs, TRPM2-AS, NSCLC, Cisplatin resistance.

Introduction

Non-small cell lung cancer (NSCLC) is one of the most frequent malignancies with a high degree of mortality and poor prognosis[1]. Unfortunately, although advances in diagnosis and treatment of NSCLC have been achieved recently, the recurrence and mortality still remains high, with 5-year survival rate less than 15%[2]. The resistance to chemotherapy drugs after surgeries remain to be one of the significant factors about the prognosis[3]. Therefore, further comprehension of the molecular mechanisms of chemotherapy resistance is required for improving the treatment of patients with NSCLC.

Long noncoding RNAs (lncRNAs), known as one type of noncoding RNA transcripts, are a lack of protein-coding capacity. It has been discovered that aberrant expression of lncRNAs can play a vital role in various cell progression, including epigenetic regulation, genomic imprinting, and alternative splicing. Anti-tumor drug resistance in various carcinomas such as colon cancer[4], chronic myeloid leukemia[5], ovarian cancer[6], gastric cancer[7] and non-small cell lung cancer[8] has been identified to be partly associated with lncRNAs. Therefore, further study of lncRNAs may be of great value in explaining the occurrence and development of drug resistance in tumors. LncRNA TRPM2-AS, an antisense of TRPM2 located at chromosome 21q22.3, was firstly discovered in prostate cancer[9]. Moreover, Li et al[10] discovered that lncRNA TRPM2-AS knockdown could upregulate SHC1 and induce cell apoptosis in NSCLC. However, until now, no research has reported the role of lncRNA TRPM2-AS in the progression of chemo-resistance in NSCLC.

SHC1 is an adaptor protein containing three isoforms, p46, p52, and p66 isoforms. It has been discovered that it functions in cell proliferation and stress response. p66^{shc} was announced to be a pro-oxidant protein in mitochondria and could negatively regulate lifespan[11]. Moreover,

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p66^{shc} was reported[12] to be a central regulator in cisplatin-resistance of renal proximal tubule cells. Recently, Xu et al[13] revealed that p53 could induce cell apoptosis in cells exposed to cisplatin by inducing p66^{shc}. Therefore, the p53-p66^{shc} pathway is a novel potential target for regulating the cisplatin-resistance in NSCLC.

Our present study revealed that downregulated lncRNA TRPM2-AS might participate in regulating cell cycle, apoptosis and chemotherapy drug resistance via activating the p53-p66^{shc} pathway in NSCLC.

Materials and Methods

Cell culture

The Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China) provided us two NSCLC cell lines, A549/DDP cells, and A549 cells. Culture medium consisted of 100 U/ml penicillin (Invitrogen Life Technologies, Carlsbad, CA, USA), 10% fetal bovine serum (FBS; Invitrogen Life Technologies) and RPMI-1640 medium (Gibco: Thermo Fisher Scientific, Inc., Waltham, MA, USA). The humidified incubator was maintained at 37°C with 5% CO_2.

Lentiviral vector construction and infection

Genewiz (Suzhou, China) was utilized to synthesize the sequence of lncRNA TRPM2-AS. According to standard protocols, all recombination was acquired from transient transfection in HEK293T cells mediated by calcium phosphate. The culture medium for HEK293T cells was 10% FBS, 100 U/ml penicillin and Dulbecco's modified Eagle's medium (DMEM). Then cell lines were infected by 10 μg lentiviral vector and 2 μg packaging lentiviral vectors pLv-GFP or pLv-TRPM2-AS in a 10 cm culture dish. On day 2 and 3 after transfection, viruses were gathered from the supernatants. 24 h after incubated with various concentrations of lentivirus, the cells were transformed to fresh culture medium DMEM with 10% FBS in it. Flow cytometry was utilized to choose out pure infected cells, 98% of which were green fluorescent protein (GFP)-positive. Then a lentiviral vector was subcloned by the plasmid. Lentiviral Packaging Mix helped transfecting the plasmid into human embryonic kidney cells HEK-293T. In addition, this lentivirus was also used for transfection of A549 cells. Cells selected with G418 were identified as cells with highly-expressed-lncRNA TRPM2-AS (A549-TRPM2-AS). Restriction digestion, *Bam*HI, and *Mlu* I (New England Biolabs, Ltd., Hertfordshire, UK), were used to subclone lncRNA TRPM2-AS sequence to pLV-GFP (Addgene, Cambridge, MA, USA) and HIV type-1 lentiviral vector. Termed as pLV-TRPM2-AS, the subcloned constructs were compared with pLV-GFP as the control. Then these cells were centrifuged for 5 min at 1000 x g after trypsinization, followed by a collection of supernatant. Subsequently. 1 x 10^6 cells were placed into six-well plates. 100 pmol siRNA was dissolved in 250 μl Opti-MEM medium respectively with 10 μl Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). Before DNA-reagent complex was added (500 μL per well), each mixture was mixed up and cultured for 5 min. 6 h after cultured at room temperature, all cells were cultivated in the medium RPMI-1640 supplement with 10% FBS Subsequently for 24 h.

Small interfering RNAs (siRNAs) Transfection

Before transfected with siRNAs, the A549/DDP cells at a density of 50-80% were placed into six-well plates. Lipofectamine®2000 (Invitrogen Life Technologies) was used for transfecting these seeded cells with 50 nM siRNAs particularly targeting lncRNA TRPM2-AS (siRNA1/TRPM2-AS sense, 5′-GGGAAGAUGUCUCAGCAGACG-3′, antisense, 5′-UCUGCUGAGACAUCUUCCCCU-3′; siRNA2/TRPM2-AS sense, 5′-CGAACCUUCCCUAAUAGAAAC-3′, antisense, 5′-UUCUAUUAGGGAAGG UUCGGG-3′; siRNA3/TRPM2-AS sense, 5′-AGACCUAUGAGGAGACAUAAC-3′, antisense, 5′-UAUGUCUCCUCAUAGGUCUCG-3′.) (GenePharma, Shanghai, China)). Subsequently, the incubator was set at room temperature with 5% CO_2, and these cells were cultivated in it for 24 h. 5'-UUAAGACGGUUGAAACUAG-3' was conducted as control siRNA sequence.

RNA extraction and qRT-PCR

As the manufacturer's instructions said, TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was utilized for extracting the total RNA in these cells. cDNAs were synthesized via reverse Transcription Kit (Takara Biotechnology Co., Ltd., Dalian, China). Quantified by SYBR Green real-time PCR. Then lncRNA TRPM2-AS mRNA level was nor-

malized to GAPDH using the primers below: for lncRNA TRPM2-AS, forwards, 5'-CGTGACCAGGTTCAGACACA-3' and reverse, 5'-TGGGCAGTTTGGTTCTGGTT-3'; and for GAPDH, forward, 5'-CCACATCGCTCAGACACCAT-3' and reverse, 5'-ACCAGGCGCCCAATACG-3'. ABI 7500 system (Applied Biosystems, Foster City, CA, USA) was used for the performance of RT-qPCR. Following were the thermal cycle: 95°C for 30 seconds, 95°C for 5 seconds for 40 cycles and then 60°C for 35 seconds.

Cell Counting Kit-8 assay

The chemo-sensitivity to cisplatin of both A549/DPP cells and the paired A549 cells was monitored with Cell Counting Kit-8 assay (CCK8, Dojindo Molecular Technologies, Inc., Kunamoto, Japan). Briefly, the cells in good status were placed in 96-well plates (4×10^3 cells/well) and then added with 0, 1, 5, 8, 10, 15, 18, 20, 22 or 24 μg/ml cisplatin. The CCK8 solution was used to assess the cell viability at 0, 24, 48, 72 and 96h after adding cisplatin. Then spectrophotometer (Thermo Scientific, Rockford, IL, USA) was used to measure the absorbance of each well at 450 nm. Every step was operated three times at least.

Cell cycle analysis

Cell cycle staining Kit (MultiSciences Biotech Co., Ltd, Hangzhou, China) was used for detecting the cell cycle according to the protocol. For details, cells (2×10^5/ml) were overnight in 75% ice-cold ethanol diluted by RNase A. Then, they were washed with PBS twice and finally stained successively for 30 min accompanied with 50 mg/ml propidium iodide (PI) at 4ºC in the dark. Flow cytometer (FACScan, BD Bioscience, San Jose, CA, USA) was used to detect the distribution of cell cycle.

Cell apoptosis analysis

Annexin V-APC/7-AAD Apoptosis Detection Kit II (KeyGEN BioTESCCH Co., Ltd, Nanjing, China) was used to evaluate apoptosis in both the A549 cells and A549/DDP cells. Briefly, 1×10^6 of these cells were collected and washed twice with cold phosphate-buffered saline (PBS). Then these cells were dissolved in 1,000 ml binding buffer 100 μl out of the solution containing 1×10^5 cells were replaced to a fresh tube with 5 μl 7-AAD 5 μl and Annexin V-APC in it. After cultured at 37°C for 15 min in the dark, each tube was added with 400 μL binding buffer. Flow cytometry (FACScan, BD Biosciences) programmed with CellQuest software (BD Biosciences, San Diego, CA, USA) was used to discriminate dead, viable, late apoptotic cells and early apoptotic, the percentages of which were used for the comparison between experimental and control groups. The test was repeated thrice at least.

Western blotting analysis

A protein assay (bicinchoninic acid method; Beyotime) was utilized for quantifying the total protein expression. The target proteins were replaced to the polyvinylidene fluoride (PVDF) membrane, which was then blocked in 5% dry milk at 37°C for 1 hour after fractionated by SDS-PAGE. Following was the immunostaining with antibodies overnight at 4°C: the rabbit anti-$p66^{shc}$ (1:1000, Cell Signaling Technology, CST, Danvers, MA, USA), the rabbit anti-p53 (1:1000, Cell Signaling Technology, CST, USA) and the rabbit anti-GAPDH (1:5000; Cell Signaling Technology, CST, Donvers, MA, USA). PBS supplement with 0.1% Tween 20 was utilized four times to wash the membranes. The membrane cultivated within a goat anti-rabbit secondary antibody (1:1000, CST) at room temperature for 1 h. After that, PBS was again used to wash the membranes three times for 15 min. The compares between relevant protein levels were conducted by Image J software.

Statistical analysis

Data analysis was conducted with SPSS.18.0 (SPSS Inc., Chicago, IL, USA). Graph PAD 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) was applied in presenting the consequence. Quantitative data was presented as mean ± SD. The method of $2^{-\Delta\Delta CT}$ was used to measure the relative expression of mRNA[14]. The independent samples *t*-test was chosen for statistical analysis. Values of $p<0.05$ were considered statistically significant.

Results

LncRNA TRPM2-AS expression is increased in A549/DDP cells

A549/DDP cells were termed as the cisplatin-resistance A549 cells. After the treatment with cisplatin, the CCK8 assay was used to monitor half of the maximal inhibitory concentration (IC_{50}) for A549 cells as well as paired A549/DDP cells. Respectively, the IC_{50} of cisplatin for A549 cells was significantly lower compared with A549/DDP cells (5.33±2.40 μg/mL

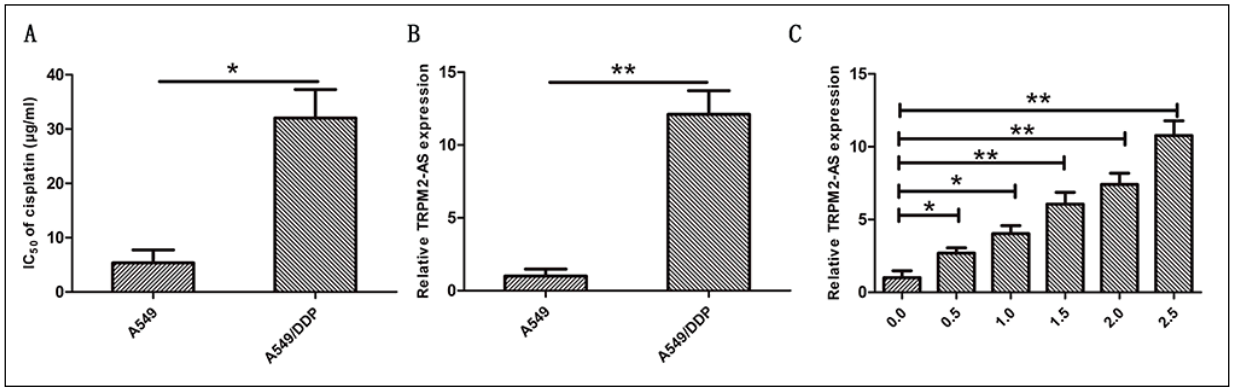

Figure 1. Expression levels of lncRNA TRPM2-AS were increased in A549/DDP cells. **A**, IC_{50} value of cisplatin was higher in A549/DDP cells, as compared with that of the A549 cells. **B**, Expression levels of lncRNA TRPM2-AS relative to GAPDH were determined in the A549/DDP and A549 cells by RT-qPCR. **C**, A549 cells were cultured in various concentrations of cisplatin (0.0, 0.5, 1.0, 1.5, 2.0 or 2.5 μg/mL) for 24 h. lncRNA TRPM2-AS expression was evaluated by RT-qPCR. GAPDH was used as an internal control. Data are presented as the mean ± standard error of the mean. *p<0.05; **p<0.01.

vs. 32.21±3.62 μg/mL) (Figure 1A). Furthermore, lncRNA TRPM2-AS expression was remarkably decreased in A549 cells compared with paired A549/DDP cells (Figure 1B).

LncRNA TRPM2-AS is upregulated in A549 cells treated with cisplatin

The above results showed that lncRNA TRPM2-AS was upregulated in A549/DDP cells. The current study focused on the changes of lncRNA TRPM2-AS expression in response to different concentrations of cisplatin. 24 h after added with 0.0, 0.5, 1.0, 1.5, 2.0 or 2.5 μg/mL cisplatin, cells were used to detect the lncRNA TRPM2-AS expression respectively. With increased cisplatin concentrations, the lncRNA TRPM2-AS expression became higher relatively (Figure 1C). The results above suggested that the increment of lncRNA TRPM2-AS expression was actively associated with cisplatin treatment in A549 cells.

Decreased apoptosis rate and alteration of cell cycle in lncRNA TRPM2-AS-overexpressed A549 cells

A549 cells were steadily transfected with a lentivirus to further explore the effect of lncRNA TRPM2-AS overexpression on the sensitivity to cisplatin. RT-qPCR was used to validate the efficiency of A549-TRPM2-AS cells transfection. Compared with A549 cells (control), the lncRNA TRPM2-AS expression levels of lncRNA TRPM2-AS-overexpressed cells (A549-TRPM2-AS cells) were remarkably higher (Figure 2A). Also, the IC_{50} of cisplatin increased by ~8.75-fold after lncRNA TRPM2-AS was upregulated (Figure 2B). Upregulation of lncRNA TRPM2-AS led to a reduced rate of apoptosis induced by increased cisplatin in A549-TRPM2-AS cells (Figure 2C). Moreover, with the increasing dosage of cisplatin, the proportion of A549-TRPM2-AS cells in G1 phase, as well as subG0/G1 phases, gradually increased, especially at G1 phase (Figure 2D).

Reversed cisplatin resistance in lncRNA TRPM2-AS knockdown A549/DPP cells

To further understand the function lncRNA TRPM2-AS on resistance to cisplatin, A549/DDP cells were infected with siRNA1/TRPM2-AS, siRNA2/TRPM2-AS, siRNA3/TRPM2-AS or siRNA/control. The expression level of siRNA3/TRPM2-AS-transfected cells was remarkably declined after transfection for 48h compared with siRNA/control-transfected cells (Figure 3A). The inhibition efficiency of siRNA3/TRPM2-AS was much higher than that of siRNA1/TRPM2-AS (36.7%) or siRNA2/TRPM2-AS (56.9%). Based on these results, siRNA3/TRPM2-AS was utilized to inhibit the lncRNA TRPM2-AS expression in A549/DDP cells. Subsequently, CCK8 assay showed that IC_{50} of cisplatin was reduced by siRNA3/TRPM2-AS in A549/DDP cells (Figure 3B). In addition, different concentrations (0.0, 1.0 and 2.0 μg/ml) of cisplatin were added to dishes with siRNA3/TRPM2-AS-transfected cells respectively. An apoptosis assay

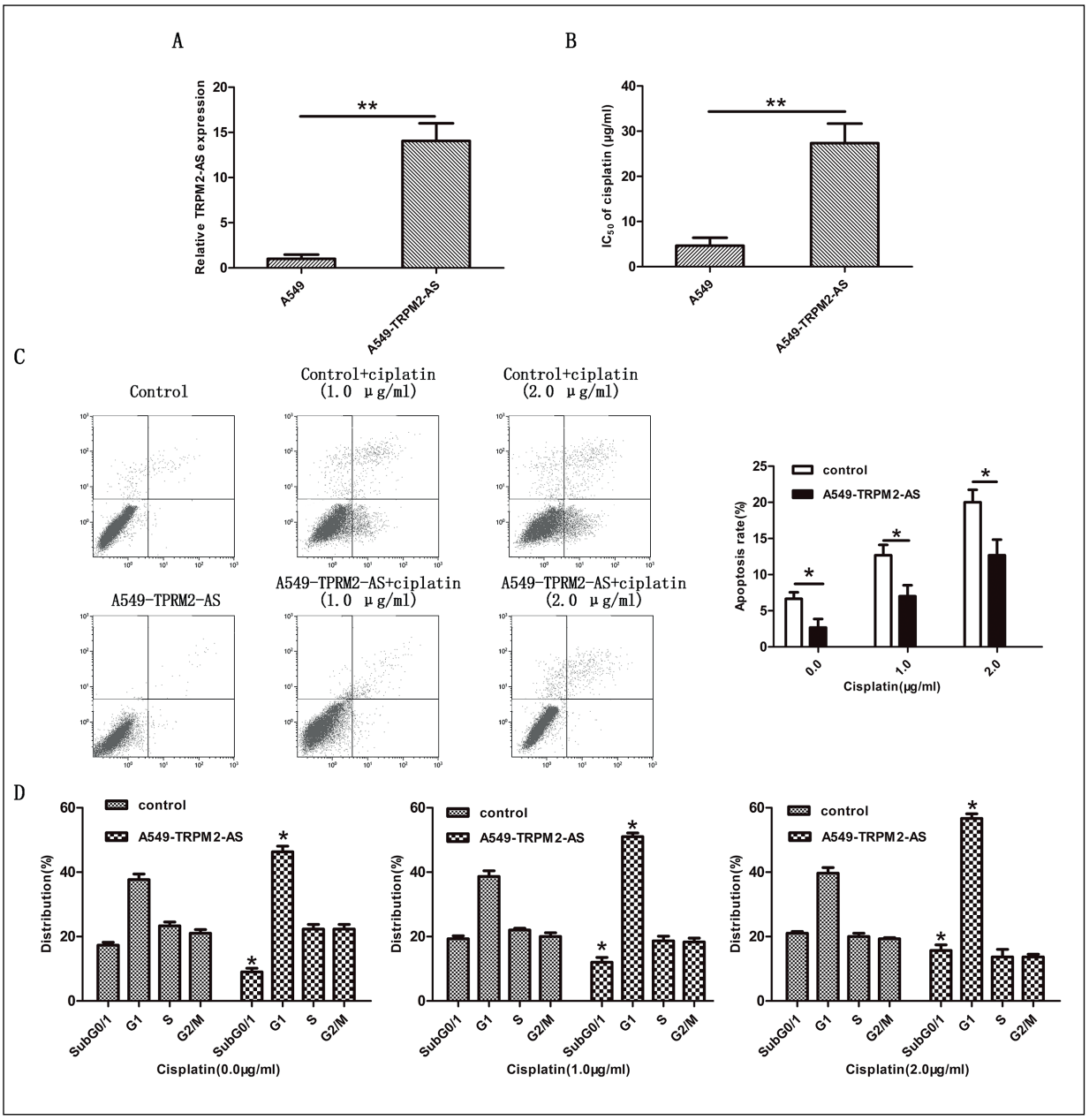

Figure 2. lncRNA TRPM2-AS enhances the cisplatin-resistance of parental A549 cells. **A**, lncRNA TRPM2-AS expression was detected in A549 cells stably transfected with a lncRNA TRPM2-AS lentivirus by RT-qPCR. GAPDH was used as an internal control. **B**, IC_{50} values of cisplatin in A549 and A549 -TRPM2-AS cells were analyzed by CCK8 assay. **C**, Flow cytometric analysis of apoptosis in A549 (control) or A549-TRPM2-AS (lncRNA TRPM2-AS overexpressing) cells treated with various concentrations of cisplatin (0.0, 1.0 or 2.0 μg/mL). **D**, Flow cytometric analysis of the cell cycle distribution in A549 or A549-TRPM2-AS cells treated with various concentrations of cisplatin (0.0, 1.0 or 2.0 μg/mL). The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

showed that the apoptosis caused via cisplatin increased with lncRNA TRPM2-AS knockdown in A549/DDP cells (Figure 3C). Moreover, as the increasing dosage of cisplatin, the percentage of siRNA3/TRPM2-AS-transfected A549/DDP cells in subG0/G1 and G1 phases increased, especially in checkpoint subG0/G1 (Figure 3D). The results indicated that downregulated lncRNA TRPM2-AS might be correlated to cell cycle arrest and increased apoptosis, thus reversing cisplatin resistance of A549/DDP cells.

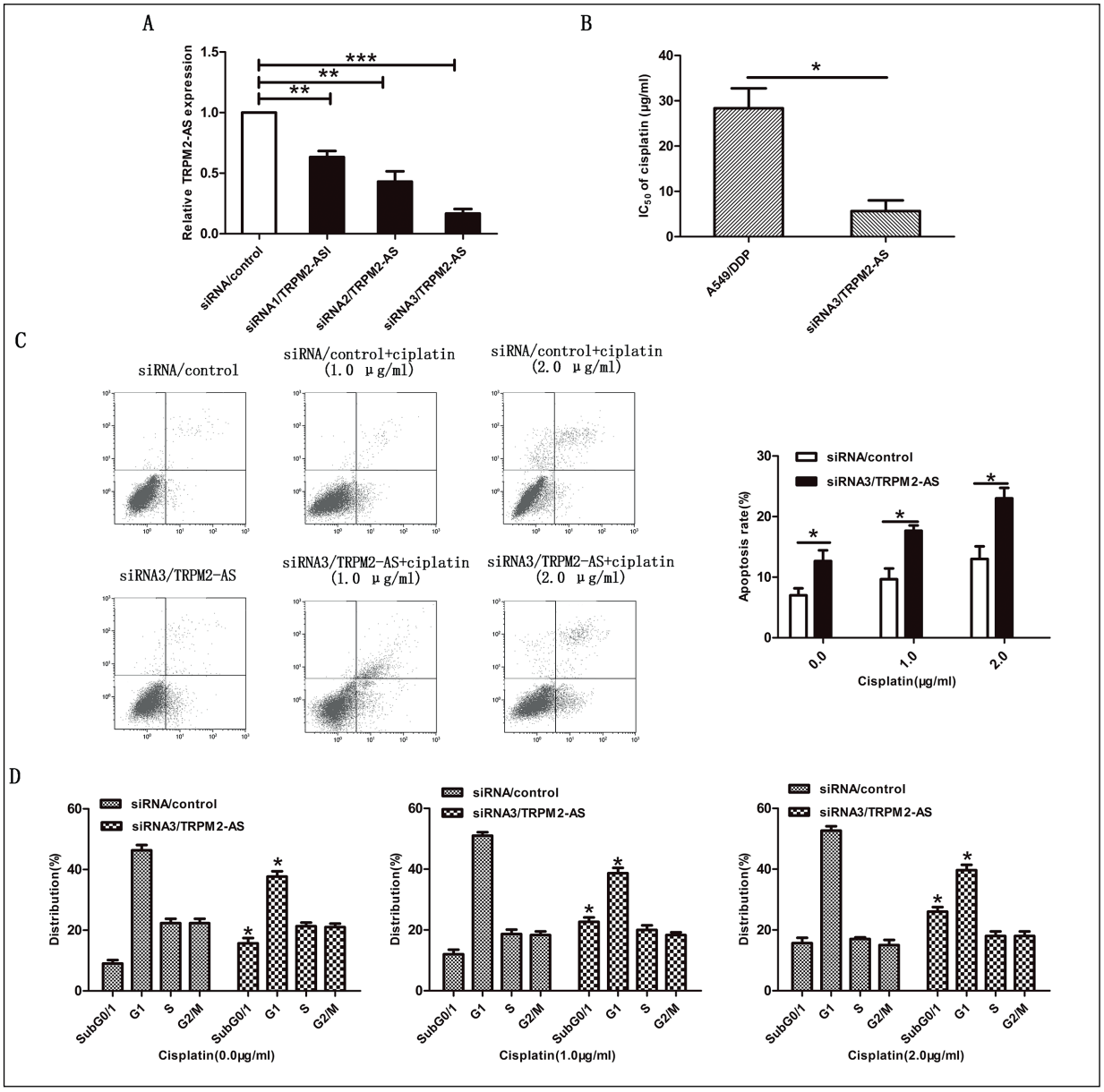

Figure 3. siRNA transfection reduces the expression of lncRNA TRPM2-AS and increases the sensitivity of A549/DDP human lung cancer cells to cisplatin. **A**, A total of 48 h after the A549/DDP human lung cancer cells were transfected with siRNA/control, siRNA1/TRPM2-AS, siRNA2/TRPM2-AS or siRNA3/TRPM2-AS, the inhibition efficiency of the siRNA were detected by RT-qPCR. GAPDH was used as an internal control. **B**, IC_{50} values of cisplatin in A549/DDP and siRNA3/TRPM2-AS cells were analyzed using CCK8 assay. **C**, Flow cytometric analysis of apoptosis of A549/DDP (siRNA/control) and siRNA3/TRPM2-AS cells treated with various concentrations of cisplatin (0.0, 1.0 or 2.0 μg/mL). **D**, Flow cytometric analysis of the cell cycle distribution of A549/DDP (siRNA/control) and siRNA3/TRPM2-AS cells treated with various concentrations of cisplatin (0.0, 1.0 or 2.0 μg/mL). The results represent the average of three independent experiments Data are presented as the mean ± standard error of the mean. *p<0.05, **p<0.01, **p<0.001.

LncRNA TRPM2-AS knockdown decreases cisplatin resistance through activating p53 and p66^{shc} in A549/DDP cells

The protein p53, as well as p66^{shc} expression, was upregulated in siRNA3/TRPM2-AS-transfected A549/DDP cells by Western blot analysis (Figure 4). All findings above suggested that cisplatin resistance in A549/DDP cells might be inhibited by lncRNA TRPM2-AS knockdown via activation of p53 and p66^{shc}.

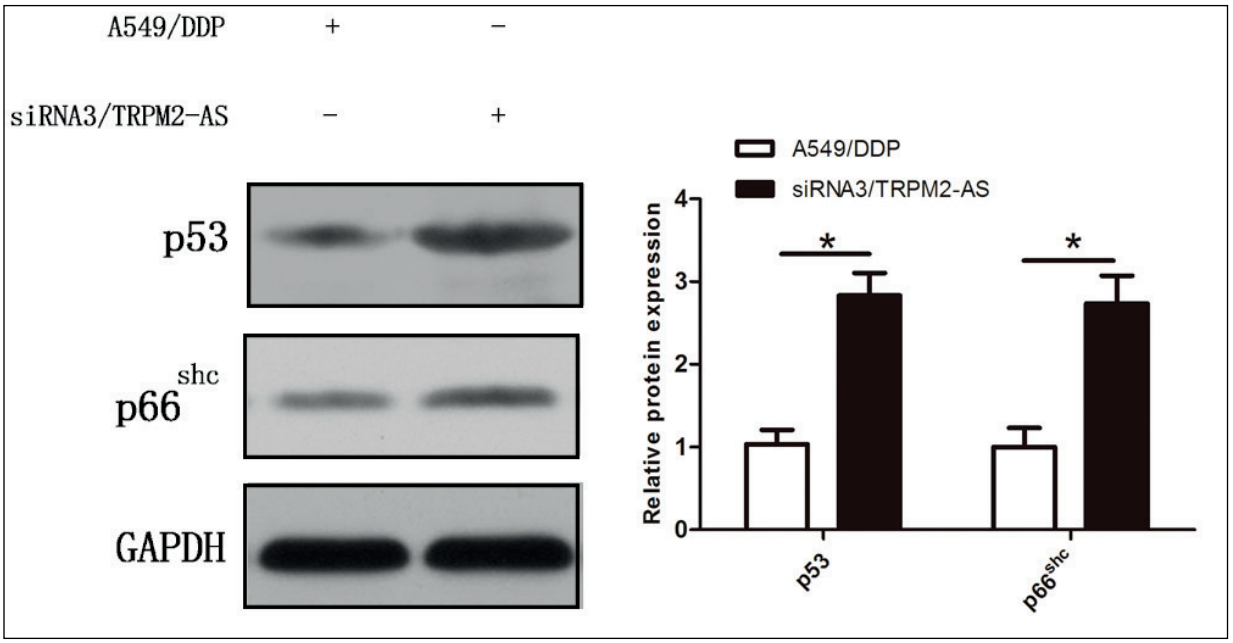

Figure 4. lncRNA TRPM2-AS regulates p53 and p66^{shc} expression. Protein expression levels of p53 and p66^{shc} in A549/DDP or siRNA3/TRPM2-AS human lung cancer cells were analyzed by western blotting. GAPDH was used as a control. Average values of integrated optical density were assessed by analyzing five times per experiment and recorded in the histograms. *p<0.05.

Discussion

NSCLC accounts for high mortality and poor survival worldwide[15]. Although various chemotherapy drugs are available for lung cancers, the resistance to these drugs is a crucial factor for the prognosis of lung cancer patients[16,17]. Therefore, the concrete mechanism of cisplatin resistance attracts more and more attention nowadays. The inhibition of the apoptosis pathway has been identified as a possible mechanism of drug resistance for cancers. Increasing evidence suggests that lncRNAs could modulate drug resistance in cancer cells partly through this mechanism[18-21].

Recently, several studies showed that downregulated p53 inhibited cell apoptosis and was associated with the resistance to anti-tumor drugs in different types of cancer[22-24]. A previous study[13] demonstrated that the activation of p53-p66^{shc} pathway induced apoptosis in cells exposed to cisplatin. Moreover, lncRNA TRPM2-AS was associated with the expression of p66^{shc} transcription[10]. However, it remained unclear whether lncRNA TRPM2-AS regulated cisplatin resistance of A549/DDP cells via the p53-p66^{shc} pathway. We first confirmed the connection between lncRNA TRPM2-AS upregulation and the establishment of cisplatin resistance in A549/DDP cells and then explored the association between lncRNA TRPM2-AS knockdown and the corresponding changes in p53 and p66^{shc} protein levels.

In this manuscript, we investigated the influence of lncRNA TRPM2-AS on cell cycle, cell apoptosis and resistance to cisplatin of NSCLC cells. The results demonstrated that A549/DDP cells exhibited higher expression of lncRNA TRPM2-AS than the parental A549 cells. Moreover, the lncRNA TRPM2-AS expression increased in treated A549 cells with increasing dose of cisplatin. Besides, overexpression of lncRNA TRPM2-AS reduced the cisplatin-induced apoptosis of A549 cells, while lncRNA TRPM2-AS knockdown raised cisplatin-induced apoptosis rate in A549/DDP cells. Accompanied with this increment in doses of cisplatin, the cisplatin-treated A549-TRPM2-AS cells (TRPM2-AS-overexpression A549 cells) at subG0/G1 and G1 phase, mainly in G1 phase, grew up. By contraries, the cisplatin-treated siRNA/TRPM2-AS-transfected A549/DDP cells at subG0/G1 and G1 phase, especially in subG0/1 phase, appeared more with increasing

doses of cisplatin. Downregulation of lncRNA TRPM2-AS activated p53 and $p66^{shc}$ expression in A549/DDP cells. Previous research identified the p53-$p66^{shc}$ pathway in the regulation of cisplatin-induced apoptosis. Therefore, we suggested that lncRNA TRPM2-AS possibly regulated cell cycle, apoptosis, and resistance to anti-tumor drugs of carcinoma cells via suppressing p53-$p66^{shc}$ pathway in NSCLC.

Conclusions

Our findings demonstrated that lncRNA TRPM2-AS enhanced the resistance of A549 cells lung cancer cells to cisplatin. Furthermore, lncRNA TRPM2-AS could suppress p53-$p66^{shc}$ pathway. These findings implied that lncRNA TRPM2-AS can act as a prospective therapeutic target for NSCLC. The exact biological functions of lncRNA TRPM2-AS need to be further explored in more NSCLC cell lines and tissues in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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