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# Interferon-γ Upregulates SOCS3 Expression to Reduce Cisplatin Chemoresistance in Non-Small Cell Lung Cancer A549 Cells

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## Abstract

**Background:** Cisplatin (DDP)-based chemotherapy is the mainstay of first-line therapeutic strategy for the treatment of advanced non-small cell carcinoma (NSCLC). However, the anticancer efficacy of DDP is often limited by the existence or development of chemoresistance. Thus, we investigated the effect of interferon-γ on DDP-resistant A549 cell.

**Methods**: Semi-quantitative RT–PCR was used to compare the differences of SOCS3 mRNA expression in both cisplatin-resistant A549 (A549/DDP) cell and the parental A549 cell. The cellular sensitivity to cisplatin, cell viability and apoptosis were detected by MTT, flow cytometry and Western blotting.

**Results**: Semi-quantitative RT–PCR and western blotting showed that SOCS 3 expression was significantly down-regulated in A549/DDP cell compared to the parental A549 and normal human bronchial epithelial cells BEAS-2B. IFN-γ treatment could restore SOCS3 expression, resulting in an increased sensitivity of these resistant A549 cells to DDP. In addition, p53 and Bcl-2 signaling pathways were also involved in regulating IFN-γ-induced cell death in DDP-resistant A549 cells.

**Conclusion:** Our study indicates that SOCS 3 may play a crucial role in the progress of cisplatin resistance of A549. Moreover, IFN-γ could induce SOCS3 expression and potentiate the re-sensitization of these resistant A549 cells to DDP.

## Key Words:

Interferon-γ; SOCS3; Cisplatin; Chemosensitivity; Lung cancer

## Introduction

Lung cancer is the leading cause of cancer deaths worldwide in both men and women [1]. Lung cancer is classified into two major categories: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) [2]. For advanced NSCLC, cisplatin (DDP)-based chemotherapy is the main therapeutic strategy. However, their efficacy is often limited by the existence or development of chemoresistance.

Suppressor of cytokine signaling 3 (SOCS3) is a key negative feedback regulator of cytokine signalling [3]. SOCS3 not only suppresses cytokine-mediated JAK/STAT signaling pathway, but also inhibits other downstream signaling by degradation of receptors or associated proteins via the proteasomal pathway [4]. In addition, SOCS3 plays a critical role in cancer initiation and progression. SOCS3 deficiency induces development of gastric tumors in mice [3] and the restoration of SOCS3 expression in several cancer cell lines was found to effectively suppress tumorigenicity [5,6]. However, the role of SOCS3 in chemoresistance has not been well recognized.

Previous studies show that the treatment of interferon-γ (IFN-γ) can inhibit cell growth and induce cytotoxicity in lung epithelial malignancies [7,8]. Moreover, IFN-γ can also induce SOCS 3 expression and exhibit high cytotoxic effect in human melanoma cell lines [9]. However, no relationship has been reported between IFN-γ and DDP resistance in lung cancer cells.

We investigated SOCS 3 gene expression in response to IFN-γ treatment in DDP-resistant A549 cells. Here we demonstrate that SOCS3 was decreased in DDP-resistant A549/DDP cells compared with parental A549 cells, suggesting that SOCS3 might be associated with DDP-resistant in NSCLC. Furthermore, we found that IFN-γ could induce SOCS3 expression and potentiate the re-sensitization of these resistant A549 cells to DDP. In addition, p53 and Bcl-2 signaling pathways were also involved in regulating IFN-γ-induced cell death in DDP-resistant A549 cells. Therefore, our data suggest a novel and promising strategy for the increase in clinical efficacy of DDP in NSCLC patients.

## Methods

### Reagents and antibodies

Human IFN-γ and DDP were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Proteins were reacted with one of the following: anti-cleaved caspase-3 (#9661), anti-SOCS3 (#2923) and anti-Bcl-2 (#286) were purchased from Cell Signaling Technology. Mouse anti-p53-HRP was purchased from Santa Cruz Biotechnology.

### Cell lines

The normal human bronchial epithelial cell BEAS-2B was purchased from Shanghai MEIXUAN Biological Science and Technology Ltd (Shanghai, China). The parental lung cancer A549 cell was purchased from Shanghai Institute of Cell Biology (Shanghai, China). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA). For A549/DDP, 2 μg/mL DDP was added. All media were supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) of 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were cultured at 37°C in a humidified 5% CO2 incubator.

### Measurement of cell viability

Cell viability was determined by MTT assay. Cells were seeded in 96-well flat bottom microtiter plates at a density of $1\times10^4$ cells per well. 24 hrs later, DDP was added at the concentrations indicated for 24 hrs. The absorbance was measured on a microplate reader (Synergy HT, Bio-Tek, USA) at 570 nm.

### Measurement of apoptosis

Phartmingen annexin V-FITC Apoptosis Ddtection Kit I (BD, USA) was used to detect apoptosis and the estimation procedure was performed according to the manufacturer's instructions. $2 \times 10^6$ cells were seeded into a 6 cm dish. After attachment overnight, cells were washed twice with PBS and the medium was replaced medium with DDP for 24 hrs. All cells including the floating cells in the culture medium were harvested. The cells were resuspended in ice-cold 1 × binding buffer at a concentration of $1 \times 10^6$ cells/ml 100 μl of cell suspension were each mixed with 5 μl FITC Annexin V and 5 μl PI. The mixture was incubated for 15 min at room temperature in the dark and then analyzed by FACSCalibur Flow Cytometer (BD Biosystems, Heidelberg, Germany).

### Cell cycle analysis

Cell cycle analysis was performed with flow cytometry assay. A549 and A549/DDP cells were harvested, fixed overnight at 4°C, and stained with a propidium iodide solution for 30 minutes, and were then measured their relative DNA content by FACSCalibur Flow Cytometer (BD Biosystems, Heidelberg, Germany). In this way, cells that are in S phase will have more DNA than cells in G1. They will take up proportionally more dye and will fluoresce more brightly until they have doubled their DNA content. The cells in G2 will be approximately twice as bright as cells in G1.

### Semi-quantitative RT–PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Approximately 2 μg of total RNA was converted to cDNA by First-strand cDNA synthesis kit (OriGene Technologies, MD, USA). Semi-quantitative RT–PCR was performed by using the primers SOCS3 F: 5'-TCAAGACCTTCAGCTCCAAG-3', R: 5'-TTGACGCTGAGCGTGAAGAA-3'. Cycling conditions initial denaturation at 95°C for 2 minutes was followed by 35 cycles with denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The GAPDH mRNA sequence was also amplified as an internal control.

### Western blot analysis

Cells were harvested from cultured dishes and were lysed in a lysis buffer [20 mM Tris-HCl pH 7.6, 1 mM EDTA, 140 mM NaCl, 1% NP-40, 1% aprotinin, 1 mM phenylemethylsulfonyl fluoride (PMSF), 1 mM sodium vanadate]. Protein concentration was determined using a BCA Protein Assay Kit (Pierce). Cell lysates (40 μg protein/line) were separated on a 5 to 20% Tris-Tricine Ready Gel SDS-PAGE (Bio-Rad) for nitrocellulose membrane blotting. The blotted membranes were blocked with 5% skim milk for 1 h and were incubated with primary antibodies. The immunoreactive bands were visualized by enhanced chemiluminescence using horseradish perox-idase-conjugated IgG secondary antibodies. Band density was measured by densitometry, quantified using gel plotting macros of NIH image 1.62, and normalized to an indicated sample in the identical membrane.

### Statistical analyses

Results are expressed as values of mean ± standard deviation (SD). Statistical analysis was performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). The correlation coefficient of two factors was evaluated using two-tailed, homoscedastic t test. A P-value less than 0.05 is considered significant.

## Results

### The different biological effect between BEAS-2B, A549 and A549/DDP cells

To better investigate the chemoresistance of DDP in lung cancer cells, we established a DDP-resistant human lung adenocarcinoma cell line A549/DDP by subjecting A549 cells to drug pressure. The cell viability assay was performed on lung cancer cell line A549 and A549/DDP, which produced IC50 values for DDP of 4.61 ±0.72 μg/mL and 21.31 ±1.96 μg/mL ($P<0.01$), respectively (Figure 1A). The cell cycle analysis by flow cytometry showed that A549/DDP cells displayed a predominant accumulation in the S phase but a reduction in the G2 phase compared with A549 cells (Figure 1B). In addition, we detected the apoptosis in A549 and A549/DDP cells after the treatment with 4 μg/mL DDP. As a result, a greater percentage of apoptotic cells was found in A549 cells than in A549/DDP cells (Figure 1C). Therefore, A549/DDP cells displayed higher resistance for DDP than their parental A549 cells.

### The different expression of SOCS3 between A549 and A549/DDP cells

Then we study the expression of SOCS3 in the normal human bronchial epithelial cell BEAS-2B and lung cancer cell line A549 and A549/DDP by RT-PCR and western blotting. We found that both mRNA and protein levels of SOCS3 were inhibited in A549/DDP cells,

compared with their parental A549 cells and normal human bronchial epithelial cells BEAS-2B (Figure 2A and B). This data indicate that SOCS3 may be a critical mediator for DDP resistance in lung cancer cells.

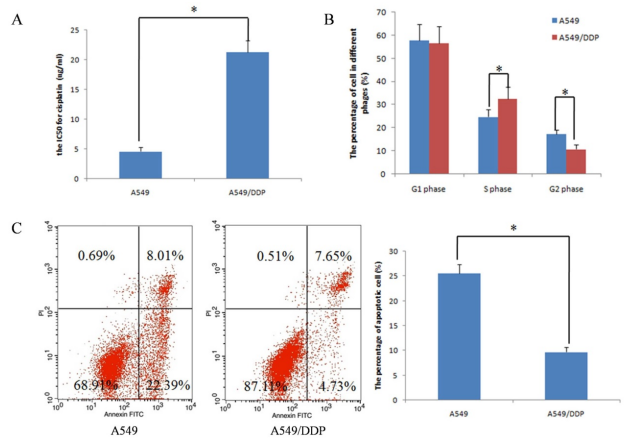


**Figure 1:** The different characteristics of A549 and A549/ DDP cells. (A) MTT was used to measure cell toxicity (IC50) of DDP for A549 and A549/ DDP cells;(B) Cell cycle was investigated by flow cytometry. $p<0.05$; (C) Cell apoptosis was investigated by flow cytometry. $p<0.05$. The bars represent three experiments realized in triplets.

## IFN-γ could induce SOCS3 expression to re-sensitize A549/DDP cells to DDP

The previous study show IFN-γ treatment can also induce SOCS 3 expression [9], so we want to know whether IFN-γ could induce SOCS3 expression to overcome the DDP resistance in A549/DDP cells. Then, A549/DDP cells were treated by the combination of DDP with or without IFN-γ (10 ng/ml). After the treatment with IFN-γ, RT-PCR showed SOCS3 expression was activated at the transcriptional level (Figure 2C) and western blotting displayed the protein expression of SOCS3 was also increased (Figure 2D). Moreover, IFN-γ treatment not only induced SOCS3 expression but also enhanced DDP-induced apoptosis which can be demonstrated by the increased expression of active caspase-3 (Figure 2D). To obtain objective quantification of apoptosis, we performed an Annexin V–FITC dual staining assay followed by flow cytometry. As a result, the percentage of apoptotic cells was significantly higher in A549/DDP cells treated with the combination of DDP and IFN-γ than that in their respective controls (Figure 2E). Whereas, when A549/DDP cells were treated with the combination of IFN-γ and SOCS3 inhibitor zoledronic acid (ZA) [10], the percentage of apoptotic cells was remarkably reduced (Figure 2F), which suggested the γsuppression of SOCS3 by ZA could decrease the toxicity of the treatment with IFN-γ in DDP-treated A549/DDP cells. Taken together, IFN-γ could induce SOCS3 expression and potentiate the re-sensitization of these resistant A549 cells to DDP.

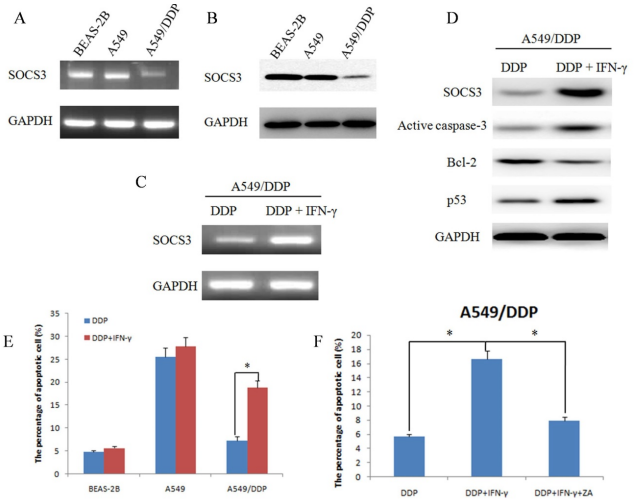


**Figure 2:** The expression of SOCS 3 and the effect of IFN-γ on the apoptosis of A549/ DDP cells; (A) The different expression of SOCS3 in BEAS-2B, A549 and A549/DDP cells is detected by RT-PCR; (B) The different expression of SOCS3 in BEAS-2B, A549 and A549/DDP cells is detected by western blotting; (C) after the treatment with IFN-γ, the expression of SOCS3 in A549/DDP cells is detected by RT-PCR; (D) after the treatment with IFN-γ, the expression of SOCS3, active caspase-3, Bcl-2, p53 in A549/DDP cells is detected by western blotting; (E) after the treatment with IFN-γ, the apoptosis in BEAS-2B, A549 and A549/DDP cells was investigated by flow cytometry. ($p<0.05$); (F) after the treatment with the combination of IFN-γ and SOCS3 inhibitor ZA, the apoptosis in A549/DDP cells was investigated by flow cytometry. ($p<0.05$). The bars represent three experiments realized in triplets.

## p53 and Bcl-2 are also involved in in regulating IFN-γ-induced cell death in DDP-resistant A549 cells

Deficiency or mutation in the p53 tumor suppressor gene commonly occurs in human cancer and can contribute to disease progression and chemotherapy resistance [11]. Bcl-2 as an antiapoptotic protein is widely associated with tumor initiation, progression, and chemoresistance [12]. Bcl-2 inhibition can overcome intrinsic drug resistance in cancer cells. Therefore, we investigated the expression of p53 and Bcl-2 in DDP-resistant A549 cells by the combination of DDP with or with IFN-γ. We found that IFN-γ can enhance the p53 expression but reduce Bcl-2 expression in A549/DDP cells (Figure 2D), indicating p53 activation and Bcl-2 inhibition are associated with IFN-γ-induced cell death in DDP-resistant A549 cells.

## Discussion

Despite the great advances in cancer therapy, chemotherapy is still one of the most effective treatments for advanced lung cancer. Among those chemotherapeutic agents, DDP is first-line therapeutic strategy for treating NSCLC. Unfortunately, the majority of tumors will eventually develop acquired drug resistance of DDP, which restricts the clinical application of DDP. Therefore, how to overcome DDP resistance will be a key issue for the design of more effective individualized therapeutic strategies.

SOCS 3, one of the best characterized molecules of the SOCS family, functions as an important role in immune and inflammatory processes [13,14]. However, the study regarding the relationship between activation of SOCS 3 and the sensitivity of DDP has been not reported. Our data provide the evidence that SOCS 3 play a crucial role in the resistance of cisplatin for A549. The suppression of SOCS 3 is significantly associated with DDP resistance in A549/DDP cells.

The discovery of the IFN family has considerably contributed to the treatment not only in viral infections but also in cancer. Recently, treatment of IFN-γ was found to cause cell growth inhibition and cytotoxicity in lung epithelial malignancies [7]. However, whether IFN-γ can reduce DDP resistance in lung cancer cell is still unclear. Our study showed that IFN-γ treatment not only induced SOCS3 expression but also enhanced DDP-induced apoptosis in A549/DDP cells and inhibiting SOCS3 expression reduced the percentage of apoptotic cells in A549/DDP cell line, indicating IFN-γ could re-sensitize of these resistant A549 cells to DDP by inducing SOCS3 expression. Mechanistically, p53 activation and Bcl-2 inhibition are also involved in IFN-γ-induced cell death in DDP-resistant A549 cells.

Taken together, we demonstrate that SOCS 3 may play a crucial role in the resistance of cisplatin for A549 and IFN-γ treatment could induce SOCS3 expression and potentiate the re-sensitization of these resistant A549 cells to DDP.

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## Author contributions

XS, DX and HP designed the study. XS wrote the main manuscript text. YX, HT, LG, HH, and LC performed experiments. YX and HT prepared figures. LG and HH did the statistical analyses. All authors reviewed the manuscript.

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