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Effects of gemcitabine on radiosensitization, apoptosis, and Bcl-2 and Bax protein expression in human pancreatic cancer xenografts in nude mice

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ABSTRACT. The aim of this study was to evaluate the radiosensitizing effects of gemcitabine towards human pancreatic cancer xenografts. A human pancreatic cancer xenograft model was established in nude mice, 36 of which were randomly divided into 6 treatment groups. Tumors were measured every 2 days, and the tumor volumes, growth delays, and inhibition rates were compared to evaluate the gemcitabine enhancement factor. The apoptotic index was determined by terminal deoxynucleotidyl transferase dUTP nick end-labeling assay, and apoptosis inhibitory protein Bcl-2 and apoptosis-related protein Bax expression were detected by immunohistochemistry. Compared with the control group, xenograft growth was significantly inhibited in the 25 (G25) and 50 mg/kg gemcitabine (G50) groups ($P < 0.05$). In the 25 (G25R) and 50 (G50R) mg/kg gemcitabine + radiotherapy groups, local tumor growth was significantly inhibited, with inhibition rates of 88.22 and 91.23%, respectively, significantly higher than those of

the simple radiotherapy (SR), G25, and G50 groups (44.11, 72.88, and 77.53%, respectively; $P < 0.05$). The tumor growth delay in the G25R and G50R groups were 9 and 15 days, respectively, higher than the SR, G25, and G50 groups (each 4 days, $P < 0.05$). The apoptosis of tumor cells in the intervention groups significantly increased, and the apoptotic index among the intervention groups exhibited significant differences ($P < 0.05$). The immunohistochemical results indicated that Bcl-2 was downregulated to different degrees in the intervention groups, whereas Bax was upregulated ($P < 0.05$). Therefore, gemcitabine appears to enhance the radiotherapeutic sensitivity of human pancreatic cancer xenografts significantly.

Key words: Pancreatic cancer; Gemcitabine; Radiotherapy; Radiosensitization

INTRODUCTION

Pancreatic cancer is one of the most common malignancies of the digestive system, with an incidence exhibiting an upward trend both domestically and abroad. Pancreatic cancer often shows no obvious symptoms in the early stages, and 80% of patients are not clinically diagnosed until the advanced stages (Pannala et al., 2008); therefore, the overall 5-year survival rate is <5% (Hackert and Büchler, 2013). Until recently, simply focusing on early diagnosis and surgical treatment has not resulted in significant improvement in the prognosis of pancreatic cancer; however, the combined therapy could potentially improve the survival rate or life quality of patients.

Radiotherapy is one of the most important methods utilized in the treatment of pancreatic cancer, but the low sensitivity of this disease to radiation and the damage to the cancerous tissues limit its use. For non-responsive tissues, the inhibition of tumor growth can be realized through the following: 1) highly efficient and low-toxic radiosensitizers could be used to increase the sensitivity of pancreatic cancer cells to radiation, thus improving the local control rate of tumor; or 2) a higher single dose of radiation can be used to improve the biological equivalent dose (BED) (Onishi et al., 2004). The BED is a measure of the reaction of an organism to radiation; it is important for evaluating the collateral damage after radiation therapy. A greater single dose results in a greater biological effect. The improvement in the BED is dependent on the increase of the single dose. Because of the specific anatomical site of pancreatic cancer, it would be very difficult to improve the single dose, as this would likely cause significant toxicity. Therefore, identifying an efficient and low-toxicity drug might be much more practical.

Gemcitabine, a nucleoside analogue, has been proven to have anti-tumor effects with fewer toxic side effects compared to those caused by other drugs (Van Laethem et al., 2010). However, little has been reported regarding its sensitizing effects and possible mechanisms of action when combined with radiotherapy. In this study, gemcitabine combined with single high-dose radiotherapy was applied for the treatment of xenografts of human PANC-1 pancreatic cancer cells in nude mice. Our goal was to observe the radiosensitization effects of gemcitabine on pancreatic cancer and its influence on the apoptotic index (AI) and expression of the apoptosis protein inhibitors Bcl-2 and Bax.

MATERIAL AND METHODS

Mice and xenografts

Male BALB/c-nu/nu nude mice aged 4-6 weeks and weighing 18-22 g were purchased from the Department of Comparative Medicine, Jinling Hospital, Medical School of Nanjing University (Nanjing, China). The mice were bred under strict conditions to ensure the absence of specific pathogens and to obtain specific pathogen-free animals. The human pancreatic cancer PANC-1 cell line was provided by the Hepatobiliary Institute, Nanjing Drum Tower Hospital (Nanjing, China). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Medical School of Nanjing University.

Reagents

Gemcitabine (200 mg/bottle; Eli Lilly, Indianapolis, IN, USA) was prepared by resuspension in saline. Calf serum was purchased from Sigma-Aldrich (St. Louis, MO, USA); RPMI 1640 culture medium was obtained from Gibco (Gaithersburg, MD, USA); trypsin was purchased from Difco Laboratories (Detroit, MI, USA); Bcl-2 and Bax antibodies were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China); horseradish peroxidase double-staining and diaminobenzidine chromogenic kits were purchased from Fuzhou Maixin Biotechnology Development Co., Ltd. (Fuzhou, China). An Elekta Precise linear accelerator (Elekta, Stockholm, Sweden) was used, with a dose rate of 300 cGy/min.

Establishment of a cancer-bearing nude mouse model

Nude mice were adaptively bred in a sterile chamber with a laminar airflow unit for 1 week. Human PANC-1 pancreatic cancer cells were cultured in RPMI 1640 medium with 10% fetal calf serum at 37°C in an atmosphere containing 5% CO_2 to obtain cells in the logarithmic phase. Next, 0.25% trypsin was used to digest the cell suspension, and the trypan blue exclusion test was performed to count live cells. When the number of live cells present was more than 95% of the total population, serum-free RPMI 1640 medium was used to adjust the cell concentration to obtain a final concentration of 3×10^7 cells/mL. A 0.2-mL fraction of the above cell suspension was subcutaneously inoculated into the back of 42 nude mice (6×10^6 cells/each mouse). After 10-15 days, a tumor approximately 5 mm in size developed, and the mice were randomly grouped as described below.

Grouping

Thirty-nine of the 42 inoculated nude mice exhibited tumorigenicity (93% success rate). Of these, 36 were randomly divided into 6 groups of 6 animals each. In the control group (CG), mice were injected with saline through the tail vein. In the simple radiotherapy group (SR), each nude mouse received a single dose electron irradiation locally (16 Gy) to the tumor on the back. In the G25 and G50 gemcitabine groups, mice were injected with 25 or 50 mg/kg body weight gemcitabine, respectively, through the tail vein. In the 25 (G25R) and 50 (G50R)

mg/kg gemcitabine + radiotherapy groups, mice were injected with gemcitabine (25 or 50 mg/kg body weight, respectively) through the tail vein, and after 12 h, single-electron irradiation (16 Gy) was performed locally to the tumor on the back.

Method of administration

The specific dose of gemcitabine was calculated according to the experimental grouping and the body weight of each mouse. The diluted drug was intravenously injected through the tail vein, and the nude mice in the CG were injected with an equivalent volume of saline.

Irradiation method

Three nude mice were irradiated during each procedure. First, the mice were intravenously injected with 0.2 mL ketamine. Subsequently, the nude mice were placed on an operating table, and irradiated with a 6-MeV electron beam. Only the tumor on the animal's back was irradiated, while the rest of the body was shielded with 1-cm thick rubber lead.

Evaluation method

After grouping, the general characteristics of the cancer-bearing nude mice were observed. The maximum diameter (A) and minimum diameter (B) of the tumor were measured by hand with a vernier caliper, and the tumor volume was calculated according to the formula, $V = \pi/6AB^2$. The observation period was 1 month, after which the nude mice were sacrificed. The tumor inhibition rate was calculated as [(the average tumor weight of the CG - the average tumor weight of the experiment group) / the average tumor weight of the CG] x 100%.

Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL)

The presence and degree of apoptosis in xenograft tissues from each group were detected using an *in situ* 3'-hydroxyl end-labeling method (the TUNEL assay). A positive reaction was defined as the intracellular distribution of brown coarse particles or the diffuse distribution of brownish yellow fine particles. Each slice of tissue sample was randomly selected, and 10 consecutive fields were observed under high magnification (400X) to count the ratio of positive cells to all cells within each field, i.e., the apoptotic rate. The average value was considered to be the AI.

Detection of the apoptosis inhibition protein Bcl-2 and the apoptosis-related protein Bax

Five tumor samples from each group were used for immunohistochemical staining. Immunostaining was completed according to the manufacturer instructions. The dilutions of Bcl-2 and Bax antibodies were 1:300 and 1:400, respectively. Positive expression of Bcl-2 and Bax was found to be primarily localized in the cytoplasm, as indicated by the cytoplasm staining brown. Ten fields, with a minimum of 1000 cells in each field, were randomly counted under high magnification (400X). The number of positive cells was counted and is expressed as a percentage.

Statistical analysis

The SPSS 16.0 statistical software (Chicago, IL, USA) was used to perform the *t*-test, with $P < 0.05$ considered to be statistically significant, and $P < 0.01$ considered to be highly statistically significant.

RESULTS

General observations

All 36 nude mice survived the experiment, and no lung or liver metastases were observed in any group. There were no abnormalities in the eating habits and activities of the CG mice, the gemcitabine treatment groups, or the gemcitabine + radiotherapy treatment groups. Conversely, in the radiotherapy groups, the irradiated skin surface showed redness without exudation 3 days after the radiation treatment.

Effects of different experimental treatments on xenograft inhibition

Before treatment, the growth profiles of the xenografts in each group were similar; there was no significant difference in the xenograft volumes ($P > 0.05$). After treatment, the growth of the xenografts in each intervention group was inhibited to different extents. Two different doses of gemcitabine (25 and 50 mg/kg) were administered intravenously to different groups, and the tumor volumes of these groups at the end of the experiment were smaller than those of the CG, indicating that gemcitabine possessed an inhibitory effect ($P < 0.05$). When gemcitabine was combined with radiotherapy, the tumor volumes and weights in the experimental groups were lower than those of the CG as well as those of the drug only and radiotherapy only groups, with the results of the G50R group showing the highest significance ($P < 0.01$; Table 1).

Table 1. Comparison of the tumor burdens of xenografts in nude mice for all groups.

Group	Tumor volume [mm^3 (means ± SD)]		Tumor weight [g (means ± SD)]	Inhibition rate (%)
	0 days	30 days		
A	60.32 ± 5.89	1805.9 ± 15.87	3.65 ± 0.11	-
B	61.50 ± 4.16	1161.2 ± 339.51	2.04 ± 0.11	44.11
C	62.57 ± 4.40	664.35 ± 136.52	0.99 ± 0.09	72.88
D	65.32 ± 4.64	541.10 ± 28.81	0.82 ± 0.04	77.53
E	63.53 ± 3.32	171.08 ± 21.35	0.43 ± 0.07	88.22
F	64.62 ± 3.43	122.55 ± 15.70	0.32 ± 0.05	91.23
P value	>0.05	<0.001	<0.001	-

Compared between any two groups: A) control; B) radiation; C) 25 mg/kg gemcitabine; D) 50 mg/kg gemcitabine; E) combined treatment 1; F) combined treatment 2.

Measurement of apoptosis in xenografts of each group using the TUNEL assay

Following the TUNEL assay, apoptotic cells were stained brown, and normal cells stained bluish-purple. The results showed that apoptosis was most obvious in the two combined-therapy subgroups, followed by the G50, G25, and SR groups, while it was the least

apparent in the CG. Apoptosis in the intervention groups increased significantly compared with that in the CG, and there were significant differences in the AI between the intervention groups ($P < 0.05$; Table 2).

Table 2. Comparison of the apoptosis rates for the six groups.

Group	Example	AI
A	5	2.13 ± 0.41
B	5	8.42 ± 1.30
C	5	12.62 ± 1.17
D	5	19.57 ± 0.90
E	5	24.31 ± 1.51
F	5	36.84 ± 2.29

Compared between any two groups, $P < 0.05$. AI, apoptosis index; A) control; B) radiation; C) 25 mg/kg gemcitabine; D) 50 mg/kg gemcitabine; E) combined treatment 1; F) combined treatment 2.

Expression rates of Bcl-2 and Bax in the xenografts of each group

Following immunohistochemical analysis, brown-stained cytoplasm indicated positive expression of Bcl-2 or Bax in the respective assays. Immunohistochemical results defined the expression of Bcl-2 protein as follows: highest expression in the CG, followed by the SR, G25, and G50 groups, while the positive expression rate in the combined-therapy group was lower (Figure 1 and Table 3). The expression of Bax protein was as follows: highest expression in the combined-therapy group, followed by the G50, G25, and SR groups, while the CG had the lowest expression (Figure 2 and Table 3).

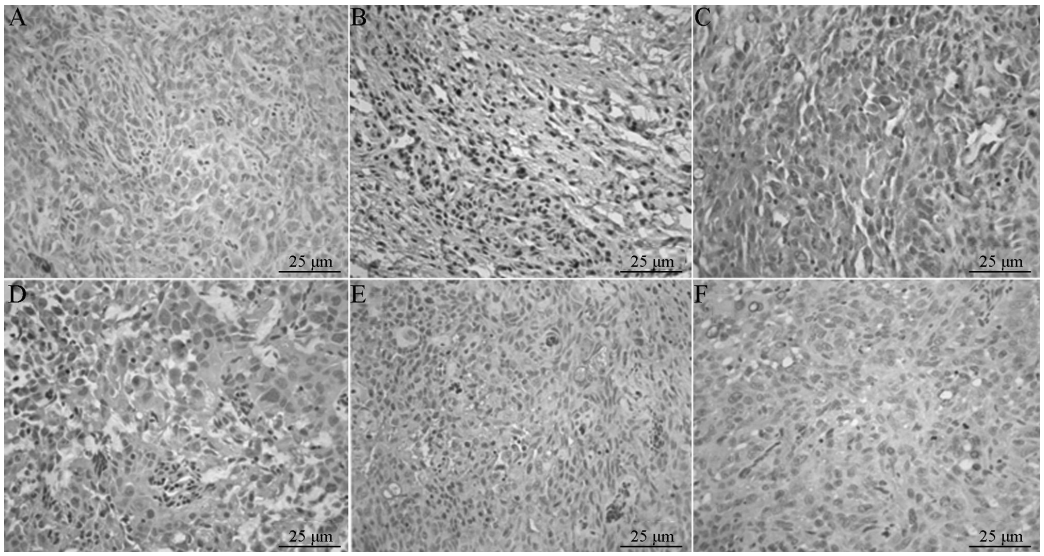

Figure 1. Bcl-2 expression in nude mouse xenografts of the six groups (400X). **A.** Control; **B.** radiation; **C.** 25 mg/kg gemcitabine; **D.** 50 mg/kg gemcitabine; **E.** combined treatment 1; **F.** combined treatment 2.

Table 3. Relative quantity of Bcl-2, Bax expression ratio in six groups.

Group	Example	Bcl-2	Bax
Control group (A)	5	90.56 ± 3.87	40.73 ± 2.69
Radiation group (B)	5	79.14 ± 2.62*#	52.52 ± 3.86*#
25 mg/kg group (C)	5	70.02 ± 3.50*#Δ	59.97 ± 1.72*#Δ
50 mg/kg group (D)	5	61.74 ± 4.23*#	68.06 ± 3.56*#
Combined treated group 1 (E)	5	46.68 ± 2.35*▲	82.72 ± 3.97*▲
Combined treated group 2 (F)	5	37.54 ± 3.92*	94.84 ± 4.59*

vs Group A, *P < 0.05; *vs* Group E, F, #P > 0.05; *vs* Group D, ΔP < 0.05; *vs* Group F, ▲P < 0.05.

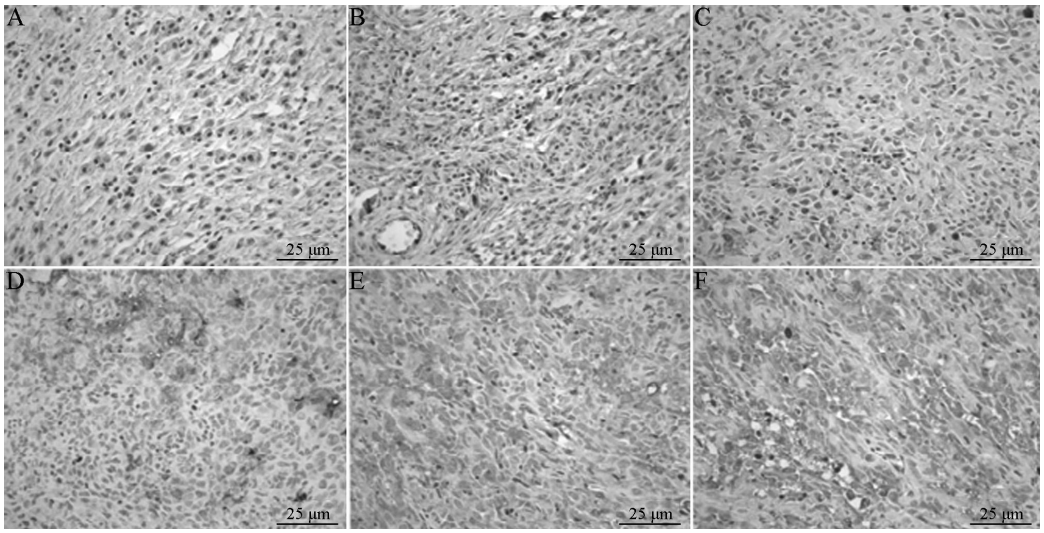

Figure 2. Bax expression in nude mouse xenografts of the six groups (400X). **A.** Control; **B.** radiation; **C.** 25 mg/kg gemcitabine; **D.** 50 mg/kg gemcitabine; **E.** combined treatment 1; **F.** combined treatment 2.

DISCUSSION

Radiosensitizers, which are currently receiving increasing amounts of attention, primarily include the following categories: 1) electrophilic radiosensitizers, represented by misonidazole and its derivatives; 2) biological reductants, namely hypoxic cytotoxic drugs; 3) chemotherapy drugs such as platinum, 5-fluorouracil, gemcitabine, taxol, camptothecin, and vinca alkaloids; antimetabolites such as gemcitabine act on cells during S-phase, and they can also prevent the progression of cells in the G1-phase to the S-phase, affect cell cycle redistribution, and inhibit the radioactive damage repair of cellular DNA (Shewach and Lawrence, 2007); 4) molecular targeted drugs such as epidermal growth factor receptor inhibitors including cetuximab and erlotinib, as well as the cyclooxygenase-2 inhibitor celecoxib (Katz et al., 2009; Herman et al., 2013); and 5) natural medicines such as irisquinone. An ideal radiosensitizer should possess the following characteristics (Kvols et al., 2005): 1) stability; it should not react easily with other substances *in vivo*, but exhibit a slow metabolic degradation and a long biological half-life; 2) the ability to selectively concentrate in the tumor tissue; 3) the effective

therapeutic dose must be below the toxic dose; 4) some solubility in water or fat; 5) the ability to selectively sensitize hypoxic cells while having little or no effect towards normal aerobic cells; 6) no demonstration of phase-dependent characteristics, i.e., it should ideally function throughout the cell cycle.

Gemcitabine, a synthetic cytarabine-like drug, belongs to the sensitizer class of chemotherapy drugs, and can be converted into its active diphosphate and triphosphate forms by cellular nucleoside kinases *in vivo*. Nucleoside triphosphate inhibits the activity of nucleoside reductase, suppressing the production of deoxynucleoside triphosphates that are necessary for DNA synthesis, thus reducing the nucleotide pool (Plunkett et al., 1995a,b). Once the nucleoside triphosphate is added to the extending DNA, it inhibits the further extension of the DNA chain, resulting in apoptosis. The radiosensitizing effect of gemcitabine arises because of the consequent significant reduction of S-phase cells in the tumor cell population, which would otherwise tolerate the radiation treatment. Treatment ultimately causes the surviving cells to enter the radiosensitive G2 and M phases synchronously. Simultaneously, the cytotoxic effects of gemcitabine reduce the cell numbers, thereby increasing the re-oxygenation of the tumor cells, such that the originally nonsensitive hypoxic cells are now sensitive to radiation (Mason et al., 1999). Gemcitabine has ideal features for consideration as a radiation sensitizer. Studies have shown that gemcitabine, administered prior to the radiation therapy, has a sensitizing effect on tumor cells (Pauwels et al., 2005; Morgan et al., 2010). Shewach and Lawrence (1996) have confirmed that radiosensitization was obvious if gemcitabine was administered prior to irradiation, while the effects were poor if it was administrated during or after irradiation. The length of drug action should be ≥2 h, with 24 h of activity being the most effective; however, there was little difference in the sensitization observed after 24 h.

This study demonstrated that gemcitabine had anti-tumor effects against xenografts of human pancreatic cancer cells, and the growth curve illustrated that a dose of 50 mg/kg was much more effective 25 mg/kg ($P < 0.05$). The comparison of G25R and G50R groups with the SR group results revealed that the anti-tumor effects of combined-therapy were stronger than those of radiotherapy alone, and that 50 mg/kg of gemcitabine + radiotherapy was stronger than 25 mg/kg of gemcitabine + radiotherapy. A month later, the average tumor weight of mice in the G50 group was significantly smaller than that in the G25 group. The tumor inhibition rates in the G25R and G50R groups also exhibited a statistically significant difference ($P < 0.05$). Studies regarding the sensitization of gemcitabine have shown that the sensitization of gemcitabine appears to increase gradually with increasing concentrations until it plateaus at a certain point (Brullé et al., 2012). Our experiments confirmed that the sensitizing effect and tumor growth inhibition of G50 were better than those of G25 and further demonstrated that G50 did not exhibit any toxic side effects.

Apoptosis is a key mechanism of tumor cell death, and the dysregulation of apoptosis is a major cause of tumor development. Apoptotic signaling pathways comprise the endogenous and exogenous pathways (Hersey and Zhang, 2003). The exogenous pathway is mediated by the death receptor on the cell surface, while the endogenous pathway is mediated by the mitochondria, of which the Bcl-2 family has received much attention (Trisciuoglio et al., 2010, 2011). Previous studies have reported that overexpression of Bcl-2 inhibited apoptosis induced by a variety of factors. *Bax* is an important member of the Bcl-2 family, and was the first *Bcl-2* homologue to be identified. The Bax protein inhibits the anti-apoptotic role of the Bcl-2 protein; it can antagonize the protective effects of Bcl-2, thus leading to apoptosis (Fennell et al., 2008). The ratio of Bax to Bcl-2 is a key factor of apoptosis inhibition; thus,

the detection of the differential expression of Bax and Bcl-2 via immunohistochemistry is an important part of identifying the mechanism of the radiosensitizing effects of gemcitabine. In this study, the apoptosis results obtained using the TUNEL assay demonstrated that apoptosis within the intervention groups was significantly enhanced when compared to that of the CG, and there were statistical differences in the AI among the intervention groups as well. Immunohistochemical results showed that compared with the CG, use of gemcitabine could downregulate the expression of the Bcl-2 protein and upregulate the expression of the Bax protein to various degrees ($P < 0.05$). Compared with the SR, G25, and G50 groups, the downregulated expression of Bcl-2 and upregulated expression of Bax in the G25R and G50R groups had greater statistical significance. These results indicate that the combination of gemcitabine and radiotherapy had significant effects towards promoting the apoptosis of tumor cells and were more effective than the single application of either radiotherapy or gemcitabine. The combination of the two therapies seemed to have multiplied the effects. The results showed that G50 and G50R each had a stronger effect of promoting apoptosis in tumor cells compared to G25, while hematoxylin and eosin staining indicated that there were no significant pathological changes in the hearts, lungs, livers, kidneys, and other organs of the experimental nude mice of each group. However, previous studies (Rübe et al., 2004; Zinner et al., 2009; Gomez et al., 2012) have reported that the combination of gemcitabine and radiotherapy in the treatment of pancreatic cancer increased lung damage, gastrointestinal symptoms, and bone marrow suppression, and that the effects were more obvious with increasing doses of gemcitabine. Therefore, in combining gemcitabine with single high-dose radiotherapy in the treatment of pancreatic cancer, it is very important to investigate the safe therapeutic window of gemcitabine.

Immunohistochemistry results indicated that administration of gemcitabine before radiotherapy could lead to downregulation of the Bcl-2 protein and relative upregulation of the Bax protein. This led to an increase in the Bax/Bcl-2 ratio, which would be expected to cause the release of cytochrome c from the mitochondrial membrane and induce the apoptotic cascade; thus, apoptosis likely occurred through the mitochondria-mediated endogenous pathway. In our study, the combination of gemcitabine with a single high dose of radiotherapy exhibited a significant synergistic effect, which was concluded to be primarily caused by DNA damage induced by radiotherapy treatment. This mechanism involves gemcitabine interference with the nucleotide pool and negatively affecting the necessary raw materials of DNA synthesis and repair, thus having synergistic anti-tumor effects. Gemcitabine is a safe and effective sensitizer with low toxicity, and the combination of gemcitabine and radiotherapy could effectively improve the therapeutic effect and the local control rate of radiotherapy for pancreatic cancer. This study found that the combination of gemcitabine and a high dose of radiotherapy influenced the ratio of Bax/Bcl-2 proteins, the associated consequences and thus likely their *in vitro* activities in nude mice with pancreatic cancer xenografts. These findings suggest a novel therapeutic model for the combination of gemcitabine and high-dose radiotherapy (stereotactic radiotherapy). However, the therapeutic window of gemcitabine and the specific model for its combined use with radiotherapy still need to be clinically evaluated.

Conflicts of interest

The authors declare no conflict of interest.

REFERENCES

Brullé L, Vandamme M, Riès D, Martel E, et al. (2012). Effects of a non thermal plasma treatment alone or in combination with gemcitabine in a MIA PaCa2-luc orthotopic pancreatic carcinoma model. *PLoS One* 7: e52653.

Fennell DA, Chacko A and Mutti L (2008). BCL-2 family regulation by the 20S proteasome inhibitor bortezomib. *Oncogene* 27: 1189-1197.

Gomez DR, Tucker SL, Martel MK, Mohan R, et al. (2012). Predictors of high-grade esophagitis after definitive three-dimensional conformal therapy, intensity-modulated radiation therapy, or proton beam therapy for non-small cell lung cancer. *Int. J. Radiat. Oncol. Biol. Phys*. 84: 1010-1016.

Hackert T and Büchler MW (2013). Pancreatic cancer: advances in treatment, results and limitations. *Dig. Dis*. 31: 51-56.

Herman JM, Fan KY, Wild AT, Hacker-Prietz A, et al. (2013). Phase 2 study of erlotinib combined with adjuvant chemoradiation and chemotherapy in patients with resectable pancreatic cancer. *Int. J. Radiat. Oncol. Biol. Phys*. 86: 678-685.

Hersey P and Zhang XD (2003). Overcoming resistance of cancer cells to apoptosis. *J. Cell Physiol.* 196: 9-18.

Katz D, Ito E and Liu FF (2009). On the path to seeking novel radiosensitizers. *Int. J. Radiat. Oncol. Biol. Phys*. 73: 988-996.

Kvols LK (2005). Radiation sensitizers: A selective review of targeting DNA and non-DNA targets. *J. Nucl. Med.* 46: 187S-190S.

Mason KA, Milas L, Hunter NR, Elshaikh M, et al. (1999). Maximizing therapeutic gain with gemcitabine and fractionated radiation. *Int. J. Radiat. Oncol. Biol. Phys.* 44: 1125-1135.

Morgan MA, Parsels LA, Zhao L, Parsels JD, et al. (2010). Mechanism of radiosensitization by the Chk1/2 inhibitor AZD7762 involves abrogation of the G2 checkpoint and inhibition of homologous recombinational DNA repair. *Cancer Res.* 70: 4972-4981.

Onishi H, Araki T, Shirato H, Nagata Y, et al. (2004). Stereotactic hypofractionated high-dose irradiation for stage I nonsmall cell lung carcinoma: clinical outcomes in 245 subjects in a Japanese multiinstitutional study. *Cancer* 101: 1623-1631.

Pannala R, Leirness JB, Bamlet WR, Basu A, et al. (2008). Prevalence and clinical profile of pancreatic cancer-associated diabetes mellitus. *Gastroenterology* 134: 981-987.

Pauwels B, Korst AE, Lardon F and Vermorken JB (2005). Combined modality therapy of gemcitabine and radiation. *Oncologist* 10: 34-51.

Plunkett W, Huang P and Gandhi V (1995a). Preclinical characteristics of gemcitabine. *Anticancer Drugs* 6: 7-13.

Plunkett W, Huang P, Xu YZ, Heinemann V, et al. (1995b). Gemcitabine: metabolism, mechanism of actions, and self-potentiation. *Semin. Oncol.* 22: 3-10.

Rübe CE, Wilfert F, Uthe D, König J, et al. (2004). Increased expression of pro-inflammatory cytokines as a cause of lung toxicity after combined treatment with gemcitabine and thoracic irradiation. *Radiother. Oncol.* 72: 231-241.

Shewach DS and Lawrence TS (1996). Radiosensitization of human solid tumor cell lines with gemcitabine. *Semin. Oncol.* 23: 65-71.

Shewach DS and Lawrence TS (2007). Antimetabolite radiosensitizers. *J. Clin. Oncol.* 25: 4043-4050.

Trisciuoglio D, Gabellini C, Desideri M, Ziparo E et al. (2010). Bcl-2 regulates HIF-1alpha protein stabilization in hypoxic melanoma cells via the molecular chaperone HSP90. *PLoS One* 5: e11772.

Trisciuoglio D, Gabellini C, Desideri M, Ragazzoni Y, et al. (2011). Involvement of BH4 domain of bcl-2 in the regulation of HIF-1-mediated VEGF expression in hypoxic tumor cells. *Cell Death Differ.* 18: 1024-1035.

Van Laethem JL, Hammel P, Mornex F, Azria D, et al. (2010). Adjuvant gemcitabine alone versus gemcitabine-based chemoradiotherapy after curative resection for pancreatic cancer: a randomized EORTC-40013-22012/FFCD-9203/GERCOR phase II study. *J. Clin. Oncol.* 28: 4450-4456.

Zinner RG, Komaki R, Cox JD, Glisson BS, et al. (2009). Dose escalation of gemcitabine is possible with concurrent chest three-dimensional rather than two-dimensional radiotherapy: a phase I trial in patients with stage III non-small-cell lung cancer. *Int. J. Radiat. Oncol. Biol. Phys*. 73: 119-127.