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EXPERIMENTAL STUDY

Effect of Jinlong capsule on proliferation and apoptosis of human pancreatic cancer cells BxPC-3

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Abstract

OBJECTIVE: To study the possible roles of Jinlong capsule (JLC) on the proliferation and apoptosis of human pancreatic cancer cells BxPC-3.

METHODS: The human pancreatic cancer cells BxPC-3 were treated with JLC at the concentration of 0.05-1.00 mg/mL for 24-120 h. The inhibition rate of JLC on human pancreatic cancer cells BxPC-3 was detected by 3-(4,5-dimethiylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Flow cytometry was employed to measure cell apoptosis using Annexin V-FITC/Propidium iodide (AV-FITC/PI) method. Cell cycles were determined by PI staining. The expression of S100 Calcium binding protein A4 (S100A4) in cell matrix was measured by enzyme-linked immunosorbent assay (ELISA). The expression levels of apoptosis-related protein such as BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), B-cell lymphoma/leukemia-2 (Bcl-2) and Cys-teinylaspartate specific proteinase 3 (Caspase-3) were detected by Western blotting.

RESULTS: JLC significantly inhibited the proliferation of human pancreatic cancer cells BxPC-3 in a dose-dependent and time-dependent manner. JLC promoted cell apoptosis and maintained cell cycle in S and G_2/M phase rather than G_1/G_0 phase. The expression of S100A4 in the cell matrix was reduced. The expression of cell apoptotic protein BNIP3 was increased while Bcl-2 was decreased.

CONCLUSION: JLC can inhibit the proliferation of human pancreatic cancer cells BxPC-3 by stimulating cell apoptosis, arresting the cell cycle at S and G_2/M phase which blocks the circulation of normal cell cycle and reducing the expression of S100A4 protein. Higher pro-apoptosis protein BNIP3 and lower anti-apoptosis protein Bcl-2 levels were found, which may be related to the apoptotic effects of JLC.

Key words: Pancreatic neoplasms; Cell proliferation; Apoptosis; Jinlong capsule

INTRODUCTION

Pancreatic cancer is a malignant solid tumor grown in internal organs, one of the hardest cancer which can be early diagnosed and treated. Five-year survival of pancreatic cancer is about 5%.[1] The morbidity and mortality of pancreatic cancer lead to the belief that pancreatic cancer is "the king of the cancer" in the 21st century. In order to improve the survival rate of patients with pancreatic cancers, the development of new effective

treatment strategies and new medicines to treat pancreatic cancer is of great importance.

Jinlong capsule (JLC), composed of Xianbaihuashe (Fresh *Bungarus*), Xianjinshe (Fresh *Agkistrodon*), Xianshougong (Fresh *Gecko*), etc, is a Traditional Chinese Medicine preparation. The active ingredients are extracted by the process of modern cryogenic and biochemical separation from raw animal drugs. The data of our previous studies has revealed that JLC is multi-targeted on tumors inhibition.[2-5] However, its role on the inhibition of pancreatic cancer has not been reported yet. In this study, we performed experiments to explore how JLC influences the proliferation and apoptosis of human pancreatic cancer cells BxPC-3 and possible mechanism involved. The results will support the fundamental roles of JLC on pancreatic cancer treatment and pave the way for further studies.

MATERIALS AND METHODS

Cell line

Human pancreatic cancer cells BxPC-3 were purchased from Shanghai institute of biochemistry and cell biology, Chinese Academy of Sciences (Shanghai, China).

Reagents and instruments

Dulbecco's modified eagle medium (DMEM) was the product of HyClone Laboratories, Inc. (Utah, USA), containing 15% fetal calf serum, 1% penicillin and streptomycin in the experiment. Fetal calf serum was from Sijiqing Company (Hangzhou, China), and was inactivated for 30 min at 56℃. JLC was purchased from Jiansheng Pharmaceutical Co., Ltd. (No. 110425, Beijing, China). The JLC powder was dissolved in the culture medium to configure the stock solution in 4 mg/mL, which was diluted to the desired concentration in the experiment. Gemcitabine hydrochloride (GEM) of 200 mg/ampoule was from Hansoh Pharmaceutical Co., Ltd. (Jiangsu, China). Anecxin-V/PI apoptosis kit and cell cycle staining solution were purchased from Biovision, Inc. (California, USA). Human S100 Calcium binding protein A4 (S100A4) ELISA kit was the product of Blue Gene company (Shanghai, China). Primary antibodies against Bcl-2 and Caspase-3 were the products of Cell Signaling Technology, Inc. (Boston, USA). Primary antibody against BNIP3 was purchased from R&D Systems China Co., Ltd. (Shanghai, China). β-actin antibody was purchased from Kangwei Century Biotech Co., Ltd. (Beijing, China). Secondary antibody conjugated to horse radish peroxidase was purchased from Beyotime institute of Biotechnology (Beijing, China). Cell-culturing box for CO_2 at constant temperature was the product of Sanyo Electric Co., Ltd. (Osaka, Japan). Microplate reader was the product of Biotek Instruments, Inc. (Vermont, USA). Flow cytometry was the product of Becton, Dickinson and Company (New Jersey, USA). Electrophoresis and electroporation instrument was the product of Bio-Rad Laboratories, Inc. (California, USA).

Cells culture

Human pancreatic cancer cells BxPC-3 were cultured in DMEM medium and maintained in the incubator at 37℃ with 5% CO_2 in a humidified atmosphere. The culture medium was replaced each two days and the cells at logarithmic growth phase were used for the following experiments.

Cell proliferation analysis

Human pancreatic cancer cells BxPC-3 were respectively seeded in 96-well culture plates for different culture period at 1×10^4 cells/well (24 h), 8×10^3 cells/well (48 h), 6×10^3 cells/well (72 h), 4×10^3 cells/well (96 h), 3×10^3 cells/well (120 h). After overnight incubation, the culture medium was replaced and JLC was added at final concentrations in 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL, respectively. Blank control group (Bg, no medium, no JLC), negative control group (Ng, no JLC) and positive control group (GEM in 0.02 mg/mL) were set up as well (six duplicates for each group). After 24, 48, 72, 96, 120 h incubation, 10 μL MTT (5 mg/mL) was added into each well at 37°C for 4 h. After the culture medium was removed, 150 μL dimethylsulfoxide (DMSO) was added and shaken to completely dissolve the formazan crystals. Optical density (OD) was measured at 570 nm with a microplate reader. The percentage of inhibition was calculated as the following formula: inhibition rate (IR) $=[1-(OD_{Sample}-OD_{Bg})/(OD_{Ng}-OD_{Bg})]\times100\%$. The data were from three independent replicates.

Assay of apoptosis by Annexin V-FITC/Propidium iodide (AV-FITC/PI) staining

After 48 h incubation with JLC at 0.4, 0.6 and 0.8 mg/mL, without JLC (negative control) or positive control group (GEM in 0.02 mg/mL), supernatants were collected and cells were digested. Cells were then mixed with supernatants at 5×10^5 cells/group and centrifuged at the speed of 1000 rpm for 5 min at 4℃. Supernatants were discarded and cells were suspended in phosphate buffered solution (PBS). This procedure was repeated twice. Cells were then suspended in 500 μL Binding Buffer. 5 μL AV-FITC and 10 μL PI were added and incubated in dark place at room temperature for 5 min. Flow cytometry was used to detect the living cells as well as early, moderate and late apoptotic cells or necrotic cells.

Determine the change of cell cycle by PI staining

After 48 h incubation with JLC at 0.4, 0.6 and 0.8 or without JLC (negative control), supernatants were collected and cells were digested. Cells were then mixed with supernatants at 1×10^6 cells/group and centrifuged

at the speed of 1000 rpm for 5 min at 4℃. Supernatant was discarded and 1 mL of PBS was added at room temperature to resuspended cells. Resuspened cells were then transferred gently to 4 mL of absolute ethanol at -20℃ with high speed vortexing and then kept at -20℃ for 10 min. The cells were pelleted by centrifugation, and then the ethanol was discarded. 5 mL of PBS was added at room temperature to rehydrate cells for 15 min. The cell suspension was centrifuged and the supernatant was discarded. 1 mL of DNA staining solution was added. Cells were incubated for 30 min at room temperature and analyzed by flow cytometry.

Detect S100 Calcium binding protein A4 (S100A4) in the cells matrix by ELISA

Supernatants collected from JLC treatment groups for 48 h (0.4, 0.6 and 0.8 mg/mL), or negative control group were mixed with buffer solution at the ratio of 10∶1, and incubated for 1 h at room temperature. 50 μL of standards or samples were added to the respective wells in the antibody pre-coated microtiter plate (4 duplicate wells for each group). 100 μL of enzyme conjugate was added to each well and mixed well. Plates were covered and incubated at 37℃ for 1 h. The microtiter plate was washed for five times. Fifty μL substrate A and 50 μL substrate B were added to each well, and then incubated for 10 min in dark place at 20℃-25℃. 50 μL of stop solution was added to each well and mixed well. The OD was read at 450 nm using a microtiter plate reader within 30 min of final incubation.

Detect expression level of proteins by western blotting

Cells collected from JLC treatment groups for 48 h (0.4, 0.6 and 0.8 mg/mL), or negative control group were lysed to extract proteins. The Bicinchoninic acid method was used to detect the concentration of protein. The Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was used to separate protein (100 μg per hole). Protein was electrically transferred to a polyvinylidene fluoride (PVDF) membrane. Western blot assay was used to detect the expression level of the Bcl-2, Caspase-3 and BNIP3. The PVDF membrane was immersed in Tris-Buffered Saline and Tween 20 (TBST) containing 5% skimmed milk powder at room temperature for 1 h to close. The corresponding primary antibody was incubated at 4°C overnight. The membrane was rinsed 3 times for 10 min each at room temperature with slow agitation in the TBST. The membrane was exposed for 2 h at room temperature to secondary antibody conjugated to horse radish peroxidase. After rinsing, the membrane was incubated in the chemical luminescence liquid and exposed onto the X-ray film in a dark room and fixed to observe targeted protein.

Statistical analysis

All statistical analysis were undertaken by SPSS 19.0 (SPSS Inc., Chicago, USA). The data are expressed as mean ± standard deviation (*SD*). LSD-*t* and Welch test was used. In all the test, $P<0.05$ was regarded as a statistically significant difference.

RESULTS

JLC inhibited the proliferation of human pancreatic cancer cells BxPC-3

The MTT assay was used to determine the effect of JLC on the proliferation of human pancreatic cancer cells BxPC-3. Table 1 and Figure 1 illustrated the inhibition rate of 0.05-1.00 mg/mL JLC and GEM (0.02 mg/mL) on the growth of human pancreatic cancer cells BxPC-3 after 24-120 h of incubation. The results showed that JLC inhibited the proliferation of human pancreatic cancer cells BxPC-3 in dose-dependent and time-dependent manner, and GEM inhibited the

Table 1 The inhibition rate of proliferation of human pancreatic cancer cells BxPC-3 affected by JLC (%, $\bar{x} \pm s$, n=6)

Group	24 h	48 h	72 h	96 h	120 h
Negative control	0	0	0	0	0
JLC (0.05 mg/mL)	-1.0±7.3[b]	0.6±10.7[b]	-2.1±6.0[b]	6.1±20.5[b]	3.6±20.6[b]
JLC (0.1 mg/mL)	-16.9±15.8[ab]	-3.9±21.0[b]	-9.5±9.2[ab]	-6.2±16.3[b]	-1.4±23.6[b]
JLC (0.2 mg/mL)	-13.1±12.4[ab]	-4.6±11.0[b]	6.8±6.5[b]	9.1±16.8[b]	28.4±24.8[ab]
JLC (0.4 mg/mL)	-8.0±6.1[b]	14.4±11.7[ab]	33.4±12.1[ab]	44.8±13.3[ab]	68.8±10.4[ab]
JLC (0.6 mg/mL)	1.8±8.1[b]	33.2±7.4[ab]	65.1±5.8[ab]	77.2±7.3[a]	91.0±5.8[a]
JLC (0.8 mg/mL)	19.2±9.3[ab]	53.3±5.4[a]	92.0±5.1[ab]	94.8±2.5[a]	99.4±1.6[a]
JLC (1.0 mg/mL)	45.2±6.2[a]	83.3±2.3[ab]	96.6±1.8[ab]	98.4±1.5[a]	99.4±1.0[a]
GEM (0.02 mg/mL)	47.1±5.4[a]	61.8±5.6[a]	80.4±2.4[a]	88.1±1.3[a]	93.8±2.0[a]

Notes: JLC: Jinlong capsule; BxPC-3: pancreatic cancer cells. Compared with the negative control group, [a]$P<0.05$; compared with the positive control group, [b]$P<0.05$.

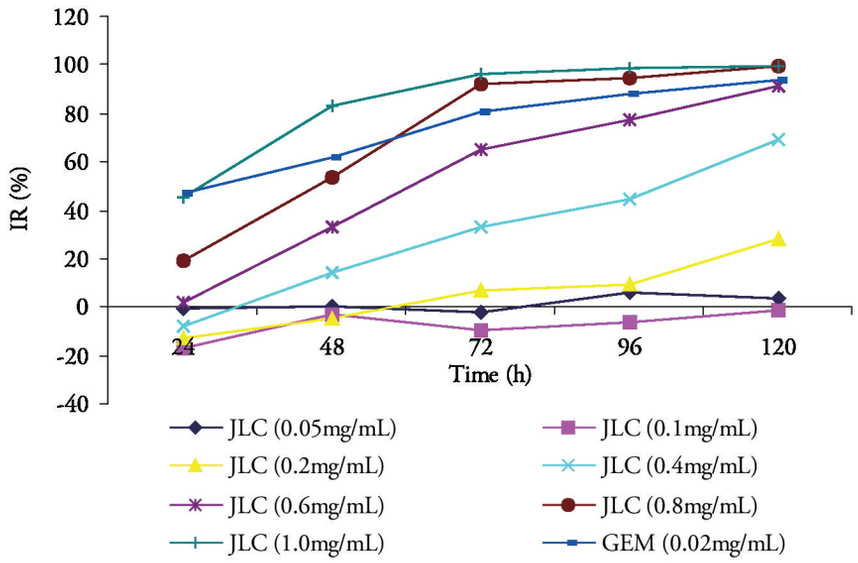

Figure 1 Inhibition rate of proliferation of human pancreatic cancer cells BxPC-3 affected by JLC
IR: inhibition rate; JLC: Jinlong capsule; GEM: gemcitabine hydrochloride.

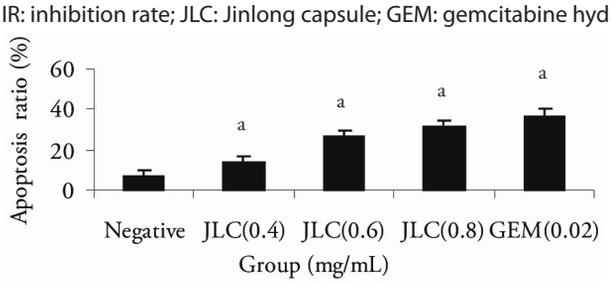

Figure 2 Apoptosis of human pancreatic cancer cells BxPC-3 in different culture media
JLC: Jinlong capsule; GEM: gemcitabine hydrochloride. Compared with the negative control group, $^aP<0.05$.

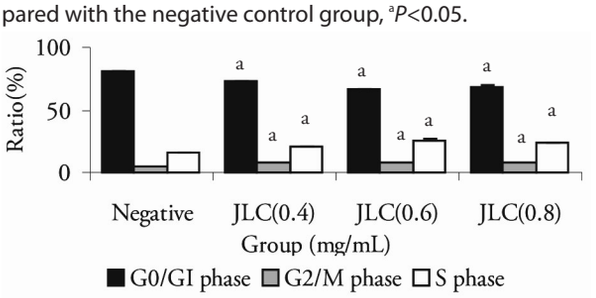

Figure 3 The cell cycle of human pancreatic cancer cells BxPC-3 in different culture media
JLC: Jinlong capsule. Compared with the negative control group, $^aP<0.05$.

proliferation of the cells in a time-dependent manner. However, after 48 h incubation, when compared to the negative control group, while JLC still showed robust effects on cell growth suppress at the concentrations of 0.4, 0.6, 0.8, 1.0 mg/mL [(14.38 ± 11.68)% to (83.33 ± 2.29)% inhibition] (all $P<0.05$), and the inhibition was not significantly different between the group of 0.8 mg/mL and the positive control group ($P>0.05$), and the 1.0 mg/mL dose group performed even better than the positive drug group ($P<0.05$). In addition, after incubation for 48 h, cell metabolite rate was relatively low which has less impact on the results. Based on these facts, the follow-up experiments were performed with JLC at the dose of 0.4, 0.6, 0.8 mg/mL and the incubation time were set at 48 h.

JLC promoted the apoptosis of human pancreatic cancer cells BxPC-3

Annexin V-FITC/PI double dyed flow cytometry was used to detect the apoptosis of human pancreatic cancer cells BxPC-3 with the treatment of JLC. As shown in Figure 2, after 48 h treatment, the apoptotic cells in the negative control group was (7.1±3.2)%, and JLC at the dose of 0.4, 0.6 and 0.8 mg/mL promoted the apoptosis by (13.6 ± 3.4)%, (27.0 ± 2.1)% and (31.0 ± 3.5)% (all $P<0.05$), and there was no variance between the group of 0.8 mg/mL and the positive control ($P>0.05$). The data suggested JLC could significantly promote apoptosis of human pancreatic cancer cells Bx-

Table 2 The OD values of cell matrix in different groups and the concentration of S100A4 ($n=4$)

Group	JLC (0.8 mg/mL)	JLC (0.6 mg/mL)	JLC (0.4 mg/mL)	Negative control
OD	0.126	0.129	0.132	0.137
	0.124	0.127	0.13	0.133
	0.13	0.131	0.126	0.136
	0.124	0.129	0.129	0.131
Mean	0.1260±0.0028[a]	0.1290±0.0016[a]	0.1293±0.0025[a]	0.1343±0.0028
Concentration (pg/mL)	16.782	17.907	17.982	19.789

Notes: OD: optical density; JLC: Jinlong capsule. Compared with the negative control group, $^aP<0.05$.

PC-3 in a dose-dependent manner. With elevated concentrations of JLC, number of late apoptotic cells increased more apparently.

JLC affected the cell cycle of human pancreatic cancer cells BxPC-3

PI-stained flow cytometry was used to detect the change of cell cycle of human pancreatic cancer cells BxPC-3 treated with JLC. As shown in Figure 3, after 48 h treatment, the number of cells in phase G_0/G_1, S and G_2/M were all significantly different between the negative control and the JLC treatment group at the dose of 0.4, 0.6 and 0.8 mg/mL (all $P<0.05$). Number of cells in S and G_2/M phase increased significantly with JLC treatment while the number of cells in G_1/G_0 phase reduced. The results illustrated that JLC affected the circulation of normal cell cycle by arresting cells at the G_2/M and S phase, thus inhibiting the cell proliferation.

JLC affected the expression of S100A4 in the cells matrix

ELISA was used to detect the change of S100A4 in the cells matrix of human pancreatic cancer cells BxPC-3 treated with JLC. As shown in Table 2, after 48 h incubation, the OD values of cell matrix in the groups of JLC in the concentrations of 0.4, 0.6 and 0.8 mg/mL were totally different as compared with the negative control (all $P<0.05$). The levels of S100A4 in the groups treated with 0.4, 0.6, 0.8 mg/mL JLC and negative control were 17.982, 17.907, 16.782 and 19.789 pg/mL respectively. The results illustrated that JLC reduced the expression of S100A4 in the human pancreatic cancer cells BxPC-3.

JLC affected the expression level of apoptosis-related proteins

Western blot analysis was used to detect the expression level of proteins of Bcl-2, Caspase-3 and BNIP3. As shown in Figure 4, after 48 h incubation, compared with the negative control, the expression level of Bcl-2 in JLC treatment group decreased, and the expression of BNIP3 increased in a dose-dependent manner. However, the expression of Caspase-3 was not significantly changed in each group. The results illustrated that JLC promotes the apoptosis of human pancreatic cancer cells BxPC-3 by both increasing the expression of pro-apoptotic protein BNIP3 and reducing the level of anti-apoptotic protein Bcl-2.

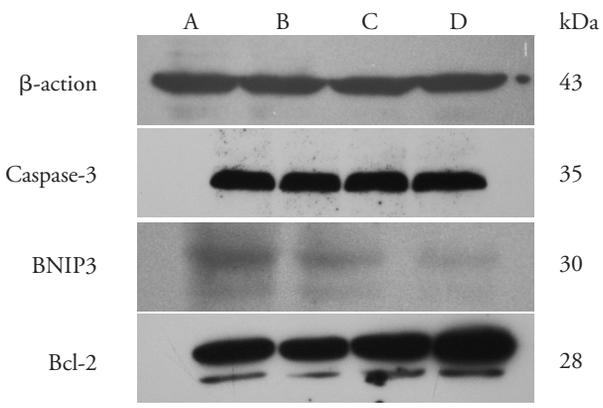

Figure 4 The expression of protein β-action, Caspase-3, BNIP3 and Bcl-2

A: JLC (0.8 mg/mL); B: JLC (0.6 mg/mL); C: JLC (0.4 mg/mL); D: negative control. JLC: Jinlong capusle; BNIP3: BCL2/adenovirus E1B 19 kDa protein-interacting protein 3.

DISCUSSION

In multicellular organisms, cell death has two different forms, namely necrosis and apoptosis. Apoptosis is the process of dying in physiological or pathological conditions under control by the gene, known as programmed cell death, and is a hot research topic of antitumor. In this study, the result of Annexin-VFITC/PI double staining flow cytometry showed that the JLC can promote apoptosis of human pancreatic cancer cells BxPC-3 in a significant dose-dependent manner which was consistent with the MTT assay and illustrated that promoting apoptosis may be one of the mechanisms involved in the suppression of human pancreatic cancer cells BxPC-3 proliferation by JLC. Furthermore, JLC affected the cell division cycle by arresting cells at the S and G_2/M phase which also contributed to the inhibition of cell proliferation.

S100A4 is a protein that has multiple functions, including enhancing the proliferation of tumor cell, inhibiting cell apoptosis, promoting cell motility, reducing cell adhesion, promoting angiogenesis as well as triggering cells invasion and metastasis.[6-8] It is closely related to the generation and development of tumor. S100A4 presents in the cell as a non-covalently bound dimer, and is secreted into the cell matrix as covalent dimer. S100A4 protein in the cell matrix is the basis of signal transduction between cells and plays a major role in mediating cell and cell matrix interaction.[9] As shown in the result of ELISA assay, S100A4 in the cell matrix decreased when the human pancreatic cancer cells BxPC-3 were treated with JLC. A large number of studies have confirmed that the downregulation of S100A4 inhibited the proliferation, migration and invasion of pancreatic cancer cell.[10] Therefore, lower S100A4 level in the cell matrix with JLC treatment may also contribute to the inhibition effects of JLC on the proliferation of human pancreatic cancer cells BxPC-3.

The molecular mechanism of apoptosis is very complicated, influenced by many factors, and involves the participation of a variety of apoptosis-related proteins, including pro-apoptosis proteins and anti-apoptosis proteins. Bcl-2 protein, as an anti-apoptotic protein, can prolong cell survival and inhibit cell apoptosis.[11] In this study, Western blot results showed that the JLC could reduce the expression of Bcl-2 protein when in BxPC-3 human pancreatic cancer cells and may contribute to the cells apoptosis.

BNIP3, as a pro-apoptotic protein, can antagonize the anti-apoptotic function of Bcl-2 and Bcl-xl through the formation of isomers with the Bcl-2 and Bcl-xl.[12] BNIP3 also enhances the pro-apoptotic function of Bax and Bak, and induces autophagy.[13] Under the normal circumstances, BNIP3 protein is not expressed in human pancreatic cancer cell lines BxPC-3.[14] The previous studies showed that the over-expression of S100A4 is one of the mechanisms related to the reduced expression of BNIP3 in pancreatic cancer.[15] Western Blot results showed that the JLC can promote the expression of BNIP3 protein in a dose-dependent manner in human pancreatic cancer cells BxPC-3. ELISA results showed that the JLC can reduce expression of S100A4 dose-dependently in human pancreatic cancer cells BxPC-3. In summary, JLC may increase the expression of pro-apoptotic protein BNIP3 by reducing the expression of S100A4 protein in the cell matrix. These two factors mutually affected, synergistically promoting the apoptosis of BxPC-3 human pancreatic cancer cells.

Caspase-3 is in the downstream of cell apoptosis, involved in apoptosis execution.[16] The result of Western blot showed that the expression of Caspase-3 protein had no significant difference between each group. Therefore, we postulate the pathway of apoptosis of human pancreatic cancer cells BxPC-3 triggered by JLC was not through Caspase-3 protein.

In summary, this study demonstrated that JLC can inhibit the proliferation of human pancreatic cancer cells BxPC-3 through the promotion of apoptosis, arresting the cell division cycle at S and G_2/M phase and reducing the expression of S100A4 protein. The mechanism of promoting apoptosis may be associated with raising pro-apoptosis protein BNIP3 and reducing the anti-apoptosis protein Bcl-2. We believe this study only reveals part of the molecular mechanisms involved in JLC's anti-tumor effects and warrants further in-depth study.

The theory of Traditional Chinese Medicine holds that the formation of tumors is closely related with blood stasis, phlegm stagnation and *Qi* stagnation. JLC composed by Xianbaihuashe (Fresh *Bungarus*), Xianjinshe (Fresh *Agkistrodon*), Xianshougong (Fresh *Gecko*), etc. has the function of promoting blood circulation, resolving masses and stagnation, and dredging collaterals,[17,18] so it has good anti-tumor function. In this study, the results showed that JLC inhibited the proliferation of human pancreatic cancer cells BxPC-3, consistenting with the relevant Chinese medicine theory. In additon, this study revealed that JLC as a compound preparation, holds the multi-targeted therapeutic effects of traditional Chinese medicine.

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