

GAS5 modulated autophagy is a mechanism modulating cisplatin sensitivity in NSCLC cells

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Abstract. – OBJECTIVE: In this study, we investigated the association between lncRNA GAS5 and cisplatin (DDP) resistance in NSCLC and further studied the regulative effect of GAS5 on autophagy and DDP resistance.

PATIENTS AND METHODS: GAS5 expression in cancerous and adjacent normal tissues from 15 NSCLC patients received neoadjuvant chemotherapy and the following surgery were measured using qRT-PCR analysis. GAS5 gain-and-loss study was performed using A549 and A549/DDP cells as an in-vitro model to investigate the effect of GAS5 on autophagy and cisplatin sensitivity.

RESULTS: NSCLC tissues had a substantially lower expression of GAS5 than adjacent normal tissues. The NSCLC tissues from patients with progressive disease (PD) had even lower GAS5 expression. GAS5 knockdown increased DDP IC50 of A549 cells, while GAS5 overexpression decreased DDP IC50 of A549/DDP cells. A549/DDP cells had significantly higher basal autophagy than A549 cells. GAS5 knockdown resulted in decreased autophagy in A549 cells, while GAS5 overexpression led to increased autophagy in A549/DDP cells. Treatment with 3-MA, an autophagy inhibitor, significantly decreased DDP IC50 and promoted DDP-induced cell apoptosis in A549 cells. In addition, 3-MA also partly reversed the effect of GAS5 knockdown. In A549/DDP cells, GAS5 showed the similar effect as 3-MA in reducing DDP IC50 and promoting DDP-induced apoptosis and also presented synergic effect with 3-MA.

CONCLUSIONS: GAS5 downregulation is associated with cisplatin resistance in NSCLC. GAS5 can inhibit autophagy and therefore enhance cisplatin sensitivity in NSCLC cells.

Key Words:

GAS5, NSCLC, Autophagy, Chemoresistance.

Introduction

Non-small-cell lung cancer (NSCLC) is the major type of lung cancer, which accounts for

about 85% of all lung cancer cases¹. Currently, platinum-based chemotherapy is still the standard first-line chemotherapy for the patients with unresectable locally advanced or distant metastatic NSCLC². In addition, it is also used as neoadjuvant chemotherapy before surgery or adjuvant chemotherapy after surgery for the operable patients³. However, the therapeutic effects of this drug is hampered due to intrinsic and acquired resistance^{4,5}. The mechanisms of chemoresistance in NSCLC is multifactorial and remain obscure^{6,7}.

Recent studies^{8,9} observed that dysregulated long non-coding RNA (lncRNA) and autophagy are also involved in regulation of chemoresistance in NSCLC. lncRNAs are non-protein coding transcripts longer than 200 nucleotides¹⁰. Growth arrest-specific transcript 5 (GAS5) is a well-recognized tumor suppressive lncRNA¹¹. GAS5 is usually downregulated in cancerous tissues and its down regulation was correlated with worse clinicopathological characteristics in NSCLC^{11,12}. GAS5 overexpression increased tumor cell growth arrest and induced apoptosis both *in-vitro* and *in-vivo* and can also enhance the effect of some chemotherapeutic reagents in NSCLC^{12,13}. However, the association between GAS5 dysregulation and chemoresistance and the downstream regulation of GAS5 in NSCLC is not quite clear.

Autophagy is a highly conserved process involving destruction and recycling of cellular components, which is important for maintaining cellular hemostasis¹⁴. The association between autophagy and chemoresistance was observed in several types of cancer, such as prostate cancer¹⁵, breast cancer¹⁶ and gastric cancer¹⁷. Recent reports^{8,18} revealed that enhanced autophagy confers increased chemoresistance in NSCLC. Enhanced autophagy acts as a protective mechanism to NSCLC cells after cisplatin incubation under both normoxia and hypoxia⁸. However, how autophagy

is dysregulated in NSCLC has not been fully revealed.

In this study, we investigated the association between GAS5 and cisplatin (DDP) resistance in NSCLC and further studied the regulative effect of GAS5 on autophagy and DDP resistance.

Patients and Methods

Tissue Collections

The study design was approved by the Ethics Committee of Henan Provincial People's Hospital, China. 15 patients with stage IIIA NSCLC and underwent primary surgical resection were recruited from the Department of Thoracic tumor surgery of the hospital. All patients signed an informed consent form. All patients received four cycles of cisplatin-based neoadjuvant chemotherapy before surgery. After completion of the chemotherapy, the patients' responses were evaluated. Then, the patients received surgical resection of the tumors. The tumor and adjacent normal tissue samples collected during the surgery were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Cell Culture and Treatment

Immortalization of human bronchial epithelial cell line HBE and human NSCLC cell lines, including H1650, H1299, H1975 and A549 and the cisplatin resistance A549/DDP cells were all purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI 1640 culture supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a humidified incubator with 5% CO₂.

GAS5 siRNA and the scramble negative control were synthesized by Ribobio (Shanghai, China). pCDNA3.1-GAS5 expression was generated by inserting the full-length GAS5 sequence (NR_002578.2) into the NheI and BamHI sites of a pCDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). A549 cells were transfected with 100 nM GAS5 siRNA, while A549/DDP cells were transfected with pCDNA3.1-GAS5 expression vector. All transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

GFP-LC3 Puncta Formation Assay

A549 cells and A549/DDP cells were seeded in 6-well plates at a density of 6×10^5 cells/well over-

night. Then the cells were transiently transfected with GFP-LC3 Expression Vector using Lipofectamine LTX Reagent (Invitrogen, Carlsbad, CA, USA). In the case of GAS5 si-RNA or GAS5 overexpression, the cells were firstly transfected with si-RNAs or GAS5 expression vectors 24 hours before transiently transfection of GFP-LC3 Expression Vector. 36 hours later, autophagy level was assessed by counting the GFP-LC3-positive puncta under a fluorescence microscope and Western blot analysis of LC3B expression.

qRT-PCR Analysis of GAS5 Expression

Total RNA in the frozen tissue or cultured cells were extracted using TRIZOL reagent (Invitrogen). Then, cDNA was synthesized using a reverse transcription kit (Takara, Dalian, China) according to manufacturer's instruction. GAS5 expression was detected using qRT-PCR with gene-specific primers: GAS5, forward, 5'-CTTGC-CTGGACCAGCTTAAT-3', reverse, 5'-CAAGC-CGACTCTCCATACCT-3' and SYBR Premix Ex Taq II (TaKaRa). GAPDH was used as endogenous control. The PCR reactions were conducted in the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The relative expression of GAS5 expression was calculated using the $2^{-\Delta\Delta CT}$ method relative to GAPDH.

Drug Sensitivity Test

A549 and A549/DDP cells after indicating treatments were seeded in a 96-well plate. After 24 hours incubation, the cells with treated with varying concentrations of DDP (0, 0.25, 0.5, 1, 5, 10 and 15 or 0, 1, 5, 10, 20, 40 and 80 μ M) for 48 hours. Then, cell viability was measured using a conventional MTT (Sigma-Aldrich, St. Louis, MO, USA) assay. Absorbance was recorded at 490 nm using a microplate reader. IC₅₀ value was determined by creating dose-response curves.

Western Blot Analysis of LC3B Expression

Cell samples were firstly lysed using a RIPA lysis buffer (Beyotime, Shanghai, China). Then, protein concentration was determined using a protein assay kit (Beyotime). The protein samples were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and then transferred onto 0.22- μ m nitrocellulose membranes (Sigma-Aldrich, St. Louis, MO, USA). Primary antibodies used included anti-LC3B (ab51520, 1:3000, Abcam, Cambridge, UK) and anti- β -actin (1:2000, ab8227, Abcam). Protein bands were de-

tected using an ECL chromogenic substrate (Bio-Rad, Hercules, CA, USA). The relative LC3-I and LC3-II level was analyzed using densitometry (Quantity One Software, Bio-Rad).

Flow Cytometry Analysis of Cells with Active Caspase-3 Expression

A549 cells with GAS5 suppression and A549/DDP cells with GAS5 overexpression were treated with DDP (10 $\mu\text{g/mL}$) for 24 hours. In the cases of inhibition, 3-Methyladenine (3-MA) (Sigma-Aldrich, 5 $\mu\text{mol/L}$) were added 1 hours before DDP treatment, for a duration of 24 hours. Caspase-3 activation in the cells was evaluated by flow cytometry using a human Active Caspase 3 FITC Staining Kit (ab65613, Abcam) according to manufacturer's instruction in a FACSCaliber (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0. The difference between groups was evaluated by unpaired, two-tailed Student t-test. $p < 0.05$ indicates statistical significance.

Results

GAS5 Downregulation is Associated with Cisplatin Resistance in NSCLC

Previous studies reported that GAS5 acts as a tumor suppressor in NSCLC. In this study, by performing qRT-PCR analysis, we also observed that the NSCLC tissues had significantly lower GAS5 expression than the adjacent normal tissues (Figure 1A). According to the responses after the completion of neoadjuvant chemotherapy, the patients could be divided into three groups, including 5 cases of pro-

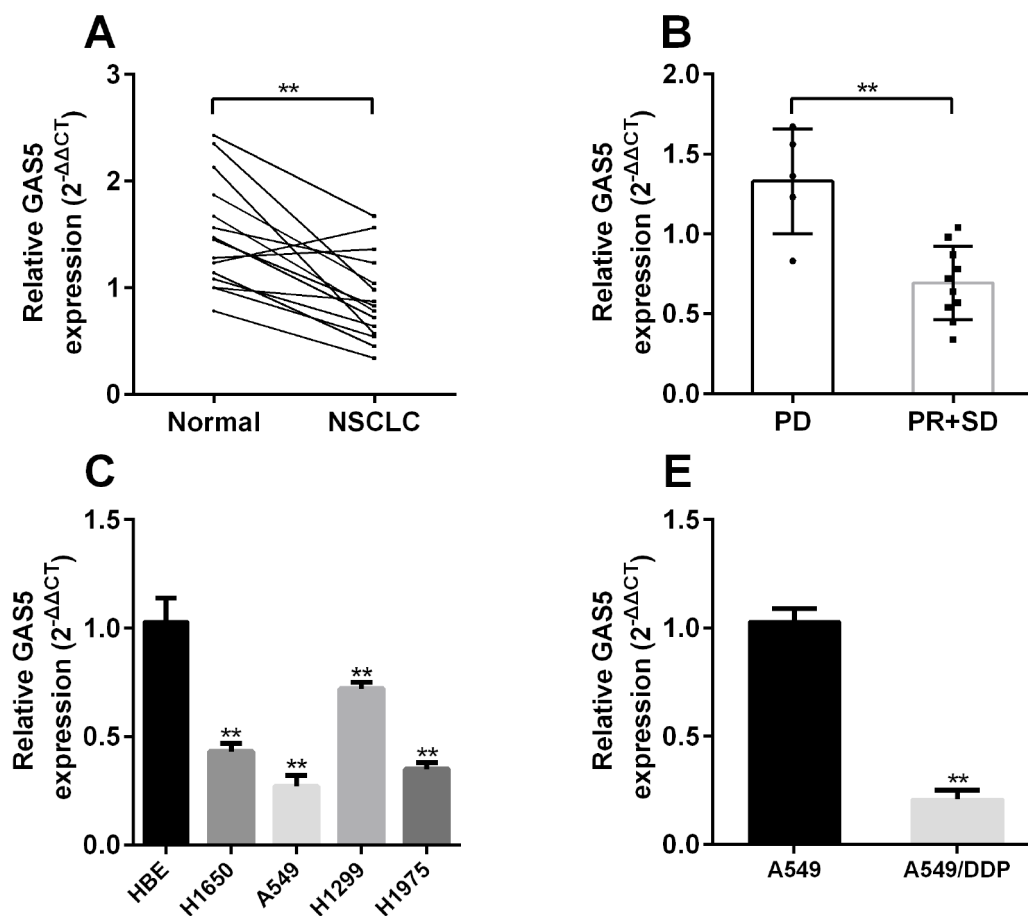


Figure 1. GAS5 downregulation is associated with cisplatin resistance in NSCLC. **A**, QRT-PCR analysis of GAS5 expression in the 15 cases of NSCLC tissues and adjacent normal tissue. **B**, Comparison of relative GAS5 expression in NSCLC tissues between patients with PD (n=5) and patients with PR or SD (n=10). **C**, QRT-PCR analysis and comparison of GAS5 expression in HBE, H1650, H1299, H1975 and A549 cells. **D**, QRT-PCR analysis and comparison of GAS5 expression in cisplatin sensitive A549 cells and cisplatin resistant A549/DDP cells. ** $p < 0.01$.

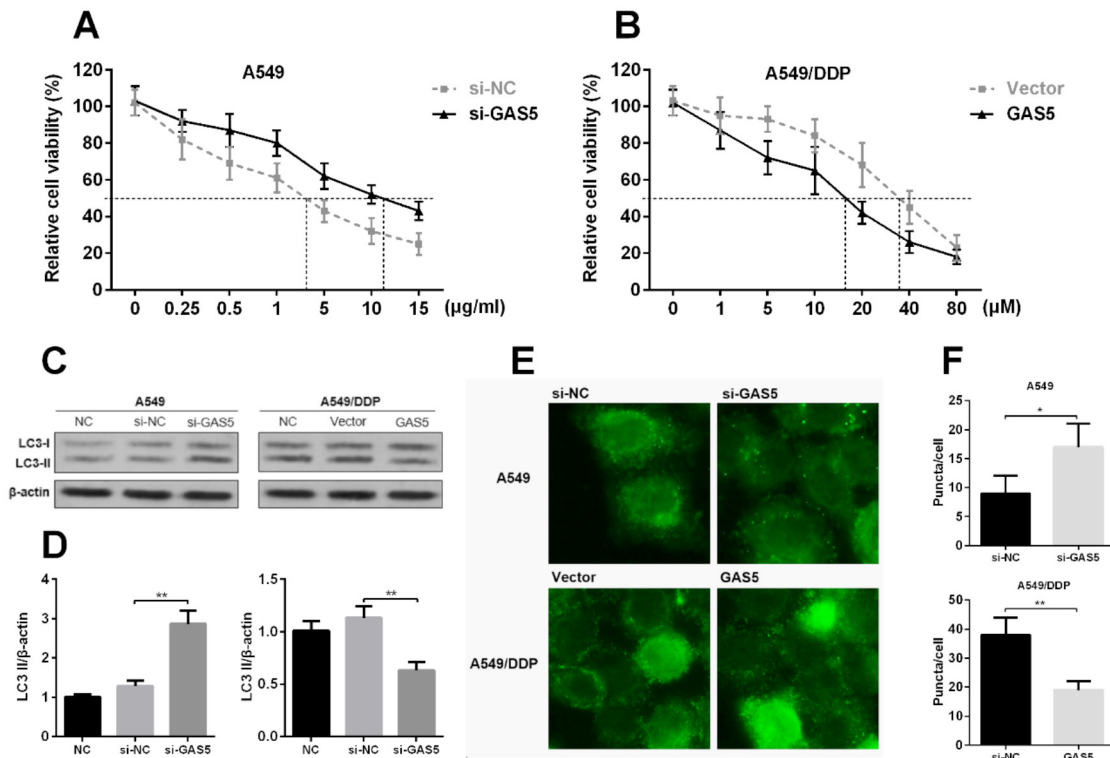


Figure 2. GAS5 modulates cisplatin sensitivity and autophagy in NSCLC cells. **A-B**, MTT assay of viability of A549 cells with or without transfection of GAS5 si-RNA (100 nM) (A) and A549/DDP cells with or without transfection of GAS5 expression vector (B) after treatment with varying concentrations of DDP for 48 hours. **C-D**, Western blot analysis (C) and quantification (D) of relative LC3-I and LC3-II expression in A549 cells and in A549/DDP cells with indicating treatment in figure A-B. **E-F**, Representative images (E) and quantification (F) of LC3-GFP puncta accumulation in A549 cells and in A549/DDP cells with indicating treatment in figure A-B. ** $p < 0.01$.

gressive disease (PD), 6 cases partial remission (PR) and 4 cases of stable disease (SD). By grouping the patients according to their responses, we found that the patients with PD had even lower GAS5 expression than those with SD and PR (Figure 1B). By comparing GAS5 expression between NSCLC cell lines and human bronchial epithelial cell line HBE cells, we found that H1650, H1299, H1975 and A549 cells all had significantly lower GAS5 expression than HBE cells (Figure 1C). The cisplatin-resistant A549/DDP cells had substantially lower GAS5 expression than the parent A549 cells (Figure 1D). These results suggest that GAS5 downregulation is associated with cisplatin resistance in NSCLC. A549 cells and A549/DDP cells were further used as an *in-vitro* model to investigate the association between GAS5 dysregulation and cisplatin resistance.

GAS5 Modulates Cisplatin Sensitivity and Autophagy in NSCLC Cells

To further investigate the regulative effect of GAS5 on cell viability of A549 and A549/DDP

cells, A549 cells with GAS5 knockdown and A549/DDP cells with GAS5 overexpression were treated with varying concentration of DDP. MTT assay showed that GAS5 knockdown increased DDP IC₅₀ of A549 cells (Figure 2A), while GAS5 overexpression decreased DDP IC₅₀ of A549/DDP cells (Figure 2B). Then we investigate whether GAS5 modulates autophagy in the cells. By detecting LC3-I and LC3-II levels in A549 with or without GAS5 knockdown and in A549/DDP cells with or without GAS5 overexpression, we observed that A549/DDP cells had significantly higher basal autophagy than A549 cells (Figure 2C-D). In addition, GAS5 knockdown resulted in decreased autophagy in A549 cells, while GAS5 overexpression led to increased autophagy in A549/DDP cells (Figure 2C-D). By observing LC3-GFP puncta formation in A549 and A549/DDP cells, we also observed that GAS5 knockdown resulted in increased number of LC3-GFP puncta in A549 cells (Figure 2E-F), while GAS5 overexpression led to decreased number of LC3-

GFP puncta in A549/DDP cells (Figure 2E-F). These results further confirmed that GAS5 can modulate autophagy in NSCLC cells.

GAS5 Modulated Autophagy is a Mechanism Modulating Cisplatin Sensitivity in NSCLC Cells

Previous studies observed that autophagy is an important mechanism of chemoresistance in NSCLC cells^{13,19,20}. Therefore, we investigated whether GAS5 modulated autophagy is a mechanism modulating cisplatin sensitivity in NSCLC cells. In A549 cells, we observed that treatment with 3-MA, an autophagy inhibitor, significantly decreased DDP IC₅₀ (Figure 3A). In addition, 3-MA also partly reversed the effect of GAS5 knockdown on increasing DDP IC₅₀ (Figure 3A). In A549/DDP cells, GAS5 showed the similar effect as 3-MA in reducing DPP IC₅₀ and also presented synergic effect with 3-MA (Figure 3B). Then, we performed flow cytometric analysis to investigate DDP-induced cell apoptosis in the cells. In A549 cells, knockdown of GAS5 significantly decreased the ratio of the cells with active caspase-3, while treatment with 3-MA significantly increased the ratio (Figure 3C). 3-MA treatment significantly abrogated the protective effect of

GAS5 knockdown on cell apoptosis (Figure 3C). In A549/DDP cells, GAS5 overexpression showed the similar effect as 3-MA on increasing the ratio of cells with active caspase-3 (Figure 3D and E). In addition, it also had synergic effect with 3-MA (Figure 3D and E). These results suggest that GAS5 mediated autophagy is a mechanism modulating cisplatin sensitivity in NSCLC cells.

Discussion

The GAS5 gene locates at 1q25, which consists of 12 exons and 11 introns. The exons transcripts can be alternatively spliced to two possible mature lncRNAs (GAS5a and GAS5b), while the introns encode 10 box C/D snoRNAs²¹. A large number of studies reported that GAS5 acts as a tumor suppressor in several types of cancer, including Breast cancer²², prostate cancer²³, gastric cancer²¹ and lung cancer²¹. In NSCLC, previous studies reported that GAS5 was significantly downregulated in the cancerous tissues compared to the adjacent normal tissues¹². In addition, GAS5 decrease might also be correlated with tumor size and clinical stage of the NSCLC¹². Since GAS5 exerts a strong regulative effect on apoptosis and

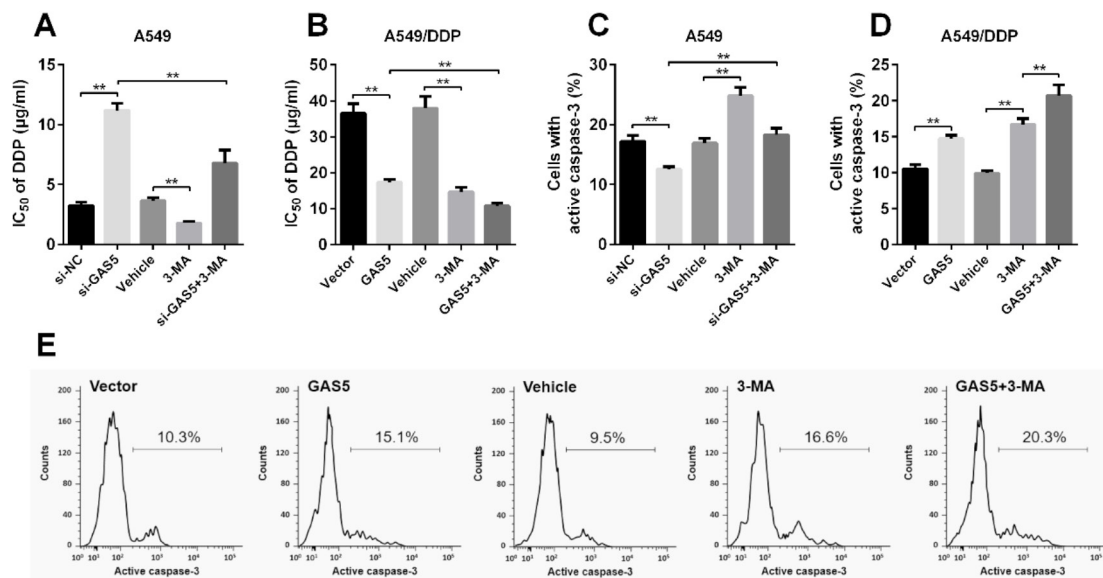


Figure 3. GAS5 modulated autophagy is a mechanism modulating cisplatin sensitivity in NSCLC cells. **A-B**, DDP IC₅₀ in A549 cells with GAS5 knockdown or 3-MA treatment alone or with GAS5 knockdown and 3-MA treatment in combination (A) and in A549/DDP cells with GAS5 overexpression or 3-MA treatment alone or with GAS5 overexpression and 3-MA treatment in combination (B). **C-D**, Quantification of A549 cells (C) and A549/DDP cells (D) with active caspase-3 after indicating treatment in figure A-B. **E**, Representative images of flow cytometric analysis of A549/DDP cells with active caspase-3 after indicating treatment in figure D. ***p*<0.01.

growth arrest in several mammalian cell lines^{22,24}, previous studies also studied its expression and regulation of chemosensitivity in NSCLC cells. One recent research⁹ investigated the regulative role of GAS5 in the resistance to the epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) in NSCLC. This work⁹ found that GAS5 expression was significantly higher in EGFR-TKI sensitive cell lines than in the resistant cell line. GAS5 overexpression reversed the gefitinib resistance in A549 cells and suppressed the growth of A549-derived tumors in nude mice treated with gefitinib⁹.

In this study, we also observed that the NSCLC tissues had a substantially lower expression of GAS5 than adjacent normal tissues. Also, we revealed that the chemoresistant NSCLC tissues had significantly lower GAS5 expression. A similar trend was also found in cisplatin sensitive A549 cells and cisplatin-resistant A549/DDP cells. GAS5 knockdown increased DDP IC50 of A549 cells, while GAS5 overexpression decreased DDP IC50 of A549/DDP cells. Therefore, we decided to further investigate its regulative effect on chemoresistance. Elevated autophagy is an important mechanism contributing to chemoresistance in NSCLC^{8,25,26}. Inhibition autophagy in NSCLC cells can directly increase cell apoptosis after cisplatin treatment^{27,28}. Actually, the mechanisms involved in autophagy regulation are quite complex in NSCLC. Since NSCLC is a type of solid tumor, the increased HIF-1 α expression can induce a higher level of autophagy, which acts a protective mechanism after cisplatin incubation⁸. MiR-24-3p can target ATG4A and modulates VP16-DDP resistance in NSCLC cells¹⁸. Interfering endogenous HMGB1 can inhibit cell autophagy and increase cell apoptosis of A549/DDP cells²⁸. One recent study²⁹ found that GAS5 can stimulate apoptosis and suppress autophagy in osteoarthritis chondrocytes via suppressing miR-21 induction. Therefore, we hypothesized that GAS5 might also modulate autophagy in NSCLC cells. By performing Western blot analysis of LC3B and observation of LC3-GFP puncta formation, we confirmed that A549/DDP cells had significantly higher basal autophagy than A549 cells. GAS5 knockdown resulted in decreased autophagy in A549 cells, while GAS5 overexpression led to increased autophagy in A549/DDP cells. Since autophagy is an important mechanism of chemoresistance in NSCLC cells^{13,19,20}, we investigated whether GAS5 modulated autophagy is a mechanism modulating cisplatin sensi-

tivity in NSCLC cells. By performing MTT assay of cell viability and flow cytometric analysis of caspase-3, we confirmed that GAS5 modulated autophagy is a mechanism modulating cisplatin sensitivity in NSCLC cells.

Conclusions

GAS5 downregulation is associated with cisplatin resistance in NSCLC. GAS5 can inhibit autophagy and, therefore, enhance cisplatin sensitivity in NSCLC cells.

Conflicts of interest

The authors declare no conflicts of interest.

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