

Ursodeoxycholic acid inhibits epithelial-mesenchymal transition, suppressing invasiveness of bile duct cancer cells: An *in vitro* study

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Abstract. Epithelial-mesenchymal transition (EMT) features are associated with pathological severity in the progression and metastasis of various cancer types, including bile duct cancer (BDC). Our previous study demonstrated that ursodeoxycholic acid (UDCA) blocked the EGFR-MAPK signaling pathway and inhibited the invasion of BDC cells. The present study was performed to determine whether UDCA inhibits EMT and promotes the expression of E-cadherin to inhibit the invasion and aggressiveness of BDC. In addition, the present study aimed to confirm that the primary mechanism of inhibition of EMT by UDCA is related to the EGFR axis. Human extrahepatic BDC cells were cultured. The effect of UDCA on cell proliferation was evaluated using MTT assays. A cell death ELISA kit was used to measure apoptosis, and western blot assays or immunofluorescence staining assays measured the expression levels of various target proteins. The mRNA expression of Slug and ZEB1 was evaluated via reverse transcription-quantitative PCR. The invasiveness of BDC cells was estimated by invasion assays and western blot assays for focal adhesion kinase (FAK). UDCA inhibited the proliferation of BDC cells as effectively as gefitinib (an EGFR inhibitor), and the combination of UDCA and gefitinib revealed an additive effect on the proliferation of cells. UDCA and gefitinib induced apoptosis, and the combination of UDCA and gefitinib demonstrated an additive effect on apoptosis in BDC cells. UDCA restored the E-cadherin expression inhibited by EGF and suppressed N-cadherin expression increased

by EGF as effectively as gefitinib. UDCA suppressed the Slug and ZEB1 mRNA expression induced by EGF in BDC cells. UDCA suppressed the invasiveness of BDC cells and FAK expression linked to the invasiveness of BDC. In conclusion, UDCA enhanced E-cadherin expression and suppressed N-cadherin expression through inhibition of the EGF-EGFR axis, contributing to the inhibition of EMT and invasiveness in BDC cells. Therefore, UDCA may be applied as an adjuvant or palliative antineoplastic agent and as a therapeutic option to enhance the effect of other chemotherapeutics.

Introduction

Bile duct cancer (BDC) is a malignant tumor with a 20~30% 5-year survival rate, even after resection, where most patients who cannot receive resection die within 2 years (1,2). This is because symptoms occur during the late stages of disease progression and an early diagnosis prior to metastasis, particularly to the lymphatic system, is challenging. Non-surgical palliative chemotherapy and radiation therapy may be considered, but the results have not been satisfactory (1).

Ursodeoxycholic acid (UDCA), an endogenous hydrophilic bile acid, protects cells by inhibiting apoptosis in various cell types, such as hepatocytes. Activation of the EGFR/MAPK survival pathway, prevention of mitochondrial dysfunction and apoptosis, and minimization of the pro-apoptotic cascade activation are all known biological mechanisms that utilize UDCA to protect cells (3-5). UDCA is known to induce, rather than inhibit, apoptosis in malignant cells (6). In particular, UDCA induces potent apoptosis through BAX gene activation and BCL2 inhibition in hepatoma cells (6). Furthermore, a mouse model study demonstrated that UDCA inhibited hepatocellular carcinoma cell growth (7). UDCA inhibits signaling of EGFR and COX-2, blocking the tumorigenic effect caused by deoxycholic acid (DCA), thereby inhibiting the progression of colon cancer cells (4,8,9). A study was conducted on whether the effects of UDCA on apoptosis and growth in malignant and normal cells. This study showed that normal oral epithelial cells were not affected by UDCA treatment up to a toxic concentration, whereas apoptosis was stimulated in oral cancer epithelial cells proportional to the treatment

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concentration (10). Studies on whether UDCA decreases the incidence of BDC in high-risk groups are controversial. However, several epidemiological studies agree that long-term UDCA treatment lowers the incidence of cancer (11,12).

Epithelial-mesenchymal transition (EMT) is a complex reversible process wherein epithelial cells increasingly change to the functional and structural properties of mesenchymal cells (13-15). Although it is the basis of physiological biogenesis and wound healing, EMT is also an early mechanism of metastasis and invasion at the primary site of tumor cells. The primary EMT mechanism alters gene expression to suppress the epithelial phenotype, activating the mesenchymal phenotype (16,17). In other words, the first step of EMT is the internalization and inhibition of E-cadherin, which induces the rupture of adherens junctions. After acquiring mesenchymal traits, EMT-transcriptional factors (ZEB1/2, Slug, Twist and Snail, etc.) regulate the expression of E-cadherin (14,15,18,19). Several studies have shown that EMT features was highly associated with pathological severity in terms of the progression and metastasis of BDC (20-25). Disappearance of epithelial markers (such as E-cadherin) and acquisition of mesenchymal markers (such as N-cadherin, S100A4, and Slug) were associated with aggressive characteristics of BDC, including metastasis, vascular and neural invasion, advanced tumor stage, and poor differentiation (20-22,24).

EGFR activation is known to destabilize the E-cadherin/ β -catenin complex in several tumors, thereby interfering with cell-cell adhesion, promoting EMT, and helping acquire a motile phenotype (25-27). Additionally, over-expressed EGFR is correlated with the tumor progression in BDC as well (28-32), and the EGFR axis triggers EMT in BDC cells, the most crucial step in the progression of the cancer (33). Recently, our studies demonstrated that UDCA suppresses the proliferation of BDC cells through the induction of apoptosis and inhibition of the EGFR-PI3K-Akt signaling pathway. Moreover, we found that UDCA blocks the EGFR-MAPK p42/44 (ERK1/2) signaling pathway and inhibits the invasion of the cancer cells (34).

Accordingly, this study was conducted to determine whether UDCA inhibits EMT and promotes the expression of E-cadherin to inhibit the invasion and aggression of BDC. In addition, the primary mechanism of inhibition of EMT by UDCA, believed to be related to the EGF/EGFR axis, was investigated.

Materials and methods

Materials. Fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin-streptomycin, trypsin, and sodium bicarbonate were supplied by Gibco. Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and UDCA were procured from Sigma Chemicals. Goat anti-rabbit IgG-horseradish peroxidase (HRP, Cat# sc2004) and human EGF were supplied by Santa Cruz Biotechnology. The Western Blot Hyper HRP substrate (Cat# T7103A) was procured from Takara. Gefitinib was procured from Roche Diagnostics. E-cadherin (Cat# 3195), N-cadherin (Cat# 4160), FAK (Cat# 3285), phosphorylated FAK (pFAK, cat# 3283), and β -actin (Cat# 4967) antibodies were purchased from Cell Signaling Technology.

Cell culture. The Korean Cell Line Bank (KCLB) supplied the SNU-245 cells (cat. #00245) obtained from distal common BDC presenting well-differentiation. They reported that the cells did not have any mutations in the genes of p53, p15, p16, hMLH1, and K-Ras. Moreover, the gene and mRNA of E-cadherin without mutation were found. The KCLB authenticated the absence of bacterial or mycoplasma contamination and the short tandem repeat (35). We cultured the SNU-245 cells in RPMI 1640 medium supplemented with 2 mM glutamine, 10% FBS, 1.5 g/l sodium bicarbonate, 100 μ g/ml streptomycin, and 100 IU/ml penicillin. The media was refreshed twice a week and the cells were incubated at 37°C in a humidified incubator with 5% CO₂. The cells were dislodged from the vessel using EDTA (1 g/l) and trypsin (2.5 g/l) when the cells were confluent.

MTT assays. MTT assays were performed to estimate cell proliferation as previously described (36). Briefly, cells were plated at a density of 5x10⁴ cells/ml in RPMI regular media in 96-wells and incubated for 24 h. The various concentrations of UDCA then treated the cells within the serum-free medium (SFM) for 24 or 48 h. MTT (0.5 mg/ml) was then loaded in each well, and the cells were incubated at 37°C for an extra 4 h. After removing of the culture media, 100 μ l of DMSO was added to each well. The colorimetric response was estimated using an ELX800 (Biotek) at 570 nm.

Cell apoptosis assays. Cell apoptosis was estimated using the Cell Death Detection ELISA Plus Kit (Roche Molecular Biochemicals) that detects histone-associated deoxyribonucleic acid (DNA) fragments as previously described (36). Cells were plated at a density of 2x10⁴ cells/ml in 96-well plates and incubated for 24 h. Various concentrations of UDCA were applied to the cells at 37°C for 24 or 48 h. After removing the media, 100 μ l lysis buffer were loaded onto the cells for 30 min and followed by centrifugation at 200 x g at 4°C for 10 min. The supernatant was placed in the wells of a streptavidin-coated plate. The cell lysate was treated with the antibodies for DNA-peroxidase and histone-biotin, and then incubated for 2 h. After washing, 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid (100 μ l) was incubated with each well for 20 min. A BioTek ELX800 microplate reader (BioTek Instruments) measured the absorbance at 405 nm.

Flow cytometry analysis. The Annexin V-FITC/PI (fluorescein isothiocyanate/propidium iodide) Apoptosis Detection kit. (Cat. #ab14085; Abcam) was applied to the identification of apoptotic cells. Cells were treated with indicated concentrations of UDCA or gefitinib at 37°C for 48 h. The cells were collected (2x10⁶ cells) and washed once with PBS, and then, resuspended in 500 μ l binding buffer (1x). The harvested cells were stained with Annexin V-FITC and PI for 20 min at RT in the dark. The stained cells were measured by CytoFLEX Flow Cytometer (Beckman Coulter), and the cell apoptosis rate was analyzed using CytExert software version 2.4 (Beckman Coulter).

Western blot assays. Western blot assays were conducted as previously described (36). Briefly, cells were treated with various concentrations of UDCA, gefitinib, or EGF when the

confluence reached 90% for 24 or 48 h. Cells were collected and washed with cold PBS (Gibco). Protein samples were extracted with RIPA buffer (Cat# R0278, Sigma Chemicals) and centrifuged at 15,000 rpm for 20 min. Bradford assays (Sigma-Aldrich; Merck KGaA) were used to estimate the amount of protein content of the cell lysate. Blots were blocked using a blocking solution at room temperature and incubated overnight at 4°C in 5% bovine serum albumin (BSA) solution with rabbit polyclonal diluted antibodies for N-cadherin (1:1,000), E-cadherin (1:1,000), focal adhesion kinase (FAK, 1:1,000), phosphorylated FAK (pFAK, 1:1,000), and β -actin (1:1,000). The nitrocellulose membranes were incubated with goat anti-rabbit IgG-HRP (1:8,000 dilution) after rinsing with TBS-T at room temperature for 1 h. The Luminata Forte Western HRP Western Blotting Detection Kit (Millipore Sigma) was used to detect the specific bands on the blots. Amersham image 600 system detected the bands automatically (Amersham Biosciences-GE Healthcare). The signal intensities of bands were measured with the ImageJ (Version 1.43; National Institute of Health).

Reverse transcription-quantitative PCR (RT-qPCR)

Extraction and quantification of RNA. BDC cells were lysed by adding 1 ml of Tri-reagent (Sigma-Aldrich; Merck KGaA) over 1 min. The lysate was treated with 200 μ l chloroform (Sigma-Aldrich; Merck KGaA) and incubated for 10 min. It was then centrifuged at 12,000 \times g at 4°C for 10 min. An equal volume of isopropanol (Sigma-Aldrich; Merck KGaA) was treated to the supernatant and incubated for 15 min. The final product was centrifuged again at 12,000 \times g at 4°C for 15 min. The pelleted RNA was rinsed with 70% ethanol and 50 μ l nuclease-free-water (Roche Diagnostics) was added to dissolve the RNA, which was quantified using ELX800 (Biotek) at 260 nm.

Synthesis of cDNA. After mixing 1 μ g of RNA [extracted using RNA to cDNA EcoDry Premix (Takara)] and 20 μ l of RT Master Mix, cDNA was synthesized under reverse transcription conditions (one cycle at 42°C for 60 min, one cycle at 70°C for 10 min, and 4°C for 10 min). After completion cDNA synthesis, 20 μ l of cDNA was saved at -70°C until use.

Relative quantitative PCR. Slug and ZEB1 gene expressions in the cDNA of SNU-245 cells were measured using relative quantitative PCR (LightCycler 480 using SYBR®-Green I Master, Cat. #5081963001, Roche Diagnostics). Relative quantitation of expression was determined by comparative C_t ($2^{-\Delta\Delta C_t}$) method (37). PCR was performed under the following conditions: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Primer sequences were as follows: *Slug*, forward, 5'-ACACATTAG AACTCACACGG-3', reverse, 5'-GAGAGACATTCTGGA GAAGG-3'; *ZEB1*, forward, 5'-ACCTGCCAACAGACCAGA CAGTGT-3', reverse, 5'-GCCCTTCCTTTCTGTTCATCCT CCCA-3'; *GAPDH*, forward, 5'-GAGTCAACGGATTGTGTC GT-3', reverse, 5'-GACAAGCTTCCCCGTTCTCAG-3'.

Immunofluorescence staining assays. Cells were cultured on glass coverslips and treated with 250 μ M UDCA, 10 nM gefitinib, or 50 nM EGF for 24 h. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) for 10 min, and then, cells were permeabilized with 0.3% triton x-100

in PBS for 10 min. Cells were incubated further with 10% goat serum (Cat. #sc-2043) (Santa Cruz Biotechnology) solution at room temperature for 1 h. Cells were incubated with an E-cadherin antibody (1:200 dilution, Cat. #3195; Cell Signaling Technology) in PBS containing 1% BSA (PBS-A) at room temperature. After reaction, this was incubated further with 1% albumin for 1 h and then goat serum solution at room temperature for 1 h. The cells were treated with a FITC-conjugated secondary antibody (1:500 dilution, Cat. #sc-36869, Santa Cruz Biotechnology) for 1 h in PBS-A and rinsed with PBS three times. The cells on coverslips were then treated with DAPI (0.5 μ g/ml) (Sigma-Aldrich; Merck KGaA) for 1 min, and images were captured (x400 optical and x3 digital magnification) using a super-resolution confocal laser microscope (Carl Zeiss).

Invasion assays. Invasion assays were performed as previously described to evaluate the invasiveness of cancer cells (34). Briefly, we coated the upper membranes of cell culture inserts (Cat# 3401, Corning Incorporated) with Matrigel (Cat# A14132-01, Gibco) for 1 h. Serum-free regular medium (described in cell culture) of 200 μ l was plated to the upper compartment, and 500 μ l regular medium containing 10% FBS was added to the lower compartment. The cells were plated at a density of 2×10^4 cells/ml in the upper inserts and incubated at 37°C for 24 or 48 h. The upper membranes containing invading cells were fixed using 100% methanol for 20 min and stained for 15 min with 0.1% crystal violet (Sigma Chemicals) at room temperature. The upper surface of the inserts was washed in PBS, and noninvasive cells were wiped with cotton swabs. The membranes containing invading cells were mounted on slides, and light microscopy (100x, magnification, Olympus BX51-p polarizing Microscope) was used to count the number of cells present.

Statistical analysis. All experiments in this study were performed at least in triplicate. All described results were representative data and expressed as the means \pm SD of duplicate cultures. The data were considered to follow parametric distribution after performing normality test (Skewness and Kurtosis statistics). One-way ANOVA followed by Tukey post hoc test for multiple comparison was used to compare three or more unpaired groups, and Student's t-test was used to compare two unpaired groups. P-values of less than 0.05 were considered statistically significant. IBM-SPSS version 27 (Armonk) was used as a statistical software.

Results

UDCA effectively inhibits proliferation and induces apoptosis in BDC cells. Suppression of BDC cell proliferation by UDCA and gefitinib, a known EGFR inhibitor, was evaluated by an MTT assay after incubation for 24 or 48 h. Both gefitinib and UDCA treatment inhibited the viability of BDC cells in a dose- and time-dependent manner (Fig. 1A and B). The combination of UDCA and gefitinib for 24 or 48 h treatment demonstrated an additive effect, although not synergistic, on the proliferation of SNU-245 cells (Fig. 1C and D). A Cell Death Detection ELISA assay measured the effect of UDCA and gefitinib on apoptosis. UDCA and gefitinib induced

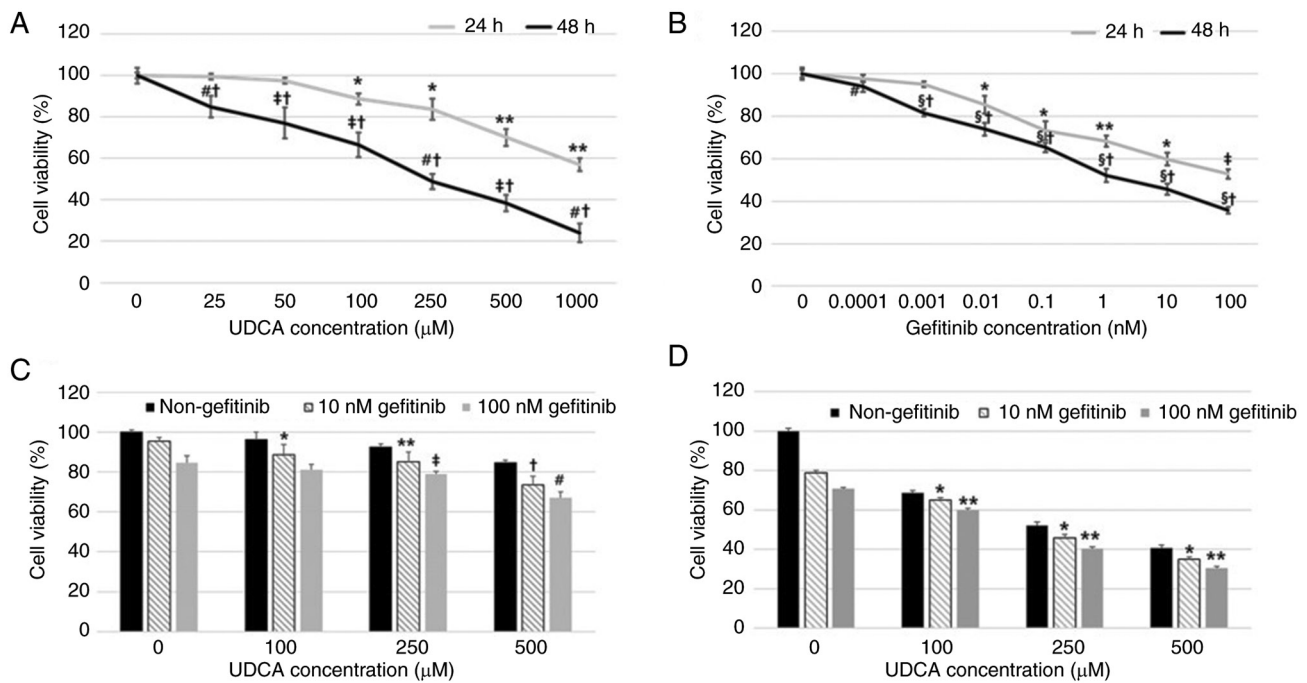


Figure 1. UDCA inhibits proliferation in BDC cells as effectively as gefitinib. Suppression of the proliferation of BDC cells by (A) UDCA and (B) gefitinib was evaluated using an MTT assay after incubation for 24 or 48 h. Both (B) gefitinib and (A) UDCA treatment suppressed the viability of BDC cells in a dose- and time-dependent manner. The combination of UDCA and gefitinib demonstrated an additive effect on the viability of SNU-245 cells for (C) 24 or (D) 48 h. The results are presented as the mean \pm SD. (A) * $P < 0.05$ vs. untreated control cells for 24 h, ** $P < 0.001$ vs. untreated control and cells treated with all lower concentrations of UDCA for 24 h, * $P < 0.001$ vs. untreated control and cells treated with all lower concentrations of UDCA for 48 h, * $P < 0.01$ vs. untreated control and cells treated with all lower concentrations of UDCA for 48 h, * $P < 0.001$ vs. cells treated for 24 h with the same concentration of UDCA. (B) * $P < 0.001$ vs. untreated control and cells treated with all lower concentrations of gefitinib for 24 h, * $P < 0.001$ vs. untreated control and cells treated with 0.01 nM gefitinib for 24 h, * $P < 0.01$ vs. untreated control and cells treated with all lower concentrations of gefitinib for 24 h, * $P < 0.01$ vs. cells treated for 24 h with the same concentration of gefitinib, * $P < 0.001$ vs. cells treated for 24 h with the same concentration of gefitinib, * $P < 0.001$ vs. untreated control and cells treated with all lower concentrations of gefitinib for 48 h. (C) * $P < 0.01$ vs. non-treatment of UDCA (10 nM gefitinib), ** $P < 0.001$ vs. non-treatment of UDCA (10 nM gefitinib), * $P < 0.001$ vs. non-treatment of UDCA and cells treated with all lower concentrations of UDCA (co-treated with 10 nM gefitinib), * $P < 0.05$ vs. non-treatment of UDCA (co-treated with 100 nM gefitinib), * $P < 0.001$ vs. non-treatment of UDCA and cells treated with all lower concentrations of UDCA (co-treated with 100 nM gefitinib). (D) * $P < 0.001$ vs. non-treatment of UDCA untreated control cells and cells treated with all lower concentrations of UDCA (co-treated with 10 nM gefitinib), ** $P < 0.001$ vs. non-treatment of UDCA untreated control cells and cells treated with all lower concentrations of UDCA (co-treated with 100 nM gefitinib). BDC, bile duct cancer; UDCA, ursodeoxycholic acid.

significant apoptosis of BDC cells after 24 or 48 h of incubation in a dose- and time-dependent manner as well (Fig. 2A and B). The combination of UDCA and gefitinib for 48 h treatment also demonstrated an additive, but not synergistic, effect on the apoptosis of SNU-245 cells (Fig. 2C). We confirmed that UDCA and gefitinib induce significant apoptosis in BDC cells using Flow cytometry assays (Fig. S1). These results revealed that UDCA induced apoptosis and inhibited cell proliferation as effectively as gefitinib, and the combination of UDCA and gefitinib had an additive effect on apoptosis.

UDCA restored E-cadherin expression inhibited by EGF and suppressed N-cadherin expression increased by EGF in BDC cells. Western blot assays were conducted to evaluate whether UDCA activates E-cadherin (primary epithelial marker) and suppresses N-cadherin (primary mesenchymal marker) in BDC cells. The BDC cells were loaded with the indicated concentrations of UDCA and/or gefitinib and co-treatment with EGF in regular media containing 1% FBS for 48 h. EGF-only treatment, as a control, inhibited E-cadherin expression and increased N-cadherin expression (Fig. S2) in a time and dose-dependent manner, as was expected. Gefitinib or UDCA treatment restored the E-cadherin expression inhibited

by EGF (50 ng/ml) and suppressed the N-cadherin expression enhanced by EGF in a dose-dependent manner as well (Fig. S3). Even though co-treatment with UDCA (250 μ M) and gefitinib (10 nM) did not show synergistic restoration of E-cadherin expression decreased by EGF (50 ng/ml), co-treatment with UDCA (250 μ M) and gefitinib (10 nM) synergistically suppressed N-cadherin expression increased by EGF (50 ng/ml) (Fig. 3).

An immunofluorescence staining study was performed to confirm that UDCA activates E-cadherin expression in BDC cells. We treated SNU-245 cells with the determined concentrations of UDCA and/or gefitinib with co-treatment of EGF in regular media containing 1% FBS for 48 h. UDCA (250 μ M) treatment restored E-cadherin expression inhibited by EGF (50 ng/ml) (Fig. 4), which was similar to what was observed for the western blot assay.

UDCA suppresses the expression of Slug and ZEB1 mRNA induced by EGF in BDC cells. Here, we evaluated whether UDCA inhibits the mRNA expression of Slug and ZEB1, main EMT-transcription factors, using qPCR. Cells were loaded with the determined concentrations of UDCA (250 μ M) and/or gefitinib (10 nM) with or without co-treatment of

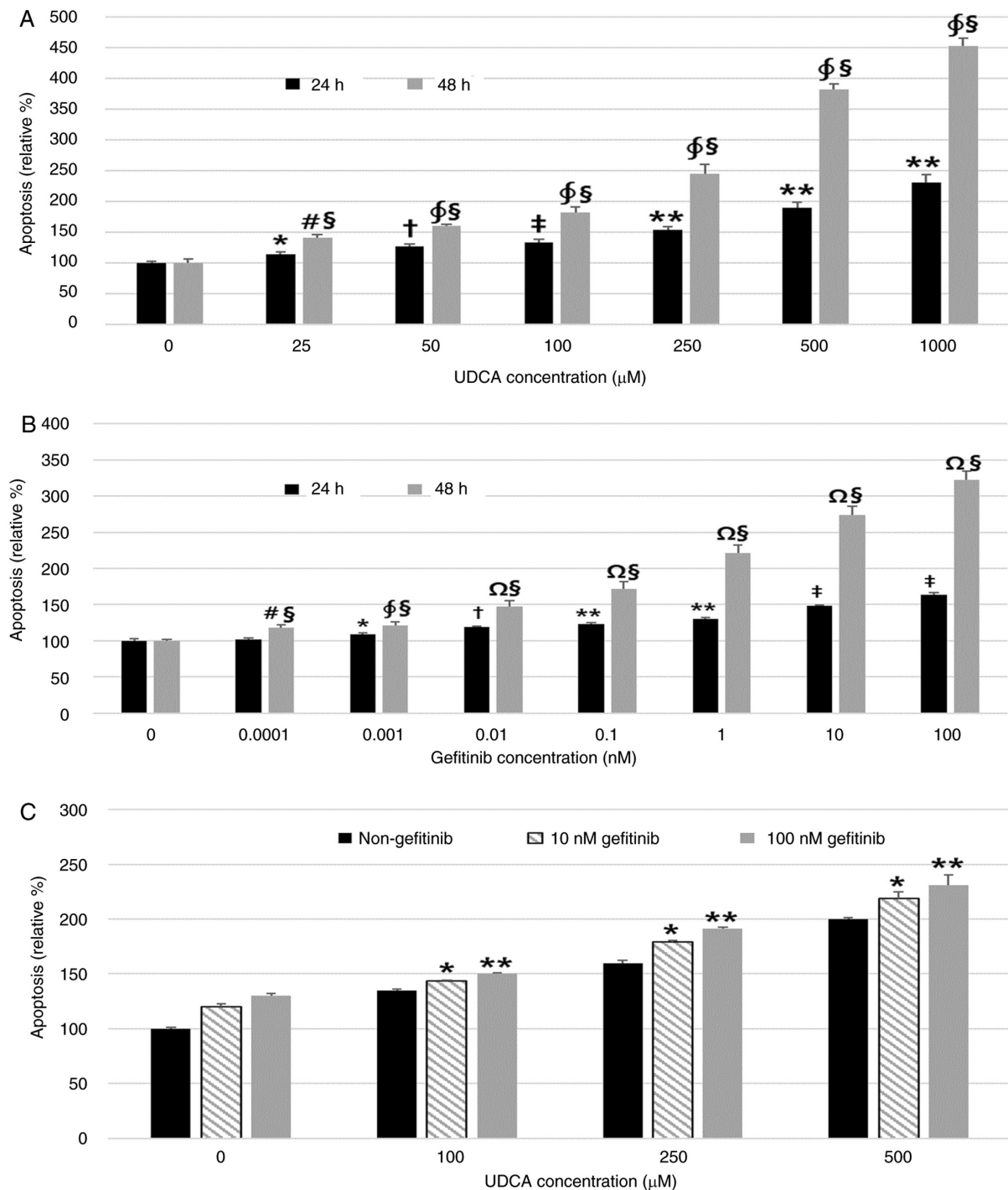


Figure 2. UDCA induces apoptosis as effectively as gefitinib in BDC cells. A Cell Death Detection ELISA assay was used to measure the effect of UDCA and gefitinib on apoptosis. (A) UDCA and (B) gefitinib dose- and time-dependently induced apoptosis of BDC cells after incubation for 24 or 48 h. (C) Treatment with a combination of UDCA and gefitinib for 48 h showed an additive effect on apoptosis of SNU-245 cells. The results are presented as the mean \pm SD. (A) * $P < 0.01$ vs. untreated control for 24 h, $^{\dagger}P < 0.05$ vs. cells treated with all lower concentrations of UDCA and untreated control for 24 h, $^{\ddagger}P < 0.001$ vs. untreated control and 25 μ M UDCA-treated cells for 24 h, ** $P < 0.001$, cells treated with all lower concentrations of UDCA and untreated control for 24 h, $^{\phi}P < 0.01$ vs. untreated control for 48 h, $^{\Omega}P < 0.001$ vs. cells treated with all lower concentrations of UDCA and untreated control for 24 h, $^{\#}P < 0.01$ vs. cells treated with the same concentration for 24 h. (B) * $P < 0.05$ vs. untreated control for 24 h, $^{\dagger}P < 0.01$ vs. untreated control and cells treated with all lower concentrations of gefitinib for 24 h, ** $P < 0.001$ vs. untreated control and 0.001 nM gefitinib-treated cells for 24 h, $^{\ddagger}P < 0.001$ vs. untreated control and cells treated with all lower concentrations of gefitinib for 24 h, $^{\Omega}P < 0.05$ vs. untreated control for 48 h, $^{\phi}P < 0.001$ vs. cells treated with same concentrations for 24 h, $^{\#}P < 0.001$ vs. untreated control for 48 h, $^{\Omega}P < 0.001$ vs. untreated control and cells treated with all lower concentrations of gefitinib for 48 h. (C) * $P < 0.001$ vs. cells treated with all lower concentrations of UDCA (co-treated with 10 nM gefitinib), ** $P < 0.001$ vs. cells treated with all lower concentrations of UDCA (co-treated with 100 nM gefitinib). BDC, bile duct cancer; UDCA, ursodeoxycholic acid.

EGF (50 ng/ml) in regular media containing 1% FBS for 24 h. UDCA treatment significantly inhibited Slug and ZEB1 mRNA expression slightly less effectively than gefitinib

(Fig. 5). Although co-treatment with UDCA and gefitinib did not show synergistic suppression of Slug mRNA expression increased by EGF (50 ng/ml), co-treatment with UDCA and

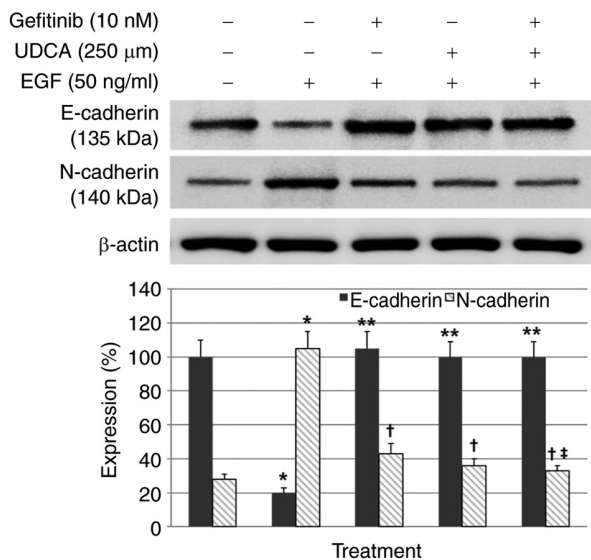


Figure 3. UDCA restores E-cadherin expression and suppresses N-cadherin expression increased by EGF in bile duct cancer cells. Cells were treated with gefitinib and/or UDCA with or without treatment of EGF in 1% FBS RPMI media for 48 h. Expression levels were measured by western blotting. All data are representative of triplicate experiments. Co-treatment with UDCA (250 μ M) and gefitinib (10 nM) synergistically suppressed N-cadherin expression increased by EGF, although co-treatment with UDCA and gefitinib did not show synergistic or additive restoration of E-cadherin expression decreased by EGF (50 ng/ml). *P<0.001 vs. untreated control, **P<0.001 vs. expression of E-cadherin treated with only EGF, †P<0.001 vs. expression of N-cadherin treated with only EGF, ‡P<0.01 vs. expression of N-cadherin treated with EGF + gefitinib and EGF + UDCA. UDCA, ursodeoxycholic acid.

gefitinib synergistically decreased the ZEB1 mRNA expression enhanced by EGF (Fig. 5).

UDCA suppresses the invasiveness of BDC cells. Invasion assays were conducted to estimate the effect of UDCA on the aggressiveness on invasion and migration of BDC cells. The cells were seeded on upper inserts of Transwell® (Corning Incorporated) and treated with the indicated concentration of gefitinib (10 nM) and/or UDCA (250 μ M) in SFM for 24 (not shown data) or 48 h. This experiment revealed that the invasiveness of bile duct cancer cells was significantly decreased after treatment with UDCA and was just as effective as gefitinib. In addition, the combination of UDCA and gefitinib had an additive or synergistic effect on the suppression of invasiveness of BDC cells (Fig. 6).

Another western blot assay was conducted to evaluate the expressional change of pFAK, known to be positively associated with cancer metastasis and invasion (38), following treatment with gefitinib and/or UDCA in SFM for 24 h with pre-treatment of IGF-1 (100 nM) for 15 min. Both UDCA and gefitinib treatment inhibited the expression of pFAK enhanced by IGF. In addition, the combination of UDCA and gefitinib had an additive or synergistic effect on the suppression of pFAK induced by IGF (Fig. S4).

Discussion

UDCA shows antineoplastic effects as a result of the induction of apoptosis, which has been demonstrated in several

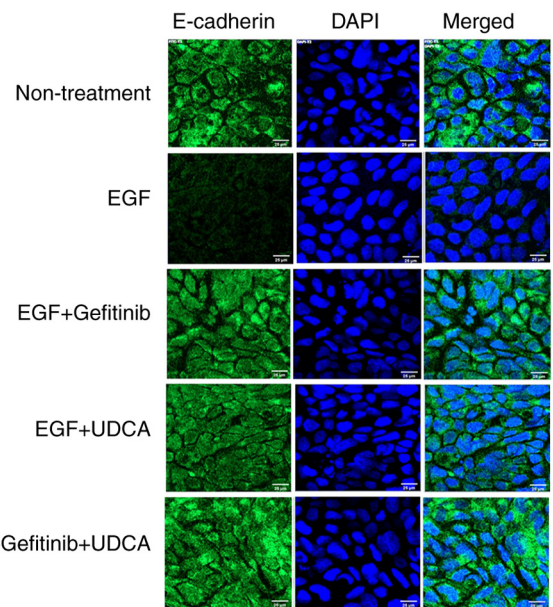


Figure 4. Immunofluorescence staining demonstrated that UDCA restores E-cadherin expression inhibited by EGF in BDC cells. Cells were treated with UDCA (250 μ M) and/or gefitinib (10 nM) with or without co-treatment of EGF (50 ng/ml) in regular media containing 1% FBS for 48 h. Immunofluorescence staining was used to analyze the expression levels of E-cadherin. UDCA treatment restored the E-cadherin expression inhibited by EGF (50 ng/ml) similar to what was observed in the western blot assays. Scale bar, 25 μ m. UDCA, ursodeoxycholic acid.

studies using cells and xenograft models of malignances (6,7). Recently, we proved that UDCA suppresses the proliferation of BDC cells via the induction of apoptosis and inhibition of the pathways of the EGFR-ERK and the PI3K-AKT, while blocking the invasiveness (34).

Epidermal growth factor receptors (EGFR, HER-1, ErbB-1) belong to the tyrosine kinase receptor family. These growth factors, such as the epidermal growth factor (EGFR), bind at their extracellular binding domain, initiating intracellular signaling involved in stimulating cell proliferation, differentiation, and survival (39). Increased signaling from EGFR linked to its overexpression and mutation is associated with various cancers, including breast, colorectal, lung, head, neck, pancreatic, and BDCs (31,40,41). Enhanced expression of EGFR is known to contribute to poor prognosis in these cancers (28,42-45). EGFR expression in total cholangiocarcinoma ranged from 10.7 to 86% (31,46-48). Among them, EGFR in intrahepatic cholangiocarcinoma is positive in 43.3±30.6% (mean ± SD) (46), and extrahepatic BDC in Korea, where the prevalence rate is high, showed 86% positivity for EGFR (48). The prognosis in gallbladder cancer is also influenced by enhanced EGFR expression (49,50).

Therefore, EGFR can be a therapeutic target for human cancer. ATP-competitive tyrosine kinase inhibitors, such as erlotinib or gefitinib, have increased the therapeutic efficacy for colorectal non-small cell lung, and pancreatic cancer treatments (51-53). In addition, studies have demonstrated that the inhibition of EGFR signaling by gefitinib effectively suppressed the proliferation of cholangiocarcinoma cells (29). The SNU-245 cells used in this study are extrahepatic bile duct cancer cells that exhibit EGFR expression (34). The aim of this

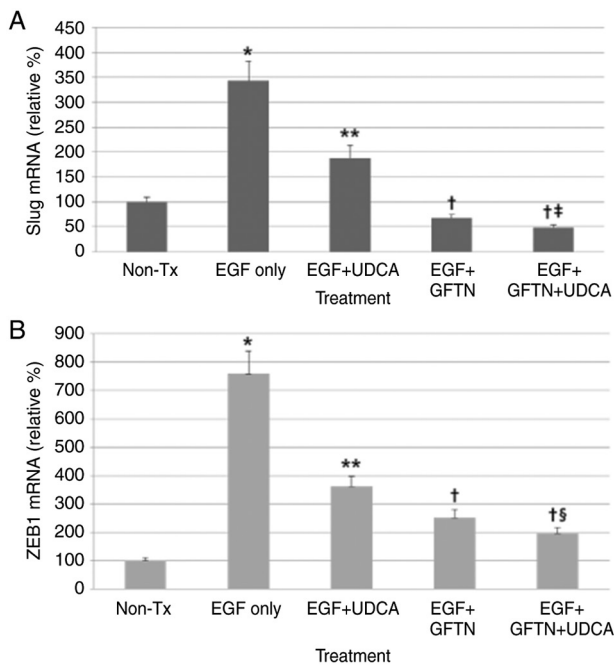


Figure 5. UDCA suppresses Slug and ZEB1 mRNA expression induced by EGF in bile duct cancer cells. Cells were loaded with the determined concentrations of UDCA (250 μ M) and/or gefitinib (10 nM) with or without co-treatment of EGF in regular media containing 1% FBS for 24 h. mRNA expression levels of Slug and ZEB1 were measured via quantitative PCR. UDCA treatment significantly inhibited (A) Slug and (B) ZEB1 mRNA expression but was less effective than gefitinib. Although co-treatment with UDCA and gefitinib did not show significant synergistic suppression of Slug mRNA expression, the co-treatment did synergistically decrease ZEB1 mRNA expression. * $P < 0.001$ vs. untreated control, ** $P < 0.001$ vs. EGF only-treated group, † $P < 0.001$ vs. EGF only-treated group and EGF + UDCA-treated group, ‡ $P < 0.05$ vs. EGF + GFTN-treated group, § $P < 0.01$ vs. EGF + GFTN-treated group. GFTN, gefitinib; Slug, snail family transcriptional repressor 2; UDCA, ursodeoxycholic acid; ZEB1, zinc finger E-box binding homeobox 1; Tx, treatment; relative %, target mRNA expression/GAPDH expression $\times 100$.

study was to evaluate how effectively UDCA inhibits tumor cell proliferation compared to gefitinib (an EGFR inhibitor) and determine whether UDCA works synergistically with gefitinib compared to the monotherapy groups. Our results revealed that UDCA-induced apoptosis and inhibited cell proliferation as effectively as gefitinib and the combination of UDCA and gefitinib had an additive effect on apoptosis. Therefore, UDCA can be suggested as an antineoplastic agent with or without combination with known chemotherapeutics in BDC.

EGFR activation is known to destabilize the E-cadherin/ β -catenin complex in several tumors, thereby interfering with cell-cell adhesion, promoting EMT, and acquiring a motile phenotype (25-27,54), through the induction of adherens junction rupture. Once mesenchymal traits are acquired, EMT-transcriptional factors, such as ZEB1/2, Slug, Twis and Snail, modulate the expression of E-cadherin (14). Weakening of epithelial markers (E-cadherin) and obtainment of mesenchymal markers (N-cadherin, S100A4, and Slug) were associated with aggressive characteristics of BDC including metastasis, vascular and neural invasion, advanced tumor stage, and poor differentiation (20-24). In addition, Clap  ron *et al* (33) proved an association between EGFR and EMT in cholangiocarcinoma

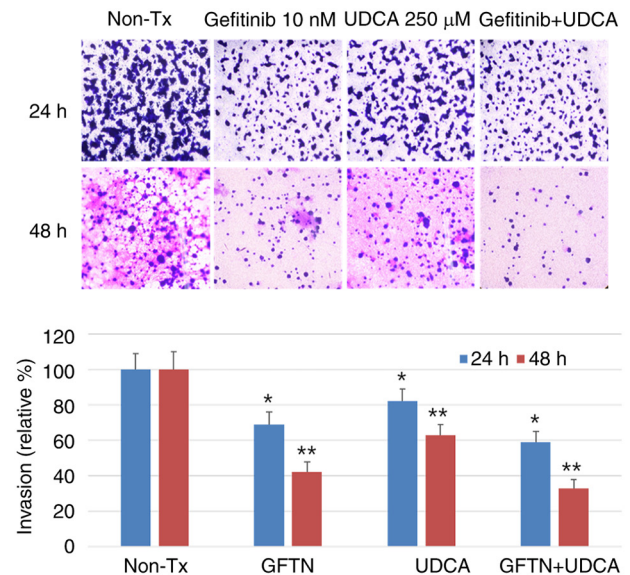


Figure 6. UDCA inhibits the invasiveness of BDC cells. Cells were treated with the indicated gefitinib (10 nM) and/or UDCA (250 μ M) concentrations in serum-free media for 24 or 48 h. This assay demonstrated that the invasiveness of BDC cells was significantly decreased after treatment with UDCA, which was just as effective as gefitinib. In addition, the combination of UDCA and gefitinib had an additive or synergistic effect on the inhibition of the invasiveness of BDC cells. The results are presented as the mean \pm SD. * $P < 0.001$ vs. all other groups treated for 24 h, ** $P < 0.001$ vs. all other groups treated for 48 h. Light microscopy (magnification, $\times 100$). BDC, bile duct cancer; GFTN, gefitinib; UDCA, ursodeoxycholic acid; Tx, treatment.

by demonstrating that EGFR is a major factor in cancer progression by triggering EMT. As UDCA effectively inhibits EGFR in bile duct cancer cells, it has the potential to also inhibit EMT (34). In addition, if UDCA can properly inhibit the EGFR axis and EMT, there is a possibility that it may contribute to the inhibition of BDC progression by suppressing aggressiveness. In this study, UDCA restored E-cadherin expression inhibited by EGF and suppressed N-cadherin expression increased by EGF as effectively as gefitinib. UDCA also suppressed the expression of *Slug* and *ZEB1* mRNA induced by EGF in BDC cells. These data implicate that UDCA suppresses EMT as effectively as gefitinib, through EGF-EGFR axis inhibition.

We demonstrated that UDCA inhibits EMT and EGFR, which are directly linked to invasiveness and metastasis in BDC cells. Additionally, we performed invasion assays and western blot assays to evaluate the expressional change of phosphorylated FAK for the purpose of verifying the suppression of BDC cell invasiveness by UDCA. The invasion assays showed that UDCA suppresses invasiveness, and the combination of UDCA and gefitinib has a synergistic or additive effect on the suppression of BDC cells invasiveness. In addition, FAK is a significant regulator of signals mediated by the growth factor receptor and integrin and modulates basic processes in cancers. Enhanced FAK expression has been noted in various metastatic cancers and is associated with a grave prognosis. Therefore, FAK is regarded as a potential determinant of aggressiveness and metastasis (38,55). In this study, both UDCA and gefitinib treatment inhibited expression of pFAK enhanced by IGF. In addition, the combination of UDCA and gefitinib had a synergistic or additive effect on the inhibition of FAK induced by IGF. Accordingly, we suggest

that UDCA-induced EMT suppression can be a significant determinant in regulating the invasiveness of BDC cells.

As this study was a cellular-level *in vitro* study, there is a limitation in proving the actual anticancer effect of UDCA in animal and human BDC. Accordingly, we intend to conduct a study to investigate the effect of UDCA, with or without combination with other existing chemotherapeutics on EGFR/EMT, and antineoplastic effects using a xenograft animal model for BDC. In addition, we hope that various future practical studies will reveal the synergistic or additive effect of UDCA with known chemotherapeutics for BDC. On the other hand, SNU-245 cells, a human common BDC cell line presenting well-differentiation, was chosen for testing in this study although there are more types of BDC cell lines, which can be another limitation of our study. We wanted to evaluate wild BDC cells that express E-cadherin and do not have mutations of p53, p15, p16, hMLH1, and K-Ras to avoid lots of elements originated from mutations. In the future study, we hope we examine other BDC cell lines.

In addition, 250 μ M UDCA treatment in the media corresponds to the dose of 98.14 mg/Kg of bodyweight. Usual dose of UDCA in the patient with primary biliary cirrhosis is up to 15~20 mg/Kg, which means that 250 μ M UDCA dose in our experiments was approximately 4.9-6.5 times higher than general therapeutic dose. Considering that we had to demonstrate definite change in experiments for short-term period (24 or 48 h) and prove anti-neoplastic effects, and that 25 or 50 μ M UDCA (0.49~0.98 times of usual dose) was also effective on suppression of BDC cell proliferation, the concentrations we loaded may be acceptable.

In conclusion, this study demonstrated that UDCA enhanced E-cadherin expression and suppressed N-cadherin expression, contributing to the inhibition of EMT and invasiveness in BDC cells, through inhibition of EGF-EGFR axis. Accordingly, UDCA may be applied as an adjuvant or palliative chemotherapeutic agent and as a therapeutic combination option that enforces the effect of other antitumor agents in BDC.

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Availability of data and materials

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

Authors' contributions

JL performed the study design, data analysis, statistical analysis, data interpretation and manuscript drafting. EMH performed the main experiments. JHaK, JHeK and JHJ performed the data interpretation and critical revision. SWP

and DHK contributed to the study design and statistical analysis. JL and EMH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable because this study was performed using cells purchased from the Korean Cell Line Bank (KCLB).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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