# Targeting Smoothened Sensitizes Gastric Cancer to Chemotherapy in Experimental Models

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: The Hedgehog pathway receptor smoothened (SMO) has critical roles in tumor progression. However, whether SMO is a key factor regulating gastric cancer chemotherapy resistance is unknown.

Material/Methods: We investigated the potential functions of SMO in inducing gastric cancer paclitaxel resistance in clinical samples, gastric cancer cell lines (424GC and AGS), and subcutaneous syngeneic mouse models.

Results: We found high SMO expression in paclitaxel-resistant gastric cancer clinical samples. Paclitaxel gastric cancer cells had higher SMO expression than in drug-sensitive cells. Upregulating SMO expression induced paclitaxel resistance in gastric cells lines via enhancing cell proliferation and inhibiting apoptosis. The combination of IPI-926, an inhibitor of SMO, with paclitaxel decreased cell viability of paclitaxel-resistant gastric cancer cells *in vitro* and controlled tumor growth in animal models.

Conclusions: The Hedgehog pathway receptor SMO is an important regulator of gastric cancer paclitaxel resistance and could be a target for sensitizing paclitaxel-resistant tumors.

MeSH Keywords: **Antineoplastic Agents • Drug Resistance • Hedgehog Proteins • Stomach Neoplasms**

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## Background

Worldwide, gastric cancer is the fifth most common cancer, with more 952 000 cases diagnosed in 2012 [1]. Gastric cancer is difficult to cure unless it is found at an early stage. However, because early-stage gastric cancers cause few symptoms, the disease is usually detected in late stages [2]. Treatments for advanced gastric cancer include surgery, chemotherapy, and radiation therapy [3]. Even though multiple chemotherapy plans were developed, gastric cancer only responds moderately to chemotherapeutic drugs [4]. Therefore, understanding the mechanisms of gastric cancer-resist chemotherapy is the basis for developing novel treatment strategies.

The Hedgehog signaling pathway has roles in embryonic cell differentiation and cancer cell proliferation [5]. The major receptor of the Hedgehog signaling pathway is smoothened (SMO), a protein that is encoded by *SMO* gene [5]. Stimulation of the patched receptor by the sonic Hedgehog ligand leads to translocation of SMO, followed by accumulation of transcriptional factor Gli in the nucleus [5]. In basal-cell carcinomas, it was found that abnormal activation of SMO via mutation could promote cell transformation and serve as an oncogene [6]. Further studies in different cancer models indicated that downregulating SMO expression alleviated liver tumor cell invasion and taxane resistance in ovarian cancer [7,8]. However, the functions of SMO in gastric cancer progression and drug resistance are still unclear.

While paclitaxel is a fundamental drug in gastric cancer treatment, resistance to paclitaxel happens rapidly after an initial response. Because the Hedgehog signaling pathway induces drug resistance in several types of tumors [7–10], we investigated the significance of SMO, a key protein of the Hedgehog pathway, in regulating gastric cancer paclitaxel resistance. Specifically, we hypothesized that paclitaxel-resistant gastric cancer has high SMO expression; therefore, Hedgehog pathway inhibitors would be suitable for use against SMO-induced paclitaxel-resistant tumors.

## Material and Methods

### Cell culture and viability assay

Murine gastric cancer cell line 424GC (obtained from Beijing Institute for Tumor Prevention and Treatment, China) and human gastric cell line AGS (Sigma-Aldrich, USA) were used in this study. The 424GC cell line was cultured with RPMI1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, non-essential amino acids, and 10% fetal bovine serum (FBS). AGS cell line was cultured in DMEM medium with 10% FBS. A final concentration of 100 U/mL penicillin and 100 µg/mL

streptomycin was used to control cell culture contamination. All the cell lines were maintained in a humidified incubator with 5% $CO_2$ at 37°C. Subculture was performed when the cells were 80–100% confluent. Paclitaxel-resistant cell lines (424GC-R and AGS-R) were induced by treating these cell lines with 100 nM paclitaxel in the medium for 1 month. After drug treatments, cell lines were subjected to test viability using the CCK-8 kit (Sigma-Aldrich, USA), following the manufacturer's procedures.

### Patient samples

A total of 54 gastric cancer patients diagnosed between Oct 2008 to Sep 2011 in Tianjin Hospital were included in this study. Informed consent was signed by each patient. This study was approved by the Ethics Committee of Tianjin Hospital. The archived formalin-fixed, paraffin-embedded (FFPE) primary tumor tissues obtained during the surgery of these patients were collected. All of these patients accepted paclitaxel treatment after the surgery, but none of them accepted chemotherapy or radiotherapy before the surgery.

### Animal model

A paclitaxel-resistant mouse model was established using the 424GC-R cell line and 5-week-old male C57BL/6 mice (18–20 g, Chinese Academy of Science, Shanghai, China). The cell suspension with $5 \times 10^5$ cells was injected subcutaneously into the flank of each mouse. Treatments were started on the 7th day after the injection. The mice were randomly distributed to 4 groups (10 in each group) to receive different treatments: paclitaxel (20 mg/kg/3 days); Hedgehog pathway inhibitor (IPI-926, 20 mg/kg/week); paclitaxel and IPI-926; and saline. The tumor volume (width$^2$×length×π/6) of each mouse were recorded every 5 days. The animal study was approved by the Experimental Animal Use and Care Committee of Tianjin Hospital.

### Enzyme-linked immunosorbent assay

The amount of BrdU and activated caspase-3 in the tumor cells and tissue were measured by using an enzyme-linked immunosorbent assay (ELISA) kit (Thermo Fisher Scientific, USA) following the manufacturer's procedures. The total protein amount was measured and normalized by BCA assay.

### Cell transfection

Mouse SMO overexpression lentivirus vector, shRNA lentivirus vector, and control vector were purchased from Origene (Rockville, MD, USA). We transfected 293 cells with either overexpression, shRNA, or control vector for producing lentivirus to infect 424GC cells. The lentivirus particles were used for infecting 424GC cells. Expression of SMO were measured by Western blot analysis in each group.
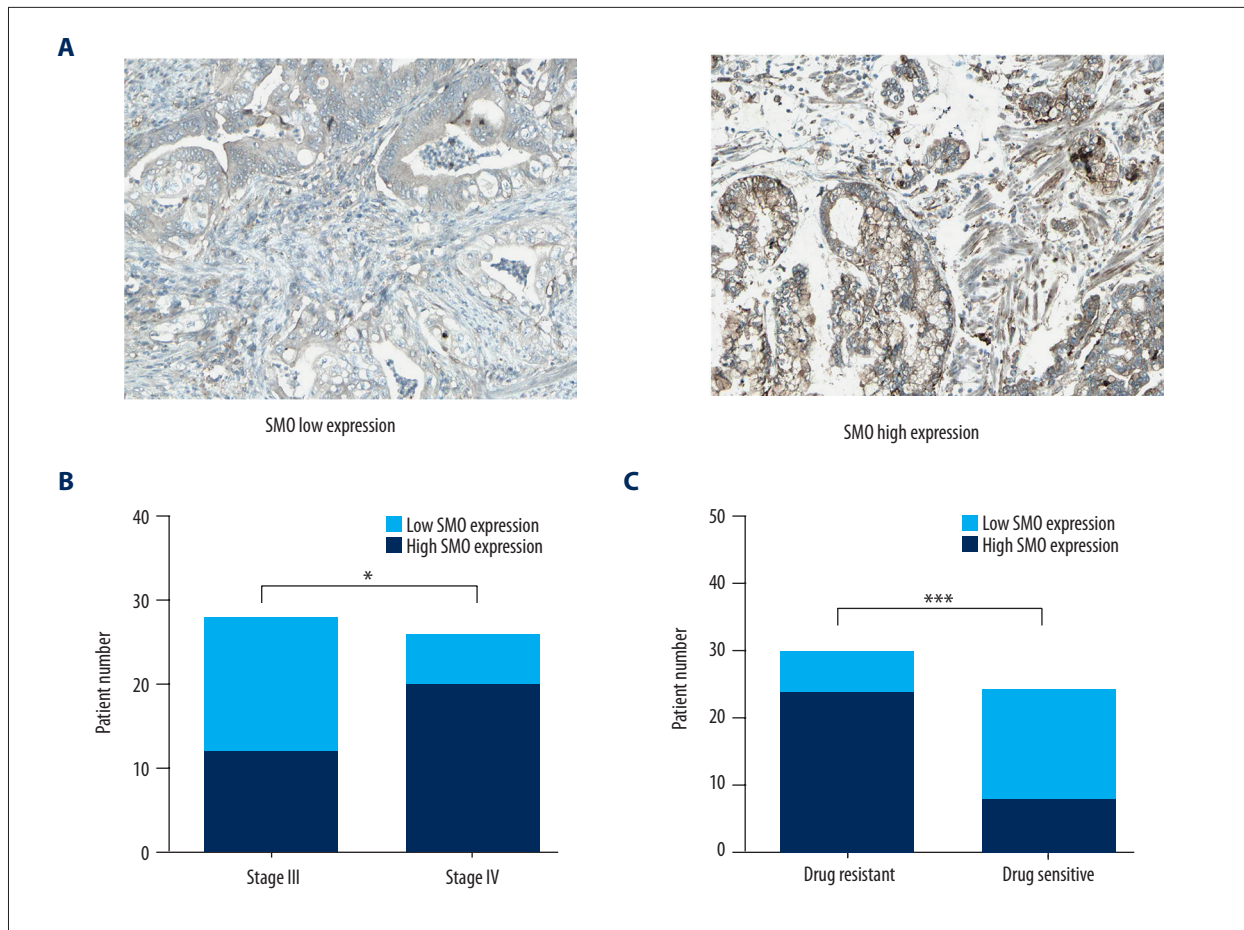
**Figure 1.** Expression of SMO protein in the tumor tissue of gastric cancer patients. (**A**) Representative images of IHC-stained human gastric cancer tissues with high SMO expression and low SMO expression, respectively. (**B**) The stage-IV patients tended to have high SMO expression, while the stage III patients tended to have low SMO expression. (**C**) The relationship between paclitaxel resistance and SMO expression level in gastric cancer patients. The patients who were resistant to paclitaxel treatment tended to have high SMO expression, but the patients who were sensitive to the paclitaxel treatment tended to have low SMO expression.

## Western blotting assay

The expression of SMO in the gastric cell lines (424GC, 424GC-R, AGS, and AGS-R) was measured by Western blotting assay. The cells were collected and lysed by RIPA buffer with proteinase inhibitor and phosphatase inhibitor. The total protein amount was measured and normalized by BCA assay. A standard procedure of Western blot assay was followed. The total protein was separated by sulfate-polyacrylamide gel electrophoresis. Then, the proteins were transferred to a PVDF membrane. The SMO protein was detected using anti-SMO antibody (1: 1000 dilution, Abcam, USA). The anti-β actin antibody (Abcam, USA) was used as internal control. Horseradish peroxidase-conjugated secondary antibody and ECL Western blotting detection reagents (ThermoFisher Scientific, USA) were used for imagining.

## Immunostaining

To detect SMO expression in the gastric cancer tissue of human patients, we performed immunohistochemistry (IHC). Briefly, the formalin-fixed paraffin-embedded sections were de-paraffined by xylene and re-hydrate with ethanol. Antigen was retrieved by heating the citric acid buffer (pH=6.0) submerged slides in a water bath for 15 min. Incubation with 5% BSA-PBS for 20 min at room temperature was performed for blocking non-specific antibodies. Primary antibodies (diluted by 1: 300) were added to the slides to incubate overnight at 4°C. Then, horseradish-peroxidase-conjugated secondary antibodies (for IHC) or fluorescent secondary antibodies (for IF, R&D Systems) were added to incubate for 1 h at room temperature. Slides were then washed by PBS and stained by DAPI. Five fields of each slide under 400× magnification were randomly chosen to calculate the positive cell percentages, and
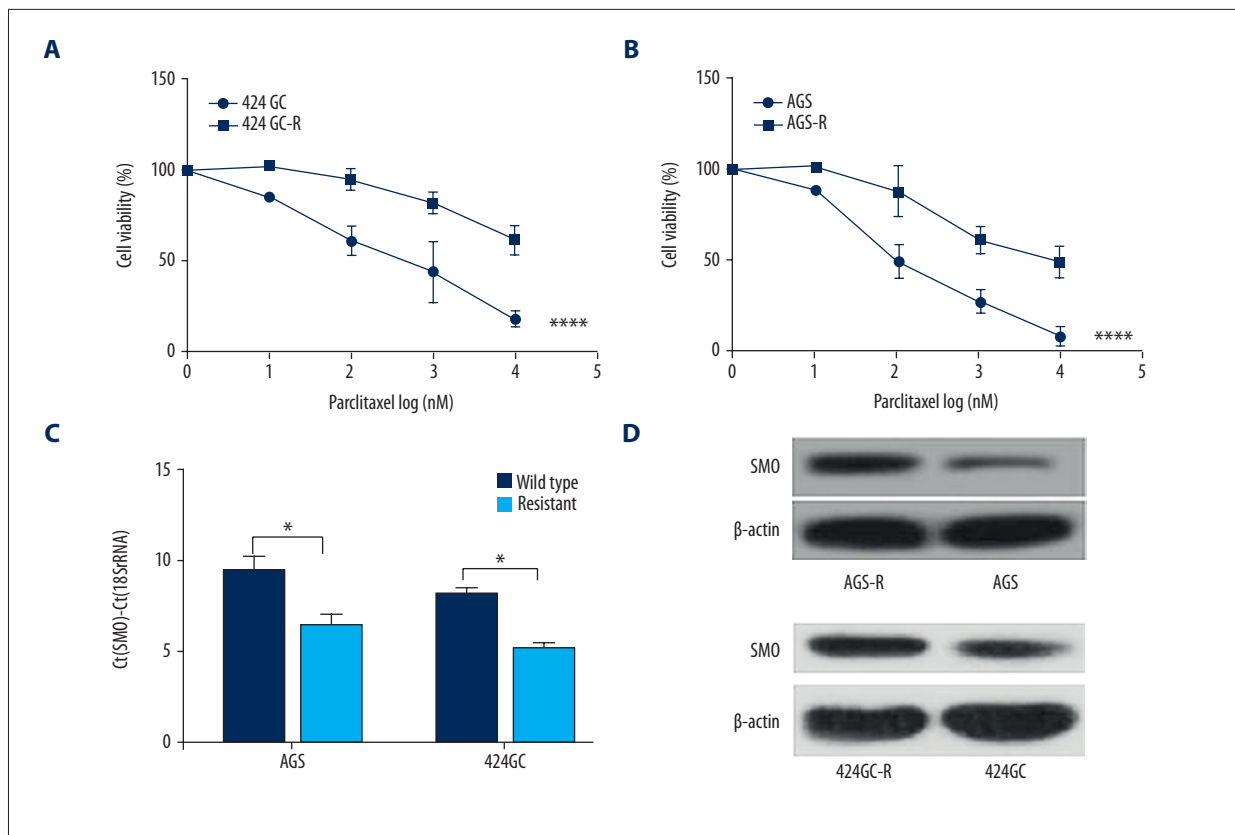
**Figure 2.** Expression of SMO in paclitaxel-resistant gastric cancer cell lines. (**A**) With the treatment of paclitaxel, the cell viability of paclitaxel-resistant gastric cancer cell line 424GC-R was much higher than in the wild-type 424GC cell line. (**B**) The cell viability of paclitaxel-resistant gastric cell line AGS-R was higher than in the wild-type AGS cell line under increasing concentrations of paclitaxel treatment. (**C**) The qPCR assay indicated that paclitaxel-resistant gastric cancer cell line 424GC-R and AGS-R had increased levels of SMO mRNA compared with the wide-type cell lines. (**D**) Western blotting assay indicated that the SMO protein expression level in the paclitaxel-resistant 424GC-R and AGS-R cell lines were higher than that of the wild-type cell lines (424GC and AGS).

their average was used as the final percentage. The final positive cell percentage more than 50% was considered as "high expression", and otherwise was considered as "low expression".

## qPCR

mirVana miRNA Isolation Kit was used to extract total RNA. RNA was reverse-transcribed using the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany). The quantification was performed with SYBR Green (Qiagen) and the LightCycler® 480 System (Roche Life Science). The comparative threshold cycle (CT) method was used to determine gene-relative expression. We chose 18S rRNA as an internal control for mRNA expression comparison. The primers used for each gene were: SMO (Forward: 5'-GTTCTCCATCAAGAGCAACCAC-3', Reverse: 5'-CGATTCTTGATCTCACAGTCAGG-3') and 18S rRNA (Forward: 5'-GTAACCCGTTGAACCCCATT-3', Reverse: 5'-CCATCCAATCGGTAGTAGCG-3').

## Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad Software, USA). A two-sample t-test or one-way ANOVA was used to analyze the difference among means of experimental groups. Tukey's multiple comparisons test was preformed to compare the mean of each group with the mean of every other group after the one-way ANOVA analysis. The two-tailed P value of each hypothesis test less than 0.05 was considered as statistically significant.

## Results

### High SMO expression was associated with paclitaxel drug resistance in gastric cancer patients

Gastric cancer tissues were collected from 54 patients and their SMO expression levels were measured by IHC (Figure 1A). We
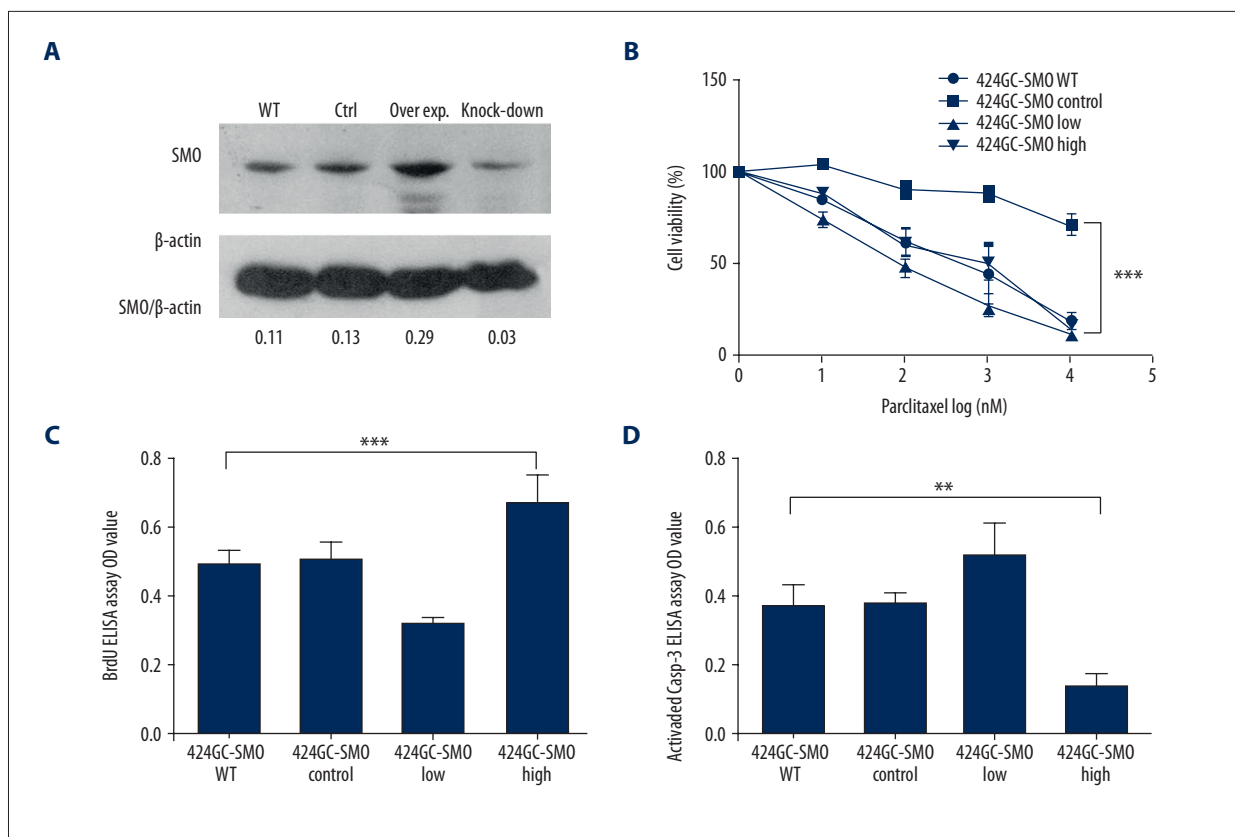
**Figure 3.** SMO overexpression induced gastric cell line resistance to paclitaxel treatment. (**A**) The Western blotting assay of 424GC cells with different SMO levels. (**B**) Cell viability curves showed that the SMO overexpression in the 424GC cell line was highly resistant to paclitaxel treatment. (**C**) The BrdU incorporation of the SMO overexpression 424GC was high. (**D**) The cells were treated with 100 nM paclitaxel for 24 h. The activated caspase-3 level was low in the SMO-overexpression 424GC cell line, even under paclitaxel treatment.

found that there was a higher ratio of high SMO expression among the stage-IV gastric cancer patients than among the stage III patients (Figure 1B). Notably, the patients with drug resistance to paclitaxel tended to have high SMO expression, but few of the patients who were sensitive to paclitaxel treatment had high SMO expression (Figure 1C). These data from gastric cancer patients suggest that the high SMO expression promotes the development of gastric cancer and might be associated with resistance to paclitaxel.

**Paclitaxel-resistant gastric cancer cell lines showed high SMO expression**

Paclitaxel-resistant gastric cancer cell lines 424GC-R and AGS-R were established by culturing the gastric cancer cell lines 424GC and AGS with 100 nM paclitaxel for 1 month. Then, they were treated with increasing concentrations of paclitaxel. The cell viability curves confirmed their resistance to paclitaxel treatment by comparing them with drug-sensitive cells (Figure 2A, 2B). Importantly, the mRNA level of SMO gene was increased in these paclitaxel-resistant cell lines (Figure 2C).

Western blotting assay also confirmed the enhanced expression of SMO protein in the 424GC-R and AGS-R cell lines compared to the sensitive cell lines (Figure 2D).

**Overexpression of SMO induced paclitaxel resistance in gastric cancer cells**

To further explore the role of SMO gene in gastric cancer development, we manipulated the SMO expression in the 424GC cell line (Figure 3A). Then, their sensitivity to the paclitaxel treatment was measured. With increasing concentrations of paclitaxel treatment, the SMO overexpression in 424GC cells had significantly higher cell viability than in the SMO low-expression cells (Figure 3B). SMO overexpression in 424GC cells showed much higher BrdU incorporation than in others (Figure 3C). On the other hand, the activated caspase-3 level in the SMO overexpression 424GC cells was significantly lower than in others after being treated with paclitaxel (Figure 3D). These data suggest that the SMO protein is important in inducing paclitaxel resistance and promoting the development of gastric cancer cells.
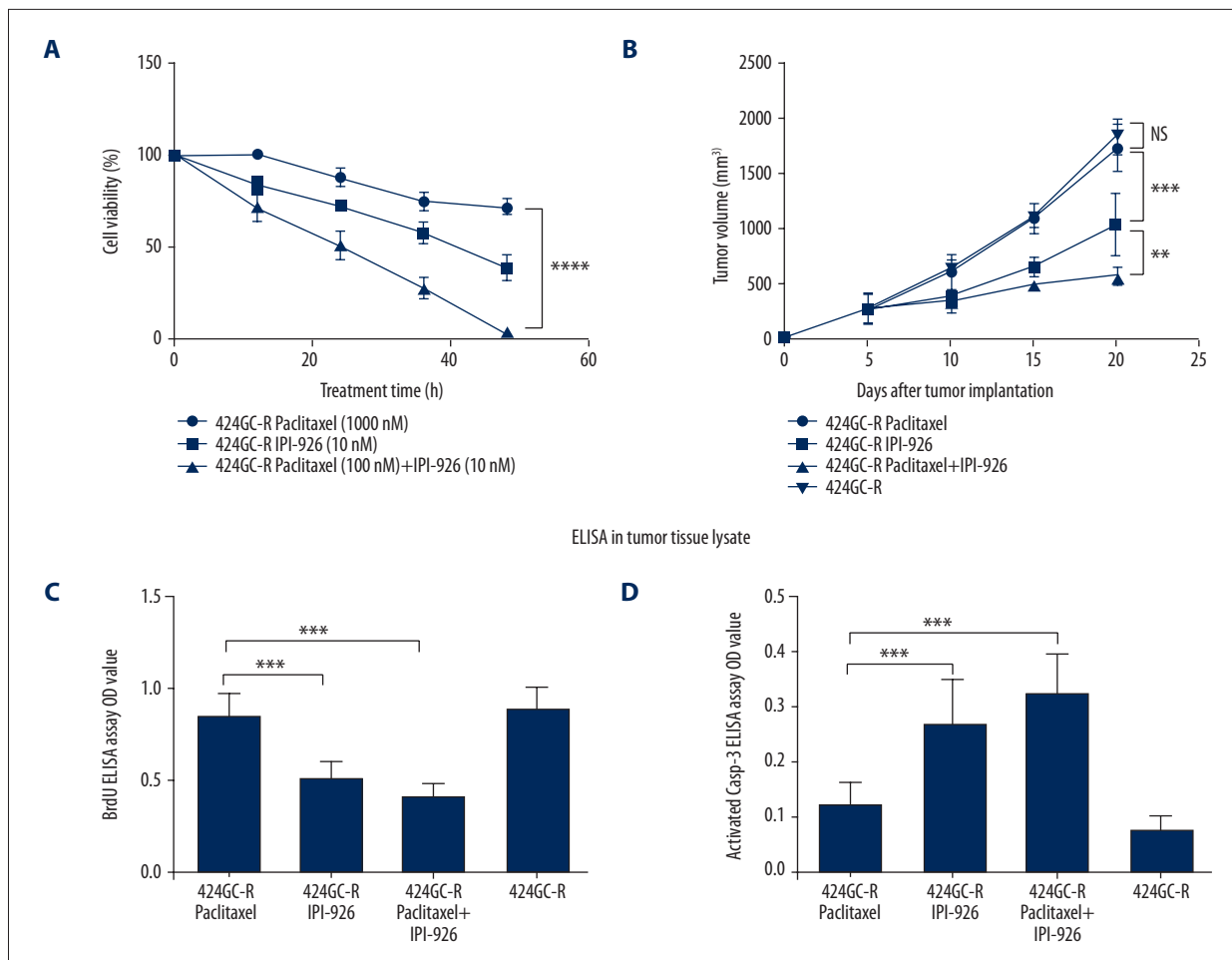
**Figure 4.** SMO inhibitor reversed paclitaxel resistance in gastric cancer models. (**A**) The cell viability curves indicated that the 424GC-R cell line was resistant to high-dose paclitaxel (1000 nM) treatment, but when combined with IPI-929, a smaller dose of paclitaxel (100 nM) achieved significant inhibition of cell viability. (**B**) Tumor growth of the 424GC-R subcutaneous mouse models treated with paclitaxel + IPI-929 was much slower than in the mice treated with paclitaxel alone. (**C**) BrdU incorporation of the tumor tissue from the mouse models treated with paclitaxel + IPI-929 was much less than that of the mice treated with paclitaxel alone. (**D**) The level of activated caspase-3 was greatly increased in the tumor tissue from the mice treated with paclitaxel + IPI-929 compared with the mice treated with paclitaxel alone.

**SMO inhibitor decreased the paclitaxel resistance in gastric cancer experimental models**

Knowing that the SMO expression level was increased in the paclitaxel-resistant 424GC-R cell line, we used the SMO inhibitor IPI-926 to check the targeting value of SMO in gastric cancer. As shown in Figure 4A, the 424GC-R had high drug resistance to the paclitaxel single treatment, while IPI-926 decreased cell viability. When combined with IPI-926 treatment, the 424GC-R cells became sensitive to paclitaxel again, even in a low-dose treatment (Figure 4A). The 424GC-R subcutaneous mouse model was established and treated with paclitaxel with or without IPI-926 (Figure 4B). The tumor volume of the mice treated with paclitaxel alone was very close to that of the mice that did not receive any drug treatment. However,

the tumor growth of the mice treated with paclitaxel + IPI-926 was much slower than in the mice treated with paclitaxel alone. The tumor tissue BrdU incorporation in the paclitaxel + IPI-926 treatment group significantly decreased compared to the non-treatment group or the paclitaxel group (Figure 4C). In contrast, the activated caspase-3 level of the tumor tissues treated by the paclitaxel + IPI-926 combination was higher than in other groups (Figure 4D).

## Discussion

Although gastric cancer can be cured in early stages, it is still among the leading causes of cancer-related death worldwide [1,2]. Recent advances in gastric cancer include

identification of novel diagnostic and prognostic biomarkers, and testing of targeted therapies in both preclinical and clinical settings [9,11–14]. However, due to the extreme complexity of gastric cancer pathogenesis and molecular heterogeneity, these findings alone are insufficient to advance clinical practice. Therefore, to enhance overall survival of gastric cancer, continuing efforts should be made to increase the efficacy of current standard therapies, including chemotherapy. In the present study, we aimed to illustrated the function of SMO, a key protein of the Hedgehog pathway, in regulating gastric cancer drug resistance.

We started with an analysis of gastric cancer tissue samples that have different sensitivities to paclitaxel treatment. Interesting, we found that in the drug-resistant gastric cancer cases, higher SMO expression was detected. This observation provided the basis for our further analysis in cellular and animal models to determine if SMO is the driving factor in gastric cancer drug resistance. As expected, in paclitaxel-resistant cell lines, we found higher SMO expression. When we upregulate SMO expression in gastric cancer cell lines, drug resistance increased dramatically, while downregulation of SMO expression sensitized gastric cancer cell lines to paclitaxel. In line with previous reports that the Hedgehog pathway promotes cell proliferation [15,16] we also found that SMO overexpression upregulates BrdU in a gastric cancer cell line, suggesting its drug-resistance regulating role may be related to cell proliferation. In paclitaxel-treated gastric cancer cells, overexpression of SMO decreased activated caspase 3 level, thereby preventing cancer cell death. Our data show that SMO expression is correlated with paclitaxel resistance in gastric cancer in different experimental systems.

Because SMO overexpression was tightly correlated with paclitaxel resistance in gastric cancer cells, we further hypothesized that inhibiting the Hedgehog pathway would kill the drug-resistant cells, and acting synergistically with paclitaxel in treating gastric cancer. Previous studies using the Hedgehog pathway inhibitor have successfully eliminated medulloblastoma in a mouse model [17,18]. In pancreatic cancer, administration of the Hedgehog pathway inhibitor enhanced chemotherapy drug delivery and response in animal models [19,20]. In line with these findings, we found that in the SMO-overexpressing paclitaxel-resistant gastric cancer cells, administration of IPI-926, an inhibitor of the SMO protein, significantly decreased their viability. When combined with IPI-926, paclitaxel became effective again in drug-resistant gastric cancer cells. In our subcutaneous syngeneic tumor model, SMO overexpression in paclitaxel-resistant tumors was sensitive to IPI-926. The combination of IPI-926 with paclitaxel can control tumors that were originally non-responsive to paclitaxel alone.

## Conclusions

Our study clearly indicates that SMO overexpression is a major mechanism of gastric cancer paclitaxel resistance. Based on the biological relationship between SMO expression and paclitaxel resistance, we successfully administrated IPI-926, a SMO inhibitor, to control gastric cancer tumors in combination with paclitaxel. Translation of our findings from preclinical models to personalized therapeutic plans for SMO-mediated drug-resistant gastric cancer would benefit some gastric cancer patients.

### Conflict of interests

None.

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