

SiRNA interfering STAT3 enhances DDP sensitivity in cervical cancer cells

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Abstract. – **OBJECTIVE:** Signal transducer and activator of transcription 3 (STAT3) is an important regulatory protein in the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, which regulates cell proliferation, apoptosis, and other biological processes. It is closely related to tumor occurrence, progress, and resistance. Cisplatin (DDP) resistant cervical cancer cell line was established by shock induction to investigate the role of STAT3 in cervical cancer cells drug resistance.

PATIENTS AND METHODS: The expression of STAT3 in different chemotherapy response cervical cancer tissues was compared. The cervical cancer was divided into two groups upon STAT3 median level. Cervical cancer cell inhibitory rate by DDP treatment was compared. Western blot was used to detect STAT3 and phosphorylated STAT3 (p-STAT3) expressions in CaSki and CaSki/DDP cells. Cell apoptosis rate was tested by flow cytometry. CaSki/DDP cells were divided into small interfere RNA-normal control (siRNA-NC) group and siRNA-STAT3 group. Cell proliferation was evaluated by EdU staining.

RESULTS: The rate of STAT3 over-expression in cervical cancer patients with no significant chemotherapy response was markedly higher than that with a significant response. The inhibitory effect of DDP on tumor cells derived from patients with low STAT3 expression was significantly higher, while the apoptosis rate was apparently lower than that of CaSki/DDP cells from patients with high STAT3 expression. siRNA-STAT3 transfection significantly reduced the expressions of STAT3 and p-STAT3, decreased cell proliferation, and enhanced cell apoptosis in CaSki/DDP cells.

CONCLUSIONS: STAT3 over-expression is associated with DDP resistance in cervical cancer. Decreasing STAT3 can significantly promote the apoptosis of cervical cancer CaSki cells and decrease the DDP resistance.

Key Words:

STAT3, DDP, Cervical cancer, Drug resistance.

Introduction

Cervical cancer (CC) is one of the most common clinical tumors with the 4th leading mortality in China. The incidence of cervical cancer is second only to that of breast cancer, which ranks the second among female malignant tumor patients and the first among female genital malignancies. It poses a serious threat to the life and health of female patients^{1,2}. Due to the development of cervical cancer screening, the diagnostic rate of cervical cancer has also been greatly improved. Chemotherapy is an important adjuvant treatment in clinical practice in addition to surgery. However, because of the emergence of cervical cancer resistance, a considerable number of clinically accepted chemotherapy patients often cannot obtain satisfactory effect^{3,4}. Cisplatin (DDP) shows a positive effect in the treatment of cervical cancer. However, numerous patients exhibit varying degrees of resistance in DDP chemotherapy^{5,6}.

Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway protein is widely expressed in many tissues and cells of mammals. The JAK-STAT signaling pathway is widely involved in the regulation of biological processes, such as cell proliferation, apoptosis, migration, and invasion. It is associated with the occurrence, progression, distant metastasis, drug resistance, and prognosis of many cancers⁷⁻⁹, including lung cancer⁷, bowel cancer⁹, and prostate cancer⁸. STAT3 is the most important member of STAT protein family that belongs to the transcription factor. The abnormal expression or functional activity of STAT3 may mediate the over-activation of JAK-STAT3 signaling pathway, which is related to the drug resistance of many tumors¹⁰⁻¹². Fan et al¹³ observed that compared with the parental cervical cancer cells, STAT3 expression was significantly increased in

drug-resistant cervical cancer cells, suggesting that STAT3 abnormalities may be related to the resistance of cervical cancer. In this study, we compared the expression of STAT3 in cervical cancer tissues with different chemo-sensitivity to explore the potential relationship between STAT3 and DDP resistance in cervical cancer and investigate whether STAT3 regulates the proliferation, apoptosis, and DDP resistance of cervical cancer cells *in vitro*.

Materials and Methods

Main Reagents and Instruments

Human cervical squamous carcinoma cells CaSki were purchased from BeNa Culture Collection (Jiangsu, China). Roswell Park Memorial Institute-1640 (RPMI-1640) medium, fetal bovine serum (FBS), and streptomycin were purchased from Gibco (Grand Island, NJ, USA). Rneasy MiNi Kit and QuantiTect SYBR Green Real-time PCR (RT-PCR) Kit were purchased from Qiagen (Hilden, Germany). Lipofectamine 2000 was purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). Rabbit anti-human p-STAT3 and STAT3 monoclonal antibodies were purchased from Abcam Biotechnology (Cambridge, MA, USA). Mouse anti-human β -actin polyclonal antibody, siRNA-NC, and siRNA-STAT3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibody was purchased from Bio-Rad Laboratories (Hercules, CA, USA). DDP was purchased from Qilu Pharmaceutical (Shandong, China). Cell Counting Kit-8 (CCK-8) detection kit was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Annexin V/PI apoptosis detection kit and caspase-3 activity detection kit were purchased from Beyotime Biotechnology (Shanghai, China). EdU staining cell proliferation detection kit was purchased from RiboBio (Guangzhou, China). BB15 CO2 cell incubator was purchased from Thermo Scientific Pierce (Rockford, IL, USA). Real-time PCR instrument was purchased from Bio-Rad Laboratories (Mode: CFX96, Hercules, CA, USA). Gallios flow cytometer was purchased from Beckman Coulter Inc. (Brea, CA, USA).

Clinical Information

Fifty-eight patients with cervical cancer underwent surgical resection in Jiangxi Maternal

and Child Health Hospital from August 2016 to January 2017 were enrolled. The mean age was 47.2 ± 14.5 years old. No patients received radiotherapy, chemotherapy, or immunotherapy before the operation. The specimens of cervical cancer tissue removed during the operation were collected. Among them, there were 19 cases of poorly differentiated carcinoma, 24 cases of moderately differentiated carcinoma, and 15 cases of well-differentiated carcinoma. According to TNM staging, there were 23 cases in stage II, 21 cases in stage III, and 14 cases of stage IV. Another 24 cases of normal cervical mucosa epithelial tissue from uterine fibroids patients who underwent hysterectomy were selected as control. The mean age of control was 44.6 ± 15.1 years old. No statistical difference was observed on age between two groups.

Cervical cancer tissues were collected and divided into significant chemotherapy response and no significant chemotherapy response according to the chemotherapy effect. Chemotherapy effects were evaluated according to World Health Organization (WHO) entity tumor response evaluation standard (RECIST1.1). The maximum tumor diameter reduced more than 30% or the product of the maximum diameter and vertical diameter reduced more than 50% after chemotherapy was judged as a significant response to chemotherapy, the rest were considered to be no significant response. The study protocol was approved by the Research Ethics Committee of Jiangxi Maternal and Child Health Hospital, and all patients gave their informed consent before study commencement.

CCK-8 Assay

The tumor tissue was prepared as cell suspension and seeded in 96-well plates at 2×10^4 cells per well. The cells were added to a final concentration of 10 mg/l DDP. The other with no DDP treated as control. After 48 h incubation, the cells were added with 20 μ l CCK-8 solution. After further 4 h incubation, the absorbance value of each well at 450 nm was measured. Inhibition rate = $(1 - \text{dosing group A450 value}) / \text{control group A450 value} \times 100\%$.

CaSki/DDP Resistant Cell Line Establishment

The CaSki cells were routinely cultured in RPMI 1640 medium containing 10% FBS and

1% penicillin-streptomycin. The cells were passaged at a ratio of 1:4. CaSki cells were treated with DDP at a starting concentration of 1 mg/l. The DDP was removed after 24 h, and the cell was continued cultured until the cells could grow stably in this concentration of DDP followed by a gradual increase at 2 mg/l, 4 mg/l, and 8 mg/l. The DDP concentration was increased until the cells grew stably in medium containing 10 mg/l DDP. At last, the DDP resistant cell line CaSki/DDP was obtained.

Resistance Index Determination

CaSki and CaSki/DDP cells were seeded in 96-well plates. After 24 h, the cells were treated with different concentrations of DDP. After 48h incubation, CCK-8 was added to detect the absorbance of each well at 450 nm. Inhibition rate = $(1 - \text{dosing group A450 value}) / \text{control group A450 value} \times 100\%$. The half maximal inhibitory concentration (IC50) calculator software was used to calculate the concentration of the drug required for 50% inhibition of cell growth. Resistance index (RI) = IC50 of CaSki/DDP cells/IC50 of CaSki cells.

Cell Transfection and Grouping

CaSki/DDP cells were divided into small interfere RNA-normal control (siRNA-NC) group and siRNA-STAT3 group. si-NC or si-STAT3 fragment and Lipofectamine 2000 were diluted by option minimum essential media (Opti-MEM) at room temperature for 5 min. Then, they were mixed and incubated at room temperature for 30 min. Next, the mixture was added to the cells and incubated for 6 h. After that, the medium was changed back to RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin. After 72 h incubation, the cells were digested for test.

qRT-PCR

Total RNA was extracted from tissues or cells by Rneasy MiNi Kit method and reverse transcribed into complementary DNA (cDNA) using QuantiTect SYBR Green RT-PCR Kit. The relative expression level of the gene was detected by quantitative RT-PCR (qRT-PCR). The 20 μ l qRT-PCR reaction system contained 10.0 μ l of 2 \times QuantiTect SYBR Green RT-PCR Master Mix, 1.0 μ l of 0.5 μ l/0.5 μ l primer, 2 μ l of template primer, 0.5 μ l of template RNA, 0.5 μ l of QuantiTect RT Mix, and ddH2O. The

reverse transcription reaction condition was at 50°C for 30 min. The PCR reaction conditions contained pre-denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s. The product was detected on a Real-time PCR machine.

Western Blot

Sodium dodecyl sulfate (SDS) was used to extract the cell proteins. After quantification, 40 μ g of protein was separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred to nitrocellulose (NC) membrane by at 100 V for 1 h and then blocked by 5% bovine serum albumin (BSA) in skim milk powder at room temperature. The membrane was incubated in primary antibody (p-STAT3 1:1000, STAT3 1:2500, and β -actin 1:10000, respectively) at 4°C overnight. Then, the membrane was incubated with horseradish peroxidase (HRP) conjugated secondary (1: 15000) for 1 h after phosphate buffered solution Tween-20 (PBST) washing. After performing the enhanced chemiluminescence (ECL), the membrane was scanned and saved for analysis.

Cell Apoptosis

The cells were digested and resuspended in Binding Buffer. Next, the cells were stained by 5 μ l Annexin V-FITC and 5 μ l propidium iodide (PI). At last, the cells were analyzed by flow cytometry.

EdU Staining

After digested by the enzyme, the cells were fixed by paraformaldehyde, neutralized by glycine, and permeabilized by Triton X-100. After stained by 500 μ l Apollo at room temperature for 10 min, the cells were analyzed by flow cytometry.

Caspase-3 Activity Detection

According to the instructions of caspase-3 activity detection kit, the pNA standard substance was prepared to make a standard curve. The cells were lysed on ice and the supernatant was transferred to a new pre-cooled EP tube. The sample was inserted to a 96-well plate and added with Ac-DEVD-pNA. After incubated at 37°C for 2 h, the sample was measured on a microplate reader for the absorbance value at 405 nm to reflect caspase-3 activity.

Statistical Analysis

All data analyses were performed on SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation and compared by *t*-test. *p*<0.05 was considered as statistical significance.

Results

STAT3 Expression Increased in Cervical Cancer Tissue

qRT-PCR showed that the expression of STAT3 mRNA in cervical cancer tissues was significantly higher than that in normal cervical mucosa (Figure 1A). STAT3 expression decreased following TNM upstage and tissue differentiation deterioration (Table I). Western blot demonstrated that the expressions of STAT3 and p-STAT3 protein in cervical cancer tissues were markedly higher than that in normal cervical mucosa epithelial tissues. Their levels in stage IV were markedly higher than in stage III, followed by stage II (Figure 1B).

The Relationship Between STAT3 Expression Before Chemotherapy and Chemotherapy Effect

There were 38.2% (13/34) patients with obvious chemotherapy response that showed high STAT3 expression before chemotherapy, while there were 66.7% (16/24) patients with no obvious chemotherapy response that exhibited high STAT3 expression before chemotherapy ($\chi^2=4.549, p=0.033$) (Table II).

According to the different STAT3 mRNA expression in tumor tissue, the median of STAT3 mRNA level was used as the boundary to divide the cervical cancer tissues into STAT3 high expression group and low expression group. The results showed that the cells derived from cervical cancer cells with high STAT3 expression exhibited lower sensitivity to DDP (Table III).

CaSki/DDP Cells Showed Reduced Sensitivity to DDP and Elevated STAT3 Expression

The IC50 of CaSki cells was 3.87 mg/L, while it was 52.98 mg/L in CaSki/DDP cells. The RI of CaSki/DDP cells to CaSki cells was 13.69. 3.87 mg/L DDP treatment significantly up-regulated Caspase-3 activity (Figure 2A) and promoted apoptosis in CaSki cells (Figure 2B). However, it showed little effect on caspase-3 activity and apoptosis in CaSki/DDP cells (Figure 2A, B). qRT-PCR demonstrated that STAT3 mRNA expression in CaSki/DDP cells was markedly higher than that in CaSki cells (Figure 2C). Western blot revealed that STAT3 and p-STAT3 protein expressions in CaSki/DDP cells was significantly increased compared with CaSki cells (Figure 2D).

siRNA-STAT3 Transfection Decreased DDP Resistance in CaSki/DDP Cells

qRT-PCR showed that the expression of STAT3 mRNA in CaSki/DDP cells was significantly decreased compared with the siRNA-NC group (Figure 3A). Western blot demonstrated that siRNA-STAT3 transfection significantly downregulated STAT3 and p-STAT3 expressions (Figure 3B). EdU staining revealed that siRNA-STAT3 transfection apparently inhibited CaSki/DDP cell proliferation (Figure 3C). Flow cytometry exhibited that siRNA-STAT3 transfection significantly promoted CaSki/DDP cells apoptosis and decreased drug resistance (Figure 3D).

Discussion

Human papillomavirus (HPV) infection is an important causative factor in the pathogenesis of cervical cancer. In recent years, with the increase of HPV infection rate, the incidence of cervical cancer is increasing year by year¹⁴⁻¹⁶. Early sta-

Table I. The relationship of STAT3 mRNA expression with TNM stage and tissue differentiation.

Group	Cases	miR-214 expression	p-value
Tissue differentiation			<0.001
Poor	19	3.03±0.19	
Moderate	24	2.28±0.14	
Well	15	1.73±0.10	
TNM stage			<0.001
Stage II	23	1.69±0.11	
Stage III	21	2.39±0.17	
Stage IV	14	2.88±0.21	

Table II. The relationship between STAT3 expression before chemotherapy and chemotherapy effect.

Group	High expression cases (%)	χ^2 -test	<i>p</i> -value
Obvious chemotherapy response group	13 (38.2%)	4.549	0.033
No obvious chemotherapy response group	16 (66.7%)		

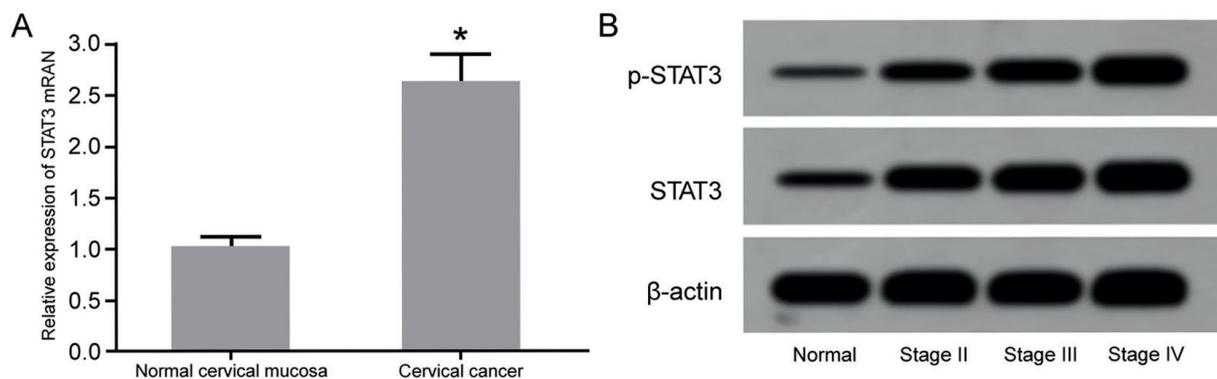
Table III. The relationship between STAT3 expression and DDP inhibitory rate.

Group	Inhibitory rate (%)	<i>t</i> -test	<i>p</i> -value
High expression	39.6±14.9	2.543	0.007
Low expression	51.3±19.8		

ge cervical cancer is mainly treated by surgery. However, most patients are in the advanced stage when admitted to the hospital, thus missing the best timing of treatment. Recently, the role of radiotherapy and chemotherapy in the treatment of cervical cancer has gradually been taken seriously. Therefore, concurrent chemoradiation after surgery is the main treatment for patients with advanced cervical cancer. DDP is a platinum complex that produces antitumor effects by disrupting the structure of DNA. In the neoadjuvant chemotherapy regimen of cervical cancer, DDP is one of the most widely used chemotherapeutic drugs. The DDP-based chemotherapy regimen is the first-line of advanced cervical cancer³. In clinical treatment, there are some differences in the DDP chemotherapy sensitivity of cervical cancer patients. A considerable number of patients cannot benefit from DDP chemotherapy and show resistance, therefore easily leading to the distant and relapse of cervical cancer, which is an impor-

tant limiting factor affecting the efficacy, survival, and prognosis^{5,6}.

There are many abnormalities in the process of drug resistance, which refers to multi-pathway, multi-mechanism, and multi-step. It was found that various pathways participate in the chemotherapy resistance of cervical cancer, including PI3K/AKT¹⁷, Wnt/ β -catenin¹⁸, and MAPK¹⁹. The JAK-STAT signaling pathway is composed of the JAK kinase and STAT family proteins. At present, four JAK kinases have been found, including JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2), all of which are activated by tyrosine phosphorylation. The JAK-STAT signaling pathway can respond to a variety of signal pathways, such as extracellular growth factor and mitogen signal stimuli. Cell membrane receptor may dimerize by the stimulus of JAK-STAT signaling pathway activation, thus activates JAK kinase to phosphorylate the receptor tyrosine. It further promotes STAT substitute to the tyrosine

**Figure 1.** STAT3 expression increased in cervical cancer tissue. **A**, qRT-PCR detection of STAT3 mRNA expression. **B**, Western blot detection of STAT3 and p-STAT3 protein expressions. **p*<0.05.

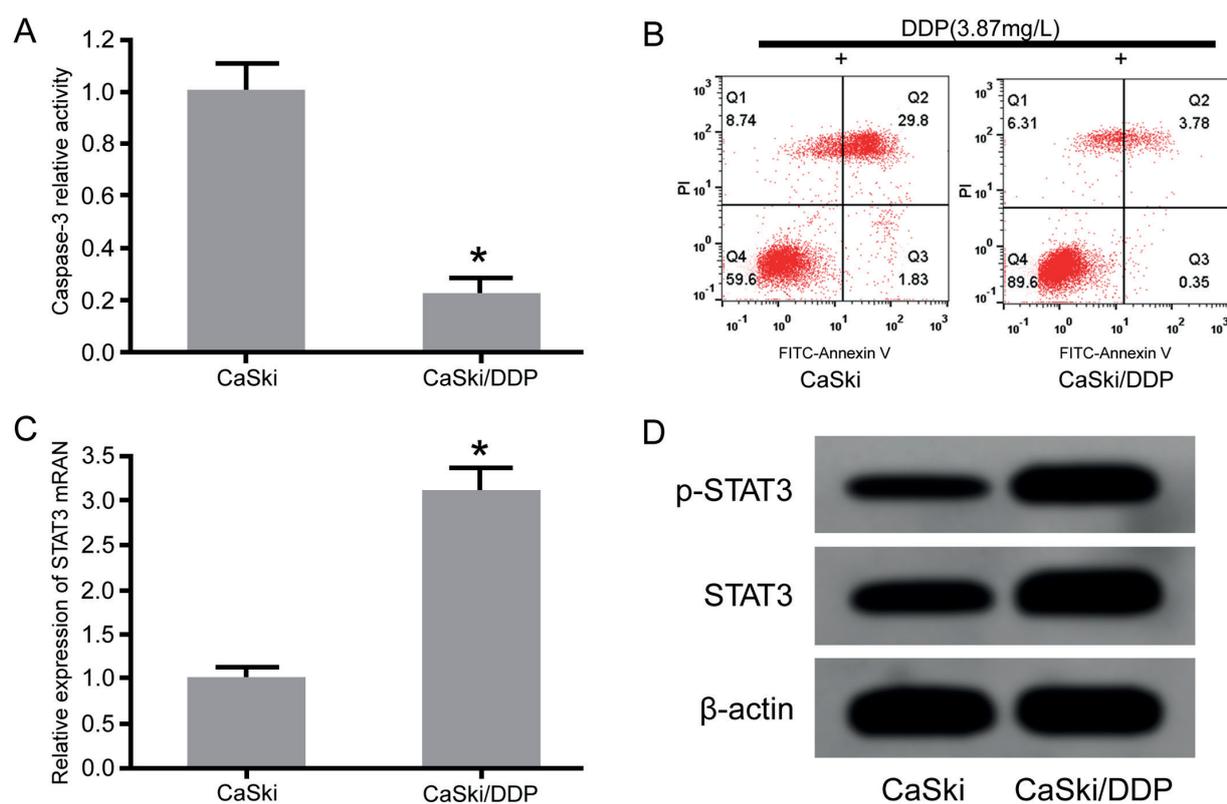


Figure 2. CaSki/DDP cells showed reduced sensitivity to DDP and elevated STAT3 expression. **A**, Spectrophotometry detection of caspase-3 activity. **B**, Flow cytometry detection of cell apoptosis. **C**, qRT-PCR detection of STAT3 mRNA expression. **D**, Western blot detection of STAT3 and p-STAT3 protein expression. * $p < 0.05$.

phosphorylation site of the receptor complex via the SH2 domain. At this time, JAK kinase phosphorylates the STAT protein near its spatial location. Upon activation, STAT separates from the receptor complex to form dimers and transport from the cytoplasm to the nucleus, thereby promoting cell proliferation, survival, and apoptosis regulation related gene transcription and expression²⁰. STAT family contained seven members as STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6, of which STAT3 is the most widely studied and most closely related to the tumor. Fan et al¹³ showed that compared with the parental cervical cancer cells, STAT3 expression was significantly increased in drug-resistant cervical cancer cells, suggesting that STAT3 abnormalities may be related to the resistance of cervical cancer. This study investigated whether STAT3 plays a role in regulating proliferation, apoptosis, and DDP resistance in cervical cancer cells.

Our results revealed that STAT3 expression was significantly elevated in cervical cancer tissue compared with normal cervical mucosa with cell differentiation and TNM staging dependence,

suggesting that STAT3 abnormal over-expression was not only related to the incidence of cervical cancer, but also to the degree of malignancy. Moreover, it was found that the high STAT3 expression before chemotherapy proportion of patients with no obvious chemotherapy response was markedly higher than that in patients with obvious chemotherapy response, indicating that the abnormal expression of STAT3 may be involved in the DDP resistance of cervical cancer patients. To further confirm the relationship between STAT3 and DDP resistance in cervical cancer, we separated cervical cancer tissues with high and low expressions of STAT3 and cultured the cells *in vitro*. It was demonstrated that the inhibitory effect of DDP was markedly stronger to tumor cells derived from patients with low STAT3 expression than that of tumor cells derived from patients with high STAT3 expression, thus confirming that STAT3 plays a role in the regulation of DDP resistance in cervical cancer.

In vitro investigation showed that the expressions of STAT3 and p-STAT3 in DDP-resistant CaSki/DDP cells were significantly higher than

that in parental CaSki cells. Apoptosis was significantly reduced in DDP resistant cells treated by DDP. Fan et al¹³ observed that the expression of STAT3 in HeLa/PR and CaSki/PR cells was significantly higher than that of HeLa and CaSki cells. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a commonly used anti-tumor drug. Nakamura et al²¹ showed that TRAIL treatment markedly inhibited STAT3 activity in TRAIL-sensitive Caski cells but exhibited no effect on STAT3 in TRAIL-resistant Siha cells, indicating that STAT3 plays a key role in maintaining the resistance of cervical cancer cells. Hu et al²² demonstrated that Erbin loss of function significantly enhanced STAT3 phosphorylation and led to the decrease of anoikis in cervical cancer cells. However, the application of JAK2/STAT3 pathway inhibitor WP1066 treatment obviously promoted cervical cancer cells anoikis and reduced the survival rate. Since anoikis is closely related to the distant metastasis and drug resistance of tumor cells, it is speculated that STAT3 may be

related to anoikis and drug resistance of cervical cancer. In this study, the expression and activity of STAT3 in cervical cancer cells were significantly increased, which may be associated with the resistance of cervical cancer.

Further test revealed that siRNA down-regulating STAT3 expression in CaSki/DDP cells markedly elevated sensitivity to DDP, accelerated cell apoptosis, and attenuated cell proliferation. Nakamura et al²¹ found that siRNA interference or small molecule inhibition of STAT3 apparently enhanced TRAIL-resistant Siha cell apoptosis and reduced drug resistance. Fan et al¹³ demonstrated that down-regulation of STAT3 expression can promote cervical cancer cell apoptosis and reduce the resistance of cervical cancer cells to paclitaxel and DDP. Han et al²³ showed that piperine (PP) treated mitomycin C-resistant cervical cancer cells significantly attenuated STAT3 activity, decreased anti-apoptotic factors Bcl-2 expression, and elevated cell apoptosis. Lirdrapamongkol et al²⁴ revealed that Chrysin can

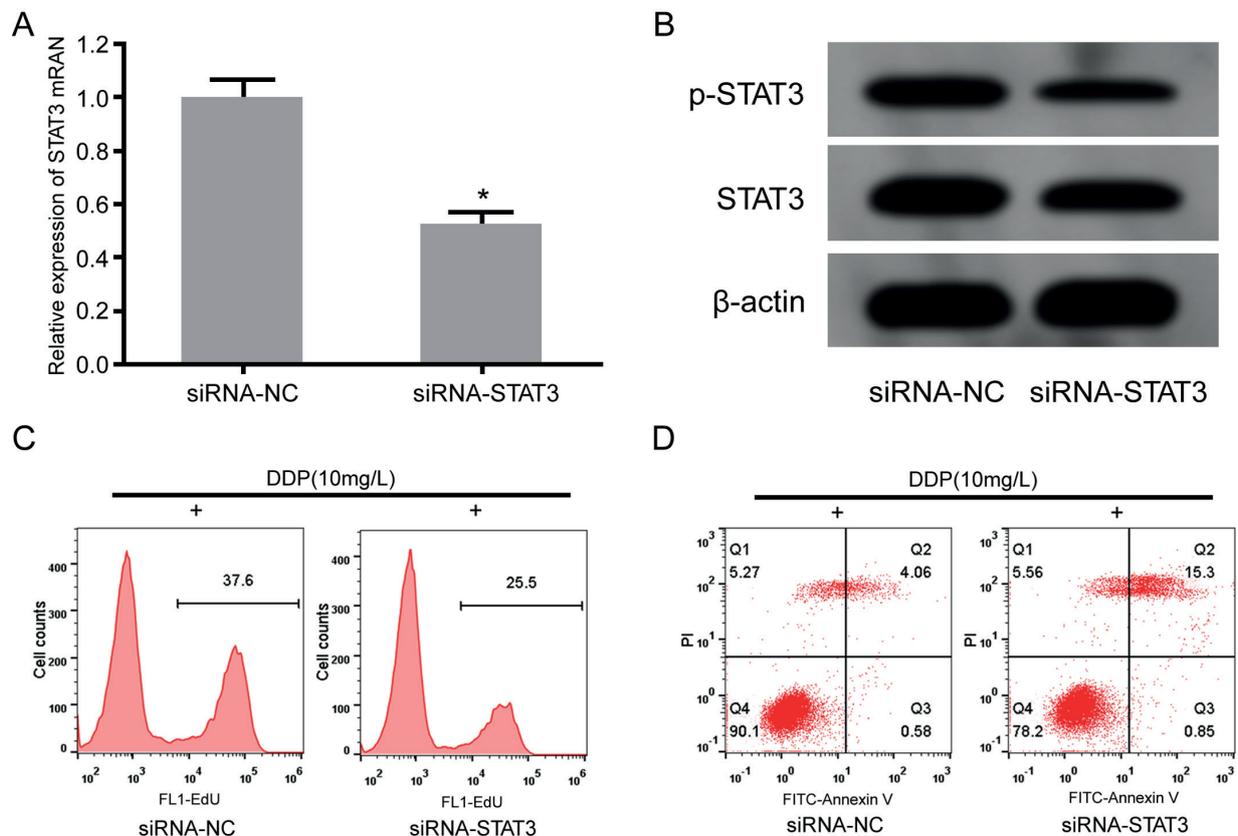


Figure 3. SiRNA-STAT3 transfection decreased DDP resistance in CaSki/DDP cells. **A**, qRT-PCR detection of STAT3 mRNA expression. **B**, Western blot detection of STAT3 and p-STAT3 protein expressions. **C**, EdU staining detection of cell proliferation. **D**, Flow cytometry detection of cell apoptosis. * $p < 0.05$.

inhibit Mcl-1 expression, promote cell apoptosis, and enhance sensitivity to TRAIL in HeLa cells by down-regulating STAT3 activity. At present, there is still lack of report about the relationship between STAT3 and DDP resistance in cervical cancer. This work adopted siRNA to interference STAT3 expression, thus directly confirming that STAT3 is associated with DDP resistance in cervical cancer. This study did not investigate the role of STAT3 in the regulation of DDP resistance in cervical cancer in clinical patients, which is a limitation of the study.

Conclusions

We showed that the STAT3 abnormal expression was associated with DDP resistance in cervical cancer. Down-regulating STAT3 can significantly promote cervical cancer CaSki cell apoptosis and decrease DDP resistance.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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