Emodin potentiates the antitumor effects of gemcitabine in PANC-1 pancreatic cancer xenograft model *in vivo* via inhibition of inhibitors of apoptosis

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Abstract. Pancreatic cancer is a highly aggressive malignant disease. Gemcitabine is currently the standard first-line chemotherapeutic agent for pancreatic cancer. As members of apoptosis inhibitors, Survivin and XIAP play an important role in chemotherapy resistance in pancreatic cancer. Emodin has therapeutic potential against cancers. This study was designed to investigate whether combination therapy with gemcitabine and emodin enhanced antitumor efficacy in pancreatic cancer. The application of the combination therapy triggered significantly higher frequency of pancreatic cancer cell apoptosis. Our research demonstrated that the combination of emodin and gemcitabine resulted in significantly reduced tumor volumes compared to gemcitabine or emodin treatment alone. Immunohistochemistry and western immunoblot analyses showed that Survivin and XIAP expression were downregulated in emodin and the combination groups compared to the other two groups. Reverse transcriptase polymerase chain reaction analyses showed that Survivin and XIAP mRNA expression in emodin and the combination groups were downregulated significantly compared to the other two groups. Furthermore, the expression of the nuclear transcription factor κB (NF-κB) protein and NF-κB mRNA were downregulated in the emodin and the combination groups. DNA-binding activity of NF-κB was inhibited in emodin and combination groups compared to the other groups. This study suggests that emodin potentiates the antitumor effects of gemcitabine in PANC-1 cell xenografts via promotion of apoptosis and IAP suppression.

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Introduction

Pancreatic cancer is a highly aggressive malignant disease. It is the fourth leading cause of cancer related mortality in the USA, with ~43,140 new diagnoses in 2010 (1). Because of the deep location, difficulties in early diagnosis, rapid progression and poor curative effect, the 5-year survival rate of pancreatic cancer is very low (1), only <5% survive 5 years (2). Surgical resection represents the only curative treatment, but due to late diagnosis most patients present in an advanced stage and only a minority (10-20%) are amenable to surgical intervention (3-5).

Previous research has shown that gemcitabine (2',2'-difuorodeoxycytidine), as a DNA nucleotide analogue, is effective against many solid tumors including pancreatic cancer, and as an adjuvant drug after curative-intent resection, can prolong disease-free survival to at least 6 months (6). Research also demonstrated that gemcitabine represents the standard and preferred chemotherapeutic agent for advanced pancreatic cancer, but this treatment still only results in a 5-week increase in survival time (7).

Moreover, combination treatment consisting of gemcitabine and 5-fluorouracil (5-FU) (8), oxaliplatin (9), cisplatin (10) or irinotecan (11) has also resulted in improved clinical responses in patients with advanced pancreatic cancer. Although numerous doublet or triplet gemcitabine combination therapies have been shown to provide modest survival benefits compared to monotherapy, the response and median survival in patients with advanced pancreatic cancer has not improved (12). Researchers have shown that a decrease in human equilibrative nucleoside transportor-1 (hENT-1) expression (13) and presence of multidrug

resistance protein 5 (MRP5) (14) may contribute to gemcitabine resistance in pancreatic cancer. It has been reported that NF-κB may also play an important role in the chemoresistance of pancreatic cancer to gemcitabine (15). Therefore, development of novel drugs or therapies with high antitumor activity, and minimal toxicity that are readily available and at low cost is greatly needed.

Traditional Chinese Medicine (TCM) holds an important position in primary health care in China and has been recently recognized by other countries as a source for novel lead molecules in modern drug discovery (16). Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a natural anthraquinone derivative isolated from the root of Chinese rhubarb (Rheum palmatum). It has been shown to have antibacterial (17), immunosuppressive (18) and anti-cancer (16) effects. Current research has demonstrated that emodin can inhibit the growth of pancreatic (19,20), ovarian (21), lung (22) and leukemic (23) cancers. Studies have suggested that emodin improved the antitumor effects of gemcitabine through enhancement of apoptosis possibly by downregulating the ratio of Bcl-2/Bax and promoting cytochrome c release from the mitochondria (24). Additionally, other studies have demonstrated that emodin enhanced the antitumor activity of gimcitabine via inhibition of Akt and NF-κB activation, thus promoting mitochondrial-dependent apoptosis (25).

Researchers have demonstrated that NF-κB and XIAP are upregulated as a consequence of several weeks of chemotherapy such as gemcitabine, which contributes to chemoresistance (26). Therefore, the purpose of the present study was to demonstrate whether emodin could potentiate the antitumor effects of gemcitabine in pancreatic cancer via suppression of survival signaling and thus promotion of apoptosis. Our study demonstrates that emodin potentiates gemcitabine-induced apoptosis on pancreatic cancer Panc-1 cell xenografts in athymic nu/nu mice as a result of inhibition of NF-κB DNA-binding activity, and downregulation of Survivin and XIAP protein levels. In addition, caspase-9 and -3 expression was upregulated.

Materials and methods

Reagents. Emodin (lot no. 043K35051, purity: >98%) was purchased from Sigma-Aldrich (St. Louis, MO USA) and dissolved in dimethylsulfoxide (DMSO, Invitrogen, Carlsbad, CA). Gemcitabine was purchased from Eli Lilly (Indianapolis, IN) and dissolved in 0.9% sodium chloride. NF-κB, Survivin, XIAP, caspase-9 and cleaved caspase-3 antibody were purchased from Abcam (Cambridge, MA).

Cell culture. The human pancreatic cancer cell line PANC-1 was obtained from American Type Culture Collection (Manassas, VA). Cells were established in RPMI-1640 (Gibco BRL, Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 mg/ml streptomycin in a humidified incubator containing 5% CO_2 in air at 37˚C. Subculture: the cells were digested with trypsin (Gibco BRL) upon 70-80% confluency.

In vivo experiments. Schematic overview of the study protocol was shown in (Fig. 1A). All procedures for animal experimentation were approved by Wenzhou Medical College Experimental Animal Center (Wenzhou, China). Six-week-old, male, athymic, BaLB/c nu/nu mice were purchased from the Shang Hai Cancer Institute (Shanghai, China). All animals were maintained in a specific-pathogen-free environment in Animal Center. Housing temperature was maintained at 25±1˚C and relative humidity was controlled at 40-60%.

PANC-1 cells in log-phase were digested with trypsin. The digestion was suspended by culture medium supplemented with 10% fetal bovine serum and the mixture was centrifuged. The cells were re-suspended with serum-free culture medium and counted using a cell counting chamber. Tumor xenografts were established by subcutaneous inoculation of $5x10^6$ PANC-1 cells into the right abdominal flank of all 32 BaLB/c male mice.

After 3 weeks of implantation, when tumor grew to the size of largest diameter ≥5 mm, mice received different treatments every three days. A total of 32 nude mice were randomized into four treatment groups with 8 mice per group: group N = 0.9% sodium chloride (n=8), group G = 125 mg/kg gemcitabine (n=8), group E = 40 mg/kg emodin (n=8) and group G+E = 80 mg/kg gemcitabine + 40 mg/kg emodin in combination (n=8). Treatments were administered intraperitoneally once every 3 days for a total of 9 treatments. The body weight and tumor size of mice were measured every six days after drug treatments. Tumor size was measured by a caliper and calculated using the following formula: Volume = (4/3) π [(length + width)/4]^3 (25).

Immunohistochemical analysis of NF-κB, Survivin, XIAP, caspase-9, active-caspase-3 and Ki-67. Formalin-fixed, paraffin-embedded tumor tissues were sectioned and blocked with goat serum and immunostained after deparaffinization in xylene, followed by rehydration in a graded series of ethanol solutions. Sections were incubated with anti-human anti-NF-κB, Survivin, XIAP, caspase-9, active caspase-3 and Ki-67 primary antibodies in a moist chamber overnight at 4˚C with the appropriate dilutions (Ki-67, 1:300, NF-κB, 1:400, active-caspase-3, 15 μg/ml, caspase-9, 1:80, Survivin, 1:800, XIAP, 2 μg/ml). After three rinses with phosphate buffered saline (PBS), specimens were incubated for 30 min at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody, then peroxidase labeling was developed by incubating the sections in diaminobenzidine tetrahydrochloride (DAB) for 3 min. Finally, Mayer hematoxylin solution was used for nuclear counterstaining. Sections were mounted and evaluated via microscopy (Olympus BX51, Japan). Non-specific primary antibody staining was evaluated by substitution of the primary antibody with PBS. At least 10 fields were randomly selected from each section. Images were analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD).

TUNEL staining. To assess the degree of apoptosis, we used the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining method after two weeks of therapy. TUNEL staining was performed by standard methods with 4-μm tissue section. Operated as shown in the *In Situ* Cell Death Detection Kit (Roche, Indianapolis, IN), tissue section was added to TUNEL reaction solution at 37˚C for 1 h. Laser scanning confocal microscope under 400-fold magnification was used, with excitation wavelength 488 nm and emission wavelength 568 nm. We examined 10 fields on each slide for the strongest fluorescent signal.

Table I. Primer pairs used in semiquantitative polymerase chain reaction.

Genes	Primer pairs	Products (bp)
NF-κB	Sense 5'-AGCACAGATACCACCAAGACCC-3' Antisense 5'-CCCACGCTGCTCTTCTATAGGAAC-3'	300
Survivin	Sense 5'-GGCATGGGTGCCCCGACGTTG-3' Antisense 5'-CAGAGGCCTCAATCCATGGCA-3'	440
XIAP	Sense 5'-TTCCTCGGGTATATGGTGTCTGAT-3' Antisense 5'-CCGTGCGGTGCTTTAGTTGT-3'	292
Caspase-9	Sense 5'-GGTTCTGGAGGATTTGGTGA-3' Antisense 5'-GACAGCCGTGAGAGAGAATGA -3'	325
Caspase-3	Sense 5'-AGCAAACCTCAGGGAAACATT-3' Antisense 5'-GTCTCAATGCCACAGTCCAGT-3'	309
GAPDH	Sense 5'-AACGGATTTGGTCGTATTGGG-3' Antisense 5'-TCGCTCCTGGAAGATGGTGAT-3'	216

Western blot analyses. Proteins were extracted from tumor using radioimmunoprecipitation assay (RIPA) buffer (Pierce, Rockford, IL). Total protein concentration was measured using the BCA assay kit (Sigma) with bovine serum albumin as a standard, according to the manufacturer's instructions. Tumor tissue extracts containing 30 μg total proteins were subjected to 10% SDS/PAGE, and the resolved proteins transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (Invitrogen). Equal protein loading was confirmed by Coomassie (Bio-Rad, Hercules, CA) staining of the gel. After blocking with PBS (phosphate buffered saline) containing 0.2% casein for 1 h at room temperature, membranes were incubated with 3-5 μg/ml antibody (NF-κB, Survivin, XIAP, caspase-9 and active caspase-3) in PBS containing 0.1% Tween-20 overnight at 4˚C. The Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used for 3,3'-diaminobenzidene chromogenic detection (Sigma), according to the manufacturer's instructions.

Reverse transcription polymerase chain reaction (RT-PCR): measurement of NF-κB, XIAP, Survivin, caspase-9 and -3 mRNA levels. Total RNA was extracted from tumor tissues using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The RNA was dissolved using 20-50 μl of diethylpyrocarbonate (DEPC, Generay, Shanghai, China) treated distilled water. The amount and purity of the extracted RNA was quantitated by spectrophotometry. The value of A260/A280 was measured to evaluate the quality of RNA. cDNA was synthesized with 5 μg of total RNA and oligo(dT) primer (Fermentas, Lithuania). In a sterile RNase-free microcentrifuge tube, 0.5 μg of oligo(dT) primer and 5 μg RNA sample were added. The tube was heated at 70˚C for 5 min, and cooled immediately on ice. 200 U of M-MLV RTase (Moloney murine leukemia virus reverse transcriptase) (Promega, Madison, WI), 25 U rRNasin ribonuclease inhibitor (Promega), 5 μl 5X RT buffer, and 2 mM dNTP were added to the tube, to obtain a 25-μl solution using 0.1% DEPC. The tube was gently mixed, incubated for 60 min at 42˚C, and heated for 5 min at 95˚C. The cDNA was stored at -20˚C.

The amplification reaction (semiquantitative) was carried out in 25 μl of the PCR mixture. The PCR mixture was amplified using the GeneAmp PCR System 9600 (Perkin-Elmer Corp., Norwalk, CT). The primer pairs used for the amplification are listed in Table I. The amplified PCR products were identified by electrophoresis using a 1% agarose gel containing ethidium bromide visualized with ultraviolet (UV) illumination. The housekeeping gene GAPDH was used as a control for the semiquantitative analysis of Survivin, XIAP caspase-9 and -3. A negative control used H_2O instead of cDNA. The gene transcripts were quantified based on the ratio of the intensity of the target gene to the intensity of the GAPDH control gene.

Electrophoretic mobility shift assay (EMSA). Electrophoretic gel mobility shift assay was performed to determine the DNA-binding activity of transcription factor NF-κB. In brief, nuclear extracts were assayed for DNA-binding activity using the double-stranded DNA oligonucleotide probes (biotin-labeled). The sequence of the NF-κB probe was: 5'-AGT TGA GGG GAC TTT CCC AGG C-3', 5'-GCC TGG GAA AGT CCC CTC AAC T-3'. The binding reaction contained 2 μl biotin end-labeled target DNA in a final volume of 20 μl. After the reaction mixture was incubated for 20 min at room temperature, DNA-protein complexes were electrophoresed and transferred to nylon membranes (Biodyne B membrane, Pierce). The samples were then incubated in a luminol/enhancer solution mixed with stable peroxide solution (Pierce) (chemiluminescent detection method) and exposed to X-ray film for 2-5 min prior to development. The bands were scanned with a BioSenSC820 (Shanghai Bio-tech Co. Ltd., Shanghai, China), and relative intensity was analyzed using an NIH Image 1.62 package (US National Institutes of Health).

Statistical analysis. Data are expressed as the means ± standard error (SE). Statistical analysis was performed using SPSS (Statistical Product and Service Solutions, IBM, Armonk, NY) software. Differences in mean values between groups were

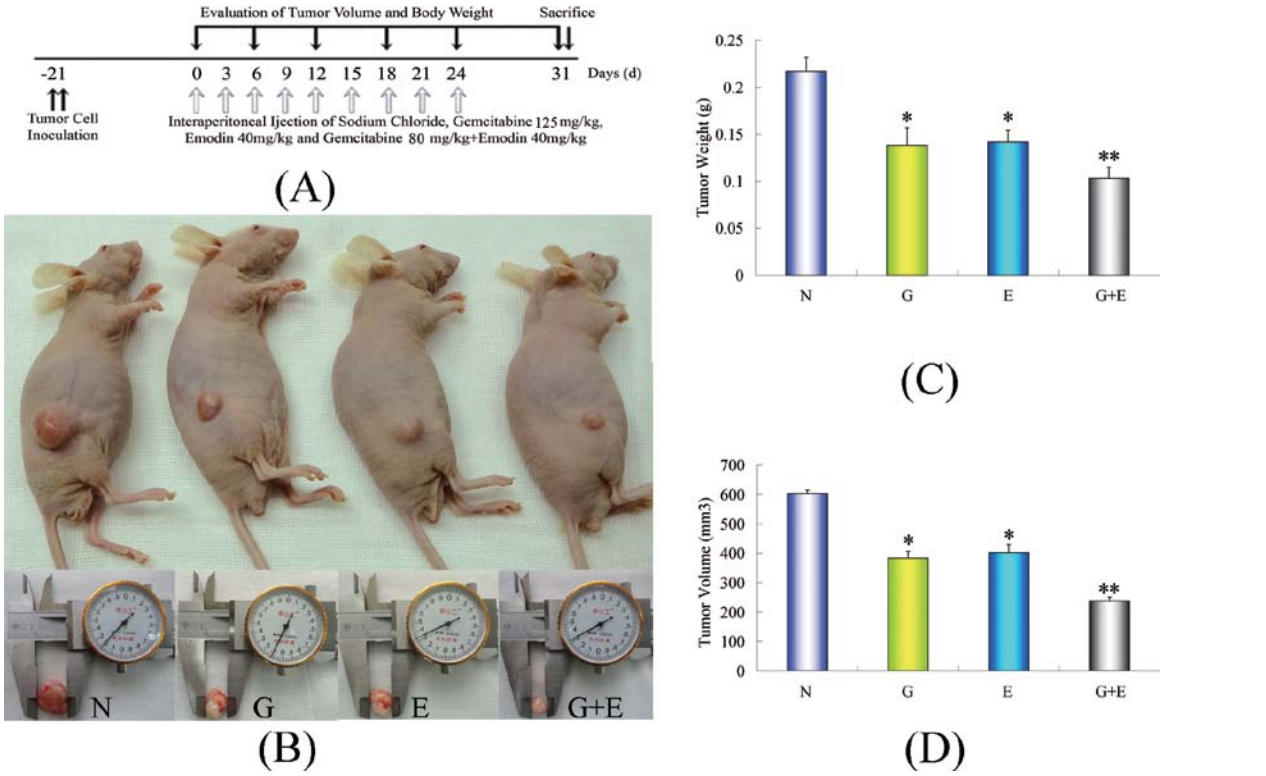

Figure 1. The effect of treatment on tumor size (N, sodium chloride; G, gemcitabine; E, emodin; G+E, gemcitabine + emodin). (A) Schematic overview of the study protocol. (B) Photographs of mice bearing subcutaneously implanted pancreatic tumors and removed tumors. (C) Tumor weight in the four groups: *when compared to group N, the difference was statistically significant ($p<0.05$); **when compared to groups N, G and E, the difference was statistically significant ($p<0.05$). (D) Tumor volume in the four groups: *when compared to group N, the difference was statistically significant ($p<0.05$); **when compared to groups N, G, E, the difference was statistically significant ($p<0.05$).

Table II. Emodin enhances the antitumor effect of gemcitabine.

Group	Dosage (mg/kg)	Tumor weight (g)	Tumor volume (mm^3)
N	0	0.217±0.025	602.4±13.2
G	125	0.138±0.033[a]	383.6±23.2[a]
E	40	0.142±0.041[a]	402.6±27.3[a]
G+E	40+80	0.103±0.026[b]	238.6±12.2[b]

One week following the final treatment (day 31), mice were sacrificed, and the tumor weight and volume were measured. [a]$p<0.05$ compared with sodium chloride treatment; [b]$p<0.05$ compared with sodium chloride, emodin or gemcitabine treatment.

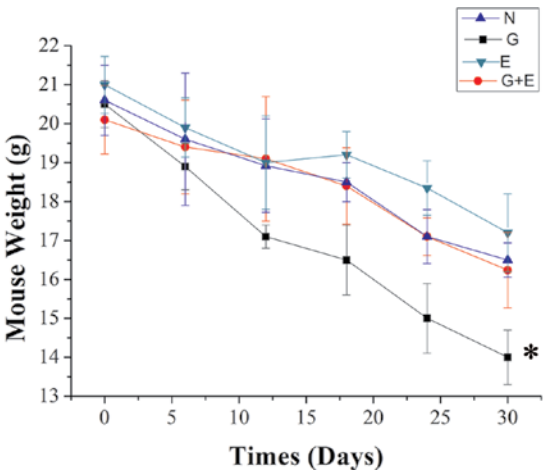

Figure 2. Variation of mouse weight. Mouse body weight decreased significantly in group G ($p<0.05$), and insignificantly in groups N, E and G+E ($p>0.05$).

determined by one-way analysis of variance (ANOVA) followed by two-tailed Student's t-test for unpaired samples, assuming equal variances. $p<0.05$ was considered statistically significant.

Results

Changes in tumor volume, tumor weight and mouse body weight (Table II). When the mice were assigned to treatment groups, the mean tumor volume was 96.3±8.6 mm^3 and the mean mouse body weight was 21.3±2.3 g. There was no statistical difference in tumor volume and body weight between the four groups. One week following final treatment, photographs were taken of mice bearing subcutaneously implanted pancreatic tumors and removed tumors (Fig. 1B), mean tumor volume was 602.4±13.2 mm^3 in the saline control group, 383.6±23.2 mm^3 in the gemcitabine group, 402.6±27.3 mm^3 in the emodin group and 238.6±12.2 mm^3 in the combination group (gemcitabine + emodin). When compared to the control group, mean tumor volume was significantly smaller ($p<0.05$) in both the G and E groups. Tumor volume in group G+E was significantly ($p<0.05$) smaller than groups G and E (Fig. 1D). Tumor weight in group G+E was also downregulated significantly when compared with the other groups ($p<0.05$) (Fig. 1C). Weight variation of

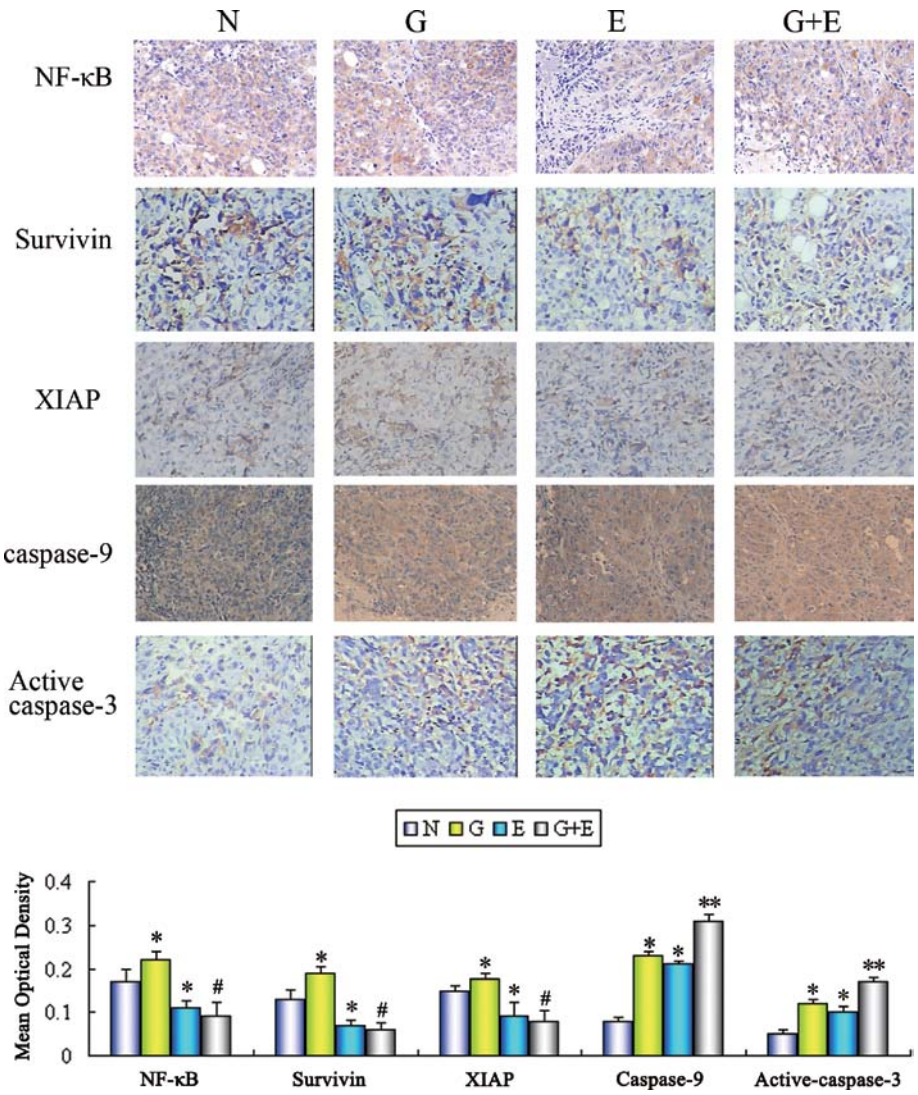

Figure 3. One week following the final treatment (day 31), protein expressions of NF-κB, Survivin, XIAP caspase-9 and active caspase-3 were assessed using immunohistochemistry (magnification x400). NF-κB, Survivin and XIAP expression was downregulated in group G+E when compared to groups N and G ($^{\#}$p<0.05). Caspase-9 and -3 expressions were upregulated significantly in group G+E when compared with the other three groups (**p<0.05).

the mice one week following the final treatment injection is shown (Fig. 2). One week following the final treatment injection, the order of the mouse weight between the four groups was: E (17.2±1.0)g > N (16.5±0.4)g > G+E (16.3±0.97)g > G (14.0±0.7)g. Body weight in group G decreased significantly when compared with group N (p<0.05).

Immunohistochemistry analysis of NF-κB, Survivin, XIAP, caspase-9 and active caspase-3. Activation of caspase-3 is recognized as a key event that mediates cellular apoptosis. Caspase-9 can active caspase-3, but Survivin and XIAP can also inhibit it. To clarify the mechanism of emodin when combined with gemcitabine, we evaluated apoptotic cell death in tumor tissues by examining the expression of inhibitors of apoptosis (IAP) Survivin, and XIAP in addition to caspase-9 and active caspase-3 levels using immunohistochemistry. The combination treatment of emodin and gemcitabine reduced NF-κB, Survivin, and XIAP expression compared with the control group (p<0.05) (Fig. 3). Importantly, combination therapy further promoted the expression of caspase-9 and active caspase-3 (p<0.05 when compared with group N). Immunohistochemistry analysis confirmed the reduction of NF-κB, Survivin and XIAP levels and elevation of caspase-9 and activated caspase-3 protein expression in the combination treatment group (p<0.05 compared with gemcitabine group) (Fig. 3).

Variation of Ki-67 expression. We next examined the expression of the cell proliferation factor Ki-67. Emodin in combination with gemcitabine significantly downregulated the cell proliferation factor Ki-67 in tumor tissues compared with the other groups (p<0.05) (Fig. 4). *In vivo*, we found inhibition of cell growth was also correlated with apoptotic cell death. Ki-67 proliferation index was counted according to the following formula: (Ki-67 positive cell number / total cell number) x 100%. The results suggested that the Ki-67 positive cell ratio was downregulated significantly in group G+E (17±2.2)% (p<0.05), when compared with group N (67±5.2)%, group G (34±4.4)%, and group E (38±3.6)%.

TUNEL staining shows emodin potentiated tumor cell apoptosis by gemcitabine. As shown (Fig. 5), increased TUNEL-positive cells were observed in drug therapy groups, and combina-

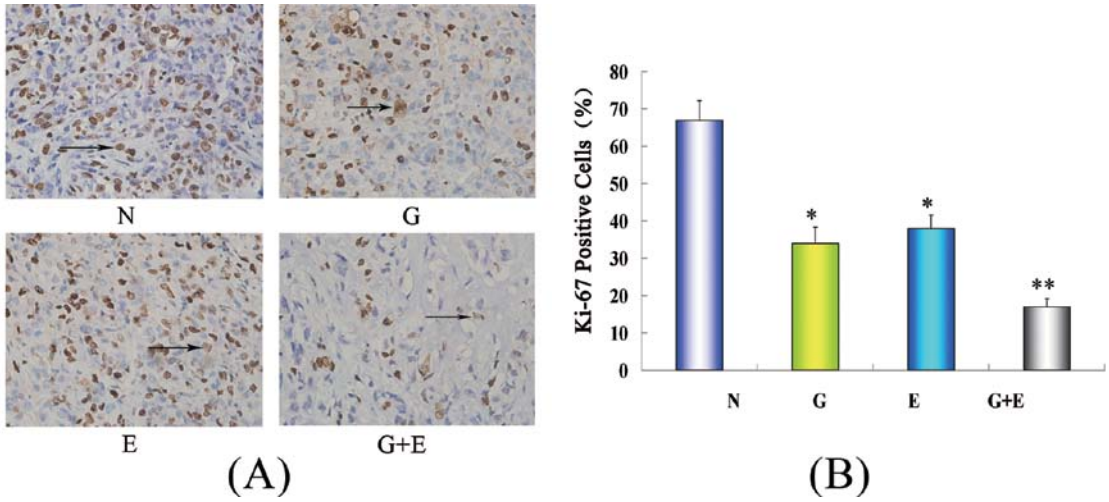

Figure 4. Determination of proliferation suppression by emodin in tumor sections of treated mice. Immunohistochemical analyses of the proliferation marker Ki-67 indicate inhibition of pancreatic cancer cell proliferation in emodin alone or in combination with gemcitabine-treated groups of animals. $^{*}p<0.05$ when compared to group N, $^{**}p<0.05$ when compared to the other groups.

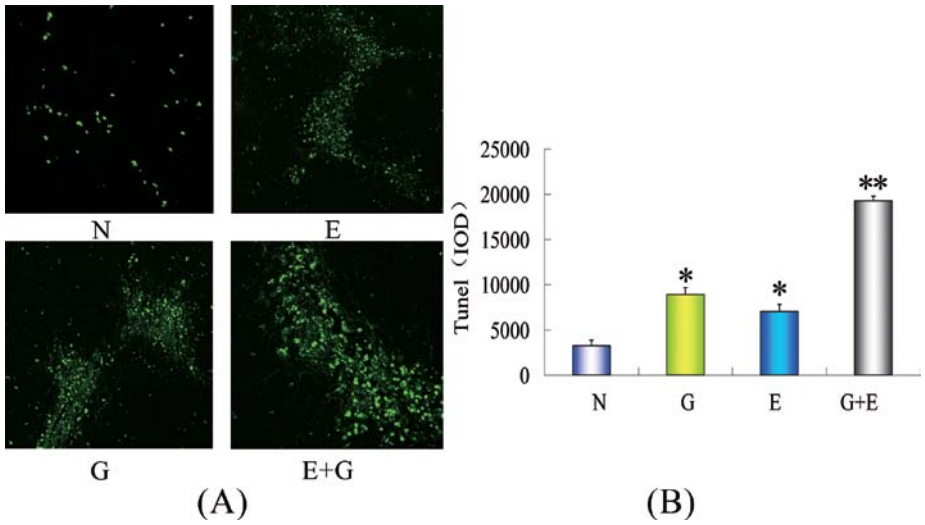

Figure 5. Tumor cell apoptosis was evaluated by TUNEL assay of the tumor sections of treated mice. Increased TUNEL-positive cells were found in the combination treatment group (magnification x400). TUNEL staining was further quantified and presented IOD values. The cell apoptosis was significantly increased in group G+E when compared with the other groups ($p<0.05$).

tion treatment remarkably promoted apoptotic cell death. Combination therapy of emodin and gemcitabine dramatically elevated integrated optical density (IOD) as compared with other groups ($p<0.05$) (Fig. 5).

Further detection of NF-κB, Survivin, XIAP, caspase-9 and active-caspase-3. We also used western immunoblot analyses to evaluate the expression of NF-κB, Survivin, XIAP, caspase-9 and active-caspase-3. Similar results to those obtained in the IHC analysis were observed. One week following the last injection, the expression of NF-κB, Survivin and XIAP were upregulated significantly in the gemcitabine treatment group compared with the sodium chloride treatment group ($p<0.05$). The expression level was significantly lower in the emodin treatment group compared to the gemcitabine treatment group. However, the combination of emodin and gemcitabine had the lowest expression of IAPs when compared with group N ($p<0.05$). At the same time, the expression of caspase-9 and active caspase-3 were upregulated significantly in the gemcitabine and emodin combination treatment group as compared to individual agents ($p<0.05$) (Fig. 6).

Emodin reduced NF-κB activation. Emodin or emodin plus gemcitabine in combination significantly decreased the DNA binding activity of NF-κB (Fig. 7). When compared with group G, DNA binding activity was decreased significantly in groups E and G+E ($p<0.05$).

The mRNA levels of NF-κB, Survivin, XIAP, caspase-9 and -3. RT-PCR assay showed that NF-κB, Survivin and XIAP mRNA levels were all significantly lower in the emodin and gemcitabine combination treatment group compared to gemcitabine as a single agent ($p<0.05$). Additionally, the mRNA levels of caspase-9 and -3 were upregulated in the emodin and gemcitabine combination group as compared to single agents and controls ($p<0.05$) (Fig. 8).

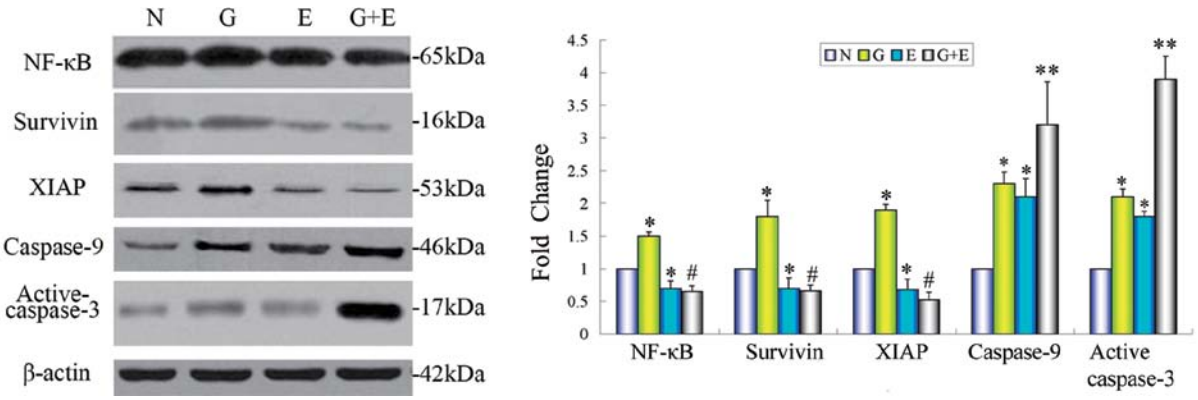

Figure 6. One week following the final treatment injection (day 31), protein expression of NF-κB, Survivin, XIAP, caspase-9 and active caspase-3 were assessed using western immunoblot analyses. For NF-κB, Survivin and XIAP, caspase-9 and active caspase-3, *p<0.05 when compared with group N, $^{\#}$p<0.05 when compared with groups N and G. For caspase-9 and active caspase-3, *p<0.05 when compared with group N, **p<0.01 when compared with groups N, G and E.

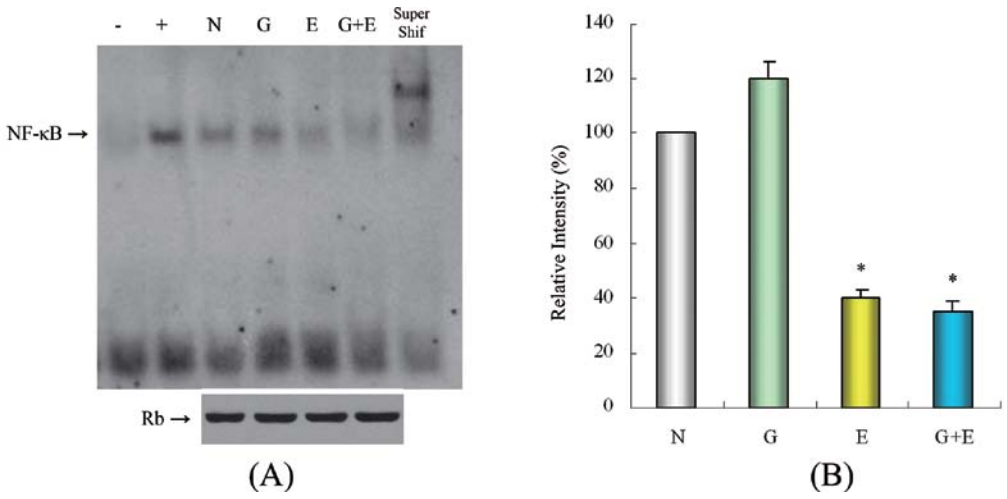

Figure 7. Emodin or emodin plus gemcitabine combination treatment significantly decreased the DNA binding activity of NF-κB. When compared with group G, the DNA binding activity was decreased significantly in groups E and G+E (p<0.05).

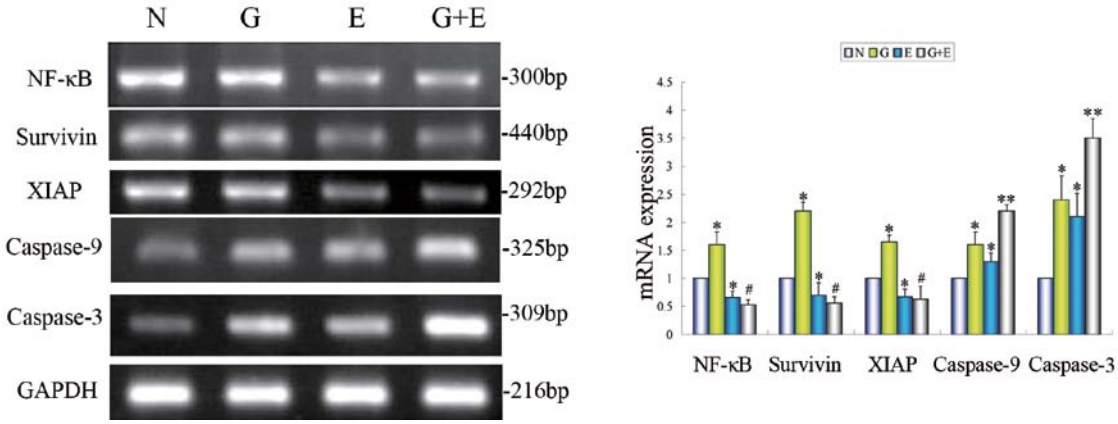

Figure 8. The mRNA levels of NF-κB, Survivin, XIAP, caspase-9 and -3 in tumor tissue from different treatment groups were detected using RT-PCR. NF-κB, Survivin and XIAP mRNA was downregulated in group G+E when compared to group N and group G ($^{\#}$p<0.05). Caspase-9 and -3 expression were upregulated significantly in group G+E when compared with the other three groups (**p<0.05).

Discussion

Programmed cell death (apoptosis) is the cell's intrinsic death program that is involved in the regulation of many physiological and pathological processes, and it is evolutionary highly conserved (27). Two principle apoptosis signaling pathways have been delineated, i.e., the death receptor pathway (extrinsic pathway) and the mitochondrial pathway (intrinsic pathway)

(27). Caspases, a family of cysteine proteases that function as common death effector molecules by cleaving a range of cytoplasmic or nuclear substrates, play important roles in both pathways (28). One of the characteristic features of human cancers is the inability to undergo apoptosis in response to stimuli that otherwise trigger apoptosis in sensitive cells (29). The activated caspases are subject to inhibition by the inhibitor of apoptosis family of proteins (IAPs) (30). Our study results showed that caspase-9 and -3 expression was relatively low in groups N, and the expression of Survivin and XIAP was highly expressed in groups N and G.

As a natural medicine, emodin was reported to enhance the antitumor effects of gemcitabine on pancreatic cancer cell xenografts in mice, with the hypothesized mechanism of action of downregulation of IAPs or NF-κB expression thus promoting apoptosis (24-26). It was reported that the antitumor efficacy of gemcitabine on pancreatic cancer could be enhanced when the activity of NF-κB was inhibited (31). In our present study, we established the pancreatic tumor model in nude mice which were randomized into four treatment groups. The mice were treated for 4 weeks with emodin, gemcitabine or the combination. We found that all treatment groups could reduce the growth of tumors, however, the decrease in tumor growth of the combination group was much more significant than that of the single agents ($p<0.05$). TUNEL staining and Ki-67 expression suggested that emodin enhanced the antitumor effects of gemcitabine by induction of apoptosis and inhibition of cell proliferation. The results also demonstrated that emodin potentiated the antitumor effects of gemcitabine by inhibition of survival signaling.

The transcription factor nuclear factor-kappa B (NF-κB) is a crucial regulator of many physiological and pathophysiological processes, including control of the adaptive and innate immune responses, inflammation, proliferation, tumorigenesis, and apoptosis (32). A prevalent mechanism by which activated NF-κB induces chemoresistance in PDAC (pancreatic ductal adenocarcinoma) is by increased expression of cellular inhibitors of apoptosis, including cIAP1, cIAP2, TRAF1, TRAF2, and Survivin (33,34). Activation of the transcription factor NF-κB after gemcitabine treatment has been found in several studies of pancreatic cancer (35,36).

We examined the effects of emodin in combination with gemcitabine as a means to improve tumor response, as the activation of NF-κB may induce resistance of pancreatic cancer to gemcitabine treatment (37). Studies have shown that emodin is involved in the inhibition of NF-κB transcriptional activity (17,38). We assessed the DNA-binding activity of NF-κB by EMSA as well as the mRNA expression of NF-κB using RT-PCR. The results demonstrated that emodin reduced the DNA-binding activity of NF-κB, as well as the mRNA expression of NF-κB in groups E and G+E.

Survivin is a functional protein that acts as a suppressor of apoptosis. Survivin is selectively expressed in most human neoplasms including pancreatic cancer, but not in normal adult tissue (39). Several preclinical studies have demonstrated that downregulation of Survivin expression reduced tumor growth potential, increased apoptotic rate and sensitized tumor cells to chemotherapeutic drugs (40). The inhibitor of apoptosis proteins (IAPs) are a class of key apoptosis regulators and are characterized by the presence of one or more baculoviral IAP repeat (BIR) domains (30). Among a total of eight mammalian IAPs, X-linked IAP (XIAP) inhibits apoptosis by directly binding to and effectively inhibiting three caspases: caspase-3, -7 and -9 (30).

Research has demonstrated that XIAP expression was upregulated as a consequence of several weeks of chemotherapy of gemcitabine on the pancreatic cancer SW1990 cells *in vivo* and emodin was able to reduce XIAP expression (26). However, the specific mechanism as to how this was achieved was unknown. Research also suggested that gemcitabine upregulated NF-κB expression and evodiamine potentiates gemcitabine antitumor activity through direct or indirect negative regulation of the PI3K/Akt pathway (31).

We also examined the mRNA expression of Survivin and XIAP by RT-PCR as related to single agent and combination treatments. The results demonstrate that emodin reduced the mRNA expression of Survivin and XIAP in groups E and G+E. IHC and WB results indicated that gemcitabine upregulated the expression of Survivin and XIAP expression and emodin, as a single agent and in combination, downregulated Survivin and XIAP expression in pancreatic tumors. Survivin and XIAP expression level decreased significantly in pancreatic cancer cells in the combination group. These results are consistent with inhibition of NF-κB DNA-binding activity as we have demonstrated.

Caspases are a family of cysteine proteases that play important roles in regulating apoptosis. While Survivin and XIAP were downregulated, IHC, WB and RT-PCR showed that caspase-9 and active-caspase-3 expression was upregulated significantly in gemcitabine and emodin combination group in our study.

Collectively, the present study demonstrates that combination treatment consisting of emodin and gemcitabine efficiently suppressed tumor growth in Panc-1, human pancreatic cancer, xenografts compared to single agent treatment. Combination treatment promoted apoptotic cell death in pancreatic tumor cells through deactivation of NF-κB, and subsequently reduced Survivin and XIAP, and in turn promoted caspase-9 and -3 activation. Hence, these results demonstrate that emodin potentiates the antitumor activity of gemcitabine and may provide new alternatives for the clinical treatment of patients with pancreatic tumors.

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