

# N,N-diethylaminobenzaldehyde targets aldehyde dehydrogenase to eradicate human pancreatic cancer cells

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**Abstract.** Pancreatic cancer is a common cause of worldwide cancer-related mortality with a poor 5-year survival rate. Aldehyde dehydrogenase (ALDH) activity is a possible marker for malignant stem cells in solid organ systems, including the pancreas, and N,N-diethylaminobenzaldehyde (DEAB) is able to inhibit ALDH activity. In the present study, the role of DEAB in the treatment of pancreatic cancer cells and the potential underlying mechanisms were investigated. The ALDH activities of pancreatic cancer cell lines treated with or without DEAB were analyzed by an ALDEFLUOR™ assay. The Cell Counting Kit-8 and colony formation assays, and cell cycle analysis were used to evaluate the viability, colony-forming ability and cell quiescence of cell lines under DEAB treatment, respectively. DEAB and/or gemcitabine-induced cell apoptosis was assessed by flow cytometry. DEAB reduced ALDH activity and inhibited the proliferation, colony-forming ability and cell quiescence of pancreatic cancer cell lines. Compared with respective controls, DEAB alone and the combination of gemcitabine and DEAB significantly decreased cell viability and increased cell apoptosis. Moreover, reverse transcription-PCR and western blotting were used to measure the expressions of B cell lymphoma 2 (Bcl2) associated X protein (Bax) and Bcl2 mRNA and protein. The anti-cancer effect of DEAB was associated with upregulation of Bax expression. Therefore, targeting ALDH with DEAB may be a potential therapeutic choice for pancreatic cancer, demonstrating a synergic effect with gemcitabine.

## Introduction

The close association between incidence and mortality indicates an extremely poor outcome in patients with pancreatic cancer. Gemcitabine alone or gemcitabine-based combination with other chemotherapeutic drugs is still the front-line standard chemotherapy of advanced pancreatic cancer (1). However, reversing the observed chemoresistance and enhancing the chemosensitivity of cancer cells to gemcitabine remains a challenge in improving the prognosis of patients with cancer (1,2).

The concept of cancer stem cells (CSCs) is used to define a tiny number of malignant cells with the characteristics of self-renewal, multi-potency and tumor formation (3). The theory regarding CSCs suggests that these stem-like cancer cells possess the capability to survive therapy and induce early resistance, which can lead to a later relapse (3,4). The accumulation of pancreatic CSCs after gemcitabine treatment has been continually observed (2,5). Therefore, targeting and eradicating cells with CSC markers represents a promising strategy to treat cancer.

Aldehyde dehydrogenase (ALDH) is a family of enzymes involved in the metabolism of intracellular aldehydes to acids by an NAD(P)<sup>+</sup> dependent reaction (6). ALDH activity is regarded as a marker for both normal and malignant stem cells in the hematological system and in the solid organ system, including pancreas, lung, liver, breast, colon and ovary organs (6-13). As an ALDH isoform, ALDH1 is a detoxifying enzyme responsible for oxidizing intracellular retinaldehyde to retinoic acid (6,10). N,N-diethylaminobenzaldehyde (DEAB), utilized as a negative control in an ALDEFLUOR™ assay, is generally considered as a selective ALDH1A1 inhibitor (12,14). Further previous studies demonstrated that DEAB is additionally able to block other ALDH1 isoforms, including ALDH1A2, ALDH1A3, ALDH1B1, and even ALDH2 and ALDH5A1 (14,15).

In the present study, three pancreatic cancer cell lines with different ALDH activities were examined and an ALDH inhibitor (DEAB) was used on these cells to test whether blocking ALDH expression triggers cancer cell elimination and sensitizes the cytotoxic effect of gemcitabine.

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## Materials and methods

**Culture of cell lines.** MiaPaCa2 and Panc1 cell lines were obtained from The Cell Center of Zhongda Hospital, and the BxPC3 cell line was obtained from The Cell Center of Jiangsu Province Hospital. Cells were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO<sub>2</sub>.

**Flow cytometry.** Cells were stained with ALDEFLUOR™ reagent (Stemcell Technologies, Inc.) and propidium iodide (PI; BD Biosciences) as previously described (16). Activated ALDEFLUOR™ reagent was aliquoted and stored at -20°C according to the manufacturer's instructions (Stemcell Technologies, Inc.). Cells were suspended with ALDEFLUOR™ Assay Buffer to a concentration of 1x10<sup>6</sup> cells/ml. In total, 1 ml cell suspension was transferred to a new tube and 5 µl activated ALDEFLUOR™ reagent per ml was added to the cell suspension. A total of 0.5 ml ALDEFLUOR™ reagent/cell suspension mixture was transferred immediately to a new tube with 10 µl DEAB (1.5 mM). DEAB, used as the negative control, inhibits the enzymatic reaction of ALDH. All samples were incubated at 37°C for 30 min. After incubation, cells were washed in cold ALDEFLUOR™ Assay Buffer, centrifuged at 250 x g for 5 min at 4°C to remove supernatant, and resuspended in 0.5 ml cold ALDEFLUOR™ Assay Buffer on ice. PI (2/100 µl reaction) was added before measurement. Cells were recorded and analyzed with a BD LSR II flow cytometer and FlowJo 10.4 (BD Biosciences). A total of three independent analyses were performed.

Cell cycle analysis was performed as described previously (17). Cells were first incubated with Human BD Fc Block (BD Pharmingen; BD Biosciences) at a final concentration of 1 µg/100 µl for 15 min at 4°C. Cells were fixed in 100 µl PBS/1.6% paraformaldehyde (Electron Microscopy Sciences) for 10 min at room temperature in the dark and then permeabilized in 300 µl PBS/90% methanol (Carl Roth) for 30 min on ice. Fixed cells were stained with Ki67-Alexa Fluor® 647 (clone B56; BD Biosciences) for 1 h at room temperature in the dark and incubated with DAPI (0.5 µg/ml; Sigma Aldrich; Merck KGaA) for 40 min in the dark on ice. Finally, cells were recorded and analyzed with a BD LSR II flow cytometer and FlowJo 10.4 (BD Biosciences). In total, four independent analyses were performed.

**Colony-forming assay.** Cells were trypsinized and re-plated at 500 cells per well in 6-well plates in triplicate and incubated for 2 weeks without medium change. After medium was removed, cells were washed twice with PBS (HyClone; GE Healthcare Life Sciences) and were fixed with 2 ml 4% paraformaldehyde (Electron Microscopy Sciences) for 15 min at room temperature. Then cells were washed three times with water and stained with 0.05% coomassie blue (Beijing Solarbio Science & Technology Co., Ltd.) for 5 min at room temperature. After being washed with water, plates were dried overnight. The number of colonies with >50 cells was counted under a Nikon Eclipse E400 polarizing light microscope under 4, 10 and 20X objective. A total of three independent experiments were performed.

**Cell Counting Kit-8 (CCK-8) assay.** The cell viability in the cytotoxicity test was quantified with CCK-8 (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. A density of 10<sup>4</sup> cells per well was seeded in 96-well plates. Cells were treated with DEAB (Sigma-Aldrich; Merck KGaA) at concentrations of 0, 50, 100, 150 and 200 µM for 1, 3 and 5 days in quadruplicate. As DEAB was dissolved in ethanol, the DEAB-untreated well was added to the same amount of ethanol (Sigma-Aldrich; Merck KGaA) as a control. Cells in each well were mixed with 10 µl CCK-8 reagent per 100 µl medium and incubated at 37°C and 5% CO<sub>2</sub> for 2 h in the dark. The absorbance of each well was measured at 450 nm using an ELISA plate reader. In total, two independent experiments were performed.

**Cell treatment and apoptosis analysis.** Cells of the three cell lines were cultured at a density of 5x10<sup>5</sup> cells per ml medium and treated in the following ways: i) Ethanol for 3 days; ii) 200 µM DEAB for 3 days; iii) ethanol for 1 day, then 50 nM gemcitabine (Dailan Meilun Biology Technology Co., Ltd.) was added for another 2 days; and iv) 200 µM DEAB for 1 day, then 50 nM gemcitabine was added for another 2 days.

Cells were stained with Annexin V-FITC and PI (Beyotime Institute of Biotechnology) for 15 min at room temperature in the dark, according to the manufacturer's instructions. Cells were recorded and analyzed using a BD LSR II flow cytometer and FlowJo 10.4 (BD Biosciences). Annexin V<sup>+</sup> cells were calculated as apoptotic cells and Annexin V-PI cells were calculated as living cells. A total of three independent experiments were performed.

**Western blot analysis.** Proteins were extracted from cells with RIPA buffer (Cell Signaling Technology, Inc.), and the protein concentration was quantified using a BCA Protein Assay kit (Cell Signaling Technologies, Inc.). Equal amounts of protein (20 µg) from each sample were loaded to the wells and separated by SDS-PAGE on 10% gels and then transferred onto a polyvinylidene fluoride membrane (EMD Millipore). After blocking with 5% skimmed milk for 1 h at room temperature, the membrane was incubated with primary antibodies at 4°C overnight. The primary antibodies of target proteins were as follows: Anti-B cell lymphoma 2 (Bcl2) associated X protein (Bax; Cell Signaling Technology, Inc.; cat. no. CST 5023T; 1:1,000), anti-Bcl2 (Cell Signaling Technology, Inc.; cat. no. CST 2872; 1:1,000) and anti-β-actin (Affinity Biosciences; cat. no. T0022; 1:1,000). Thereafter, the membrane was incubated with goat anti-rabbit IgG (H+L) HRP (cat. no. S0001) or goat anti-mouse IgG (H+L) HRP (cat. no. S0002) secondary antibodies (Affinity Biosciences; 1:5,000) at room temperature for 1 h. The enhanced Chemiluminescence Western blot kit (EMD Millipore) was used to visualize immunoreactive protein bands. Optical density was measured and relative protein expression was calculated by normalization to β-actin using ImageJ v1.51 (National Institutes of Health). A total of four independent experiments were performed.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the

manufacturer's instructions. RNA was reverse transcribed to cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) at 42°C for 1 h followed by inactivation at 70°C for 5 min. PCR was performed with FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics) according to the manufacturer's instructions. PCR was performed with denaturation at 95°C for 10 min followed by amplification by 40 cycles of 95°C for 15 sec, 60°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 min in triplicate.  $\beta$ -actin was used as an internal control. The relative expression levels of target genes were calculated using the  $2^{-\Delta\Delta C_q}$  method (18). The sequences of primers (Sangon Biotech Co., Ltd.) were as follows: Bax (forward, 5'-ATGGACGGTCCGGGGAGCAGCCC-3' and reverse, 5'-GGTGAGCACTCCCGCCACAAAGAT-3'), Bcl2 (forward, 5'-AAGAGCAGACGGATGGAAAAAGG-3' and reverse, 5'-GGGCAAAGAAATGCAAGTGAATG-3') and  $\beta$ -actin (forward, 5'-CTACCTCATGAAGATCCTCACC-3' and reverse, 5'-AGTTGAAGGTAGTTTCGTGGAT-3'). A total of two independent experiments were performed.

**Statistical analysis.** Statistical analysis was performed using SPSS Statistics 19 (IBM Corp.). At least two independent experiments were performed and all values are presented as the mean  $\pm$  SEM. One-way ANOVA was performed to compare multiple groups with Tukey's as the post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**ALDH activities vary among cell lines and are inhibited under DEAB treatment.** The ALDH activities of three pancreatic cell lines, including BxPC3, MiaPaCa2 and Panc1, were assessed; different patterns within these three cell lines were identified. On average, 38.5% of MiaPaCa2 cells were ALDH<sup>+</sup> followed by BxPC3 cells with 28.33% ALDH<sup>+</sup>, by contrast in Panc1 cells only 5.59% were ALDH<sup>+</sup>. BxPC3 and MiaPaCa2 have three sub-populations with different levels of ALDH expression, ALDH<sup>-</sup> includes ALDH-dim and intermediate ALDH, and the third sub-population is ALDH<sup>+</sup>. While Panc1 only has ALDH<sup>-</sup> and ALDH<sup>+</sup> two subpopulations; the ALDH<sup>-</sup> sub-population cannot be further divided, unlike BxPC3 and MiaPaCa2. DEAB demonstrated an ALDH-inhibition effect on all three cell lines (Fig. 1).

**Malignant proliferation of cancer cells is weakened under the existence of DEAB.** To investigate whether DEAB inhibits proliferation potential of pancreatic cancer cells *in vitro*, two adherent cell lines (BxPC3 and Panc1) were selected for colony-forming assays. A previous study demonstrated that MiaPaCa2 cells had no colony- and spheroid-forming potential (19). A colony-forming assay with MiaPaCa2 cells was conducted and it was observed that the numbers of colonies were extremely low because most were non-adherent cells (Fig. S1); therefore, MiaPaCa2 was excluded from this analysis. It was identified that DEAB alone was able to significantly prevent the colony formation ability of cancer cells (Fig. 2A).

To further confirm these results, a CCK-8 assay was performed with the three cell lines in the presence of DEAB with a range of doses from 0 to 200  $\mu$ M for 1, 3 and 5 days.

When a 1 day-treatment on MiaPaCa2 (100, 150 and 200  $\mu$ M), Panc1 (100, 150 and 200  $\mu$ M), a 3 day-treatment on BxPC3 (200  $\mu$ M), MiaPaCa2 (100, 150 and 200  $\mu$ M), Panc1 (50, 100, 150 and 200  $\mu$ M), and a 5 day-treatment on BxPC3, MiaPaCa2, Panc1 (100, 150 and 200  $\mu$ M) were performed, significant cytotoxicity with increased concentrations of DEAB was observed (Fig. 2B). Treatments of cells with 50  $\mu$ M DEAB at day 1 (MiaPaCa2), day 3 (Panc1) and day 5 (MiaPaCa2 and BxPC3); 100  $\mu$ M DEAB at day 1 (MiaPaCa2 and Panc1), day 3 (MiaPaCa2 and Panc1) and day 5 (MiaPaCa2, Panc1 and BxPC3); 150  $\mu$ M DEAB at day 1 (MiaPaCa2 and Panc1), day 3 (MiaPaCa2 and Panc1) and day 5 (MiaPaCa2, Panc1 and BxPC3); 200  $\mu$ M DEAB at day 1 (MiaPaCa2 and Panc1), day 3 (MiaPaCa2, Panc1 and BxPC3) and day 5 (MiaPaCa2, Panc1 and BxPC3) demonstrated significant cytotoxicity and the cytotoxicity was more enhanced with longer incubation time of DEAB (Fig. 2C). The proliferation of cancer cell lines was significantly inhibited by DEAB, and both dose- and time-dependent manners were observed in DEAB-induced-cytotoxicity.

**DEAB treatment promotes cell cycle.** As cell quiescence has been suggested to be associated with chemotherapy resistance (17), the cell cycle of pancreatic cancer cells under DEAB was analyzed. Cells treated with DEAB contained significantly decreased proportions of quiescent cells (defined as the G<sub>0</sub> phase) and an accumulation of cells at S-G<sub>2</sub>-M phases, compared with the controls (Fig. 3).

**DEAB induces cancer cell apoptosis and sensitizes the cytotoxic effects of gemcitabine.** In order to evaluate the effect of DEAB on pancreatic cancer cells and gemcitabine-induced cell death, the cell viability and apoptosis were measured. In all three cell lines analyzed, it was observed that compared with their respective controls (ethanol and gemcitabine treatments), both DEAB alone and DEAB-gemcitabine combination treatments induced a significant decrease of living cells and a significant increase of apoptotic cells (Fig. 4).

**DEAB effect is associated with upregulation of Bax expression.** To understand the underlying mechanisms of the anti-cancer effects of DEAB, the expressions of Bax and Bcl2 at the protein level were comparatively analyzed. When compared with the control, BxPC3 and MiaPaCa2 cells treated with DEAB demonstrated a significant increase of Bax protein expression and no significant regulation of Bcl2 protein expression (Fig. 5A and C).

Cells undergoing gemcitabine single treatment and DEAB-gemcitabine combination treatment were compared, and it was identified that DEAB exposure significantly upregulated Bax expression of BxPC3 and MiaPaCa2 cells; however, did not significantly regulate Bcl2 expression at the protein level (Fig. 5B and C). To further examine the mechanisms, the expressions of Bax and Bcl2 at the mRNA level were analyzed using RT-qPCR. It was observed that in the presence of DEAB, Bax mRNA was significantly upregulated in BxPC3 and MiaPaCa2 cells compared with the controls. Moreover, in Panc1 cells with DEAB treatment, the present results demonstrated a significant increase of Bax and Bcl2 mRNA (Fig. 5D).

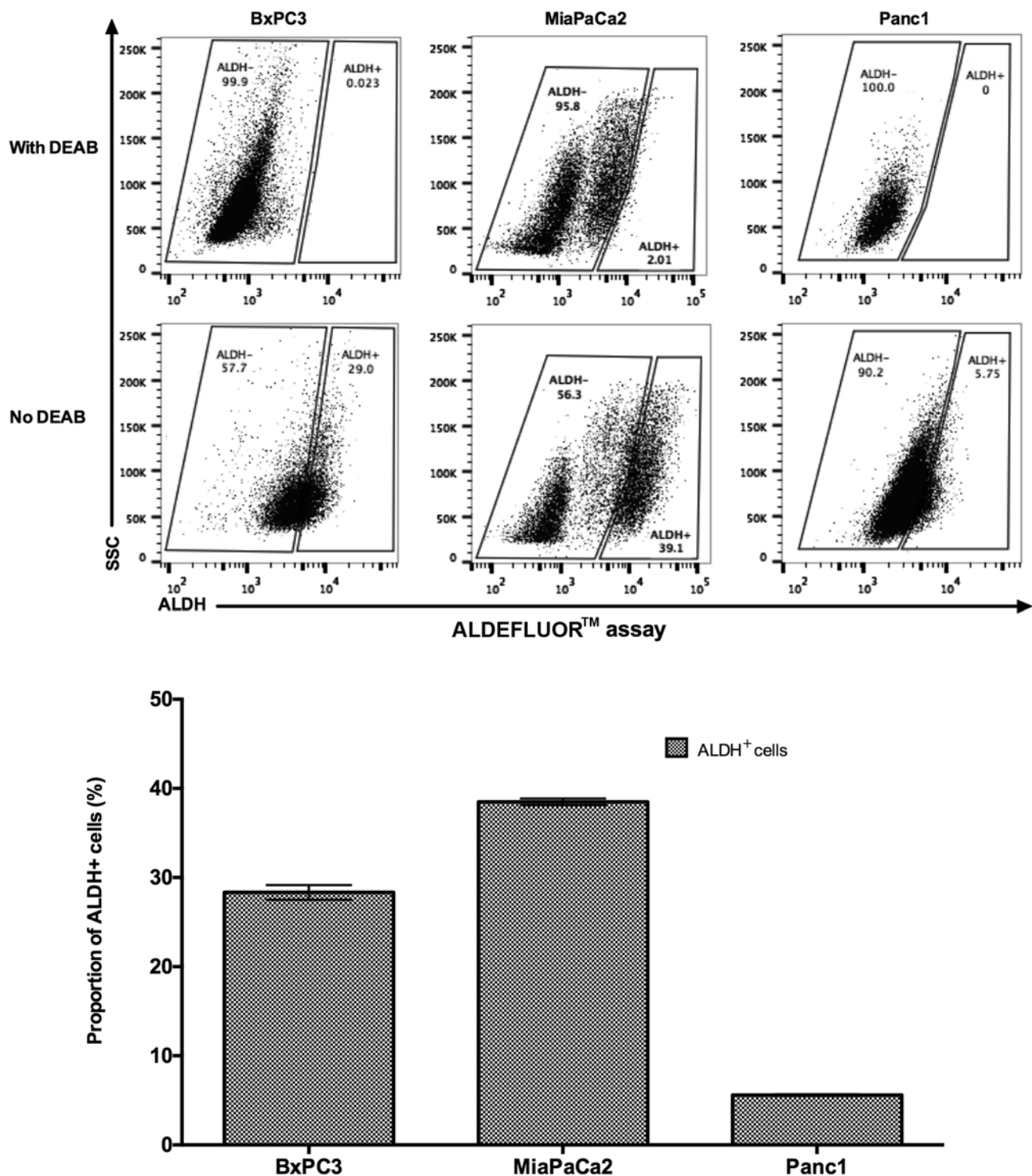


Figure 1. ALDH expression patterns in pancreatic cancer cell lines. ALDH activities were analyzed by ALDEFLUOR™ assay. MiaPaCa2 demonstrated the highest average frequency of ALDH<sup>+</sup> cells (38.5%), followed by BxPC3 (28.33%) and Panc1 (5.59%). DEAB inhibited ALDH activity in these three cell lines. Data are representative of three independent experiments. Data are presented as the mean  $\pm$  SEM. ALDH, aldehyde dehydrogenase; DEAB, N,N-diethylaminobenzaldehyde; SSC, side scatter.

In contrast to cells treated with gemcitabine, BxPC3, MiaPaCa2 and Panc1 cells treated with DEAB-gemcitabine demonstrated a significant increase of Bax mRNA (Fig. 5E).

## Discussion

In the present study, the role of the ALDH inhibitor DEAB in anti-cancer efficacy was investigated. To evaluate the

inhibitory ability of DEAB on ALDH activity, three pancreatic cancer cell lines with inconsistent ALDH activities were utilized. Similar to the cell lines, the ALDH expressions vary among patients with pancreatic cancer (20). The inhibition of ALDH activity induced by DEAB was independent of the ALDH levels of cells; this finding suggested the possibility of a universal clinical application of DEAB with unselected patients with pancreatic cancer.

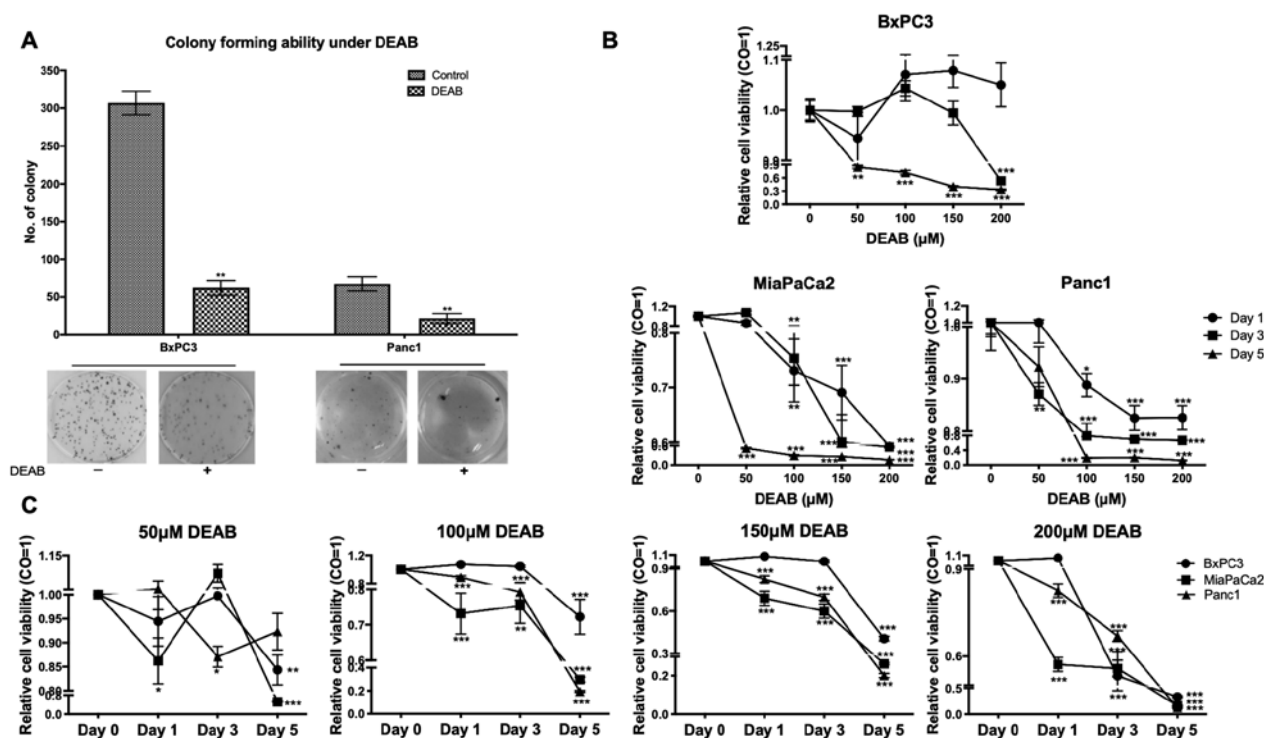


Figure 2. DEAB inhibits colony-forming ability and cell proliferation. (A) Colony-forming ability of BxPC3 and Panc1 cells treated or untreated with DEAB was detected by a cell colony formation assay. Data are representative of three independent experiments. (B) Proliferation of BxPC3, MiaPaCa2 and Panc1 cells treated with 0, 50, 100, 150 and 200  $\mu$ M DEAB were compared by a CCK-8 assay. (C) Proliferation of BxPC3, MiaPaCa2 and Panc1 cells at day 0, 1, 3, and 5 were compared using a CCK-8 assay. Cells treated with 0  $\mu$ M DEAB were used as the control. Data are representative of two independent experiments. Viability of the control was set at 1. Data are presented as the mean  $\pm$  SEM. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. respective control. DEAB, N,N-diethylaminobenzaldehyde; CCK-8, Cell Counting Kit-8; CO, control.

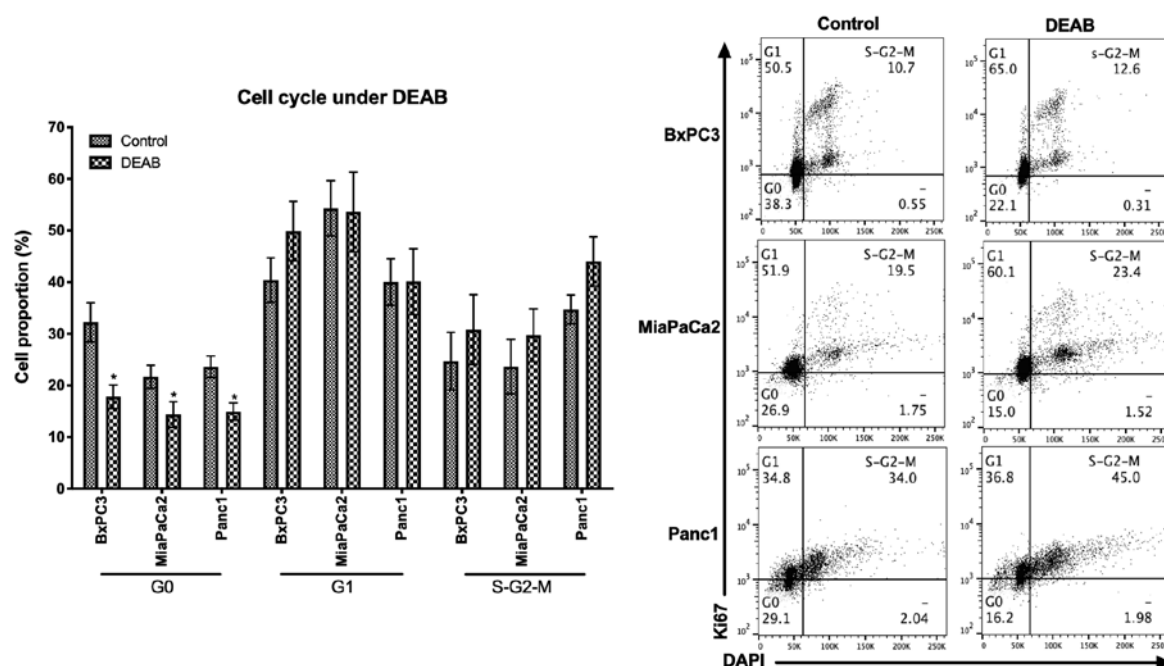


Figure 3. Cell cycle of BxPC3, MiaPaCa2 and Panc1 cells treated with or without DEAB is evaluated by Ki67-DAPI staining by flow cytometry. DEAB exposure significantly decreased the frequency of cells at G<sub>0</sub> phase. Data are representative of four independent experiments. Example of Ki67 and DAPI staining for the analysis of cell cycle in different conditions is presented. Data are presented as the mean  $\pm$  SEM. \* $P$ <0.05 vs. respective control. DEAB, N,N-diethylaminobenzaldehyde.

Gemcitabine preferentially targets cells characterizing rapid proliferation and differentiation; to some extent, the quiescence

and other stem cell-like characteristics of ALDH<sup>+</sup> cancer cells may explain their gemcitabine resistance (21,22). The

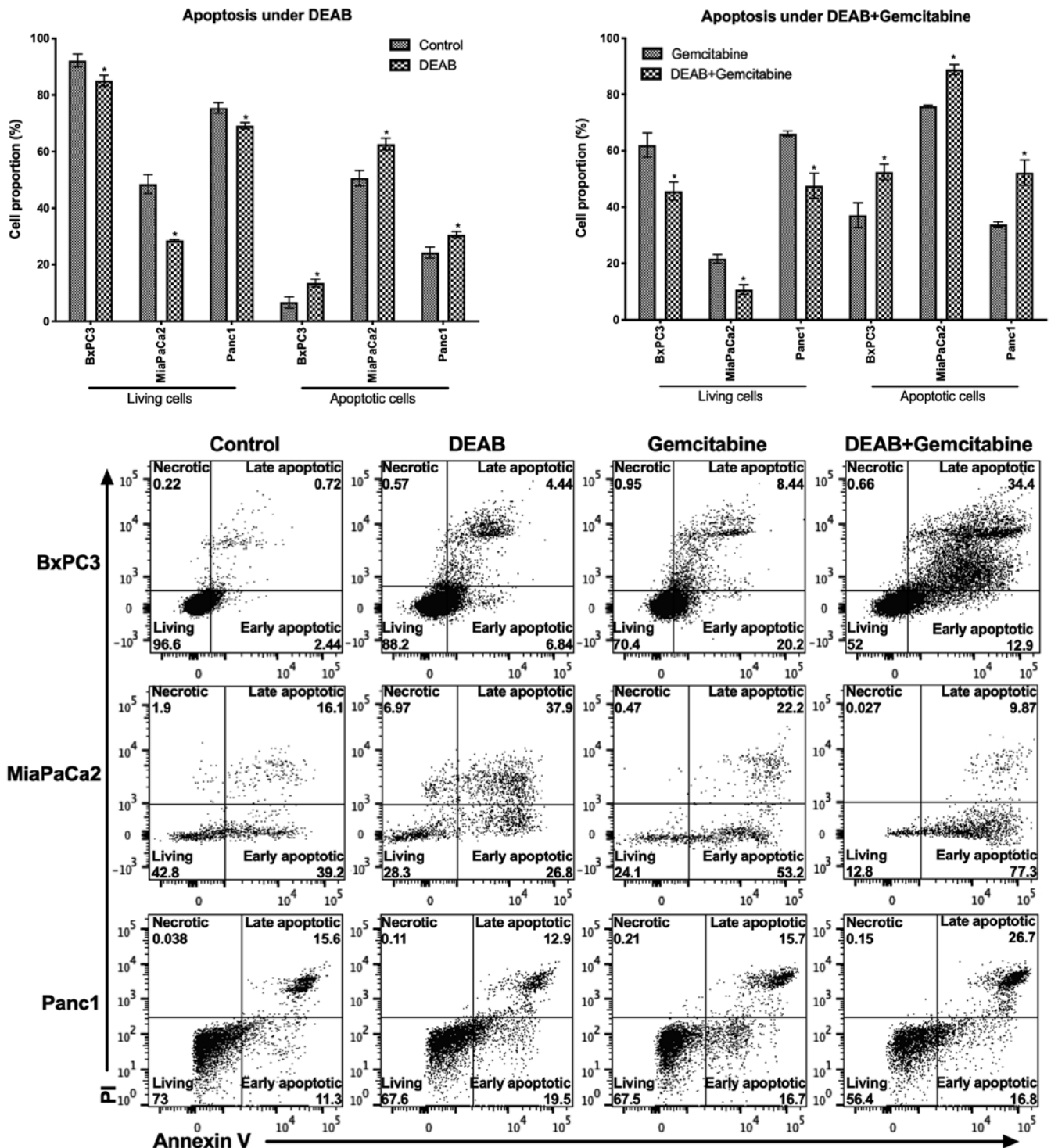


Figure 4. DEAB induces the apoptosis of cells with or without gemcitabine. Apoptosis of BxPC3, MiaPaCa2 and Panc1 cells treated with DEAB, gemcitabine and DEAB + gemcitabine was evaluated by Annexin V-PI staining by flow cytometry. Data are representative of three independent experiments. Example of Annexin V and PI staining for the analysis of apoptosis in different conditions. Data are presented as the mean  $\pm$  SEM. \* $P < 0.05$  vs. respective control or gemcitabine. DEAB, N,N-diethylaminobenzaldehyde; PI, propidium iodide.

contribution of high ALDH activity to gemcitabine resistance indicates that inhibiting ALDH may increase the sensitivity of tumor cells to gemcitabine (2,5,19). Consequently, studies regarding ALDH inhibition in pancreatic cancer treatment have emerged. Knockdown of the ALDH gene of MiaPaCa2 cells using small interfering RNAs reduced cell proliferation and overcame gemcitabine resistance in a previous study (2). Disulfiram, an irreversible ALDH inhibitor, inhibited *in vitro*

pancreatic cancer cell proliferation and *in vivo* tumor growth combined with gemcitabine (5). Sulforaphane enriched in broccoli compound suppressed the enrichment of ALDH<sup>+</sup> cells induced by gemcitabine and enhanced the cytotoxic effect of gemcitabine (19). The therapeutic potential of ALDH inhibition was also demonstrated in other solid cancer types. In cholangiocarcinoma, the reduction of ALDH activity in gemcitabine-resistant cells by metronidazole resulted in

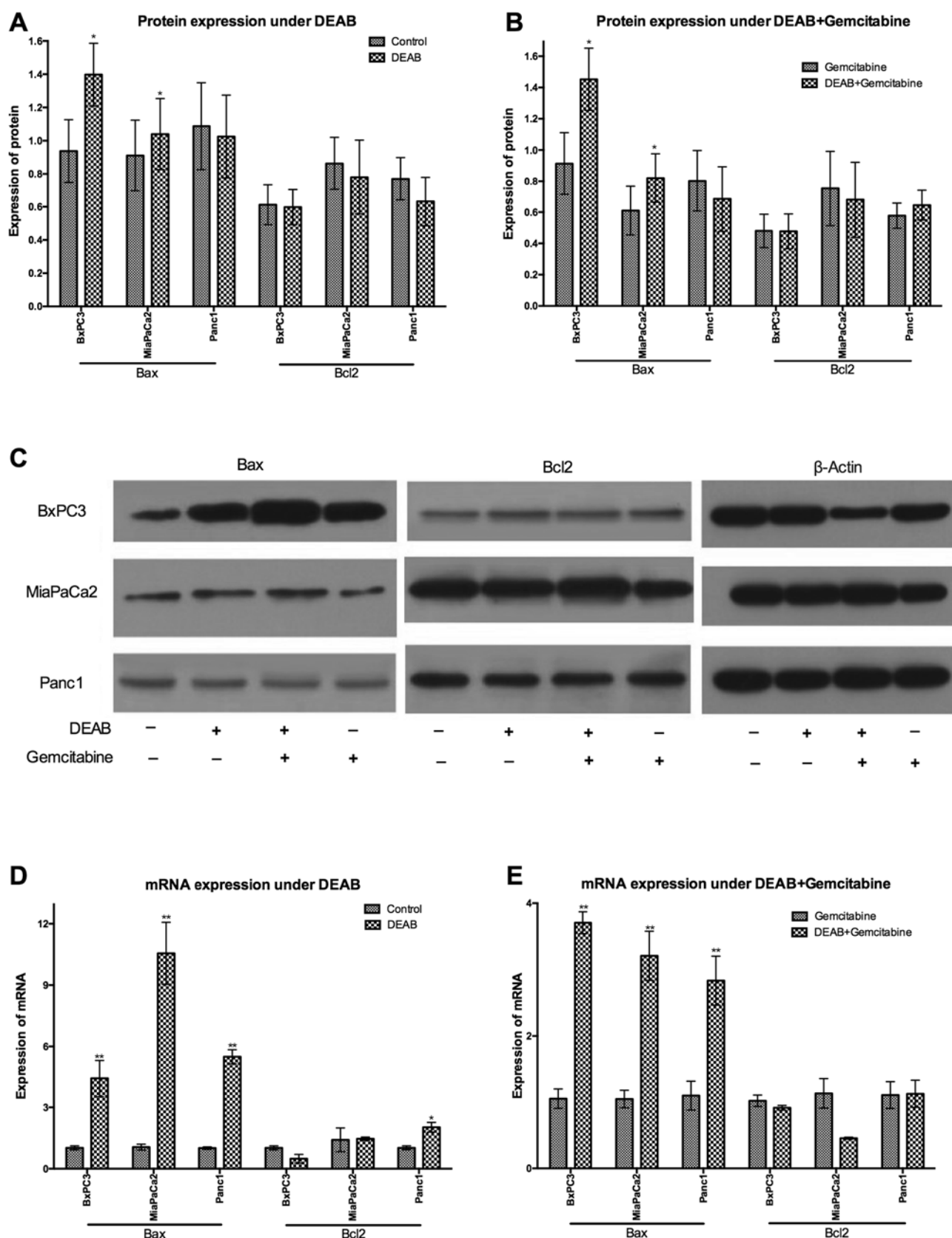


Figure 5. Apoptosis related pathway is involved in DEAB-induced anti-cancer effect. (A) Expressions of Bax and Bcl2 in BxPC3, MiaPaCa2 and Panc1 cells treated or untreated with DEAB were detected by western blotting.  $\beta$ -actin was used as a control. Data are representative of four independent experiments. \* $P < 0.05$  vs. respective control. (B) Expressions of Bax and Bcl2 in BxPC3, MiaPaCa2 and Panc1 cells treated with gemcitabine alone or combined with DEAB were detected by western blotting.  $\beta$ -actin was used as a control. Data are representative of four independent experiments. \* $P < 0.05$  vs. respective gemcitabine. (C) Western blots are presented. (D) Relative expressions of Bax and Bcl2 genes in BxPC3, MiaPaCa2 and Panc1 cells treated or untreated with DEAB were detected by reverse transcription-PCR. Data are representative of two independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs. respective control. (E) Relative expressions of Bax and Bcl2 genes in BxPC3, MiaPaCa2 and Panc1 cells treated with gemcitabine alone or combined with DEAB were detected by reverse transcription-PCR. Data are representative of two independent experiments. Data are presented as the mean  $\pm$  SEM. \*\* $P < 0.01$  vs. respective gemcitabine. DEAB, N,N-diethylaminobenzaldehyde; Bax, Bcl2 associated X protein; Bcl2, B cell lymphoma 2.

the enhancement of chemosensitivity (23). In lung cancer, inhibiting ALDH with DEAB and disulfiram suppressed the viability of cancer cells and sensitized the cancer cells to chemotherapy (10,11). Consistent with these data, in the present study, after comparing untreated cells and cells treated with DEAB, it was observed that an ALDH inhibitor (DEAB) reduced cell viability, cell quiescence and furthermore, enhanced gemcitabine-induced cytotoxicity *in vitro*. Taken together, the present results established the status of DEAB as a potential chemotherapeutic reagent or at least a chemosensitizer to overcome gemcitabine resistance.

Recently, ALDH-targeting based treatment has attracted increasing attention; however, at present, the mechanisms involved are still undetermined. The decrease of lung cancer cell viability induced by disulfiram (through ALDH inhibition) was attributed to cell cycle arrest in the G<sub>2</sub>/M phase (11). ALDH1A1-knockdown stimulated taxane-resistant ovarian cancer cells to enter the S and G<sub>2</sub> cell cycle phases (12). In pancreatic cancer, it was observed that the proportion of G<sub>0</sub> cells was decreased by DEAB and more cells entered S-G<sub>2</sub>-M phases; a previous study demonstrated that cells with ALDH1A1 knockdown were enriched at the S phase (2). It was hypothesized that the cell cycling entry of quiescent cancer cells induced by ALDH inhibition strengthens the cytotoxicity of cell cycle specific chemotherapeutic drugs, such as gemcitabine, leading to increased apoptosis. Inhibition of ALDH activity delayed the process of retinaldehyde to retinoic acid mediated by ALDH to increase the production of reactive oxygen species (ROS) (10). The induction of ROS promoted gemcitabine-related cytotoxicity in pancreatic cancer (2). Moreover, ROS-induced DNA damage and p53 activation contributed to increased apoptosis accompanied by the accumulation of retinaldehyde (10,24).

The induction of cancer cell apoptosis is a critical hallmark of anti-cancer therapy; therefore, the present focused on mitochondrial apoptosis (intrinsic pathway) related Bax and Bcl2 to elucidate the mechanisms of DEAB-induced-apoptosis (25). Although the apoptosis of all cell lines analyzed was promoted by DEAB, the latent mechanisms were not completely the same among the tested cell lines. DEAB-induced-apoptosis in BxPC3 and MiaPaCa2 is associated with the mitochondrial pathway induced by significantly upregulated pro-apoptotic Bax at the protein level, and a significant consistent trend of mRNA alteration reflected the regulation at the gene level; however, no significant downregulation of anti-apoptotic Bcl2 was observed (25,26). In addition, although Bax and Bcl2 mRNA increased in Panc1 under DEAB, Bax and Bcl2 proteins did not contribute to DEAB-induced-apoptosis in Panc1. In a previous study, ALDH1A1-knockdown upregulated the expression of Bax and induced Bax-mediated apoptosis in ovarian cancer (27). S-methyl 4-amino-4-methylpent-2-ynethioate, a synthetic suicide inhibitor of ALDH1, stimulated Bcl2-overexpressing cell apoptosis (28). However, in the present study, the effect of DEAB on Bcl2 protein expression was not observed. Serving as an indispensable entry point of the mitochondrial apoptosis pathway, the abnormal suppression of Bax results in therapeutic resistance in various cancer types; therefore re-activating Bax is considered as a strategy in the anticancer field (29). In a recent study, adaptor related protein complex 5 subunit mu 1 failed to induce apoptotic death

in Bax<sup>-/-</sup> knockout cells (30); and Bax<sup>-/-</sup> mice exhibited increase of some cell types including lymphocytes, certain neurons and immature germ cells (26). Therefore, it was hypothesized that the enhancement of mitochondrial apoptosis-related Bax is responsible for DEAB-mediated apoptosis of tumor cells. Notably, compared with Panc1 cells, both BxPC3 cells and MiaPaCa2 cells have relatively higher ALDH activity in the present study; the apoptosis of these cells induced by DEAB is likely associated with mitochondrial apoptosis, by contrast, mitochondrial apoptosis-related proteins measured were not influenced by DEAB in Panc1. Different patterns of ALDH expression in pancreatic cancer cell lines have been observed in the present study and in previous studies (5,7). Based on the findings that ALDH<sup>+</sup> cells possess stem/progenitor properties, it was suggested that the pancreatic cancer cell lines with high ALDH activity appear to represent a sub-population with stem cell-like characteristics, including an effect on the apoptosis-related pathway (3-5). The present results suggested that the mechanisms of DEAB-induced-apoptosis might be associated with the individual characteristics of a cell line, including the ALDH expression pattern. To test this hypothesis, future studies could measure the ALDH activities of more pancreatic cancer cell lines, and compare additional apoptosis-related proteins and genes of grouped cell lines based on ALDH activity.

To further elucidate the function of DEAB in an anti-cancer aspect and clarify the underlying mechanisms, subsequent studies are required. A future study could knock down Bax to investigate the direct role of Bax in apoptosis and widely screen cell cycle, proliferation and apoptosis-related proteins and genes of cancer cells with different ALDH expressions by microarray.

The present study demonstrated that DEAB may inhibit ALDH to suppress cell proliferation, promote apoptosis, activate the cell cycle, and sensitize pancreatic cancer cells to gemcitabine by activating apoptosis pathway-related proteins and genes.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

WW designed the research. WW, SZ and HG performed experiments. WW, SZ, HH and BRS analyzed the data. WW,



HH and BRS wrote the manuscript with input from all other authors. All authors read and approved the final version of the manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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