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Potentiation of the effect of gemcitabine by emodin in pancreatic cancer is associated with survivin inhibition

Qingqu Guo, Ying Chen, Bo Zhang, Muxing Kang, Qiuping Xie, Yulian Wu *

Department of Surgery, The Second Affiliated Hospital, Zhejiang University College of Medicine, Cancer Institute of Zhejiang University, #88 Jiefang Road, Hangzhou City, 310009, PR China

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ABSTRACT

Pancreatic cancer is one human malignancy which has chemoresistant behavior to gemcitabine treatment. In this study, we revealed that emodin, an active component from Chinese medicinal herbs, could enhance pancreatic cancer cells apoptosis induced by gemcitabine. Survivin, a member of the inhibitor of apoptosis gene family, is involved in control of cell division and inhibition of apoptosis and described as a β-catenin/Tcf/Lef target gene. Western blot and PCR analysis showed that emodin suppressed survivin expression in a dose- and time-dependent manner. We further demonstrated survivin expression could be up-regulated by gemcitabine. Surprisingly, survivin expression induced by gemcitabine could be inhibited in combination with emodin treatment. Moreover, cells treated with gemcitabine and emodin showed a preferential peri-plasmamembrane position of β-catenin, blocking the translocation of β-catenin to nucleus induced by gemcitabine. In addition to these in vitro results, we also found that emodin potentiates the antitumor effects of gemcitabine in vivo by down-regulating the expression of survivin and β-catenin. Taken together, these results suggest that emodin potentiates gemcitabine antitumor activity through suppression of survivin gene in pancreatic cancer.

1. Introduction

Pancreatic cancer is a highly aggressive malignant disease. Despite therapeutic advances, the prognosis of patients with pancreatic cancer is extremely poor, with a median survival of 6 months [1]. The poor prognosis of pancreatic cancer is attributable to its tendency for late presentation, aggressive local invasion, early metastases, and poor response to chemotherapy [2].

Gemcitabine is a strong and specific deoxycytidine analog with activity in a variety of solid tumors including pancreatic cancer [3]. Currently, gemcitabine remains the best chemotherapeutic agent available for the treatment of advanced pancreatic cancer. However, single gemcitabine treatment has a response rate of less than 20% and is associated with drug resistance [4,5]. Therefore, it is desirable to find new combination treatment strategies for synergistic therapeutic effects. In recent years, drug combinations of gemcitabine with botanic chemicals have gained considerable attention because of their beneficial effects in overcoming intrinsic tumor cell resistance to apoptosis [6].

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a natural anthraquinone derivative isolated from Rheum palmatum L. Pharmacological studies have demonstrated that emodin possesses variously biological function, such as anti-bacterial [7], anti-inflammatory [8], anti-cancer and a potent inhibitor of the casein kinase 2 [9]. Previous studies have demonstrated that emodin inhibits cell growth in several types of tumor cells [10–13] and regulates genes related to the control of cell proliferation, cell apoptosis, oncogenesis, and cancer cell invasion and metastasis [14–19].

Survivin is a member of the inhibitor of apoptosis gene family, which is involved in control of cell division and inhibition of apoptosis. This protein, which is expressed in the most common human cancers, exerts its anti-apoptotic activity and chemoresistance by interfering with the processing and activity of caspases [20,21].

In this study, we investigated our hypothesis emodin combined with gemcitabine could show a more synergistic effect on pancreatic cancer cell proliferation and apoptosis. The results presented in the current study suggest that emodin treatment in combination with gemcitabine caused greater antitumor activity in vitro and in vivo, which was correlated with down-regulation of survivin levels.

* Corresponding author. Tel.: +86 571 87784604; fax: +86 571 87783568.
E-mail addresses: wuyulian@medmail.com.cn, autumngqq@gmail.com (Y. Wu).

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting.

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2. Materials and methods

2.1. Cell culture

The human pancreatic cancer cell lines Panc-1, Mia Paca-2 and Bxpc-3 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Rat normal skeletal muscle cell lines L6 was preserved in our laboratory. Panc-1, Mia Paca-2 and L6 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin in a humidified incubator containing 5% CO_2 in air at 37 °C. Bxpc-3 was maintained in RPMI 1640 containing supplements as above. For all experiments, the cell lines were treated in serum-free medium (0.1% bovine serum albumin, 5 mg/mL transferrin, and 5 ng/mL sodium selenite and antibiotics).

2.2. Reagents

Emodin was purchased from Sigma (St. Louis, MO, USA), dissolved in dimethylsulfoxide (DMSO) to make 0.2 mmol/L stock solution. Gemcitabine was purchased from Ely Lilly (Bad Homburg, Germany) and dissolved in sterile 0.9% sodium chloride to make 50 g/L stock solution. The final concentration of DMSO was <0.1%.

2.3. Cell growth assay

To assess the viability of cells, cell numbers were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) (Sigma, St. Louis, MO, USA), performed according to the method of Gerlier and Thomasset [22]. Briefly, cells were plated at a density of 5×10^3 cells/well in 96-well microtiter plates. After treatment, 20 μL of MTT solution (5 mg/mL in phosphate-buffered saline (PBS)) was added to each well and the plates were incubated. The supernatant was aspirated and the MTT formazan was dissolved in 150 μL of DMSO. The plates were mixed for 10 min on a gyratory shaker, and absorbance was measured with an ELISA reader (BIO-Tek ELx800, Winooski, VT, USA) at a wavelength of 490 nm.

2.4. Caspase 3 activity assay

Caspase-3 activities were assayed using a Colorimetric Protease Assay Kit according to the manufacturer's instructions (Keygen Biotech, Nanjing, China). Briefly, 1×10^6 cells were harvested after treatment, washed three times with PBS and resuspended in 50 μL of the lysis buffer, kept on ice for 60 min, and then centrifuged (10,000 × *g*, 1 min at 4 °C). Supernatants containing 100 μg of protein were incubated with 5 μL of enzyme specific colorimetric substrates Ac-DEVD-*p*NA (2 mM), at 37 °C for 4 h. The colorimetric release of *p*-nitroaniline from the Ac-DEVD-*p*NA substrate was measured using a light wave of 405 nm with an ELISA reader (BIO-Tek ELx800, Winooski, VT, USA).

2.5. Flow cytometric assessment of apoptosis

The measurement of phosphatidylserine redistribution in a plasma membrane was conducted according to the protocol outlined by the manufacturer of the Annexin V-FITC/PI apoptosis detection kit (Abcam, Cambridge, MA, USA). After 24 h treatment, harvested 1×10^5 cells were suspended in 500 μL of 1× Annexin V binding buffer. 5 μL of Annexin V-FITC and 5 μL of PI were added and incubated with for 15 min in dark. 400 μL binding buffer (1×) was added to each sample. The stained cells were analyzed directly by flow cytometry using the Cell Quest program (Becton Dickinson, San Jose, CA, USA).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) analysis and quantitative real-time RT-PCR

Total RNA was isolated with the reagent TRIzol (Invitrogen, Carlsbad, CA, USA) following instructions provided by the manufacturer. RNA concentrations were determined by spectrophotometry. cDNAs were synthesized by moloney murine leukemia virus reverse transcriptase with oligo(dT)16 primer. Each reverse transcript was amplified with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. RNA templates were used to generate cDNAs for survivin (sense, 5′-GGCATGGGTGCCCC-GACGTTG-3′; antisense, 5′-CAGAGGCCTCAATCCATGGCA-3′) and GAPDH (sense, 5′-AACGGATTTGGTCGTATTGGG-3′; antisense, 5′-TCGCTCCTGGAAGATGGTGAT-3′) by PCR. RT-PCR was performed by a DNA thermal cycler (BIO-RAD, Hercules, CA, USA). The PCR products were visualized on 2% agarose gels with ethidium bromide staining under UV transillumination.

Fluorescent PCR analysis was performed using the BIO-RAD iCycler 5 (BIO-RAD, Hercules, CA, USA). RNA was amplified by qPCR in 25 μL reactions using SYBR® Premix Ex TaqTM (TAKARA Biotechnology, Dalian, China) with 10 μmol of each appropriate primer (sense, 5′-CAAGAACTGGCCCTTCTTGGA-3′; antisense, 5′-GTCGTCATCTGGCTCCCAGC-3′). Assays were performed in triplicate and analyzed using I cycler version 3.1.7050 (BIO-RAD, Hercules, CA, USA). The gene expression levels obtained were normalized by mRNA expression of GAPDH. The relative mRNA expression was then presented in relation to the control.

2.7. Protein extraction

For total protein extraction, cells were washed with PBS, and total cells were prepared by scraping in 200 μL of lysis buffer (50 mM Tris–HCL (pH 7.4), 1.0 mM ethylenediaminetetraacetate (EDTA), 150 mM NaCl, 0.1% SDS, 1% NP40, 0.25% sodium deoxycholate, 1 mM phenylmethanesulfonylfluoride (PMSF) and 1 mM activated sodium orthovanadate [all from Sigma Chemical Company] supplemented with 1× Protease Inhibitor Cocktail I [EMD Biosciences-Calbiochem, San Diego, CA, USA]). The suspension was centrifuged and collected.

For nuclear protein extraction, cells were prepared by scraping in 150 μL of ice-cold buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF [all from Sigma Chemical Company] and 1× Protease Inhibitor Cocktail I [EMD Biosciences-Calbiochem, San Diego, CA, USA]] and incubated on ice for 30 min, followed by centrifuging at 5000 × *g* at 4 °C for 10 min. The crude nuclear pellet was suspended in 50 μL ice-cold of buffer B [20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/DTT, 0.5 mmol/L PMSF [all from Sigma Chemical Company] and 1× Protease Inhibitor Cocktail I [EMD Biosciences-Calbiochem, San Diego, CA, USA]] and incubated on ice for 30 min. The suspension was centrifuged at 16,000 × *g* at 4 °C for 30 min. The supernatant (nuclear proteins) was collected and kept at −70 °C until use.

2.8. Western blotting

Equal micrograms of proteins determined by using BCA protein assay kit (Pierce Biotechnology, Rockford, USA). For protein analysis, total cellular lysates were separated on SDS-polyacrylamide gel and transferred to polyvinylidene fluoride. The filters were blocked in TBS with 5% skim milk and incubated overnight with primary antibodies specific for caspase3, PARP, β-catenin and survivin (Santa Cruz Biotech, Santa Cruz, CA, USA) as a primary antibody. The filters were then incubated with anti-rabbit or anti-mouse secondary antibody conjugated with

horseradish peroxidase (Santa Cruz Biotech, Santa Cruz, CA, USA). Immunoreactive bands were detected by the enhanced chemiluminescence (ECL) kit for Western blotting detection with hyper-ECL film. Equal loading was confirmed by probing the blots with the laminA/C, β-actin antibody (Santa Cruz Biotech, Santa Cruz, CA, USA).

2.9. Immunofluorescence analysis

For immunofluorescence analysis, cells were plated in six-well chamber slides for 24 h before treatment. Afterwards, cells were fixed in 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 in 0.01 M PBS (pH 7.4) containing 0.2% bovine serum albumin, air dried and re-hydrated in PBS. Then cells were incubated with a rabbit polyclonal antibody against-β-catenin (Santa Cruz Biotech, Santa Cruz, CA, USA), diluted 1:200 in PBS containing 1% normal goat serum for 2 h at room temperature. Negative controls were performed by omitting the primary antibody. After three washings in PBS for 10 min, an anti-rabbit IgG FITC-conjugated secondary antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) diluted 1:250 in PBS was added for 2 h at room temperature. Cells were then washed in PBS and stained with 10 μg/mL Hoechst 33258(Sigma, St. Louis, MO, USA) for 10 min at room temperature to counterstain DNA. Cells were observed using a Zeiss Axiophot fluorescence microscope (AxioCam MRc, Zeiss, Germany). Microphotographs were acquired using a digital video camera (AxioCam MRc, Zeiss, Germany) and Axiovision Zeiss software.

2.10. Animals study

4–6 weeks old male athymic nu/nu mice were obtained (Zhejiang Chinese Medical University) for tumor implantation. All animals were maintained in a sterile environment and cared for within the laboratory animal regulations of the Ministry of Science and Technology of the People's Republic of China (http://www.most.gov.cn/kytj/kytjzcwj/200411/t20041108_32465.htm). The food, water, and bedding for these immunocompromised mice were sterilized and changed at least once weekly. 7×10^6 Bxpc-3 cells collected in 100 μL serum-free 1640 media in log phase growth were injected subcutaneously in the backs of 20 g athymic nu/nu mice. Once tumor masses became established and palpable, animals were randomized to receive intraperitoneal (IP) injections of vehicle (0.9% sodium chloride), emodin (40 mg/kg) [14] alone, gemcitabine (125 mg/kg) [23] alone, or emodin and gemcitabine in combination twice per week for 2 weeks. Tumor volumes and body weight were measured weekly. Tumor volume was measured along the longest orthogonal axes and calculated using the formula: volume = (length × width^2)/2, where width was the shortest measurement. At the end of the experiment, subcutaneous xenografts were excised and stocked in 4% formaldehyde and embedded in paraffin.

2.11. Immunohistochemical analysis

Sections were cut from paraffin embedded pancreatic tumor tissues. Immunostaining was performed using primary antibodies specific for β-catenin and survivin with appropriate dilutions and using normal host serum for negative controls, followed by staining with appropriate HRP-conjugated secondary antibodies. The slides were developed in diaminobenzidine and counterstained with a weak solution of haematoxylin solution stain. The stained slides were dehydrated and mounted in permount and visualized on an Olympus microscope (Olympus, Japan). Images were captured with an attached camera linked to a computer.

2.12. Statistical analysis

Data were expressed as mean values ± S.E. and analyzed by a two-tailed *t*-test or ANOVA followed by the LSD's multiple comparison with $P < 0.05$ considered significant. Analyses were performed using SPSS 11.5 statistical software package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of emodin and gemcitabine on cell proliferation

To test the effects of emodin on pancreatic cancer cell growth, cells were treated with increasing concentrations of emodin (0–160 μM) for 24 h, 48 h and 72 h. As shown in Fig. 1A, cell growth was inhibited by emodin treatment in a dose- and time-dependent manner. These results indicate that emodin was an effective inhibitor of pancreatic cancer cell growth as a single agent. Bxpc-3 and Mia Paca-2 cells were more sensitive to emodin compared with Panc-1 cells. However, emodin did not change the growth of normal rat skeletal muscle cells (Fig. 1D). We subsequently evaluated the effect of gemcitabine on cell growth in vitro and found that gemcitabine was effective in inhibiting cell growth in cell lines tested (Fig. 1B). Subsequent studies were undertaken to examine whether or not cells treated with two-drug combination were more sensitive to the cytotoxic effect of gemcitabine.

3.2. Emodin potentiates growth inhibition induced by gemcitabine in pancreatic cancer cells

We investigated the effect of a combination of emodin and gemcitabine on cell viability by MTT assay. For these studies, cells were treated with emodin (Panc-1 (80 μM), Bxpc-3 and Mia Paca-2 (40 μM), respectively), gemcitabine (Panc-1 and Mia Paca-2 (5 μg/mL), Bxpc-3 (0.5 μg/mL), respectively) or the two-drug combination for 48 h. Viable cells were evaluated by MTT assay. We found that treatment with emodin plus gemcitabine for 48 h resulted in more loss of cell viability than either emodin or gemcitabine alone in pancreatic cancer cells (Fig. 1C).

3.3. Emodin sensitizes Panc-1 and Bxpc-3 cells to apoptosis induced by gemcitabine

To determine whether emodin enhances the induction of apoptosis by gemcitabine, we observed the induction of apoptosis in pancreatic cancer cell treated with either emodin (Panc-1 80 μM and Bxpc-3 40 μM, respectively) or gemcitabine (Panc-1 5 μg/mL and Bxpc-3 0.5 μg/mL, respectively) alone. At these concentrations, relative to single agents, combination treatment with gemcitabine and emodin for 24 h induced much more apoptosis in both the cell lines as shown by Annexin V/PI flow cytometry. The controls in all tested cell lines had only background levels of apoptotic cells of 3.0–4.0%. Single treatment with gemcitabine showed an increased apoptosis-induction with 9.45 ± 0.07% in Bxpc-3 cells. However, Panc-1 was not very sensitive to this treatment leading to 4.26 ± 0.06% apoptotic cells. But, the treatment with emodin demonstrated 11.47 ± 0.14% apoptosis in Panc-1 and 17.82 ± 0.06% in Bxpc-3 cells. Furthermore, the combination of gemcitabine and emodin resulted in a strong apoptotic effect. Panc-1 cells showed 28.98 ± 0.05% apoptosis and Bxpc-3 cells 22.95 ± 0.03% (Fig. 2A). To further examine the ability of the combination therapy to induce apoptosis, caspase-3 activities in both the cell lines tested were evaluated. The combination of emodin and gemcitabine caused an additional increase in caspase-3 activity (Fig. 2B) in Bxpc-3 and Panc-1 cells by comparison with either agent alone after 24 h treatment. Next, we evaluated caspase-3 and PARP activation in both

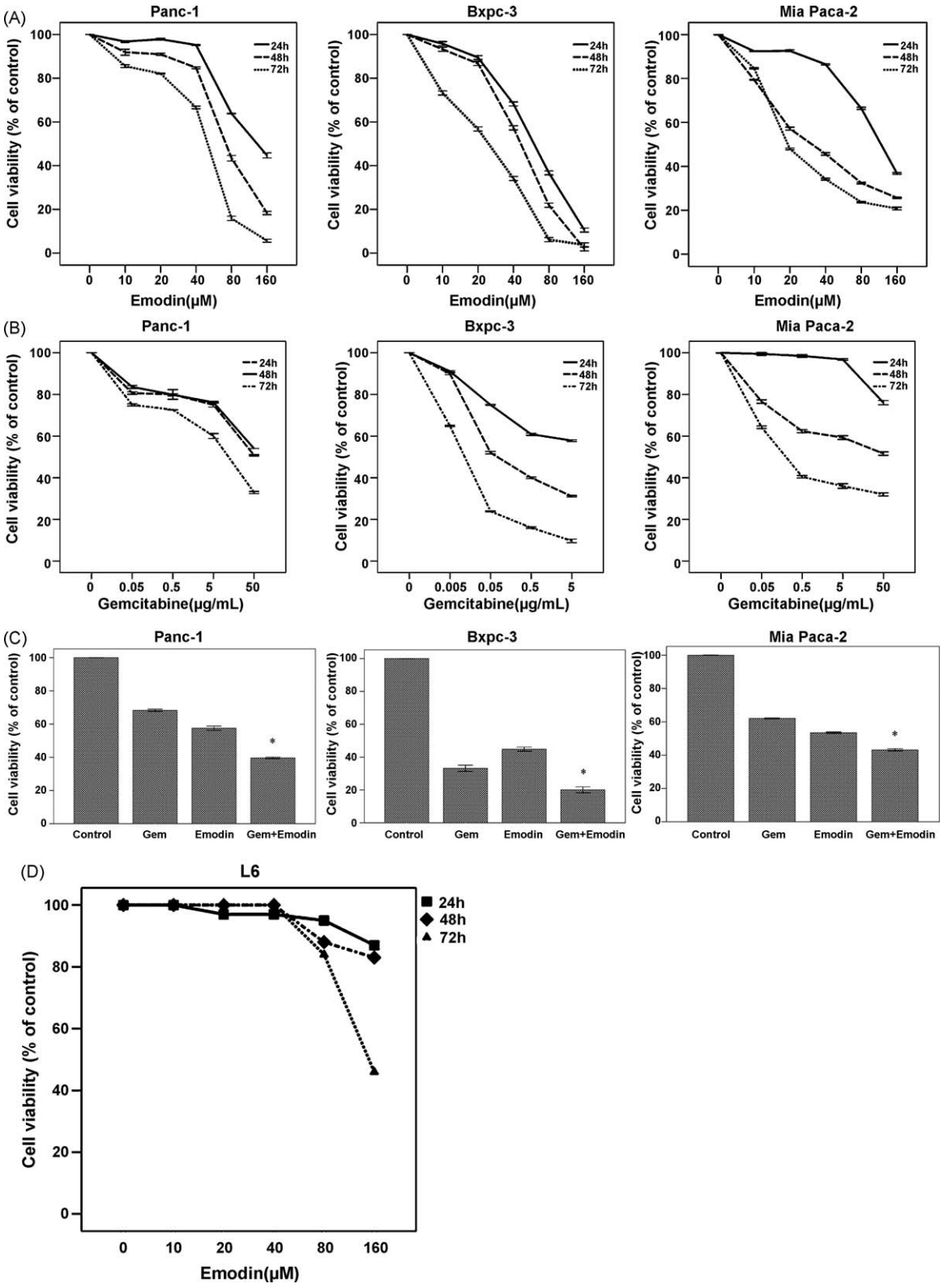

Fig. 1. Cell growth inhibition by emodin and gemcitabine. Cells were either untreated or treated with either increasing concentration of emodin (A) or gemcitabine (B), for 24 h, 48 h and 72 h and analyzed for viable cells by MTT assay as described in Section 2. Data are presented as mean ± S.E. of nine replicates from three independent experiments. (C) Pancreatic cancer cells treated with emodin (Panc-1 (80 μM), Bxpc-3 and Mia Paca-2 (40 μM), respectively), gemcitabine (Panc-1 and Mia Paca-2 (5 μg/mL), Bxpc-3 (0.5 μg/mL), respectively) or the two-drug combination for 48 h. Columns, mean of three experiments; bars, S.E. $^*P < 0.05$, combination treatment group compared with control group, single treatment with gemcitabine or emodin group. (D) Viability of normal rat skeletal muscle cells was assessed after treated with various concentration of emodin for 24 h, 48 h and 72 h. On termination of incubation, viable cells were evaluated relative to untreated control and interpreted as % viable cells. Points, averages of nine replicates from three independent experiments.

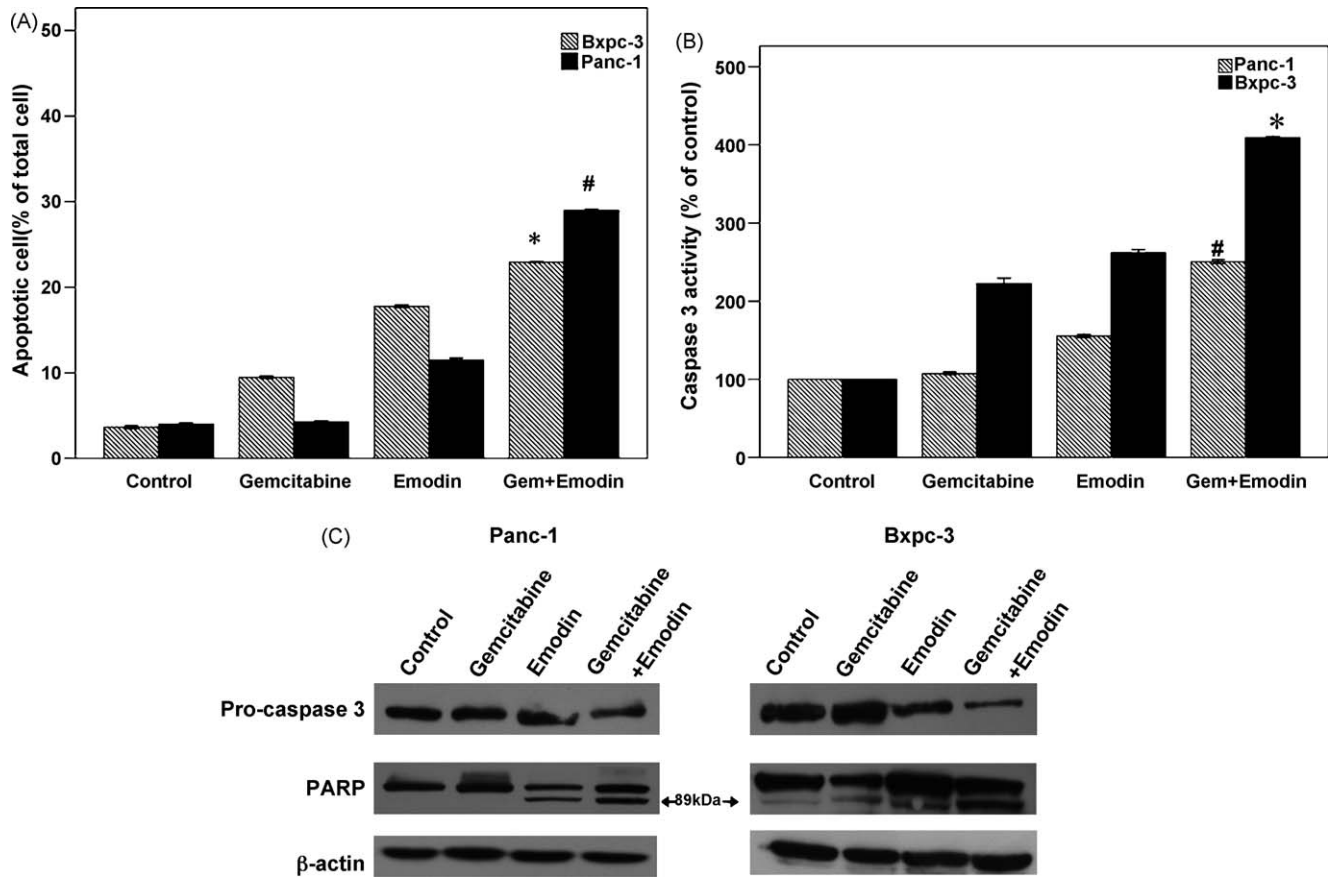

Fig. 2. Potentiation of gemcitabine-induced apoptosis by emodin. (A) Apoptosis rate analysis using Annexin V/propidium iodide flow cytometry in Panc-1 and Bxpc-3 cells treated with emodin (80 μM and 40 μM, respectively), gemcitabine(5 μg/mL and 0.5 μg/mL, respectively)and the two-drug combination for 24 h. Columns, mean of three experiments; bars, S.E. $^{\#,*}P < 0.05$, by comparison with respective group. (B) Caspase activation. Panc-1 and Bxpc-3 cells were treated with emodin (80 μM and 40 μM, respectively), gemcitabine (5 μg/mL and 0.5 μg/mL, respectively) and the two-drug combination for 24 h. Cell lysates were assayed for caspase-3 activity as described in Section 2. Data are presented as mean ± S.E. of three independent experiments. $^{\#,*}P < 0.05$, by comparison with respective group. (C) Panc-1 and Bxpc-3 cells were treated with gemcitabine (5 μg/mL and 0.5 μg/mL, respectively), emodin (80 μM and 40 μM, respectively), the two-drug combination for 24 h. Pro-caspase 3 and PARP protein levels were investigated. Western immunoblotting for β-actin protein was performed as loading control representative.

cell lines tested by Western blotting. The combined treatments also induced the activation of caspase-3 and the cleavage of its substrate PARP in Panc-1 and Bxpc-3 cells (Fig. 2C).

3.4. Emodin down-regulates survivin in Panc-1 and Bxpc-3 cells

To investigate the mechanisms by which emodin-induced apoptosis, we evaluated the expression of the anti-apoptotic gene survivin in pancreatic cancer cells after drug exposure. As shown in Fig. 3A, treatment of Panc-1 cells with emodin induced a concentration-dependent decrease in the levels of survivin protein. An emodin-induced time-dependent inhibition of survivin expression was seen in Bxpc-3 and Panc-1 cells (Fig. 3B). Furthermore, PCR analysis in Panc-1 and Bxpc-3 cells showed similar changes in survivin mRNA levels produced by emodin, suggesting that emodin modulates survivin expression at the transcriptional level.

3.5. Emodin abolishes survivin expression induced by gemcitabine

Next, we analyzed whether gemcitabine could induce survivin expression and whether down-regulate survivin by emodin could abolish chemoresistant of Panc-1 and Bxpc-3 cells, resulting in more prominent gemcitabine-induced apoptosis. We analyzed dose and time response to gemcitabine in the induction of survivin in Bxpc-3 cells. For this, protein was prepared from Bxpc-3 cells treated with increasing doses of gemcitabine for 24 h. Our results showed that relative to untreated control, gemcitabine treatment induced a concentration-dependent increasing survivin expression from 0.05 μg/mL to 5 μg/mL in Bxpc-3 cells. However, gemcitabine (0.5 μg/mL) treatment did not induce survivin expression as early as 12 h in Bxpc-3 cells (Fig. 3C). Under similar assay conditions, we also evaluated the survivin expression in Panc-1 in response to gemcitabine. A dose escalation study with gemcitabine revealed up-regulation of survivin expression at 5 μg/mL concentration after 24 h of treatment. Furthermore, the survivin expression was analyzed in different time treated with 5 μg/mL gemcitabine. The results displayed the survivin expression was significantly up-regulated after 24 h treatment (Fig. 3D). The induction in survivin mRNA was paralleled by similar increases in survivin protein levels. Moreover, we tested whether combined treatment with emodin for 24 h could abrogate gemcitabine-induced survivin expression in mRNA and protein levels. Our results showed that survivin expression induced by gemcitabine could be inhibited in combination with emodin treatment (Fig. 3E).

3.6. Emodin blocks the β-catenin/Tcf/Lef pathway

β-Catenin is a key component of the Wnt/β-catenin signaling pathway. The accumulated β-catenin promotes its translocation to the nucleus and interacts with TCF transcription factors to modulate the transcription of downstream target genes. β-catenin/Tcf/Lef pathway is frequently altered in pancreatic cancers [38] and survivin has been described as a β-catenin-Tcf/Lef target gene. We then investigated whether emodin blocked signaling

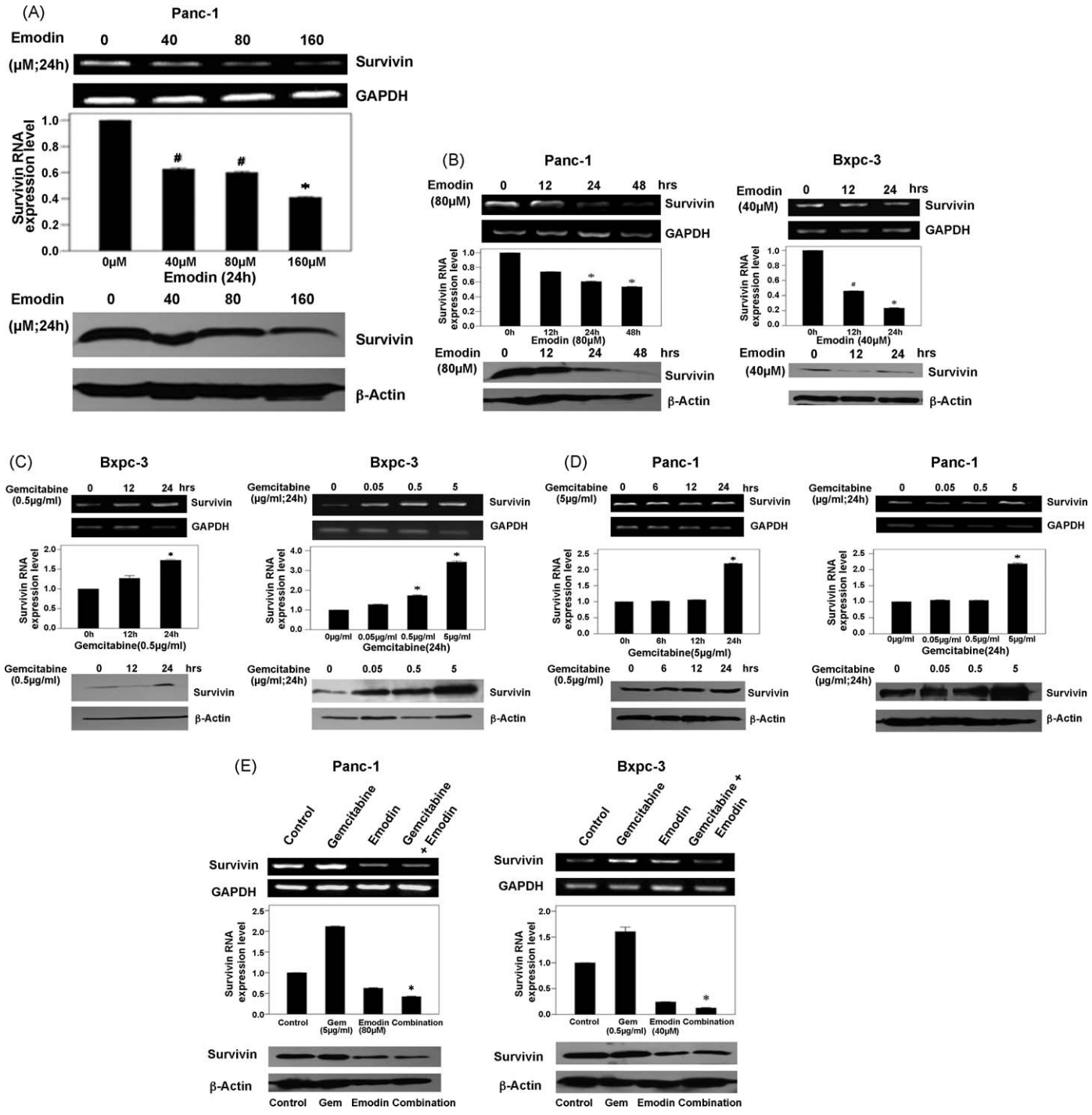

Fig. 3. Abrogation of constitutive and induced survivin mRNA and protein levels by emodin. (A) Dose response by emodin in down-regulating survivin mRNA and protein levels in Panc-1 cells. Total proteins were prepared from cells after incubation with different concentrations of emodin for 24 h as described in Section 2. (B) Time course representation of survivin down-regulation induction by emodin in pancreatic cancer cells, Panc-1 and Bxpc-3. The cells were incubated with the indicated concentration of emodin and then the extracted total protein and RNA for evaluation of survivin induction. (C and D) Survivin mRNA and protein levels induced by gemcitabine in a dose- and time-dependent manner were assessed in Panc-1 and Bxpc-3 cells, respectively. (E) Panc-1 and Bxpc-3 cells were treated with gemcitabine (5 μg/mL and 0.5 μg/mL, respectively), emodin (80 μM and 40 μM, respectively), the two-drug combination for 24 h. Survivin mRNA and protein levels were investigated. Western immunoblotting for β-actin protein was performed as loading control representative. PCR for GAPDH was used as a control for amplification. The graphic bars represent mRNA quantitation which was performed using real-time PCR. Data are presented as mean ± S.E. of three independent experiments after standardization to GAPDH. $^{\#}P < 0.05$, by comparison with control group. $^{*}P < 0.05$, by comparison with respective group.

through this pathway and thereby down-regulated survivin levels. Immunoblotting was performed to examine of β-catenin in each treatment groups in Bxpc-3 cells (Fig. 4A). No differences were noted in the total pool of β-catenin, demonstrating that emodin and gemcitabine have a no effect on the total expression of β-catenin in Bxpc-3 cells. However, former reports revealed gemcitabine increased nuclear localization of total β-catenin in pancreatic cancer cells by immunofluorescence and immunoblotting [24]. We next examined the changes of β-catenin localization following emodin, gemcitabine and in combination treatment by indirect immunofluorescence detection of the protein in Bxpc-3 cells (Fig. 4B–M). In control conditions, we observed that β-catenin

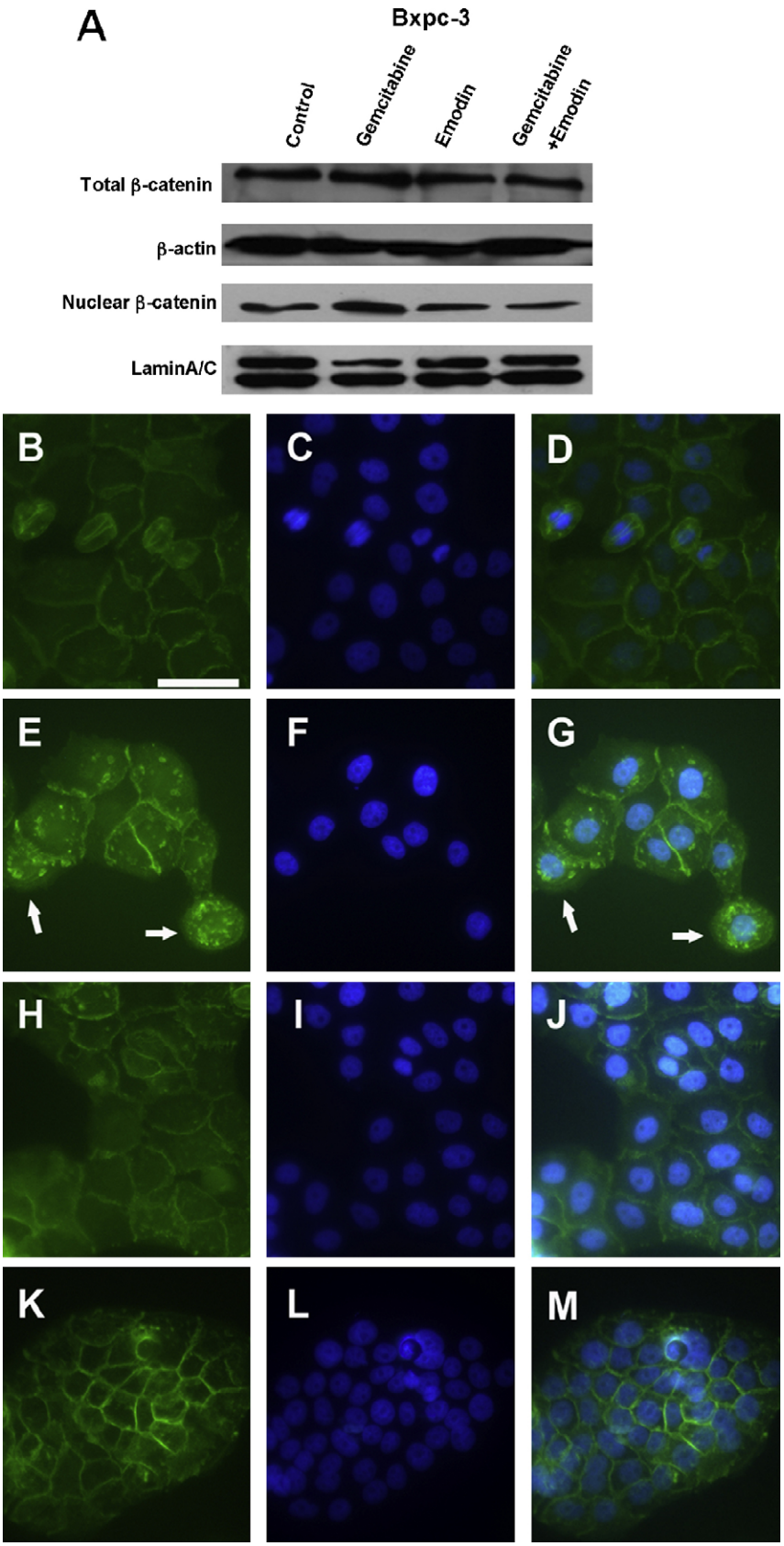

Fig. 4. Changes of β-catenin in Bxpc-3 cells. (A) Western blot analysis of total and nuclear levels of β-catenin in Bxpc-3 cell treated with gemcitabine (0.5 μg/mL), emodin (40 μM), the two-drug combination for 24 h. Western immunoblotting for β-actin and lamin A/C protein was performed as loading control representative. (B–M) Change in cellular localization of β-catenin in Bxpc-3 cells. β-Catenin cellular localization was evaluated by indirect immunofluorescence utilizing a polyclonal antibody that labels β-catenin, as described in Section 2. Immunofluorescence labeling of β-catenin in Bxpc-3 cells untreated (B), treated with 0.5 μg/mL gemcitabine (E), 40 μM emodin (H) and the two-drug combination (K) for 24 h. Nuclei were stained with Hoechst 33258 dye (C, F, I and L), and regions were merged to assess signal colocalization (D, G, J and M). Magnification for each representative picture: 400×. Scale bar: 50 μm.

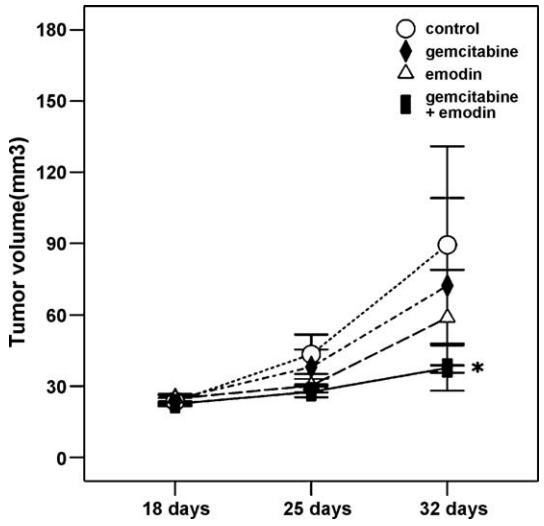

Fig. 5. Measurements of pancreatic tumor volume at various time points revealing in vivo therapeutic efficacy of emodin and gemcitabine treatment. Combination of gemcitabine plus emodin significantly decreased the mean tumor volume compared to control animals and treated with gemcitabine alone ($^*P < 0.05$) ($n = 7$). Points, mean; bars, S.E.

is present in both the cytoplasm especially in peri-plasmamembrane position (Fig. 4B). But in gemcitabine groups β-catenin preferentially accumulates in peri-nucleus and increasingly translocates to the nucleus (Fig. 4E). In contrast, cells treated with gemcitabine and emodin showed a preferential peri-plasmamembrane position of β-catenin, blocking the translocation of β-catenin to nucleus (Fig. 4K). Similarly, immunoblotting supports the result of immunofluorescence, suggesting combination treatment decreased the elevated levels of nuclear β-catenin induced by gemcitabine in pancreatic cancer cells (Fig. 4A).

3.7. Emodin augments therapeutic effect of gemcitabine in vivo

We examined the effects of emodin and gemcitabine, alone or in combination, on the growth of subcutaneous xenograft implanted pancreatic tumors. Bxpc-3 xenografts were established in athymic mice within 2 weeks and subjected to treatment with vehicle (0.9% sodium chloride), emodin (40 mg/kg), gemcitabine (125 mg/kg), or the combination of these two agents twice per week for 2 weeks. At the end of four cycles treatment, the final tumor volumes on day 14 after the start of treatment showed significant decrease in the emodin + gemcitabine group compared with control ($P < 0.05$) or with gemcitabine alone ($P < 0.05$; Fig. 5). Furthermore, the expression of survivin and β-catenin were significantly decreased in the treated with combination group compared to the control and gemcitabine alone group (Fig. 6).

4. Discussion

Due to the advanced stage of the disease and chemoresistant behavior to cytotoxic chemotherapeutic agents, pancreatic cancer remains one of the most difficult human malignancies to treat. However, in recent years, novel approach for sensitizing pancreatic cancer cells to be killed with naturally occurring dietary chemopreventive compounds have obtained considerable attention. Several studies have shown significant increase in treat response rate by the use of combinations of different type of chemopreventive compounds with a commonly used chemotherapeutic agent, gemcitabine, in pancreatic cancer cell [25,26].

Emodin has been reported to exhibit anti-tumor effects in various cancer cells including pancreatic cancer [27,28]. Former results showed that the herbal drug emodin interacted with DNA by intercalating into the double helix of DNA. Emodin can prevent cancerous cells from dividing and producing more cancer cells by inhibiting DNA reproducing [29]. In agreement with previous study, our results confirmed emodin could inhibit pancreatic cancer cell growth in a dose- and time-dependent manner. However, the growth of normal rat skeletal muscle cells did not significantly change by emodin until 160 μM for 72 h, suggesting a moderate concentration of emodin did not show cytotoxicity to normal cells.

As the best chemotherapeutic agent available for the treatment of advanced pancreatic cancer, single gemcitabine treatment is not very effective and is associated with multiple adverse events and drug resistance [5,26]. It was still unknown whether emodin could enhance the antitumor activity of gemcitabine in pancreatic cancer cells. We first investigated in human pancreatic cancer cells whether the combination of gemcitabine and emodin can enhance cell growth inhibition more than either drug alone. In this study,

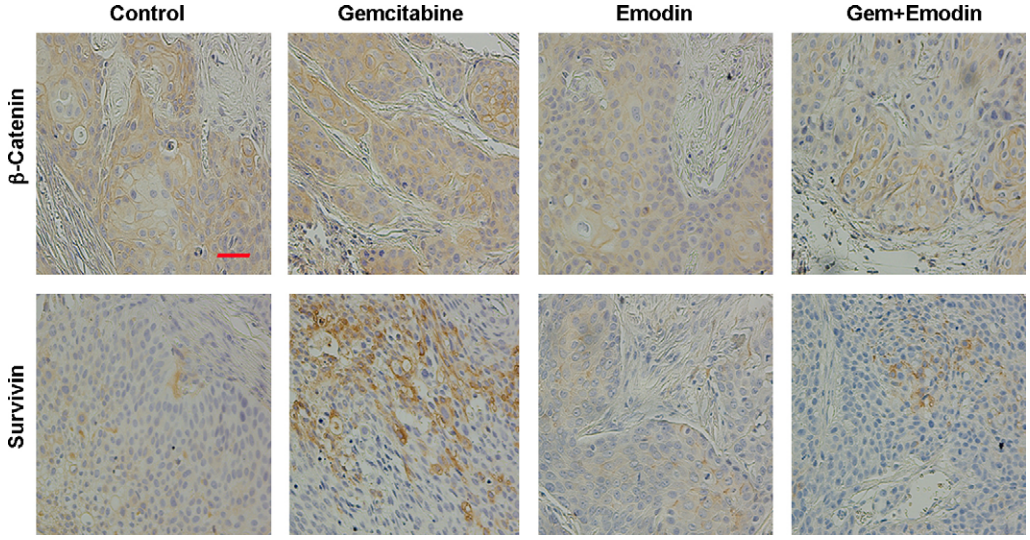

Fig. 6. Immunohistochemical analysis of survivin and β-catenin protein expression in pancreatic cancer samples. First row, representative sections immunostained with antibody recognizing β-catenin from control, gemcitabine, emodin and combination treated group. Second row, representative sections immunostained with antibody recognizing survivin. Scale bar: 50 μm.

we found that the combination of gemcitabine and emodin treatment enhanced noticeable tumor cell growth inhibition compared with either agent alone. Inhibition of cell growth was also correlated with apoptotic cell death. In cell, apoptosis was mediated by caspase pathway as evidenced by DNA fragmentation. In fact, emodin has been shown to activate caspases 3 and induce PARP cleavage [10–12]. In current study, by fluorescence-activated cell sorting analysis, our results showed emodin sensitizes Panc-1 and Bxpc-3 cells to apoptosis induced by gemcitabine. In agreement with the results of FACS, potentiation of gemcitabine-induced apoptosis by emodin in pancreatic cells was validated by enhancing caspase 3 activities and the cleavage of PARP.

The prosurvival molecule survivin, a member of the inhibitor of apoptosis protein (IAP) family, has been implicated in the control of cell division and apoptosis [30]. Survivin's anti-apoptotic function is executed via its ability to prevent caspase activation. Growing evidence suggests that survivin is responsible for drug resistance in cancer cells [31–33]. Previous studies have shown that siRNA directed against survivin could enhance pancreatic cancer cell gemcitabine chemosensitivity [34]. In our study, we showed that gemcitabine alone could up-regulate survivin supporting the conception that survivin was associated with acquired gemcitabine resistance. We also found that emodin down-regulates survivin in Panc-1 and Bxpc-3cells. In addition, our in vitro results showed that emodin alone or co-treatment with gemcitabine abrogates the expression of survivin and increases apoptotic cells, suggesting that inhibition of survivin by emodin is associated with sensitization of pancreatic cancer cells to apoptotic cell death induced by gemcitabine.

Activation of the multifunctional protein β-catenin plays a critical role in the development of cancer cells [35,36]. Accumulated β-catenin translocates to the nucleus and binds with TCF4 to regulate its target genes including myc [37], survivin [38,39] and cyclin D1 [40]. Furthermore, accumulated nuclear localization of total β-catenin increased the resistance of gemcitabine in pancreatic cancer cells [24]. Considering that survivin has been described as a β-catenin/Tcf/Lef target gene [41], we then investigated whether emodin blocked signaling through this pathway and thereby decreased survivin levels. In the present study, our results demonstrated that emodin or combination of gemcitabine and emodin treatment had no effect on the total expression of β-catenin. However, Immunofluorescence analysis revealed cells treated with emodin plus gemcitabine showed a preferential peri-plasmamembrane position of β-catenin in comparison with gemcitabine alone groups in which β-catenin preferentially accumulated in peri-nucleus and nucleus. Subsequent Western blot analysis revealed the gemcitabine-induced increase of nuclear β-catenin was blocked by combined with emodin. These results supported our hypothesis that emodin decreased survivin levels by blocked β-catenin/Tcf/Lef signaling pathway at least.

In addition to these in vitro results, we found that emodin potentiates the antitumor effects of gemcitabine in a subcutaneous xenograft pancreatic cancer. The combination treatment resulted in a visibly reduced tumor volume when compared to the control group and gemcitabine single agent treatment. Most importantly, the results in vitro were replayed in vivo, not only down-regulating the expression of survivin and β-catenin but also decreasing the translocation of β-catenin to nucleus in tumor tissues treated with combination.

In conclusion, our current findings showed that emodin potentiates the antitumor effects of gemcitabine by down-regulating survivin levels in pancreatic cancer. Blocking the translocation of β-catenin to nucleus might be playing a role in survivin down-regulation. However, further investigations are necessary to get more information before clinical use might be possible.

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