

Ursodeoxycholic acid suppresses epithelial-mesenchymal transition and cancer stem cell formation by reducing the levels of peroxiredoxin II and reactive oxygen species in pancreatic cancer cells

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Abstract. Reactive oxygen species (ROS) play a key role in cancer development and progression. Ursodeoxycholic acid (UDCA) may possess antioxidant, anti-inflammatory and chemoprophylactic effects. Therefore, we aimed to investigate the effects and mechanisms of UDCA treatment on pancreatic cancer cells. The pancreatic cancer cell lines HPAC and Capan-1 were treated with 0.2 mM UDCA. To examine alterations in the levels of intracellular ROS, the DCF-DA stain was used and both stemness and epithelial-mesenchymal transition (EMT)-related genes were quantified using qRT-PCR and western blot analysis. The pancreatic cancer sphere culture was performed following seven days of treatment with 0.2 mM UDCA, as an indicator of stemness. Following treatment with UDCA, the level of intracellular ROS was decreased in the pancreatic cancer cells. UDCA decreased both the phosphorylation of STAT3 and the expression of peroxiredoxin II (Prx2). Furthermore, the treatment resulted in the upregulation of E-cadherin and in the downregulation of N-cadherin. In addition, UDCA decreased the expression of sex determining region Y-box 2 (Sox2) and it diminished the number of pancreatic cancer spheres formed. In conclusion UDCA suppressed the levels of intracellular ROS and Prx2 and it decreased EMT and stem cell formation in pancreatic cancer cells. Therefore, UDCA may provide favorable therapeutic benefits, through its antioxidant effects, for patients with pancreatic cancer.

Introduction

Pancreatic cancer has a generally poor prognosis with a 5-year survival rate of approximately 6% (1). Pancreatic cancer is the 13th most common cancer and the 4th leading cause of cancer-related deaths in the world (2-4). Since we do not have an effective screening method for pancreatic cancer, most patients are diagnosed at an advanced stage that carries a poor prognosis (2,5). The discovery of molecular alterations in pancreatic cancer has led to the development of targeted therapies that have exhibited substantial benefits in clinical studies, however the survival rate remains poor (6).

Reactive oxygen species (ROS) consist of radicals, ions, or other molecules formed by the reduction of oxygen. The ROS-mediated damage of nucleic acids, proteins and lipids may affect the process of carcinogenesis (7). ROS may influence the cell cycle progression, proliferation, cell survival and apoptosis, angiogenesis and the maintenance of tumor stemness (8). In pancreatic cancer, ROS production is known to be increased. Contrary to other cancer cells, cancer stem cells maintain lower intracellular ROS concentrations (9), which may be the result of decreased ROS production or activated scavenging systems that remove ROS in cancer stem cells (10,11). Oncogenes that affect different pathways have been implicated in increases of ROS production. If oncogenes such as Raf, Myc and cyclin E become overexpressed, ROS production is increased (12). Epithelial-mesenchymal transition (EMT) is a crucial process toward resistance of cell death, chemoresistance, evasion of the immune system, tumor invasion and tumor metastasis (1,2). In some authoritative studies, ROS was suggested as mediators or modulators of the EMT process (3-5). However, the role of ROS in promoting EMT and cancer stem cell formation remains unclear.

Ursodeoxycholic acid (UDCA) is a hydrophilic synthetic bile acid which is the 7- β -epimer of chenodeoxycholic acid. UDCA is the standard treatment for primary sclerosing cholangitis, primary biliary cirrhosis, cystic fibrosis and intrahepatic cholestasis. Although the effect of UDCA as

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a therapeutic agent in cancer is uncertain, some studies revealed such a possibility. Xu *et al* reported that UDCA had an anticancer effect in liver cancer cell lines *in vitro* and in mice *in vivo* (13). In addition, recent studies in murine and rat models revealed that UDCA had a chemopreventive effect on colorectal cancer (14-17). UDCA prevented the formation of ROS species and inhibited the Bax protein-translocation from the cytosol to mitochondria in rat liver and human hepatocytes. In addition, anti-apoptotic effects were revealed in other cell types (18-21). However, the anti-carcinogenic mechanisms of UDCA have not been elucidated.

UDCA could play important roles via its anti-apoptotic, anti-inflammatory and chemoprophylactic effects and for this reason, we investigated the mechanisms of action of UDCA in pancreatic cancer cells.

Materials and methods

Reagents and materials. UDCA was obtained from Sigma-Aldrich (St. Louis, MO, USA) and 2',7'-dichlorofluorescein diacetate (H₂DCF-DA) was purchased from Molecular Probes (Eugene, OR, USA). The antibodies to sex-determining region Y-box 2 (Sox2) (#4900), E-cadherin (#3195), N-cadherin (#13116), anti-rabbit IgG (HRP-linked, #7074) and anti-mouse IgG (HRP-linked, #7076) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti- β -actin (LF-PA0207) and anti-peroxiredoxin II (Prx2) (LF-MA0144) were obtained from AbFrontier (Seoul, Korea).

Cell lines and culture conditions. The pancreatic cancer cell lines, HPAC and Capan-1 were obtained from Professor Si Young Song (Yonsei University College of Medicine, Seoul, Korea), cultured in a mixture of Dulbecco's modified Eagle's medium (DMEM) and F12 containing 10% fetal bovine serum (FBS) (HPAC), Iscove's modified Dulbecco's medium (IMDM) containing 20% FBS (Capan-1) with 1% penicillin-streptomycin, at 37°C in an incubator with a 5% CO₂ atmosphere.

Analysis of ROS by FACS. The level of intracellular ROS was assayed using H₂DCF-DA staining (Thermo Fisher Scientific Inc., Waltham, MA, USA). To assay the ROS level, 1x10⁶ cells were seeded in 60-mm dishes (Corning Incorporated, Corning, NY, USA). After 12 h, the seeded cells were treated with 0.2 mM UDCA. After 20 min, the cells were harvested by trypsin-EDTA and washed with phosphate buffered saline (PBS). The harvested cells were incubated with 25 μ M H₂DCF-DA at 37°C for 30 min. The fluorescence intensity was quantified using flow cytometry (BD Biosciences, Seoul, Korea).

Reverse transcription PCR and quantitative reverse transcription PCR analyses. Total RNA was extracted from harvested cells using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany). To remove genomic DNA, the extracted total RNA was digested by DNase I (New England BioLabs, Beverly, MA, USA). Purified total RNA in the amount of 1 μ g was reverse transcribed and amplified by RT-PCR using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The reactions of quantitative RT-PCR were assessed using SYBR Premix Ex Taq II (Takara, Kusatsu,

Japan) on an iCycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). The following primer pairs were used: human Prx2 (gene ID: 7001), 5'-TGTGATCGTCCGTGCGTCTA-3' and 5'-CCGATGCGCGCGTTAC-3'; human Sox2 (gene ID: 6657), 5'-TGCGAGCGCTGCACAT-3' and 5'-GCAGCGTGACTTATCCTTCTTCA-3'; human E-cadherin (gene ID: 999), 5'-CTGAGAACGAGGCTAACG-3' and 5'-GTCCACCATCATATTCAATA-3'; human N-cadherin (gene ID: 1000), 5'-TGGATGGACCTTATGTTGCT-3' and 5'-AACACCTGCTTGGGATCAA-3'; GAPDH (gene ID: 2597), 5'-AGGGCTGCTTTTAACTCTGGT-3' and 5'-CCCCACTTGATTTTGGAGGGA-3'. The thermal conditions for quantitative reverse transcription PCR assay were as follows: cycle 1, 95°C for 3 min; cycle 2 (x40), at 95°C for 10 sec and at 55°C for 30 sec. Target genes were normalized to GAPDH. The fold change from the untreated control was set at 1-fold, and the normalized fold change ratio was calculated using the $\Delta\Delta C_t$ method.

Immunoblotting. The collected cells were lysed in cold RIPA lysis buffer (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA) with protease and phosphatase inhibitors (GenDepot, Barker, CA, USA). The cell lysates were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Pall Gelman Laboratory, Ann Arbor, MI, USA). After being blocked with 8% skim milk or 5% bovine serum albumin (BSA), the membranes were incubated with primary antibodies overnight at 4°C. The dilutions used for each antibody were according to the manufacturer's instructions. After being washed in PBS with 0.1% Tween, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase. The detection step was performed using WesternBright ECL HRP substrate (Advansta, Menlo Park, CA, USA).

Floating-sphere formation assay. To assay the formation of HPAC and Capan-1 pancreatic cancer spheres, 4,000 cells/well were seeded in 6-well ultralow attachment plates (Corning Incorporated) in serum-free DMEM/F12 (HPAC) or IMDM (Capan-1) with 1X B-27 supplement (50X; Gibco, Invitrogen, Carlsbad, CA, USA), 10 ng/ml hFGF (#4114TC-01M; R&D Systems, Minneapolis, MN, USA), 10 ng/ml hEGF (#236-EG-01M; R&D Systems) and Heparin (Sigma-Aldrich). The plated cells were incubated at 37°C in a 5% CO₂ incubator. Seven days after seeding, the pancreatic cancer spheres were counted.

Results

UDCA suppresses the level of ROS in pancreatic cancer cells through the inhibition of the expression of Prx2 and a reduction of the phosphorylation of STAT3. To detect the antioxidant effect of UDCA in pancreatic cancer cells, we assessed the intracellular ROS levels using DCF-DA staining, as detected by FACS. We found that UDCA decreased the ROS levels in the HPAC and the Capan-1 cells (Fig. 1).

The change of ROS homeostasis by UDCA affected the expression of antioxidant proteins. Prx2 is an abundant antioxidant protein in cells. To determine whether UDCA affected the expression of Prx2, we assessed the level of Prx2 using

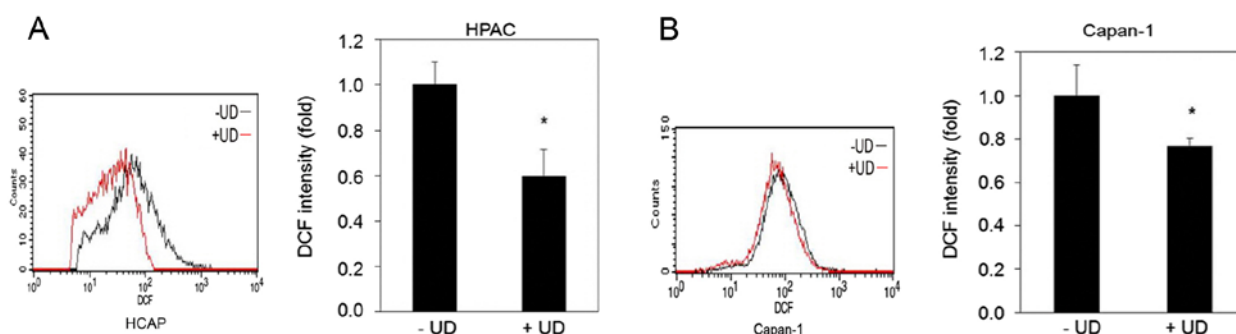


Figure 1. UDCA reduces the level of intracellular ROS in pancreatic cancer cells. (A) HPAC and (B) Capan-1 cells were treated with 0.2 mM UDCA for 20 min and stained with 25 μ M DCF-DA for 30 min. The total level of intracellular ROS was detected by FACS analysis. Asterisks indicate statistical significance (* p <0.03). UDCA, ursodeoxycholic acid; +UD, cells treated with ursodeoxycholic acid; -UD, cells not treated with ursodeoxycholic acid; ROS, reactive oxygen species.

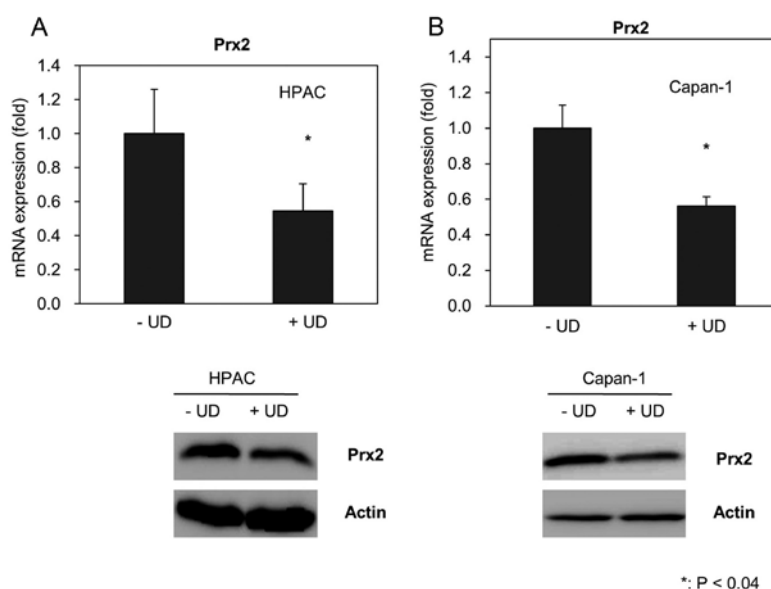


Figure 2. Prx2 expression is decreased by UDCA. (A) The HPAC cells and (B) the Capan-1 cells were treated with 0.2 mM UDCA for 24 h. The quantification of Prx2 mRNA was performed by qRT-PCR. The protein expression of Prx2 was determined by western blotting (* p <0.04). UDCA, ursodeoxycholic acid; +UD, cells treated with ursodeoxycholic acid; -UD, cells not treated with ursodeoxycholic acid; Prx2, peroxiredoxin II.

qRT-PCR and western blotting. UDCA suppressed the production of Prx2 mRNA in the HPAC and the Capan-1 cells (Fig. 2).

Correspondingly, the protein level of Prx2 was significantly reduced by UDCA. We observed that treatment of UDCA inhibited the phosphorylation of STAT3 in the HPAC and the Capan-1 cells (Fig. 3).

UDCA decreases EMT in pancreatic cancer cells. UDCA affected the expression of EMT-related proteins. The expression of E-cadherin mRNA was induced by UDCA in HPAC cells (Fig. 4A). Furthermore, the protein level of E-cadherin was increased by UDCA in the HPAC cells. N-cadherin is a representative marker of mesenchymal cells. The expression levels of N-cadherin mRNA and protein were suppressed by UDCA in HPAC cells. The effect of UDCA was replicated in the Capan-1 cells (Fig. 4B). UDCA induced the expression of epithelial marker (ZO-1) in the HPAC and Capan-1 cells. In addition, UDCA suppressed the expression of Slug and ZEB1 in the HPAC cells. The expression of Snail was suppressed by UDCA in Capan-1 cells (Fig. 4C).

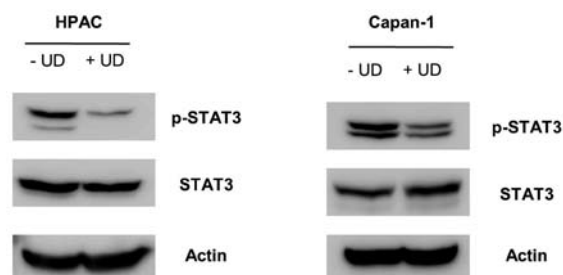


Figure 3. UDCA suppresses the phosphorylation of STAT3. The HPAC and the Capan-1 cells were treated with 0.2 mM UDCA for 24 h. The levels of STAT3, phosphorylated STAT3 and β -actin were detected by western blotting. UDCA, ursodeoxycholic acid; +UD, cells treated with ursodeoxycholic acid; -UD, cells not treated with ursodeoxycholic acid.

UDCA reduces stemness in pancreatic cancer cells. The regulation of Sox2 is synchronized with Prx2. The expression of Sox2 mRNA was suppressed by UDCA in the HPAC and Capan-1 cells (Fig. 5A). In addition UDCA reduced the

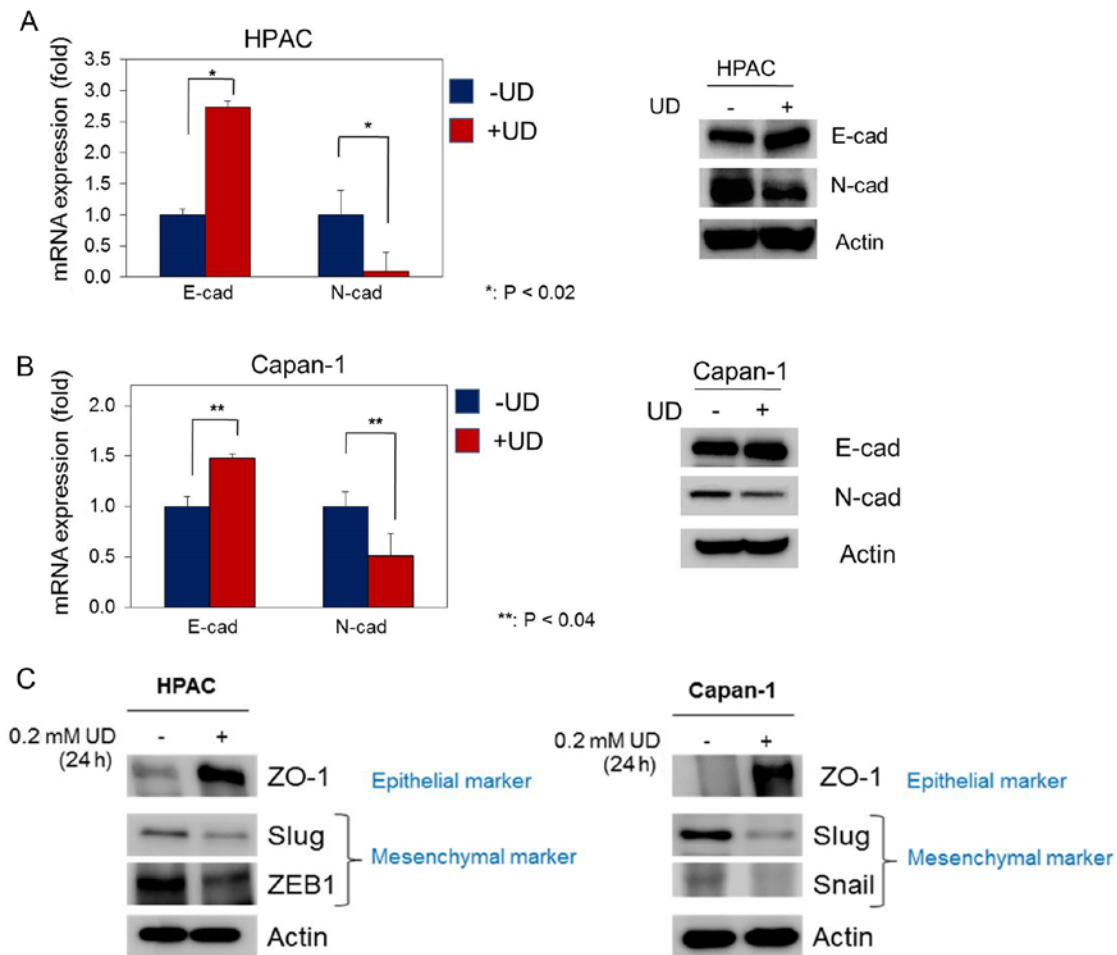


Figure 4. UDCA affects the expression of EMT markers. (A) The HPAC cells and (B) the Capan-1 cells were treated with 0.2 mM UDCA for 24 h. The quantification of E-cadherin and N-cadherin mRNA was accomplished using qRT-PCR. The protein expression of E-cadherin or N-cadherin was determined by western blotting (* $p < 0.02$, ** $p < 0.04$). (C) The protein expression of the other EMT markers was detected by western blotting after the HPAC and Capan-1 cells had been treated with 0.2 mM UDCA for 24 h. UDCA, ursodeoxycholic acid; +UD, cells treated with ursodeoxycholic acid; -UD, cells not treated with ursodeoxycholic acid; EMT, epithelial mesenchymal transition. E-cad, E-cadherin; N-cad, N-cadherin.

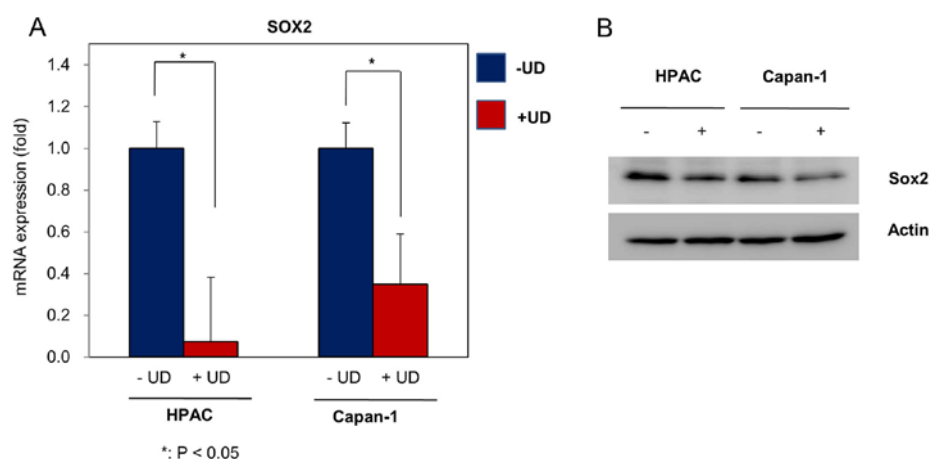


Figure 5. UDCA suppresses the expression of Sox2 in pancreatic cancer cells. (A) Sox2 mRNA was quantified using qRT-PCR. The HPAC and Capan-1 cells were treated with 0.2 mM UDCA for 24 h (* $p < 0.05$). The data are representative of at least 3 replicates. (B) The protein level of Sox2 was detected by western blotting after the HPAC and Capan-1 cells had been treated with 0.2 mM UDCA for 24 h. UDCA, ursodeoxycholic acid; +UD, cells treated with ursodeoxycholic acid; -UD, cells not treated with ursodeoxycholic acid; Sox2, sex determining region Y-box 2.

protein level of Sox2 in the HPAC and Capan-1 cells (Fig. 5B). Reduced levels of Sox2 may affect the formation and growth

of cancer stem cells. To investigate whether UDCA reduced the growth of cancer stem cells, we examined the formation

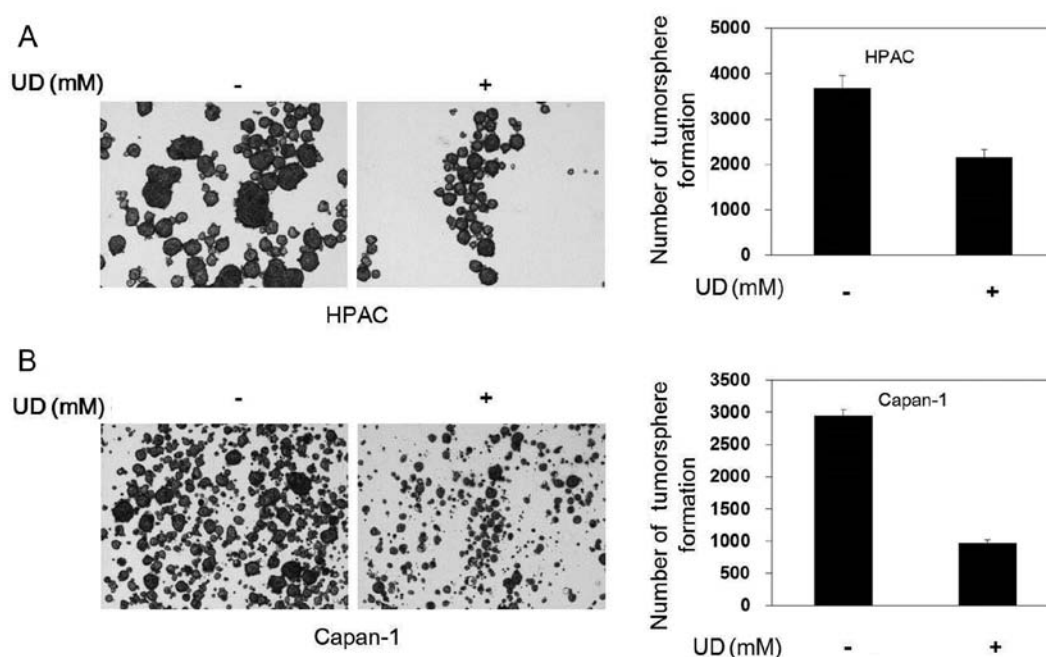


Figure 6. UDCA diminishes the formation of pancreatic tumorspheres. Representative images of tumorspheres in (A) HPAC or (B) Capan-1 cells with or without 0.2 mM UDCA treatment for seven days. All the images were captured with a 4X objective. Quantification of tumorsphere-forming potential was based upon counting tumorspheres. Error bars denote the standard error ($n=3$, $p<0.001$). UD and UDCA, ursodeoxycholic acid.

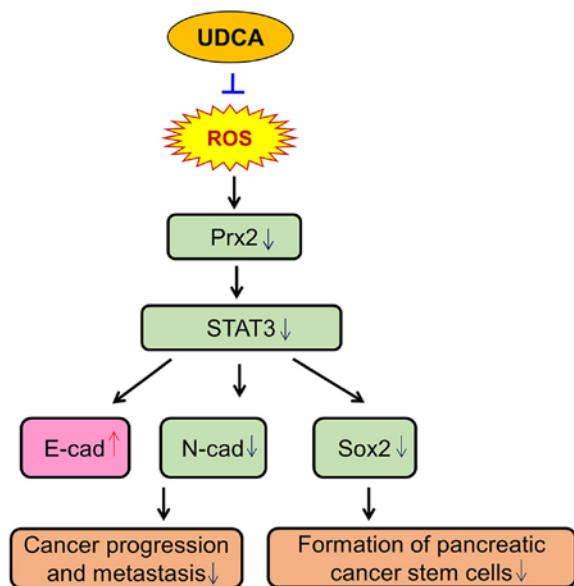


Figure 7. UDCA influences cellular signaling pathways. UDCA reduces the levels of intracellular ROS in pancreatic cancer cells. The reduced level of ROS caused by UDCA leads to a decrease in the interaction between Prx2 and STAT3 and suppresses phosphorylation of STAT3. The reduction of STAT3 activation caused by UDCA affects the expression of E-cadherin and N-cadherin. The increased expression of E-cadherin and decreased expression of N-cadherin by UDCA affects the formation of cancer stem cells. Attenuation of STAT3 activation influences the expression of Sox2. The reduction of Sox2 by UDCA affects the formation of cancer stem cells. UDCA, ursodeoxycholic acid; ROS, reactive oxygen species; Prx2, peroxiredoxin II.

of tumorspheres using culture in ultralow attachment plates. UDCA reduced the size of tumorspheres in the HPAC and Capan-1. In addition the total number of tumorspheres was reduced by UDCA (Fig. 6).

Discussion

UDCA has multifactorial mechanisms of action. Previous studies (21-24) have revealed that UDCA acts as an inhibitor of Bax protein translocation from the cytosol to mitochondria, exhibits a chemopreventive effect on colorectal cancer, demonstrates anti-apoptotic effects and inhibits the formation of ROS. UDCA is known as an antioxidant and is a well-tolerated drug (18-20). In the present study, UDCA influenced the cellular-signaling pathways (Fig. 7). Both in the HPAC and the Capan-1 cells, UDCA did not affect the same signaling pathway. In the HPAC cells, UDCA did not affect the phosphorylation of Akt, mTOR, ERK and p38. In the Capan-1 cells, UDCA reduced the phosphorylation of Akt and mTOR. However, UDCA did not affect the phosphorylation of ERK and p38 in the Capan-1 cells. Furthermore the present study revealed that treatment with UDCA reduced the levels of intracellular ROS in the HPAC and Capan-1 cells (Fig. 1).

The lowered level of ROS influenced the cellular signaling pathways (25,26). The changes in ROS levels can be related to the concentrations of the antioxidant proteins (25,27-29). Prx2 is one of the more abundant proteins able to remove ROS in the cells (27,30). In addition, a recent study revealed that Prx2 may be associated with drug resistance of cancer stem cells (31). The present study revealed that UDCA reduced the mRNA expression and protein level of Prx2 in pancreatic cancer cells (Fig. 2). Furthermore, UDCA inhibited the expression of Prx1 (data not shown). The reduced ROS level caused by UDCA did not appear to rely on the induction of antioxidant proteins.

Prx2 interacts with several molecules in a stressed environment (27,32). The altered redox state affects the association between Prx2 and its interacting proteins (33,34).

Prx2 interacts with STAT3 following exposure to H₂O₂ (35-37). This association generates STAT3 oligomerization and increased phosphorylation of STAT3, which activates the transcriptional function of STAT3. The present study revealed that UDCA reduced the amount of phosphorylated STAT3 (Fig. 3). The reduced level of ROS caused by UDCA led to a decrease in the interaction between Prx2 and STAT3 and suppressed the phosphorylation of STAT3.

The reduction of STAT3 activation caused by UDCA affected EMT (38-40). The expression of E-cadherin was induced by UDCA treatment in the HPAC and Capan-1 cells (Fig. 4). The expression of N-cadherin was suppressed by UDCA in the HPAC and Capan-1 cells (Fig. 4). Thus, the present study revealed that UDCA suppressed EMT in pancreatic cancer cells. However, the HPAC and Capan-1 cell lines may not be sufficient to study the EMT mechanism. Therefore, future experiments using more appropriate cell lines such as circulating tumor cells or more pancreatic cell lines as well as cancer patient samples are needed to improve the study of the EMT mechanism.

Attenuation of the STAT3 activation influenced the expression of Sox2 (41-43). Recently, a research group revealed that Prx2 regulated the level of Sox2 (37). As displayed in Fig. 5, the expression of Sox2 was suppressed by UDCA. Sox2 is one of the essential factors in cancer stem cell pathways. Therefore, the reduction of Sox2 by UDCA affects the formation of cancer stem cells. In the sphere forming assay, we found that UDCA reduced the formation of pancreatic cancer stem cells in the HPAC and Capan-1 cells (Fig. 6). UDCA also suppressed the maintenance of cancer stem cells in the HPAC and Capan-1 cells (data not shown).

Pancreatic cancer is a health issue worldwide and is one of the most aggressive cancers. Due to this fact novel therapeutic methods are needed for pancreatic cancer, as well as a more thorough understanding of the genetic and molecular pathways involved. The present study supports the potential usefulness of UDCA in pancreatic cancer, due to its antioxidant properties. However, the anticancer mechanism of UDCA has not been fully elucidated. Therefore, future studies are warranted to follow up our results with animal experiments and clinical trials.

In conclusion, UDCA suppressed the level of intracellular ROS and decreased EMT and stem cell formation in a pancreatic cancer cell line. Therefore, the antioxidant effects of UDCA may provide a positive therapeutic benefit for pancreatic cancer patients.

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