

Original Article

Isocorydine decrease gemcitabine-resistance by inhibiting epithelial-mesenchymal transition via STAT3 in pancreatic cancer cells

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Abstract: Gemcitabine is widely used as an anticancer chemotherapy drug for a variety of solid tumors, and it has become the standard treatment option for locally advanced and metastatic pancreatic cancer. However, pancreatic cancer cells develop resistance to gemcitabine after a few weeks of treatment, resulting in poor therapeutic effects. Isocorydine (ICD) is a typical natural aporphine alkaloid, and ICD and its derivatives inhibit the proliferation of many types of cancer cells in vitro. In this study, ICD was found to synergistically inhibit cell viability with gemcitabine in pancreatic cancer cells. A microarray analysis showed that ICD can inhibit the upregulation of STAT3 and EMT in pancreatic cancer cells induced by gemcitabine. STAT3 is closely related to tumor EMT, migration and invasion. After knocking down the expression of STAT3 in pancreatic cancer cells, the combination index (CI) of ICD and gemcitabine decreased. ICD can reverse the increase in the expression of EMT-related transcription factors and proteins caused by gemcitabine, thereby inhibiting the enhanced cell migration and invasion ability caused by gemcitabine. Finally, the synergistic treatment effect of the combination treatment of ICD and gemcitabine in pancreatic cancer cells was confirmed in established xenograft models.

Keywords: Gemcitabine, isocorydine, pancreatic cancer, EMT

Introduction

Pancreatic cancer is one of the deadliest solid malignancies with an exceedingly poor prognosis and a 5-year survival rate of < 5% [1, 2]. At present, the early diagnosis rate of pancreatic cancer is not high, the operative mortality rate is still high, and the cure rate is very low [3]. Because of the late onset of symptoms, more than 90% of pancreatic cancer patients present with advanced stage disease, with invasion and locoregional spread and/or distant metastasis at diagnosis [4]. Chemotherapy can relieve symptoms and improve survival in metastatic pancreatic cancer [5]. However, drug resistance and chemotherapy deficiency contribute to the high mortality of metastatic pancreatic cancer patients [6, 7]. Therefore, developing more effective methods for the treatment of pancreatic cancer metastasis is urgent.

Gemcitabine (dFdC: 20,20-difluorodeoxycytidine) was originally used for its antiviral effects

[8] and has been widely used as an anticancer chemotherapy drug for a variety of solid tumors, including some lymphomas [9]. Gemcitabine has become the standard treatment option for locally advanced and metastatic pancreatic cancer [10]. Although its effect on survival has been merely modest, it is notable that the clinical benefit response of gemcitabine was almost five-fold higher than that of fluorouracil [11]. However, the promising clinical response did not improve survival, in part because the drug failed to penetrate the low vascularization, dense tumor matrix [12], and sensitive tumors developed chemoresistance to gemcitabine in subsequent weeks of treatment [13]. To overcome resistance to pancreatic cancer, the combination of herbal compounds or plant products with gemcitabine has shown promising efficacy.

Isocorydine (ICD) is a typical natural aporphine alkaloid that is widely distributed in *Dicranostigma leptopodum* Fedde, *Stephania japoni-*

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ca, *Dactylicapnos scandens* Hutch and other plants. ICD and its derivatives inhibit the proliferation of many types of cancer cells in vitro, including liver cancer, gastric cancer, lung cancer and cervical cancer cell lines [14, 15]. Studies have shown that ICD can inhibit the proliferation of hepatocellular carcinoma and reduce the tumorigenic ability of CD133+ cancer stem cells by inducing G2/M cell cycle arrest and apoptosis [16]. Combined therapy showed that ICD can enhance the sensitivity of hepatoma cell lines to doxorubicin, cisplatin and other first-line anticancer drugs and plays a role in drug resistance reversal. It is a promising chemotherapy drug for liver cancer [17].

EMT endows cells with migration and invasiveness and induces resistance to traditional chemotherapy [18-20]. The abnormal activation of the EMT-related transcription factors Twist, Snail, Slug, ZEB1, etc. [21, 22] causes the deletion of E-cadherin, EpCAM, and the overexpression of Vimentin and N-cadherin, leading to spindle-like changes in cell morphology and apparent changes in enhanced mobility [23]. EMT induces drug resistance in pancreatic cancer cells, thus accelerating metastasis. Therefore, the inhibition of EMT in tumor therapy is crucial to improve drug response and block metastasis.

In the present study, we investigated the antitumor activity of gemcitabine and ICD in vitro and in vivo studies, alone and in combination, to determine whether ICD can inhibit gemcitabine-mediated EMT activation in pancreatic cancer cell migration and invasion.

Materials and methods

Materials

ICD was purchased from Herbest Company (Shanxi, China). Gemcitabine hydrochloride was purchased from Tokyo Chemical Industry (Shanghai, China). The antibodies used in this study were against E-cadherin (#3195), Vimentin (#5741), STAT3 (#4904), p-stat3 (#9145), Twist1 (#46702), Snail (#3879) (Cell Signaling Technology Inc., MA, USA), N-cadherin (ab-18203) (Abcam Inc., MA, USA), and GAPDH (AF7021).

Cell lines and cell culture

Human pancreatic cancer cell lines (CFPAC-1, PANC-1 and PaTu 8988t) were procured from

the Chinese Academy of Sciences Cell Bank. The PANC-1 cell lines were cultured in DMEM (high glucose; Gibco), and the CFPAC-1 and PaTu 8988t cell lines were cultured in RPMI 1640 medium. The medium contained 10% fetal bovine serum (FBS, qualified, Gibco, Australia origin) supplemented with 1% penicillin/streptomycin (Sigma, St. Louis, MO). The cells were placed in a humidified incubator containing 5% CO₂ at 37°C.

Cell viability assays

A Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan) was performed following the manufacturer's instructions. Cells were plated in 96-well plates, and each well contained 3000 cells and 100 µL of medium with 10% FBS. The cells were cultured for 24 h and exposed to different concentrations of drugs. After 48 h, the culture medium was discarded. Then, 90 µL of FBS-free medium and 10 µL of the Cell Counting Kit-8 reagent were added to each well. The cells were maintained at 37°C for approximately 4 h. Cell viability, shown as the fold change in absorbance at 450 nm for each well, was detected using an ELISA reader (Tecan, Männedorf, Switzerland). All experiments were conducted in triplicate.

Combination index (CI) = $E_{A+B} / (E_A + E_B - E_A \times E_B)$, CI < 0.85, = 0.85~1.15, and > 1.15 indicate antagonism and additive and synergistic effects, respectively. E_{A+B} represents the inhibition rate of the combination of two drugs; E_A represents the inhibition rate of drug A; and E_B represents the inhibition rate of drug B. ** $P < 0.01$, *** $P < 0.001$ [24].

Western blotting analysis

The expression levels of stat3, p-stat3, E-cadherin, N-cadherin, Vimentin, Snail, and Twist1 were measured by western blotting analysis, while relative protein expression levels were normalized to GAPDH. Briefly, all the proteins were extracted with RIPA buffer (Beyotime, Shanghai, China) containing a protease inhibitor (Beyotime) and phosphatase inhibitor (Roche Diagnostics GmbH, Mannheim, Germany). A BCA protein assay kit (BCA; Beyotime) was used to measure the protein concentrations of each individual group. After the proteins were denatured, equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim

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milk for 2 h at room temperature, the membranes were incubated with primary antibodies (1:1000) overnight at 4°C. The membranes were washed three times with 0.1% Tween-20 (TBST) and incubated with the appropriate HRP-conjugated secondary antibody for 1 h at room temperature. ECL Western blotting reagents were used to measure the signals by using Bio-Rad Image Lab software.

Cell migration and invasion assay

A Transwell assay and Matrigel invasion assay were performed to measure the migration and invasion ability of the three human pancreatic cancer cell lines. After different treatments, cells (2×10^5 cells for the invasion assay or 5×10^4 cells for the migration assay) were resuspended in 200 μ L of FBS-free medium and added to each upper chamber coated with (invasion assay) or without (migration assay) Matrigel (BD Biosciences, Sparks, MD) for 48 h. DMEM with 10% FBS was added to the lower compartment of the Transwell. ICD, gemcitabine or their combination was added to the upper and lower chambers at the indicated concentrations. After 48 h of incubation, the cells were fixed with 4% paraformaldehyde, stained with crystal violet, photographed, and counted.

Tumor xenografts in mice

Athymic nude mice (BALB/C-nu/nu, 5 weeks of age, male) were supplied by SLAC Co., Ltd. (Shanghai, China). To establish a pancreatic tumor xenograft model, 1×10^7 PANC-1 cells were suspended in 100 μ L of phosphate-buffered saline (PBS) and implanted subcutaneously into the right flank of each nude mouse. Two weeks after inoculation, the mice were randomly assigned to four subgroups: ICD (0.4 mg/kg), gemcitabine (100 mg/kg), gemcitabine (100 mg/kg) combined with ICD (0.4 mg/kg), and a vehicle-treated control group (equal volume of diluents) (5 mice in each group). The drug was injected intraperitoneally every other day, and the tumor volume and mouse body weight were measured. Tumor volumes were measured using Vernier calipers and calculated using the following formula: $V = (\text{length} \times \text{width}^2)/2$. The mice were closely monitored for 2 weeks. Tumor regression rate = $(1 - \text{mean tumor weight of experimental group}/\text{mean tumor weight of control group}) \times 100\%$ [25]. The ani-

mal experiments were conducted in compliance with the Guide for the Care and Use of the Animal Ethics Committee of Wenzhou Medical University (Wenzhou, PR China).

Cell transfection

For cell interference, stat3 siRNA was synthesized at the Shanghai GenePharma Company Limited. The sequences of the stat3 and negative control siRNA are as follows: sense, CCA-CUUUGGUGUUUCAUAATT; and antisense, UUA-UGAAACACCAAAGUGGTT.

Briefly, cells were seeded into 6-well plates. After 24 h, siRNA was transfected into cells using Lipo iMAX (Invitrogen, Grand Island, NY) according to the manufacturer's protocol, with a final siRNA concentration of 100 nM (CFPAC-1), 50 nM (PANC-1) or 75 nM (PaTu 8988t). The cells were harvested 48 h after transfection for subsequent analysis of RNA expression. All knockdown experiments were performed in triplicate.

Statistical analysis

All the results are presented as the mean \pm standard error of the mean, and each test was repeated at least three times. The statistical analyses were carried out with SPSS 22.0 statistical software (IBM, Armonk, NY, USA) and Prism 5.0 software (GraphPad Software, San Diego, CA, USA) using one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests to analyze the statistical significance between groups. $P \leq 0.05$ was considered statistically significant.

Result

ICD reduces pancreatic cancer cell viability and sensitizes cells to gemcitabine in vitro

To investigate whether ICD could inhibit pancreatic cancer cell proliferation and increase the sensitivity of pancreatic cancer cells to gemcitabine, CCK-8 assays were performed to measure the effects of ICD on three human pancreatic cancer cell lines, CFPAC-1, PANC-1 and PaTu 8988t. As shown in **Figure 1A-C**, the inhibition of ICD on the three types of pancreatic cancer cells increased with increasing concentration. The inhibition of gemcitabine on the proliferation of the three types of pancreatic

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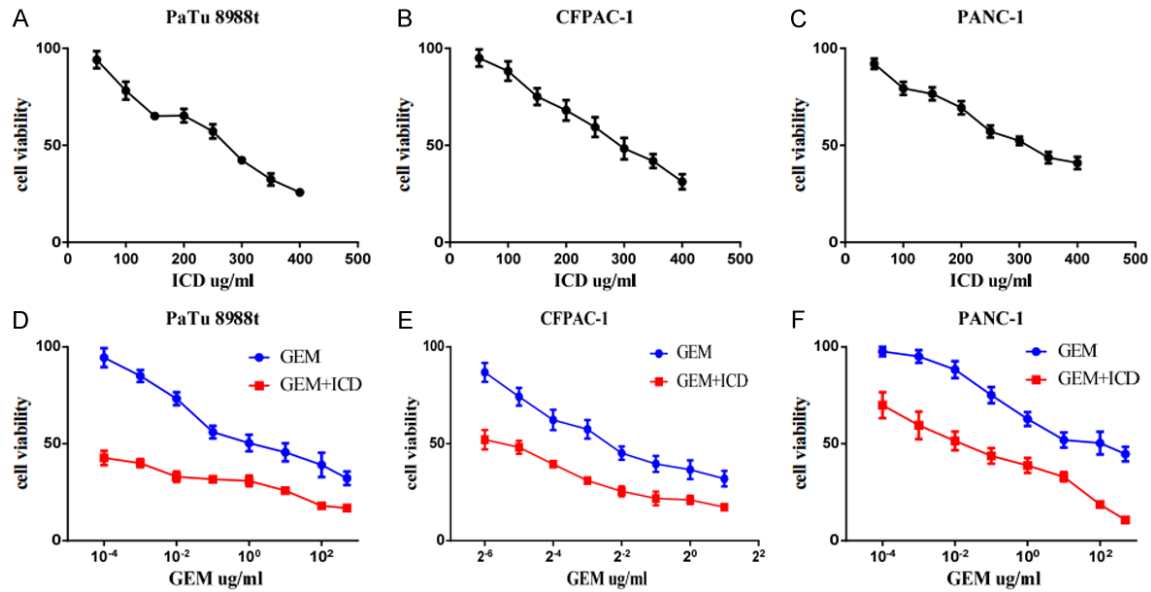


Figure 1. ICD inhibited the viability of pancreatic cancer cells, and low concentrations of ICD enhanced the cytotoxicity of gemcitabine in pancreatic cancer cells. A-C. ICD dose-dependently inhibited the viability of three types of pancreatic cancer cells: PaTu 8988t, CFPAC-1 and PANC-1. D-F. Low concentrations of ICD (100 µg/mL) enhanced the inhibitory effect of gemcitabine on pancreatic cancer cells. Cells were incubated with ICD, gemcitabine, or their combination for 48 h before being subjected to a CCK-8 assay. The data are presented as the mean ± standard error of the mean.

Table 1. IC₅₀ values for gemcitabine and the CI of ICD and gemcitabine in pancreatic cancer cells

	Gemcitabine (µg/ml)	Inhibition ratio (%)		IC ₅₀ of gemcitabine		CI
		Gemcitabine	Combination	Gemcitabine	Combination	
PaTu 8988t	50	67.877	82.632			1.185
	10	51.088	66.738	46.173	7.947	1.237
	1	12.056	44.234			2.571
PANC-1	50	49.655	81.404			1.561
	10	37.227	61.211	0.786	0.073	1.518
	1	11.681	40.479			2.521
CFPAC-1	1	63.401	78.925			1.190
	0.25	54.820	74.514	68.736	14.348	1.276
	0.03125	25.719	51.833			1.639

cancer cells was significantly enhanced by the combination of low concentration ICD (100 µg/mL) (Figure 1D-F). The combination index (CI) values calculated based on the dose-effect curves showed that ICD combined with gemcitabine can inhibit the proliferation of the three cell lines synergistically (Table 1). The half maximal inhibitory concentrations (IC₅₀) for gemcitabine in the CFPAC-1, PANC-1 and PaTu 8988t cell lines were 46.173, 0.786 and 68.736 µg/mL, respectively; when combined with the low concentration ICD, the IC₅₀ values for gemcitabine changed to 7.947, 0.073, 14.348 µg/mL, respectively. The IC₅₀ values of

gemcitabine in the three cell lines was significantly decreased by the combination with ICD.

ICD inhibits the EMT induced by gemcitabine in pancreatic cancer cells

After treatment with gemcitabine for 48 h, the western blotting results showed that the expression of Snail, N-cadherin, Twist and Vimentin in the gemcitabine-treated group increased compared with that of the control group, while the levels of E-cadherin decreased. However, compared with the gemcitabine monotherapy group, in the combination group of ICD and

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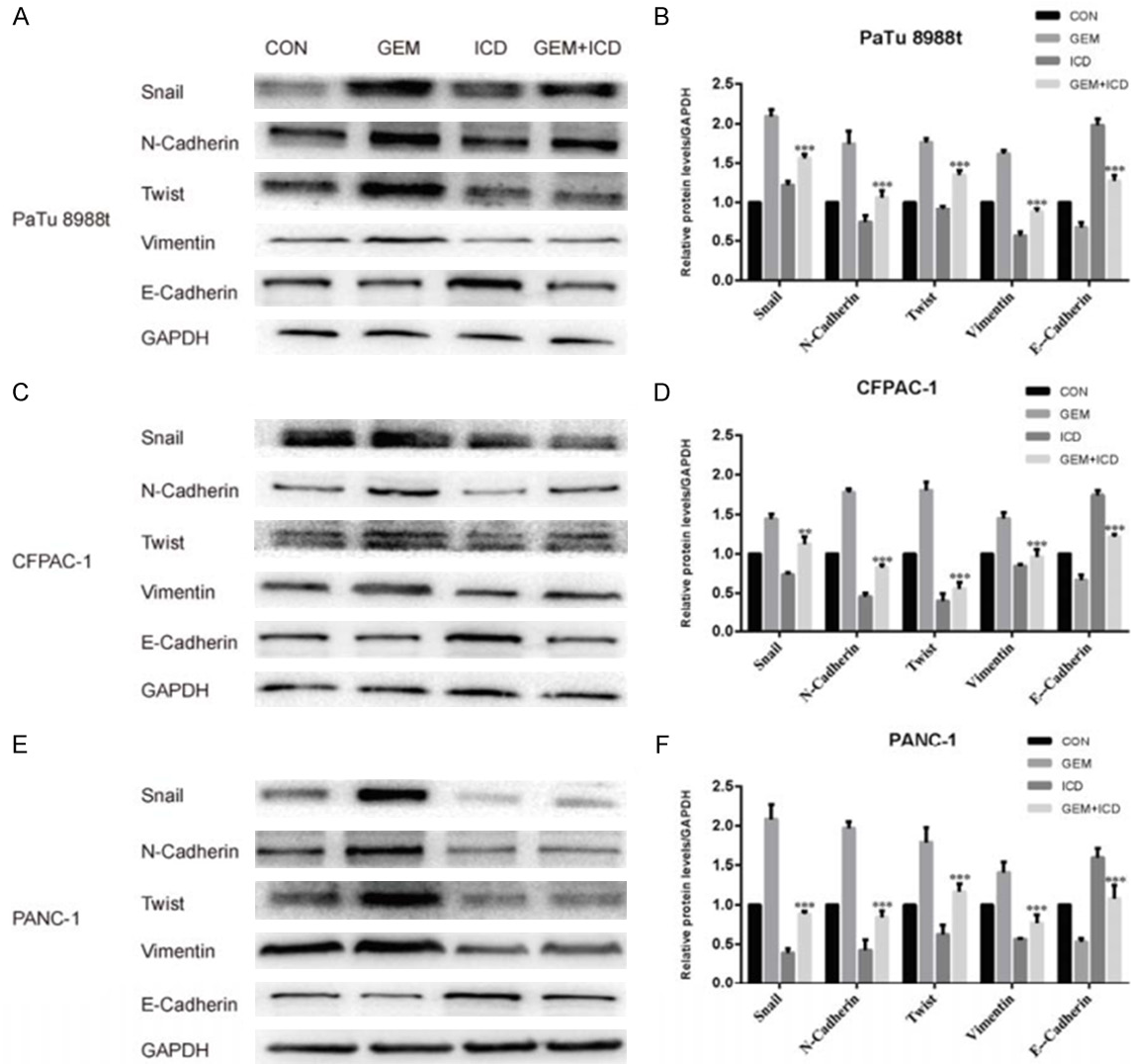


Figure 2. ICD inhibits gemcitabine-induced EMT in pancreatic cancer cells. Expression of the EMT markers E-cadherin, N-cadherin, Vimentin, Snail and Twist, as examined by western blot in PaTu 8988t (A, B), CFPAC-1 (C, D) and PANC-1 (E, F) cells treated with ICD, gemcitabine, or their combination for 24 h. The results are representative of three separate experiments. GAPDH was used as a control. The data are presented as the mean \pm standard error of the mean. $^{**}P < 0.01$, $^{***}P < 0.005$, ICD and gemcitabine combined group vs gemcitabine group. CON, control; GEM, gemcitabine (PaTu 8988t and PANC-1 25 $\mu\text{g}/\text{mL}$, CFPAC-1 0.25 $\mu\text{g}/\text{mL}$); ICD, isocorydine 100 $\mu\text{g}/\text{mL}$.

gemcitabine, Snail, N-cadherin, Twist and Vimentin were significantly downregulated and E-cadherin was upregulated. Moreover, ICD monotherapy can also reduce EMT in pancreatic cancer cells (Figure 2A-F).

ICD inhibits the migration and invasive capacity of gemcitabine-induced EMT in pancreatic cancer cells

Transwell migration assays (Figure 3A, 3B) and Matrigel invasion assays (Figure 3C, 3D) were used to detect the migration and invasion ability of pancreatic cancer cells. Compared with

the control group, gemcitabine was found to induce a certain degree of enhanced migration and invasion of pancreatic cancer cells. After treatment with ICD combined with gemcitabine, ICD downregulated the migration and invasion ability of pancreatic cancer cells.

ICD inhibited the upregulation of EMT and p-STAT3 expression in pancreatic cancer cells induced by gemcitabine

A microarray analysis was used to gain specific insight into the mechanism of the synergistic effect of ICD and gemcitabine. The results

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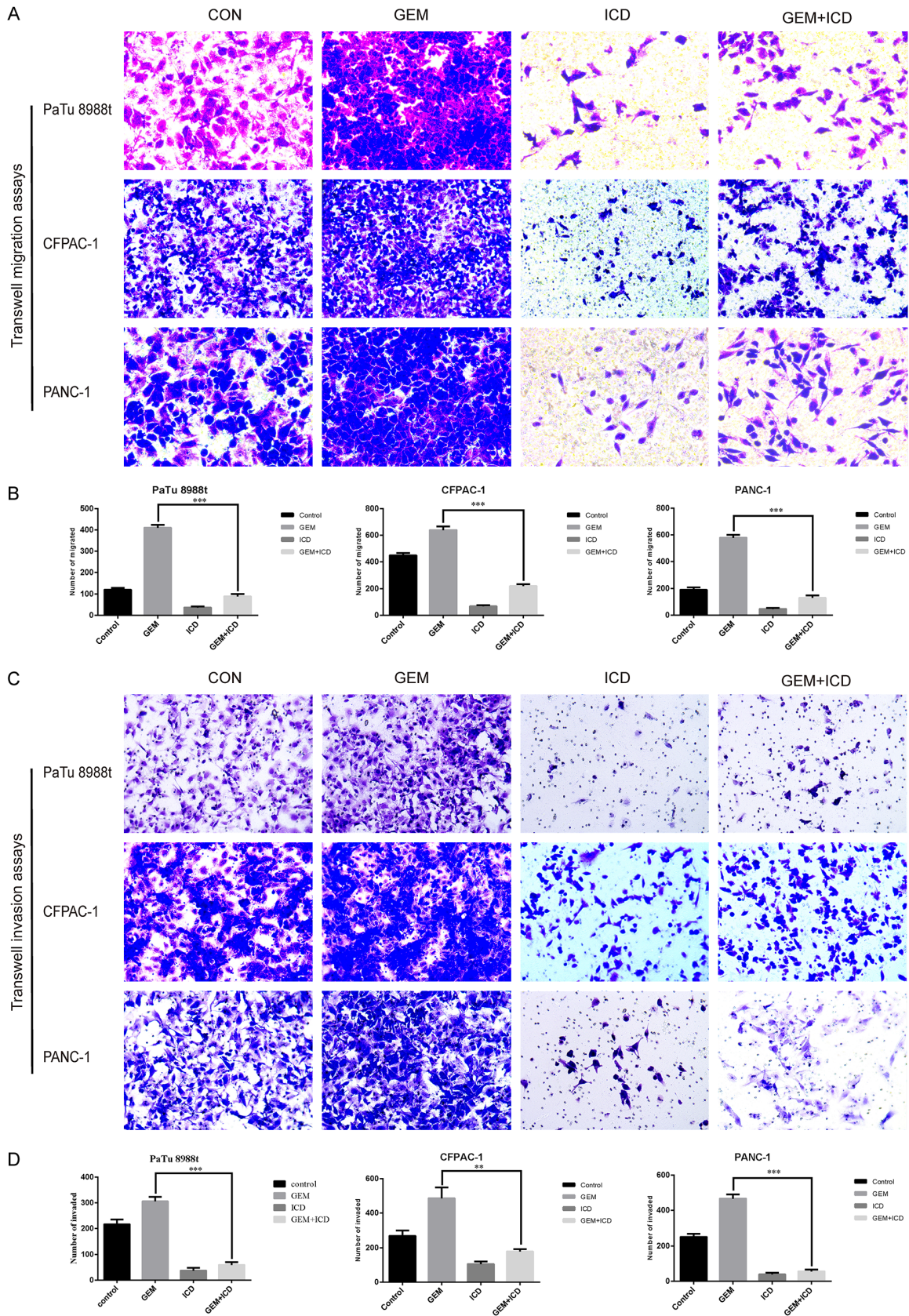


Figure 3. ICD inhibits gemcitabine-induced tumor migration and invasion in pancreatic cancer cells. Transwell assays showing the effects of ICD, gemcitabine and their combination on migration (A, B) and invasion (C, D) in PaTu

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8988t, CFPAC-1 and PANC-1 cells. Cells were pretreated with ICD, gemcitabine or their combination for 48 h. After 48 h, the cells on the lower side of the filter were counted (magnification, $\times 200$). The data are presented as the mean \pm standard error of the mean. $***P < 0.005$, CON, control; GEM, gemcitabine (PaTu 8988t and PANC-1 25 $\mu\text{g}/\text{mL}$, CFPAC-1 0.25 $\mu\text{g}/\text{mL}$); ICD, isocorydine 100 $\mu\text{g}/\text{mL}$.

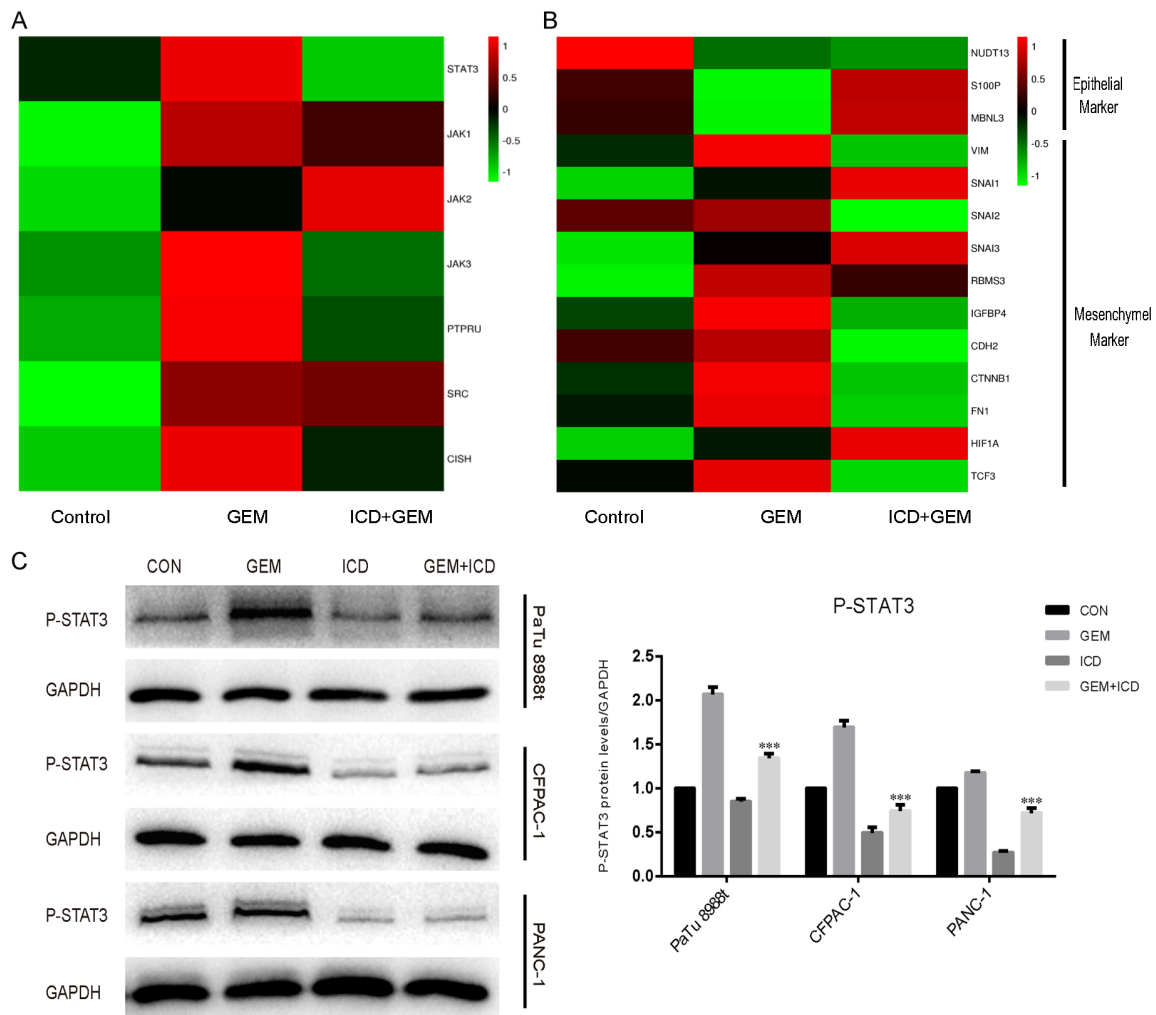


Figure 4. STAT3 is operative in ICD- and GEM-treated pancreatic cancer cells. A. Heatmap of STAT3 and related genes in the control, gemcitabine and ICD + gemcitabine samples. B. Heat map of the EMT-related genes in the control, gemcitabine and ICD + gemcitabine samples. C. Western blot analysis of p-STAT3 after ICD, gemcitabine and combination treatment for 24 h. The results are representative of three separate experiments. GAPDH was used as a control. The data are presented as the mean \pm standard error of the mean. $***P < 0.005$, ICD and gemcitabine combined group vs gemcitabine group. CON, control; GEM, gemcitabine (PaTu 8988t and PANC-1 25 $\mu\text{g}/\text{mL}$, CFPAC-1 0.25 $\mu\text{g}/\text{mL}$); ICD, isocorydine 100 $\mu\text{g}/\text{mL}$.

showed that the STAT3 gene expression level exhibited a ≥ 2 -fold change in the gemcitabine group and the ICD combined with gemcitabine group (Figure 4A). Moreover, the expression level of epithelial markers was reduced, while that of mesenchymal markers was increased in the gemcitabine group compared with the control group. The upregulation of EMT was significantly reversed in the ICD combined with

gemcitabine group (Figure 4B). STAT3 is activated by upstream factors in tumor cells, and p-STAT3 can act on target genes in the nucleus, regulate their transcription, and then regulate the expression of EMT-related genes so that the transformation of tumor cells from the epithelial phenotype to the mesenchymal phenotype increases the metastasis and invasion of malignant tumors [26-32]. The western blot-

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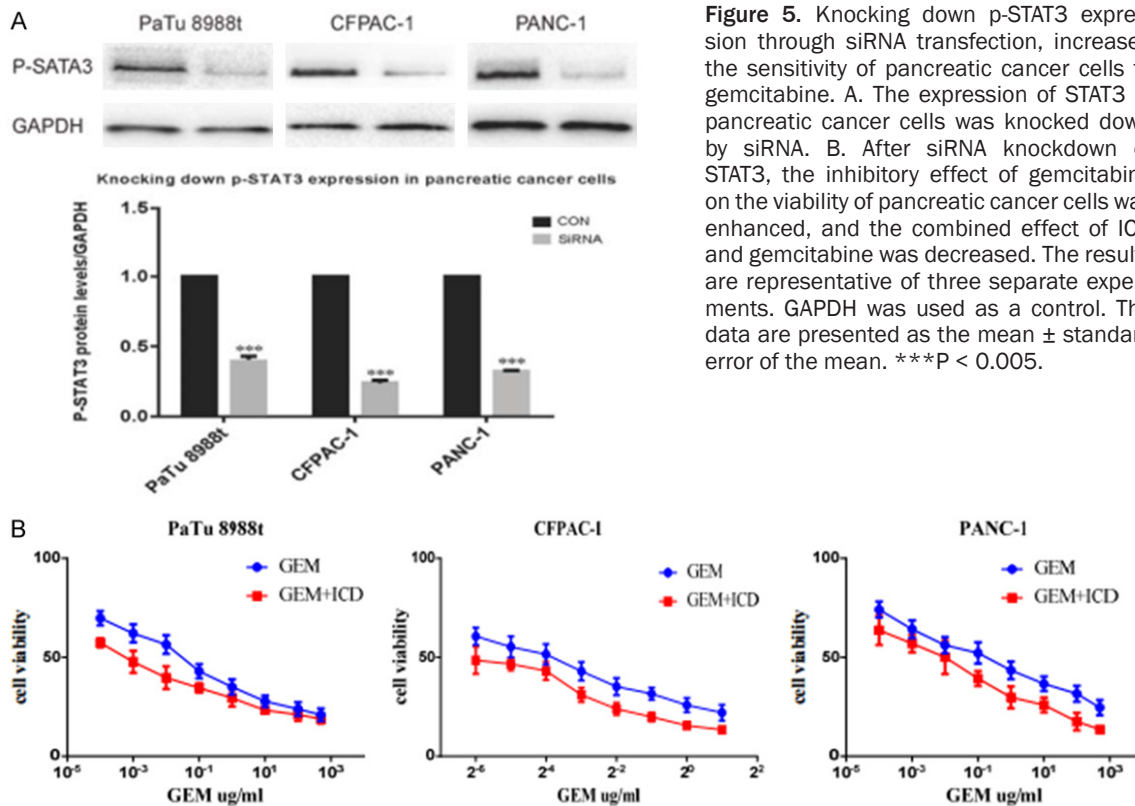


Figure 5. Knocking down p-STAT3 expression through siRNA transfection, increased the sensitivity of pancreatic cancer cells to gemcitabine. A. The expression of STAT3 in pancreatic cancer cells was knocked down by siRNA. B. After siRNA knockdown of STAT3, the inhibitory effect of gemcitabine on the viability of pancreatic cancer cells was enhanced, and the combined effect of ICD and gemcitabine was decreased. The results are representative of three separate experiments. GAPDH was used as a control. The data are presented as the mean \pm standard error of the mean. *** $P < 0.005$.

Table 2. Knockdown of STAT3 in pancreatic cancer cells. IC₅₀ values for gemcitabine and the CI of ICD and gemcitabine in pancreatic cancer cells

	Gemcitabine ($\mu\text{g/ml}$)	Inhibition ratio (%)		IC ₅₀ of gemcitabine	CI
		Gemcitabine	Combination	Gemcitabine	
PaTu 8988t	50	76.101	78.972	17.194	1.007
	10	64.992	70.576		1.031
	1	37.866	52.287		1.189
PANC-1	50	68.409	82.499	0.299	1.162
	10	56.553	70.201		1.168
	1	35.753	43.050		1.050
CFPAC-1	1	68.409	82.499	26.899	1.148
	0.25	56.553	70.201		1.040
	0.03125	35.753	43.050		0.922

ting results showed that after treatment with gemcitabine for 48 h, the expression level of p-STAT3 in pancreatic cancer cells increased, and this gemcitabine-induced increase in p-STAT3 was inhibited by ICD (Figure 4C). Furthermore, after knocking down p-STAT3 expression through siRNA transfection in pancreatic cancer cells (Figure 5A), the sensitivity of cells to gemcitabine increased, and the IC₅₀ value of gemcitabine decreased (Figure 5B). After knocking down p-STAT3 expression, the CI of the

ICD and gemcitabine combination group decreased compared with that of the group without p-STAT3 knockdown (Table 2).

ICD enhances the curative efficacy of gemcitabine for pancreatic cancer in vivo

To further confirm the in vivo effects of the ICD and gemcitabine combined treatment for pancreatic cancer, PANC-1 cells were subcutaneously inoculated into nude mice. The body

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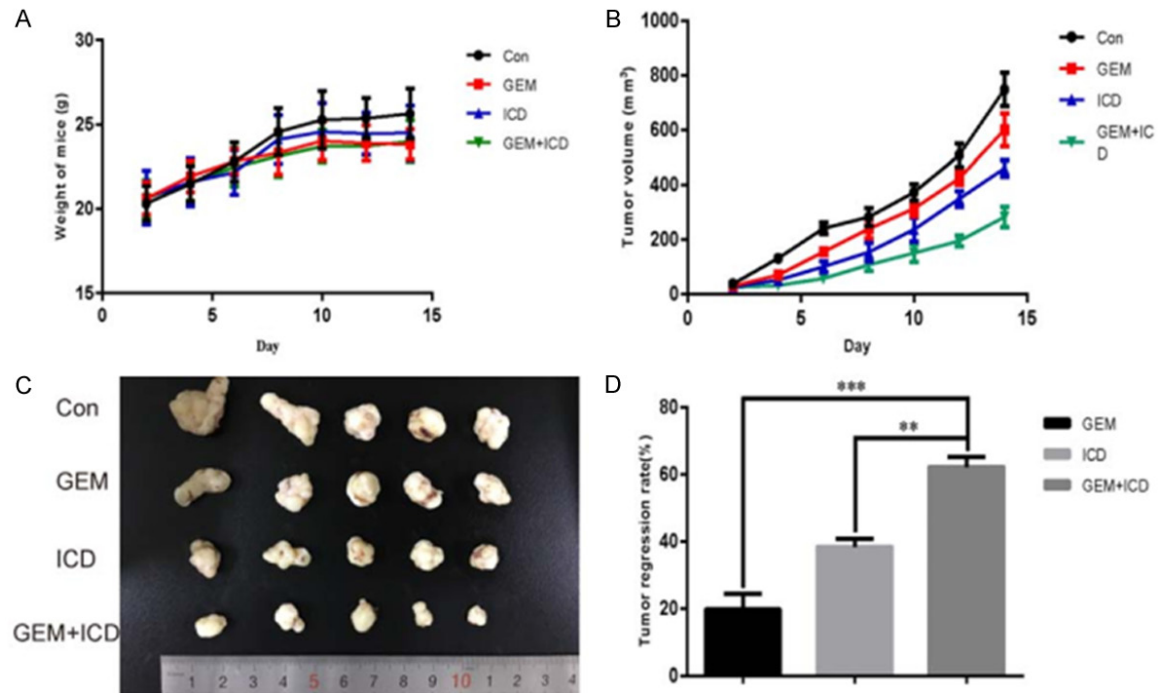


Figure 6. ICD and gemcitabine inhibit pancreatic cancer cell growth synergistically in vivo. A. 1×10^7 PANC-1 cells were inoculated into BALB/c nude mice. The mice were randomized into 4 groups ($n = 5$) and treated with PBS, isocorydine (ICD), gemcitabine (GEM) or their combination daily for 14 days. A, B. Body weight and tumor volumes were measured every 2 days. C. The tumors were excised after the last treatment. D. The tumor regression rates were calculated and compared with those of the combination group. The data are presented as the mean \pm standard error of the mean. $**P < 0.01$, $***P < 0.005$, CON, control; GEM, gemcitabine; ICD, isocorydine.

weights and tumor sizes of the mice receiving different treatments were measured every two days. The results showed that the intraperitoneal injection of ICD and gemcitabine inhibited tumor growth after two weeks, while the combination treatment significantly inhibited tumor growth (Figure 6A, 6B). Compared to the control group, after treatment with ICD and gemcitabine, there was no significant weight loss observed (Figure 6C). After two weeks of treatment, we euthanized the mice and dissected and weighed the tumors. The tumor regression rates of the different treatment schemes were calculated, and ICD was found to significantly improve the efficacy of gemcitabine in vivo in the treatment of pancreatic cancer (Figure 6D).

Discussion

Because of the late onset of the symptoms of pancreatic cancer, pancreatic cancer patients are often diagnosed at a late stage. Chemotherapy is the main method for the treatment of advanced and metastatic pancreatic cancer, and improving the effect of chemotherapy is

key in the treatment of pancreatic cancer. Gemcitabine is used to treat locally advanced or metastatic pancreatic cancer, but the effect of gemcitabine is not satisfactory. Some plant extracts combined with gemcitabine have been reported to have a synergistic antitumor effect on pancreatic cancer, which may improve the efficacy of gemcitabine in the treatment of pancreatic cancer [33-45]. ICD is a typical natural aporphine alkaloid widely present in many plants. ICD and its derivatives inhibit the proliferation of many types of cancer cells in vitro, including liver cancer, gastric cancer, lung cancer and cervical cancer cell lines [14, 15]. This study indicated the synergism of ICD with gemcitabine for pancreatic cancer cells and the underlying mechanism focused on EMT and STAT3.

In the present study, ICD inhibited the viability of the three pancreatic cancer cell lines in a concentration-dependent manner with monotherapy. We selected an ICD concentration with less effect on cell viability combined with gemcitabine to detect the effect of the com-

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bined treatment on the viability of pancreatic cancer cells. When low concentrations of ICD combined with gemcitabine were applied to the three pancreatic cancer cell lines, the inhibition of cell viability was significantly enhanced at the same concentration of gemcitabine. The IC_{50} values of gemcitabine in the three pancreatic cancer cell lines decreased significantly. The combined index of gemcitabine and ICD at high, medium and low concentrations was calculated, and the results suggest that ICD and gemcitabine have a synergistic effect on the inhibition of pancreatic cancer cells.

We investigated the expression of Snail, E-cadherin, N-cadherin, Twist and Vimentin in the three pancreatic cancer cell lines. The results showed that the expression of the EMT-related transcription factors Snail and Twist was downregulated, while the expression of the EMT-related proteins N-cadherin and Vimentin was decreased, while E-cadherin was increased. EMT is characterized by the loss of epithelial cell polarity and the acquisition of mesenchymal cell peculiarity. In the process of tumor development, tumor cells can form local diffusion through EMT and invade lymphatic vessels and blood vessels for metastasis [46]. Therefore, we simultaneously evaluated the migration and invasion ability of the three pancreatic cancer cell lines treated with ICD, gemcitabine and their combination by cell scratch assays, Transwell migration assays and Transwell matrix invasion assays. The results of the cell scratch assays and Transwell migration assays suggested that the migration ability of pancreatic cancer cells treated with gemcitabine was improved compared with the control group, while the migration ability of pancreatic cancer cells in the ICD treated group was significantly decreased, and ICD could significantly reverse the improvement of cell migration ability caused by gemcitabine. Additionally, the Transwell matrix invasion assay also showed that gemcitabine can enhance the invasion ability of pancreatic cancer, and ICD can inhibit the invasion ability of pancreatic cancer cells and reverse the enhanced effect on the invasion ability of pancreatic cancer cells caused by gemcitabine.

The microarray analysis results showed that the synergistic inhibitory effect of ICD and gemcitabine in pancreatic cancer cells was related to the expression level of STAT3 and EMT mark-

ers. The present study demonstrated that ICD enhances the inhibitory effect of gemcitabine on pancreatic cancer by reducing the expression level of p-STAT3 in pancreatic cancer cells and opposing the STAT3-related drug resistance of pancreatic cancer cells. In the three pancreatic cancer cell lines, the expression level of p-STAT3 increased in the gemcitabine treatment group and decreased significantly in the ICD treatment group, while the expression level of p-STAT3 in the ICD and gemcitabine combination group was significantly lower than that in the gemcitabine monotherapy group. Furthermore, we used siRNA to knock down the expression of p-STAT3 in the three pancreatic cancer cell lines, and the results showed that after knocking down p-STAT3, the IC_{50} value of gemcitabine in the three cell lines was significantly reduced, and the inhibitory effect of gemcitabine on cells was enhanced. This suggests that the activation of p-STAT3 is related to the resistance of pancreatic cancer cells to gemcitabine. However, after knocking down p-STAT3 expression, the CI of ICD and gemcitabine in the treatment of the three pancreatic cancer cells decreased, and the systemic effect turned to the simple summation effect. Therefore, the synergistic effect of ICD combined with gemcitabine on the activity inhibition of pancreatic cancer cells was partially carried out by inhibiting the expression of p-STAT3. The combination treatment also inhibits tumor cell growth in a synergistic manner in mouse xenograft tumors. The tumor volumes of the ICD combined with gemcitabine group were significantly smaller than those of the control group and gemcitabine group.

EMT plays a role in embryonic formation and tissue development, but in recent years, more studies have focused on its important role in malignant tumor metastasis and invasion [47]. As an oncogene, STAT3 is abnormally activated by various upstream signals in cells, which is closely related to tumor proliferation, metastasis, invasion, antiapoptosis and angiogenesis. Therefore, in recent years, STAT3 protein has been considered an ideal target for antitumor therapy, and the role of its pathway in the invasion and metastasis of malignant tumors has been closely studied. STAT3 and EMT are closely related to the occurrence of malignant tumor development, although STAT3 is not the EMT steward of transcription factors, but it participates in EMT-related gene expression and regu-

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lates the EMT process and parsing in tumor cells [48, 49]. STAT3 affects the phenotypic plasticity of tumor cells, participates in circulating tumor cell formation and the changes in phenotypic characteristics and is closely related to tumor metastasis and invasion [26, 50, 51].

In conclusion, STAT3 is involved in the expression of EMT-related genes, and the increased expression level of p-STAT3 can promote the initiation of EMT in tumor cells, which is closely related to the metastasis and invasion of tumor cells. This study demonstrated that gemcitabine can upregulate the expression of p-STAT3 in pancreatic cancer cells, upregulate the expression of EMT-related transcription factors and proteins and enhance the migration and invasion ability of pancreatic cancer cells. However, ICD can reduce the expression level of p-STAT3 in pancreatic cancer cells, downregulate the expression of EMT-related transcription factors and proteins and inhibit the migration and invasion of tumor cells. Moreover, ICD combined with gemcitabine can reverse the upregulation of p-STAT3 expression induced by gemcitabine. The ICD and gemcitabine combination treatment reduced the occurrence of EMT induced by pancreatic cancer cells and inhibited the migration and invasion of the cells. The ICD and gemcitabine combination treatment can synergistically inhibit pancreatic cancer cell viability *in vitro* and *in vivo*. Therefore, this combination may be a promising approach to the treatment of patients with pancreatic cancer.

Disclosure of conflict of interest

None.

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