

Original Article

Effect of baicalin on proliferation and apoptosis in pancreatic cancer cells

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Abstract: Background and objectives: Pancreatic cancer is one of the most lethal cancer types. Pancreatic cancer is highly malignant and characterized by rapid and uncontrolled growth. This study was designed to investigate the effect of baicalin on proliferation and apoptosis in pancreatic cancer cells. Methods: CCK-8 assay and Clone formation assay were performed to detect the effect of baicalin on proliferation in pancreatic cancer cells. Cell invasion and migration were all assessed with Wound healing assay and Transwell invasion assay. Flow Cytometry Analysis and DAPI staining were performed to detect the effect of baicalin on apoptosis in pancreatic cancer cells. Furthermore, proliferation-associated protein and apoptosis-related protein were detected to evaluate the cell proliferation and apoptosis levels. P-JNK protein, t-JNK protein, Foxo1 protein and BIM protein were examined by western blot to verify whether baicalin could regulate the proliferation and apoptosis via the JNK/Foxo1/BIM signaling pathway in pancreatic cancer cells. Results: The cell proliferation level was significantly decreased while the cell apoptosis level was significantly increased in pancreatic cancer SW1990 cells treated with baicalin. As the same, baicalin downregulated the ability of invasion and migration in pancreatic cancer SW1990 cells. Conclusion: Baicalin might inhibit cell proliferation and promote cell apoptosis via JNK/Foxo1/BIM signaling pathway in pancreatic cancer SW1990 cells.

Keywords: Baicalin, proliferation, apoptosis, pancreatic cancer cell

Introduction

Pancreatic cancer is a digestive system tumor with high degree of malignancy and poor prognosis, accounting for the fourth leading cause of cancer-related deaths worldwide [1]. The incidence and mortality rates of pancreatic cancer show a sustained increase recently, with the overall 5-year relative survival rate from 5% to 6% [2]. According to the GLOBOCAN 2012, pancreatic cancer accounts for more than 0.33 million deaths per year [3]. It is characterized by rapid disease progression and metastatic ability, high resistance to chemotherapy and radiotherapy, and difficult early diagnosis so that most patients have no chance to cure it [4]. Therefore, exploration of new strategies for the treatment of pancreatic cancer is urgently needed.

Apoptosis and proliferation, as two major mechanisms which regulates pathways to control cell state, are suggested for consideration

as the targeted therapy strategies for tumor cells [5]. Previous studies demonstrated that CDK2 and cyclinD1 genes are the most important regulators in controlling the proliferation of cells [6]. CDK2 forms a complex with cyclinE1 to promote G1/S transition and forms a complex with cyclinA to bypass the S phase and the G2/M phase [7]. P16 is known to be an important tumor suppressor gene functioning as cell cycle regulator [8], thus regulation of the function of CDK2, cyclinD1 and p16 is an appropriate strategy to inhibit the proliferation of cancer cells. The p53 tumor suppressor protein can inhibit tumor formation, in part by inducing apoptosis. It has been demonstrated that p53 is able to regulate the transcription and expression of proapoptotic and anti-apoptotic proteins, including Bax, Bcl-2 and caspase-3, resulting in cellular apoptosis [9]. It is known that JNK phosphorylates c-Jun at Ser63 and Ser73 regions to activate the pro-apoptotic effects of c-Jun [10]. Also, JNK enhances expression of FOXO-regulated genes and FOXO

Effect of baicalin on pancreatic cancer

can regulate cell apoptosis, cycle and ROS resistance by affecting Bim [11].

In recent years, many phytochemicals, with low toxicity and adverse side effects, have been observed to exhibit anti-cancer activities and thus may represent potential alternative medicine to conventional cytotoxic chemotherapy [12]. Baicalin is an active flavone isolated from *Scutellaria baicalensis* Georgi, with a chemical formula of $C_{21}H_{18}O_{11}$ and has been demonstrated to have anti-proliferation and antitumor effects [13]. It has been reported that we can inhibit the proliferation of various cancer cells through inhibition of migration and invasion and induction of apoptosis [14]. However, the effects of baicalin on pancreatic cancer cells and the underlying molecular mechanisms are still not clear. So, the present study aims to evaluate whether baicalin can exert antitumor effects on pancreatic cancer cells and investigate the underlying molecular mechanisms. In conclusion, the findings in our study suggest that baicalin may be identified as a novel regulator in inhibiting pancreatic cancer progress by JNK/Foxo1/BIM pathway. Therefore, in-depth study on the molecular mechanisms will provide new strategies for the treatment.

Materials and methods

Cell culture

Pancreatic cancer SW1990 cell lines were obtained from the Second Military Medical University (Shanghai, China). SW1990 cells was grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco) and antimycotic agents (100 units/ml penicillin, 100 µg/ml streptomycin; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an incubator with standard culture conditions (37°C, 5% CO₂ and 95% humidity).

Cell Counting Kit-8 (CCK-8) assay

Cell viability was measured by CCK-8 assay following the instructions of manufacturer. In brief, cells were digested, counted, and prepared into the cell suspension at a concentration of 5×10^3 cells/ml in 96-well plates cultured in an incubator with standard culture conditions (37°C, 5% CO₂ and 95% humidity). After treatments, 10 µL of CCK-8 solution was added to each 96-well plates and the mixture was

incubated for 3 hours in the incubator at 37°C. $\lambda=450$ nm, and the OD value of each well was read by a microplate reader (Bio-Rad, Hercules, CA, USA).

Clone formation assay

Colony-formation assays were conducted to evaluate the Cell proliferation ability. Suspensions containing 5000 cells per well were seeded into the six-well plates and individually exposed to doses of 0, 40, 80, 120 and 160 µmol/L baicalin, respectively. After treatments, the cells were incubated for 48 hours in an incubator with standard culture conditions (37°C, 5% CO₂ and 95% humidity). Next, the colonies were fixed with carbinol for 15 minutes and stained with 0.1% Giemsa for 30 minutes. Clones were counted under an inverted microscope and clones containing >50 cells were counted for statistical analysis. Cells in each group were plated in three duplicate wells and all experiments were conducted at least for three times.

Wound healing assay

Stable cells were plated into the 6-well cell culture plates and grown to near 100% confluence. Then, the cells were scratched with a 200 µL pipette tip and the detached cells were washed with phosphate-buffered saline (PBS). Images of cells migrating at the corresponding wound sites were taken at 0 and 24 h with an inverted microscope and the wound areas were determined using Image J software. Cell migration was expressed as the percentage of wound closure/initial wound area. All experiments were performed at least for three times.

Transwell invasion assay

Transwell invasion assays were performed to evaluate the cell invasive ability. Sw1990 cells were seeded on Matrigel-coated transwell inserts with 500 µL of serum free medium. The lower chamber was filled with 500 µL of medium containing 10% FBS. After incubation for 24 h, the cells remaining on upper surface of transwell inserts were wiped with cotton wool. The invaded cells were stained with crystal violet for 5 min and counted under an inverted microscope. Data were collected from three independent experiments.

Effect of baicalin on pancreatic cancer

Flow cytometry analysis of cell apoptosis

A cell apoptosis experiment was performed to determine the apoptosis rate with a FITC Annexin V Apoptosis Detection Kit according to the manufacturer's instructions. In brief, cells (5×10^5) were seeded in 6-well plates and treated with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin, respectively. The cell samples cultured for 48 h after treatment were harvested with 0.25% trypsin, then washed twice with ice-cold PBS and re-suspended in binding buffer. The cells were double-stained with FITC-conjugated Annexin V and propidium iodide (FITC-Annexin V/PI) (BD Biosciences, San Diego, CA, USA) for 20 minutes in the dark. The mixture was then analyzed on a flow cytometer (FACSCalibur; BD Biosciences) with the excitation wavelength of $\text{Ex}=488$ nm and emission wavelength of $\text{Em}=530$ nm according to the manufacturer's protocol. The experiments were performed in triplicate and repeated at least for three times.

Detection of apoptosis via DAPI staining

After treatment with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin respectively, the cells were harvested, washed with a phosphate-buffered saline (PBS) twice, and fixed in 4% formaldehyde prepared with PBS. The cells were stained with 1.0 mg/mL of 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Chemical Co.) solution for 15 min at 37°C . The stained cells were transferred to a glass slide and morphology changes in the nucleus were observed with a fluorescence microscope (Olympus BX51, Tokyo, Japan).

Measurement of reactive oxygen species (ROS) level

2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is a dye that can be oxidized by ROS to fluorescent compound dichlorofluorescein (DCF), which is monitored by fluorescence microplate spectrophotometry. ROS level is quantified by the fluorescence intensity of DCF with a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). In briefly, the cells were washed twice with phosphate-buffered saline (PBS) and then stained with DCFH-DA (10 $\mu\text{mol/l}$) at 37°C for 20 min according to the manufacturer's protocol. ROS levels of cell samples were assessed by a commercial ROS assay kit

(Nanjing Jancheng Bioengineering Institute, Nanjing, China).

Western blot analysis

Western blot analysis was used to detect the relative protein expression level including CDK2, cyclinE1, p15, bcl-2, bax, cleaved caspase3, caspase3, p53, p-JNK, JNK, Foxo1 and BIM, as described. The total protein in cell samples was extracted from pancreatic cancer SW1990 cells with a mixture of RIPA buffer (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% SDS), protease inhibitors and phosphatase inhibitor, which protect proteins from degradation by an endogenous protease. The total protein concentration was determined with an Enhanced BCA Protein Assay kit (P0010; Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. Equal amounts (20 μg) of total protein lysate per lane was used for Western blot analysis with the following method: Proteins were separated by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5%-10%) gel and then transferred to a polyvinylidene fluoride (PDVF) membrane (Sigma-Aldrich; Merck KGaA). Non-specific protein interactions were blocked by 5% fat-free milk in 1X TBST buffer at room temperature for 1 h. Following this treatment, the PVDF membranes were incubated with primary antibodies against CDK2 (1:1000, ab32147, abcam, UK), cyclinE1 (1:2000, ab33911, abcam, UK), p15 (1:2000, ab53034, abcam, UK), bcl-2 (1:2000, ab185002, abcam, UK), bax (1:1000, ab32503, abcam, UK), cleaved caspase3 (1:500, ab49822, abcam, UK), caspase3 (1:500, ab13847, abcam, UK), p53 (1:1000, ab32389, abcam, UK), p-JNK (1:500, ab47337, abcam, UK), JNK (1:2500, ab199380, abcam, UK), Foxo1 (1:5000, ab70382, abcam, UK), BIM (1:1000, ab32158, abcam, UK) and GAPDH (1:10000, ab181602, abcam, UK) at 4°C overnight. On second day, the PVDF membranes were incubated in a solution of goat anti-rabbit (1:20000, ab205718, abcam, UK) or anti-mouse IgG (1:10000, ab6708, abcam, UK) at room temperature for 1 h. GAPDH was used as an endogenous control. Protein bands were visualized by an enhanced chemiluminescence (ECL) kit (sc-2048, Sigma-Aldrich, Merck KGaA) following the manufacturer's instructions. Data were analyzed with Image Pro Plus v.6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) for densitometry.

Effect of baicalin on pancreatic cancer

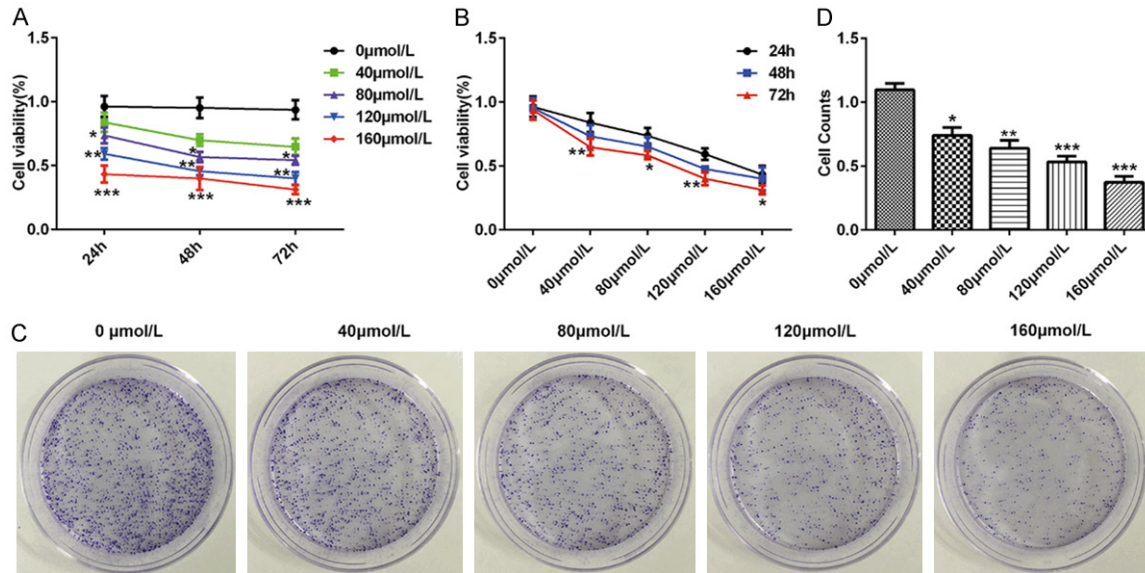


Figure 1. Baicalin inhibited proliferation of pancreatic cancer SW1990 cells. Pancreatic cancer SW1990 cells were incubated for 24, 48, 72 hours after treatment with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin, respectively. A. The time-dependent cell viability of pancreatic cancer SW1990 cells determined by CCK8; B. The dose-dependent cell viability of pancreatic cancer SW1990 cells determined by CCK8; C. The cell clone number of pancreatic cancer SW1990 cells determined by clone formation assay; D. The cell clone number of pancreatic cancer SW1990 cells determined by clone formation assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Statistical analysis

Data are processed using SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA) and presented as the mean \pm standard deviation with Graph-PAD prism software. All the experiments were performed at least for three times. A Student t-test was used for statistical comparison between means where applicable. Differences among more than two groups in the above assays were estimated using one-way ANOVA. Differences were considered significant when P -value less than 0.05.

Results

Baicalin inhibit proliferation of pancreatic cancer SW1990 cells

To further elucidate the biological role of baicalin on pancreatic cancer, we investigate the effects of baicalin on pancreatic cancer cell proliferation. Pancreatic cancer SW1990 cells were incubated for 24, 48, 72 hours after treatment with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin, respectively. CCK-8 assay data showed that baicalin could significantly inhibit pancreatic cancer cell proliferation in a dose-dependent manner ($P < 0.01$, $P < 0.05$, $P < 0.001$, **Figure 1A**) and in a time-dependent manner ($P < 0.05$, $P < 0.01$, **Figure 1B**). Clone for-

mation assay showed that baicalin could significantly inhibit pancreatic cancer cell proliferation in a dose-dependent manner ($P < 0.01$, $P < 0.05$, $P < 0.001$, **Figure 1C** and **1D**).

Baicalin influenced cell cycle of pancreatic cancer SW1990 cells

To explore whether baicalin was associated with cell cycle, we detected protein levels of CDK2, cyclinE1 and p15 by western blot. Pancreatic cancer SW1990 cells were incubated for 48 hours after treatment with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin, respectively. Baicalin obviously decreased the protein levels of CDK2 ($P < 0.01$, $P < 0.05$, $P < 0.001$, **Figure 2A** and **2B**) and cyclinE1 ($P < 0.01$, $P < 0.05$, $P < 0.001$, **Figure 2A** and **2C**) and increased the protein level of p15 ($P < 0.01$, $P < 0.05$, $P < 0.001$, **Figure 2A** and **2D**) in a dose-dependent manner.

Baicalin decreased migratory and invasive ability of pancreatic cancer SW1990 cells

To determine whether baicalin can influence the migration and invasion of pancreatic cancer SW1990 cells, wound-healing assay and trans-well assay were performed. Pancreatic cancer SW1990 cells were incubated for 48 hours after treatment with doses of 0, 40, 80,

Effect of baicalin on pancreatic cancer

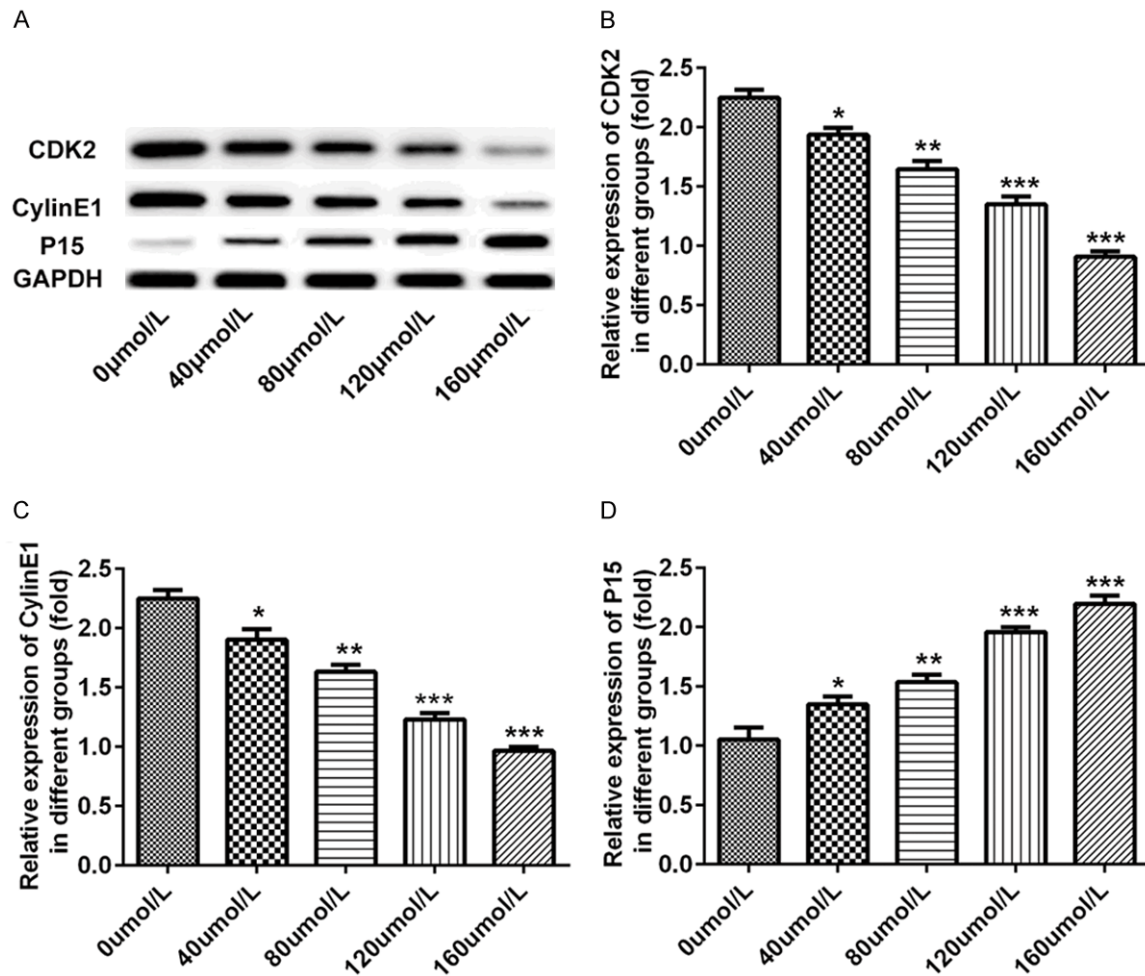


Figure 2. Baicalin influenced cell cycle of pancreatic cancer SW1990 cells. Pancreatic cancer SW1990 cells were incubated for 48 hours after treatment with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin, respectively. A. The dose-dependent protein band of CDK2, cyclinE1 and p15 determined by western blot; B. The dose-dependent protein level of CDK2 determined by western blot; C. The dose-dependent protein level of cyclinE1 determined by western blot; D. The dose-dependent protein level of p15 determined by western blot. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

120 and 160 $\mu\text{mol/L}$ baicalin, respectively. Wound-healing assay showed that baicalin could suppress the migration of pancreatic cancer SW1990 cells in a dose-dependent manner ($P < 0.01$, $P < 0.05$, $P < 0.001$, **Figure 3A** and **3B**). Similar results were obtained in trans-well assay. Trans-well assay verified that baicalin could suppress the invasion of pancreatic cancer SW1990 cells in a dose-dependent manner ($P < 0.01$, $P < 0.05$, $P < 0.001$, **Figure 3C** and **3D**).

Baicalin promoted the apoptosis of pancreatic cancer SW1990 cells

To determine whether baicalin contributed to the apoptosis of pancreatic cancer SW1990 cells, we analyzed cell apoptosis of SW1990 cells by flow cytometry, DAPI staining and west-

ern blot. Pancreatic cancer SW1990 cells were incubated for 48 hours after treatment with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin, respectively. The result of flow cytometry showed that the apoptosis levels of SW1990 cells incubated with baicalin increased in a dose-dependent manner ($P < 0.01$, $P < 0.001$, **Figure 4A** and **4B**). Also, DAPI staining assay showed that Baicalin promoted the apoptosis of SW1990 cells in a dose-dependent manner ($P < 0.05$, $P < 0.01$, $P < 0.001$, **Figure 4C** and **4D**). Besides, Baicalin obviously decreased the protein levels of Bcl-2 ($P < 0.01$, $P < 0.001$, **Figure 5A** and **5B**) and increased the levels of Bax ($P < 0.05$, $P < 0.01$, $P < 0.001$, **Figure 5A** and **5C**), Cleaved caspase-3 ($P < 0.01$, $P < 0.001$, **Figure 5A** and **5D**) and p53 ($P < 0.05$, $P < 0.01$, $P < 0.001$, **Figure 5A** and **5E**) in a dose-dependent manner.

Effect of baicalin on pancreatic cancer

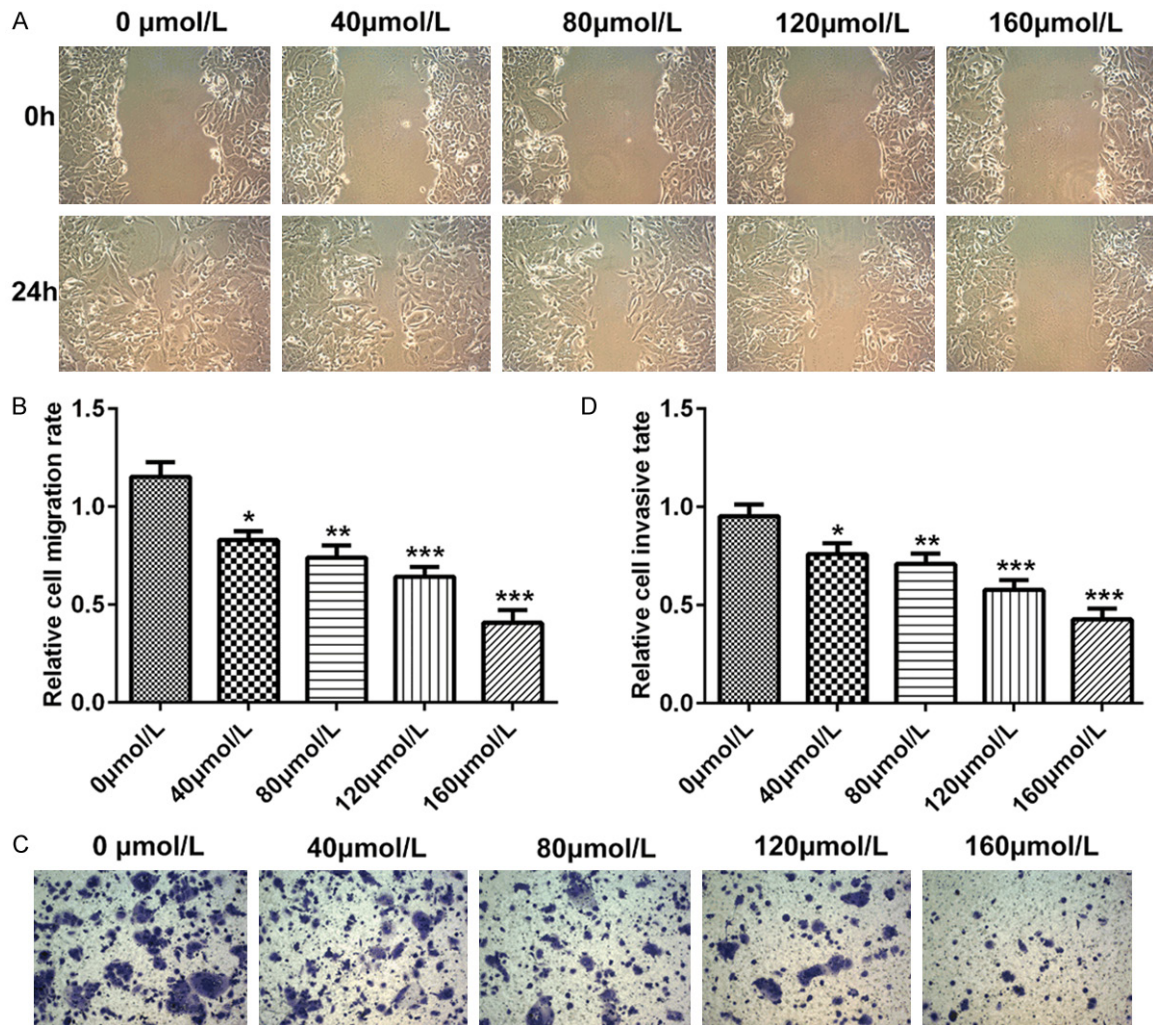


Figure 3. Baicalin decreased migratory and invasive ability of pancreatic cancer SW1990 cells. Pancreatic cancer SW1990 cells were incubated for 48 hours after treatment with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin, respectively. A. The dose-dependent migratory ability of pancreatic cancer SW1990 cells determined by wound-healing assay; B. The dose-dependent cell counts of pancreatic cancer SW1990 cells determined by wound-healing assay; C. The dose-dependent invasive ability of pancreatic cancer SW1990 cells determined by transwell assay; D. The dose-dependent cell counts of pancreatic cancer SW1990 cells determined by transwell assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Baicalin increased the ROS level of pancreatic cancer SW1990 cells

To analyze whether Baicalin would affect the ROS level in pancreatic cancer SW1990 cells, we detected the ROS level with DCFH-DA ROS assay. Pancreatic cancer SW1990 cells were incubated for 48 hours after treatment with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin, respectively. The result showed that baicalin could increase the ROS level of pancreatic cancer SW1990 cells in a dose-dependent manner, and NAC could obviously decrease the ROS level ($P < 0.001$, **Figure 6**).

Baicalin activated JNK/Foxo1/BIM pathway

To investigate the influence of baicalin on JNK/Foxo1/BIM pathway in pancreatic cancer SW1990 cells, we assessed the protein levels of JNK, p-JNK, Foxo1 and BIM. Pancreatic cancer SW1990 cells were incubated for 48 hours after treatment with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin, respectively. As expected, baicalin obviously increased the protein levels of p-JNK ($P < 0.05$, $P < 0.01$, $P < 0.001$, **Figure 7A** and **7B**), Foxo1 ($P < 0.05$, $P < 0.01$, $P < 0.001$, **Figure 7A** and **7C**) and BIM ($P < 0.01$, $P < 0.001$, **Figure 7A** and **7D**) in a dose-depen-

Effect of baicalin on pancreatic cancer

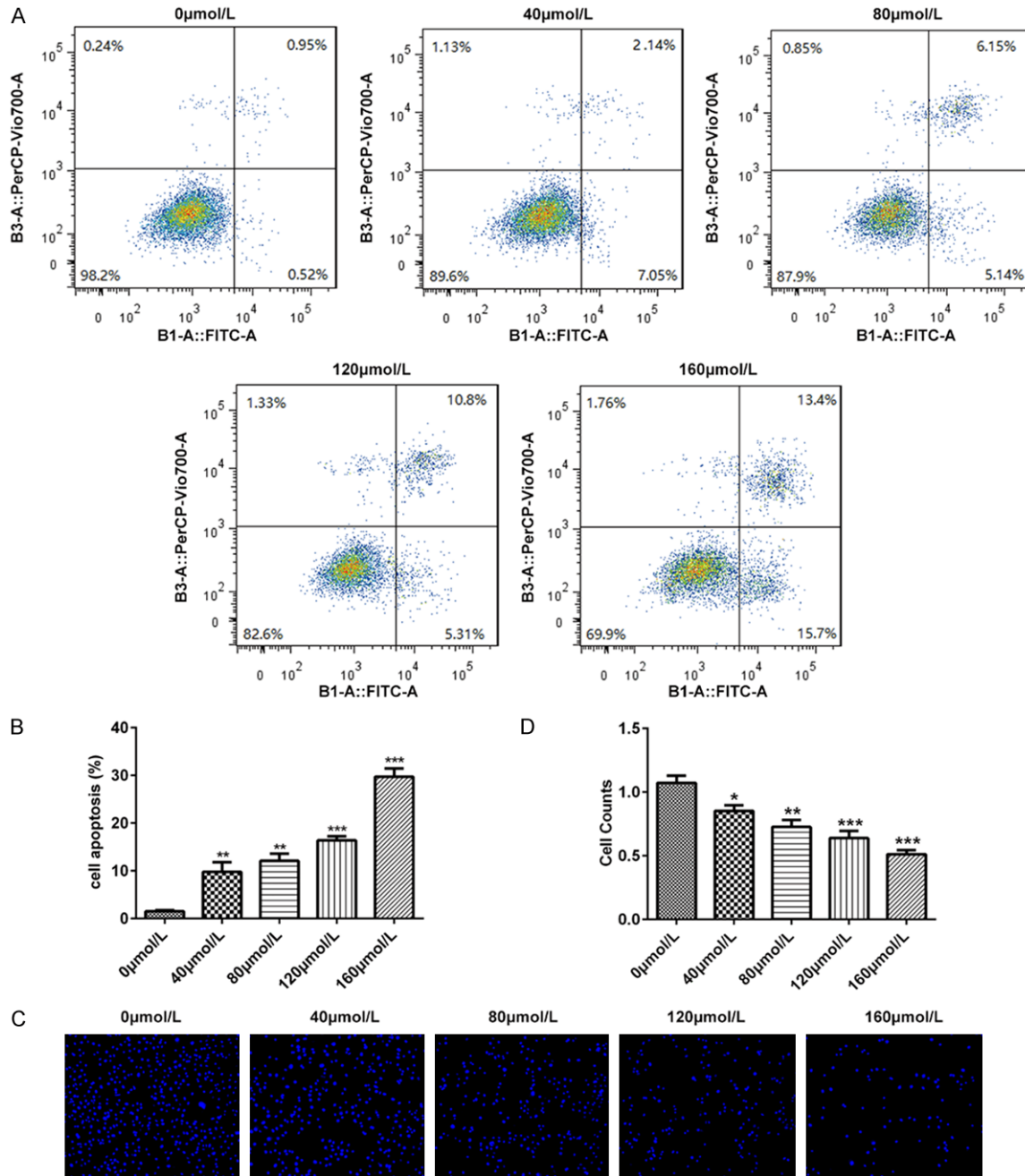


Figure 4. Baicalin promoted the apoptosis of pancreatic cancer SW1990 cells. Pancreatic cancer SW1990 cells were incubated for 48 hours after treatment with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin, respectively. A. The pancreatic cancer SW1990 cell apoptosis determined by flow cytometry; B. The dose-dependent cell apoptosis counts of pancreatic cancer SW1990 cells determined by flow cytometry; C. The pancreatic cancer SW1990 cell apoptosis determined by DAPI staining; D. The dose-dependent cell apoptosis counts of pancreatic cancer SW1990 cells determined by DAPI staining. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

dent manner. However, free radical scavenger (NAC) reversed the effect of baicalin. NAC significantly decreased the protein levels of p-JNK ($P < 0.01$, **Figure 7A** and **7B**), Foxo1 ($P < 0.001$, **Figure 7A** and **7C**) and BIM ($P < 0.001$, **Figure 7A** and **7D**).

Discussion

Recently, the use of plant extracts to prevent the progress of cancer is getting more and more attention [15]. In our study, we revealed that baicalin could significantly inhibit pancre-

Effect of baicalin on pancreatic cancer

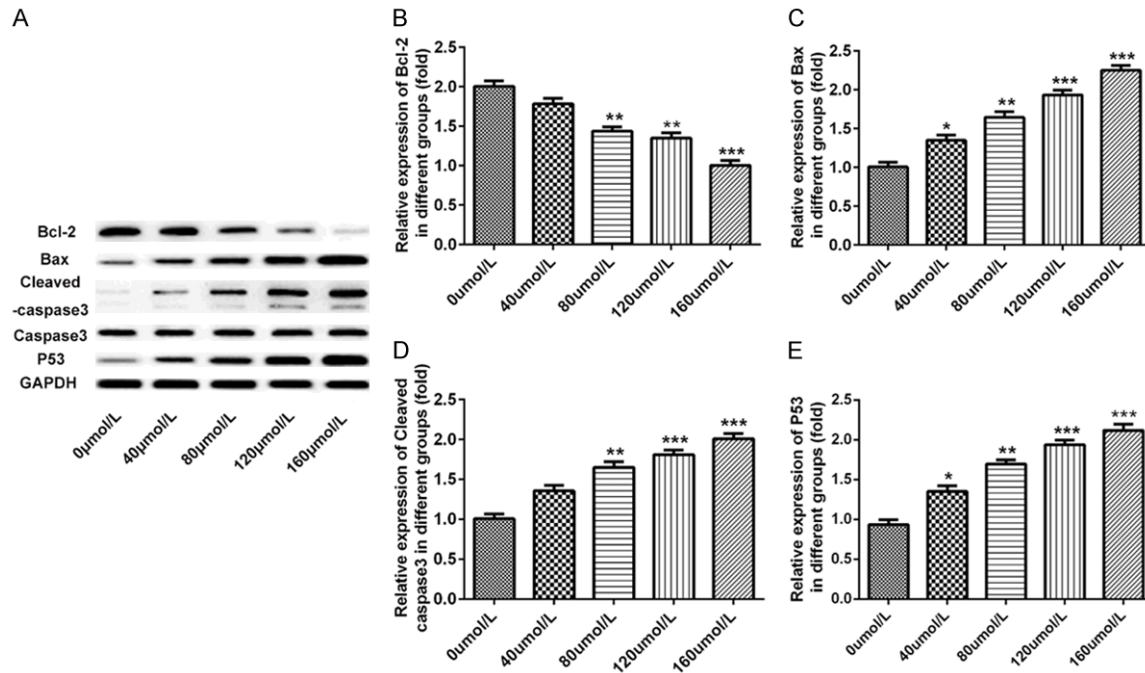


Figure 5. Baicalin significantly promoted apoptosis of pancreatic cancer SW1990 cells. Pancreatic cancer SW1990 cells were incubated for 48 hours after treatment with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin, respectively. A. The dose-dependent protein band of Bcl-2, Bax, Cleaved caspase-3, caspase-3 and p53 determined by western blot; B. The dose-dependent protein level of Bcl-2 determined by western blot; C. The dose-dependent protein level of Bax determined by western blot; D. The dose-dependent protein level of Cleaved caspase-3 determined by western blot; E. The dose-dependent protein level of p53 determined by western blot. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

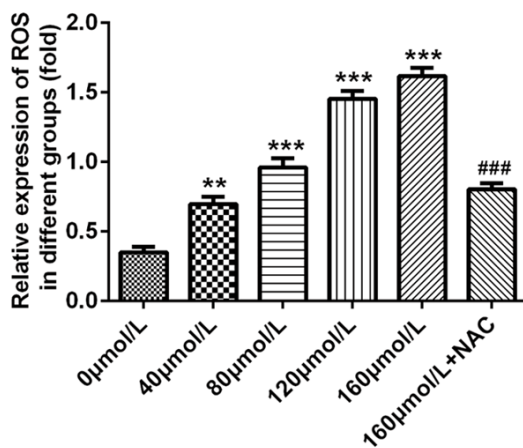


Figure 6. Baicalin increased the ROS level of pancreatic cancer SW1990 cells. Pancreatic cancer SW1990 cells were incubated for 48 hours after treatment with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin, respectively. The ROS level of pancreatic cancer SW1990 cells determined by DCFH-DA ROS assay. ** $P < 0.01$, *** $P < 0.001$, ### $P < 0.001$.

atic cancer cell proliferation and influence cell cycle in a dose-dependent manner. Besides, we demonstrated that baicalin was a positive regulator of pancreatic cancer cell apoptosis.

We also evaluated that the ROS level of pancreatic cancer cells increased after incubating with baicalin. Finally, we find that baicalin influenced pancreatic cancer cells through JNK/Foxo1/BIM pathway.

It has been well reported that baicalin have extensive antitumor activity [16]. A research showed that baicalin can inhibit the viability and proliferation of hepatic cancer cell lines in a dose-dependent manner [17]. In the present study, we found that baicalin inhibit proliferation and clone of pancreatic cancer cells. It has been reported that baicalin suppresses human osteosarcoma cells' migration, invasion, and anoikis [18]. Results revealed that baicalin markedly increased apoptosis of pancreatic cancer by influence cell cycle and apoptosis proteins. Also, we found that baicalin could inhibit pancreatic cancer cell' migration and invasion. Recently, it has been reported in the literature that baicalin triggered a significant generation of reactive oxygen species (ROS) [19]. Previous studies demonstrated that baicalin can increase the ROS level in pancreatic cancer cells. JNK/Foxo1/BIM pathway has been reported to regulate cell apoptosis, cycle

Effect of baicalin on pancreatic cancer

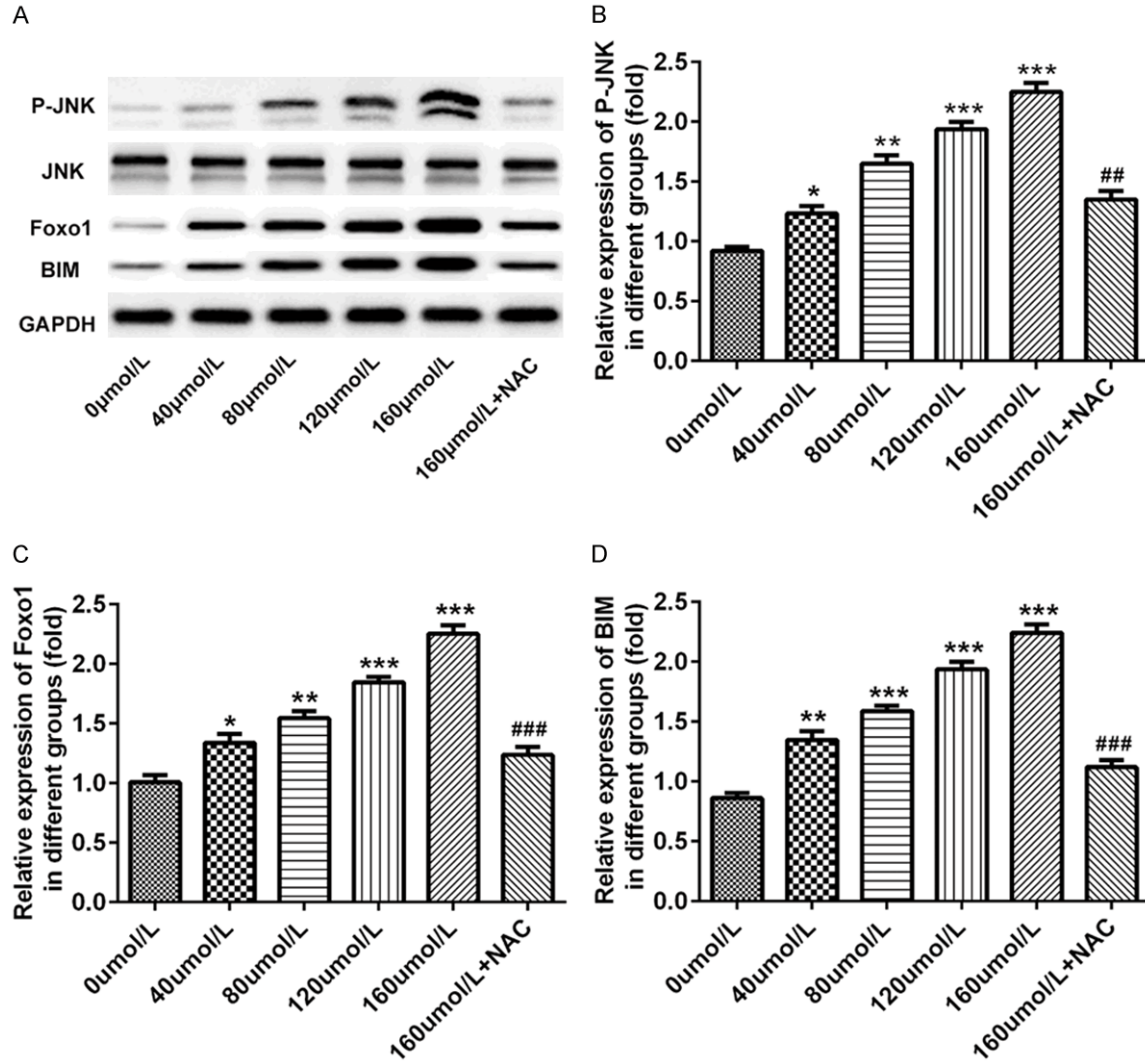


Figure 7. Baicalin activated JNK/Foxo1/BIM pathway. Pancreatic cancer SW1990 cells were incubated for 48 hours after treatment with doses of 0, 40, 80, 120 and 160 $\mu\text{mol/L}$ baicalin, respectively. A. The dose-dependent protein band of JNK, p-JNK, Foxo1 and BIM determined by western blot; B. The dose-dependent protein level of p-JNK determined by western blot; C. The dose-dependent protein level of Foxo1 determined by western blot; D. The dose-dependent protein level of BIM determined by western blot. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ### $P < 0.001$.

and ROS resistance [13, 20]. And our research proved that baicalin can activate JNK/Foxo1/BIM pathway. Here, results confirmed that baicalin influence pancreatic cancer cells by activating JNK/Foxo1/BIM pathway.

In conclusion, baicalin was identified as a novel regulator in inhibiting pancreatic cancer progress by JNK/Foxo1/BIM pathway. Therefore, this study provided new insights into the possibility of baicalin being a potential therapeutic target in pancreatic cancer.

Disclosure of conflict of interest

None.

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