

Establishment and characterization of gemcitabine-resistant human cholangiocarcinoma cell lines with multidrug resistance and enhanced invasiveness

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Abstract. To establish and characterize the gemcitabine-resistant cholangiocarcinoma (CCA) cell lines, CCA KKU-M139 and KKU-M214 cell lines were exposed stepwisely to increasing gemcitabine (GEM). The resultant drug-resistant cell lines, KKU-M139/GEM and KKU-M214/GEM, retained the resistant phenotype in drug-free medium at least for 2 months. Sulforhodamine B assay demonstrated that KKU-M139/GEM and KKU-M214/GEM were 25.88- and 62.31-fold more resistant to gemcitabine than their parental cells. Both gemcitabine-resistant cell lines were cross-resistant to 5-fluorouracil (5-FU), doxorubicin and paclitaxel indicating their multidrug-resistant nature. Using reverse transcriptase-polymerase chain reaction (RT-PCR), real-time PCR and western blot analyses, gemcitabine-resistant cells showed upregulation of *RRM1* and downregulation of *hENT1* and *dCK*. In relation to multidrug resistance, these cell lines showed upregulation of multidrug resistance protein 1 (MRP1) leading to an increase of drug efflux. Using cell adhesion and Boyden chamber transwell assays, these cell lines also showed higher cell adhesion, migration and invasion capabilities via the activations of protein kinase C (PKC), focal adhesion kinase (FAK), extracellular signal-regulated kinase-1/2 (ERK1/2) and nuclear factor- κ B (NF- κ B). Higher activity of matrix metalloproteinase-9 (MMP-9) and urokinase plasminogen activator (uPA) was also observed by a gelatin zymography assay and a casein-plasminogen zymography assay. Flow cytometry analysis indicated the G2/M arrest regulated by

downregulation of cyclin B1 and cyclin-dependent kinase 1 (Cdk1) resulted in an extended population doubling time. Using Annexin V/propidium iodide staining, evasion of apoptosis via an intrinsic pathway was observed in both cell lines in association with upregulation of Bcl-2 and downregulation of Bax. Interestingly, Fas was additionally downregulated in KKU-M214/GEM supporting the view of its higher GEM resistant characteristics. These findings indicate that long-term exposure of CCA cell lines to gemcitabine induce not only multidrug resistance but also enhance their invasiveness.

Introduction

Cholangiocarcinoma (CCA) is a cancer originating from the biliary epithelial cells. Its incidence and mortality rate are increasing worldwide (1). Surgical resection of the tumor is the most effective treatment, but only 25% of patients are resectable at presentation (2). Unresectable CCA patients are recommended for chemotherapy, using either gemcitabine alone or gemcitabine-based regimens (3). Unfortunately, the response rate and median survival time of gemcitabine treatment are only 17.5-30% over 7.6-14 months (4). The combinations of gemcitabine and cisplatin or oxaliplatin give a response rate of ~35%, and median survival times of 11-15 months (4). A major problem of chemotherapy for human carcinoma, including CCA, is the development of chemoresistance, especially multidrug resistance (MDR) (5).

Gemcitabine (2', 2'-difluorodeoxycytidine, dFdC) is a deoxycytidine analogue that has been used widely in the treatment of various solid tumors including CCA (6,7). The expression level of human equilibrative nucleoside transporter 1 (hENT1), the major gemcitabine transporter in human cancer cells, has been reported to correlate with sensitivity to gemcitabine (8). Inside the cell, gemcitabine is phosphorylated by the rate-limiting enzyme deoxycytidine kinase (dCK) to its monophosphate form (dFdCMP) and subsequent to its di- and triphosphate forms (dFdCTP) (9). The incorporation of an active metabolite, dFdCTP, into the DNA or RNA strand, causes chain termina-

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tion, leading to apoptosis (6). Ribonucleotide reductase (RR) is mainly responsible for the conversion of ribonucleoside diphosphate to deoxyribonucleoside diphosphate, which is essential for DNA synthesis. dFdCDP is an inhibitor of RR, thereby causing a decrease in dNTP pools and decrease feedback inhibition of dCK, leading to increase phosphorylation of gemcitabine (10). RR consists of two subunits, RRM1 and RRM2 (10). Overexpression of RRM1 was reported to associate with gemcitabine resistance in non-small cell lung cancer cell lines (11). Gemcitabine kills cancer cells by triggering apoptosis through both intrinsic and extrinsic pathways and the defective apoptotic pathway is considered to be associated with resistance to a wide range of chemotherapeutic drugs of cancer cells (12,13). In the mitochondrial (intrinsic) apoptosis pathway, the Bcl-2 family proteins of both anti-apoptotic proteins (i.e., Bcl-2) and pro-apoptotic proteins (i.e., Bax) play a critical role (14). In the death receptor (extrinsic) apoptosis pathway, the Fas (CD95) system plays a crucial role (12). Increased expression of Bcl-2 protein while decreased expression of Bax and Fas proteins have been reported in chemotherapeutic drug-resistant cancers (12,15).

The mechanism of gemcitabine resistance is well characterized in pancreatic cancer cells, it involves alteration of gemcitabine metabolism, decreased intracellular drug accumulation, inhibition of apoptosis pathways and aberrant activity in signaling pathways that modulate the cell cycle and apoptosis (16).

The mechanism of gemcitabine resistance in CCA is not studied well and the available information is still limited (17). Therefore, better understanding of gemcitabine resistance mechanisms would allow us to improve therapeutic strategies for CCA. In this study, we established high and stable gemcitabine-resistant CCA cell lines (KKU-M139/GEM and KKU-M214/GEM) and investigated gene expressions associated with gemcitabine transport, metabolism and characterized the biological properties compared with their parental cells. Moreover, the molecular mechanisms and signaling pathways of both cell lines were also evaluated.

Materials and methods

Cell culture. Two human intrahepatic CCA cell lines, KKU-M139 and KKU-M214 were established in the Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Thailand (18-20). Both cell types were cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum, 100 U/ml of penicillin and 100 mg/ml streptomycin (Gibco BRL) at 37°C in 5% CO₂ humidified atmosphere.

Cytotoxicity assay. The parental CCA cells (KKU-M139 and KKU-M214) and their drug-resistant cells (KKU-M139/GEM and KKU-M214/GEM) were seeded at 1x10⁴ cells/well in triplicate into 96-well culture plates (Costar, Cambridge, MA, USA) for 24 h. KKU-M139 and KKU-M139/GEM were treated with 0.025-16 µM of gemcitabine (Lilly Pharmaceuticals, Fegersheim, France), 0.5-64 µM of 5-FU (Boryung Pharm, Seoul, Korea), 0.5-100 µM of doxorubicin (Dabur Pharma Ltd., New Delhi, India), and 0.1-8 µM of paclitaxel (Bristol-Myers-Squibb Co., Princeton, NJ, USA) while KKU-M214

and KKU-M214/GEM were treated with 0.025-64 µM of gemcitabine, 0.5-128 µM of 5-FU, 0.05-16 µM of doxorubicin, and 0.01-0.8 µM of paclitaxel, in triplicate for 72 h. Cells treated with 0.9% normal saline was used as the solvent-control cells. Cytotoxicity was determined using the sulforhodamine B (SRB) assay (21). Drug concentrations required to inhibit 50% cell growth (IC₅₀) were calculated from concentration-effect curves after linear regression analysis. The extent of the resistance of drug-resistant cells to chemotherapeutic drugs was determined as the resistance ratio (RR), which is a ratio of the IC₅₀ value of drug-resistant cells to parental cells.

Induction of gemcitabine-resistant CCA cell lines. KKU-M139 and KKU-M214 cell lines were exposed to the graded concentrations of gemcitabine as described previously (22). In brief, cells (1x10⁵) were cultured in 25-cm² flasks (SPL Life Sciences, Pocheon, Korea) for 24 h and then exposed to gemcitabine at a concentration of 1X IC₅₀ value (0.41 µM for KKU-M139, 0.45 µM for KKU-M214) for 72 h. Surviving cells were cultured in drug-free medium to allow cells to attain 80% confluence. These cells were cultured at this drug concentration until they grew steadily and then the IC₅₀ values of these two CCA cell lines were determined by the SRB assay (21). These surviving cells were then exposed to gemcitabine at 2-fold increase of IC₅₀ concentration for 6 rounds. Two human gemcitabine-resistant CCA cell lines, KKU-M139/GEM and KKU-M214/GEM were obtained after 14 months of culture. KKU-M139/GEM and KKU-M214/GEM grew steadily under the presence of gemcitabine at 6 and 14 µM, respectively. Both drug-resistant CCA cell lines were grown in drug-free medium for two weeks then harvested, frozen in the liquid nitrogen, and stored at -80°C until analyzed. These drug-resistant cells were cultured in drug-free medium for 2 weeks before performing the experiments.

Doubling time. Doubling time of both parental and gemcitabine-resistant CCA cell lines was determined using the trypan blue dye exclusion test. In brief, cells were plated in triplicate at a density of 1x10⁵ cells/well in 6-well plates (SPL Life Sciences) in drug-free medium for 24, 48 and 72 h. At each time-point, cells were harvested, stained with trypan blue and counted on a hemocytometer. Doubling time was calculated using the formula $[(T-T_0) \times \log 2]/(\log N - \log N_0)$ where T is the total time at mid log-phase, T₀ is the time zero, N is the total number of cells at mid log-phase, and N₀ is the total number of cells at the day of plating (19).

Cell cycle analysis. Cells were plated at a density of 1x10⁵ cells/well in 6-well plates for 24 h. Cells were harvested, washed twice with cold phosphate buffered saline (PBS), fixed overnight in 70% ethanol at 4°C, incubated for 30 min in the dark with RNase A (final concentration 2 µg/ml) and propidium iodide (PI) (final concentration 2.4 µg/ml) at room temperature. The cell cycle distribution was examined using a FACScanto™ II flow cytometer (Becton-Dickinson, San Jose, CA, USA) and the data were analyzed using FACS Diva™ software (Becton-Dickinson).

Apoptosis assay. Cells (1x10⁵ cells/well) were plated in triplicate into 6-well plates for 24 h. KKU-M139 and

Table I. Primer sequences used for real-time PCR.

| Gene | Forward | Reverse | Refs. |
|--------------|--------------------------------|--------------------------------|-------|
| <i>hENT1</i> | 5'-TGTGCTTCGGGCCCAAGAA-3' | 5'-TTGCCCCGGAACAGGAAGGA-3' | (19) |
| <i>dCK</i> | 5'-GATGATGTATGAGAAACCTGAACG-3' | 5'-CCAGTCTTGATAAATTGTCCACTC-3' | (24) |
| <i>RRM1</i> | 5'-CACATCAGAACACACATACGAC-3' | 5'-GCACTCTCAAAAGGGTATCTCA-3' | (24) |
| <i>GAPDH</i> | 5'-TCATCAGCAATGCCTCCTGCA-3' | 5'-TGGGTGGCAGTGATGGCA-3' | (19) |

hENT1, human equilibrative nucleoside transporter-1; *dCK*, deoxycytidine kinase; *RRM1*, ribonucleotide reductase M1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

KKU-M139/GEM were then treated with 9 μ M gemcitabine, whereas KKK-M214 and KKK-M214/GEM were treated with 16 μ M gemcitabine for 48 h. Apoptotic cells were determined by the Annexin V-FLUOS staining kit (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions. After treatment, floating and adherent cells were collected, washed twice with cold PBS, resuspended in 100 μ l binding buffer containing 2 μ l Annexin V-FITC and 2 μ l PI (50 μ g/ml) and incubated at room temperature for 15 min in the dark. Then, flow cytometric analysis was performed. Data acquisition and analysis were performed using a FACScanto II flow cytometer and the data were analyzed using FACS Diva software.

Cell adhesion assay. Flat-bottom 96-well plates were coated overnight at 37°C with 30 μ g/well of Matrigel, then washed twice with PBS and blocked at room temperature for 90 min with 3% bovine serum albumin (BSA) (Pierce Biotechnology Inc., Rockford, IL, USA) in serum-free medium. Cells (1×10^4 cells/well) were plated and allowed to adhere for 1 h. The non-adherent cells were removed by washing with PBS and the adherent cells were fixed for 30 min in 20% trichloroacetic acid (TCA) (Sigma Chemical Co., St. Louis, MO, USA). The adherent cells were stained with an SRB dye. The cells were counted under a light microscope, the mean value of 5 random low-power fields ($\times 10$ magnification) was determined. Data were presented as the percentage of adherent cells to total cells. The experiments were performed in triplicate with three independent experiments.

Cell migration and invasion assays. A cell migration and invasion assay were performed using the modified Boyden chamber system. In brief, cells (1×10^5 cells/well) in serum-free medium were plated onto the upper chamber of Transwell® pre-coated Matrigel-culture inserts (8- μ m pore size polycarbonate membrane filters; Becton-Dickinson). The lower chamber contained RPMI-1640 medium supplemented with 10% FBS as a chemo-attractant. After incubation for 48 h, cells on the upper surface of the membrane were wiped away. The invaded cells on the undersurface of the membrane were fixed with 20% TCA, and stained with SRB dye (Sigma Chemical Co.). Cells were counted under a light microscope, and the mean value of 5 randomly selected low-power fields ($\times 20$ magnification) was determined. The migration assay was performed similar to the invasion assay without Matrigel coating on the membrane. The experiments were performed in triplicate using two independent experiments.

Zymographic analysis. MMP-9 activity was determined by gelatin zymography (23). Cells (1×10^5 cells/well) were plated in 6-well plates in RPMI-1640 medium containing 10% FBS for 24 h. Cells at 70% confluence were incubated in serum-free RPMI-1640 medium for 24 h, and the supernatants were collected. The protein concentration of the supernatants was determined by the Coomassie protein assay kit (Pierce Biotechnology). The supernatant protein (20 μ g) was electrophoresed in 7.5% polyacrylamide gels containing 0.1% gelatin and sodium dodecyl sulfate (SDS) under non-reducing conditions. Gels were washed twice at room temperature in 2.5% Triton X-100 with gentle agitation and subsequently incubated in developing buffer (50 mM Tris-HCl, pH 8.0; 5 mM CaCl_2 and 0.02% NaN_3) at 37°C for 20 h. Gels were stained with staining solution (0.5% Coomassie brilliant blue, 30% methanol and 10% acetic acid) and were destained in the same solution without Coomassie brilliant blue. Gelatinase activity of MMPs appeared as clear bands on blue background. Digestion bands were quantitated using the Scion Image program (Scion Corp., Frederick, MD, USA).

The uPA activity was determined by casein-plasminogen zymography (23). In brief, the supernatant was electrophoresed in 7.5% polyacrylamide gels containing 2% casein (w/v) and 20 μ g/ml plasminogen, and then processed as described in the gelatin zymography.

RNA extraction, reverse transcription (RT) and real-time polymerase chain reaction (PCR). Total RNA was extracted from parental and drug-resistant cells using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The first-strand cDNA was synthesized from RNA by reverse transcription at 42°C for 60 min in a 20- μ l reaction mixture containing 3 μ g of total RNA, 0.5 μ g of oligo(dT)₁₅ primer (Amersham Pharmacia Biotechnology Inc., Piscataway, NJ, USA), 20 U RNasin ribonuclease inhibitor and 20 U of Moloney murine leukemia virus (MMLV) reverse transcriptase (Fermentas, Ontario, Canada) in 5X polymerase chain reaction (PCR) buffer, 10 mM dNTPs and 3 mM MgCl_2 .

Real-time PCR was conducted according to the manufacturer's instruction in a final volume of 25 μ l containing 2X SYBR Green PCR Master Mix (Roche Applied Science, Mannheim, Germany), cDNA template and 5 pmol of each primer using a LightCycler® 480 Instrument (Idaho Technology, Inc., UT, USA). The PCR primers for *hENT1*, *dCK*, *RRM1* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Integrated DNA Technologies, Coralville, IA,

Table II. Primary antibody used in western blot analysis.

| Target | Type | Host species/targeted species | Dilution | Second antibody |
|--------------------|------------|-------------------------------|----------|------------------|
| Cyclin B1 | Polyclonal | Rabbit anti-human | 1:400 | Goat anti-rabbit |
| Cdk1 | Polyclonal | Rabbit anti-human | 1:400 | Goat anti-rabbit |
| p21 | Polyclonal | Rabbit anti-human | 1:400 | Goat anti-rabbit |
| Bcl-2 | Polyclonal | Rabbit anti-human | 1:200 | Goat anti-rabbit |
| Bax | Polyclonal | Rabbit anti-human | 1:200 | Goat anti-rabbit |
| FAK | Polyclonal | Rabbit anti-human | 1:400 | Goat anti-rabbit |
| p-FAK | Polyclonal | Rabbit anti-human | 1:200 | Goat anti-rabbit |
| MMP-9 | Monoclonal | Mouse anti-human | 1:1,000 | Goat anti-mouse |
| NF- κ B/p65 | Polyclonal | Rabbit anti-human | 1:500 | Goat anti-rabbit |
| PKC | Polyclonal | Rabbit anti-human | 1:500 | Goat anti-rabbit |
| Fas | Polyclonal | Rabbit anti-human | 1:400 | Goat anti-rabbit |
| FADD | Polyclonal | Rabbit anti-human | 1:400 | Goat anti-rabbit |
| MRP1 | Polyclonal | Rabbit anti-human | 1:200 | Goat anti-rabbit |
| p53 | Monoclonal | Mouse anti-human | 1:1,000 | Goat anti-mouse |
| ERK1/2 | Polyclonal | Rabbit anti-human | 1:400 | Goat anti-rabbit |
| p-ERK1/2 | Polyclonal | Rabbit anti-human | 1:400 | Goat anti-rabbit |
| β -actin | Monoclonal | Mouse anti-human | 1:3,000 | Goat anti-mouse |
| Histone H1 | Monoclonal | Mouse anti-human | 1:500 | Goat anti-mouse |

Cdk1, cyclin-dependent kinase 1; p21, protein 21; Bcl-2, B-cell lymphoma protein 2; Bax, Bcl-2 associated X protein; FAK, focal adhesion kinase; p-FAK, phosphorylated FAK; MMP-9, matrix metalloproteinase-9; NF- κ B/p65, nuclear factor- κ B/p65; PKC, protein kinase C; Fas, fatty acid synthetase; FADD, Fas-associated death domain; MRP1, multidrug resistance-associated protein 1; p53, protein 53; ERK1/2, extra-cellular signal-regulated kinase-1/2; p-ERK1/2, phosphorylated ERK1/2.

USA) are given in Table I (19,24). Real-time PCR started with a denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min, then final extension at 72°C for 10 min. To verify amplification products, a melting curve was constructed from 60 to 99°C. The comparative Ct method was used to calculate a relative expression of target mRNA as described previously (25). The mRNA expressions of target genes were normalized to the *GAPDH* gene and given as fold change in gene expression level between parental and drug-resistant cells. All data were analyzed using LightCycler 480 software, version 1.5 (Roche Applied Science).

Preparation of total cell, cytosol or nuclear lysates or membrane proteins. To prepare total cell lysate, cells were rinsed twice with PBS and lysed by vigorous shaking at 4°C for 20 min with cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate) containing protease inhibitors cocktail (Pierce Biotechnology) and phosphatase inhibitors cocktail (Pierce Biotechnology). Cell lysates were centrifuged at 13,000 g at 4°C for 30 min, and the supernatants were collected. To prepare cytosol lysate, cells were lysed with ice-cold buffer A (10 mM HEPES, 0.1 mM EDTA, 10 mM KCl, 0.2% NP40, 1.5 mM MgCl₂, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride), followed by vortexing and centrifugation at 13,000 g at 4°C for

30 min. Supernatants were collected as cytosolic fraction. The nuclear pellets were extracted with ice-cold buffer B (20 mM HEPES, 1.5 mM MgCl₂, 25% glycerol, 0.1 mM EDTA, 1 mM DTT, 420 mM NaCl, and 0.5 mM phenylmethylsulfonyl fluoride). Nuclear proteins in the supernatant were collected after centrifugation at 13,000 g for 30 min at 4°C. The final pellets were resuspended in buffer C (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 250 mM sucrose, 10% glycerol, 0.1% sodium deoxycholate, 0.5% NP-40, 1 mM sodium orthovanadate) and membrane proteins in the supernatant were collected after centrifugation at 13,000 g at 4°C for 30 min. All lysates were stored at -80°C until use. Protein concentration was determined using the Coomassie protein assay kit (Pierce Biotechnology).

Western blotting. Equal amounts of proteins were separated in a 7.5% or 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes (Millipore, Billerica, MA, USA). After blocking with Tris-buffered saline containing 0.1% Tween-20 and 5% skimmed milk at room temperature for 1 h, the blots were incubated at 4°C overnight with primary antibodies against Cyclin B1, Cdk1, p21, Bcl-2, Bax, FAK, p-FAK, MMP-9, NF- κ B/p65, PKC, Fas, FADD and MRP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or p53 (Upstate, CA, USA) or ERK1/2, p-ERK1/2 (Cell Signaling Technology Inc., Beverly, MA, USA) or β -actin (Sigma Chemical Co.) or Histone H1 (Abcam, Cambridge, UK). These

Table III. IC₅₀ values and resistant ratio in parental and gemcitabine-resistant cell lines to various chemotherapeutic drugs.

| Drugs | IC ₅₀ value (μM) | | | | | | | |
|-------------|-----------------------------|--------------|-------|---------|------------|--------------|-------|---------|
| | KKU-M139 | KKU-M139/GEM | RR | p-value | KKU-M214 | KKU-M214/GEM | RR | p-value |
| Gemcitabine | 0.41±0.006 | 10.16±0.10 | 25.88 | <0.001 | 0.45±0.006 | 28.04±0.68 | 62.31 | <0.01 |
| 5-FU | 3.31±0.08 | 26.12±0.17 | 7.89 | <0.01 | 3.76±0.16 | 79.73±2.15 | 21.20 | <0.001 |
| Doxorubicin | 3.59±0.40 | 27.69±0.40 | 7.71 | <0.01 | 0.74±0.06 | 13.48±1.3 | 18.22 | <0.05 |
| Paclitaxel | 0.45±0.06 | 3.13±0.35 | 6.96 | <0.05 | 0.05±0.006 | 0.36±0.04 | 7.2 | <0.01 |

NS, not significant difference. RR, resistant ratio.

primary antibodies are listed and characterized in Table II. Blots were incubated at room temperature for 1 h with the horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology), washed, and were visualized by an enhanced chemiluminescence kit (Pierce Biotechnology). Band intensity was visualized and captured using the Imagequant™ LAS 4000 (GE Healthcare, Piscataway, NY, USA) and analyzed using the Scion Image program. Relative intensity was determined and normalized to β-actin (total cell, cytosolic and membrane lysates) or Histone H1 (nuclear lysate).

Statistical analysis. Statistical analyses of parental and gemcitabine-resistant cells were performed using Student's t-test, *p<0.05 indicates a significant difference. Comparison between two CCA cell lines were analyzed by Mann-Whitney test, #p<0.05 indicates a significant difference.

Results

Establishment of gemcitabine resistance in cholangiocarcinoma cell lines. Gemcitabine-resistant cell lines were established from parental KKU-M139 and KKU-M214 cell lines by exposure to stepwisely increasing concentrations of gemcitabine over a period of 14 months. The increment of gemcitabine concentration was from 0.40 to 12.80 μM for KKU-M139 cells, and from 0.45 to 14.40 μM for KKU-M214 cells. The established resistant cells were designated as KKU-M139/GEM and KKU-M214/GEM. No apparent morphological differences were observed between parental and gemcitabine-resistant cells. The growth rate of both gemcitabine-resistant cells was slightly slower than that of their parental cells. The population doubling time of parental KKU-M139 and resistant KKU-M139/GEM cell lines were 17.36±1.26 and 20.32±2.79 h, and that of parental KKU-M214 and resistant KKU-M214/GEM cell lines were 22.68±2.20 and 25.21±1.88 h, respectively.

The sensitivity of the parental and gemcitabine-resistant cells to gemcitabine, 5-FU, doxorubicin and paclitaxel was examined. The IC₅₀ values and the resistance ratios for the gemcitabine-resistant cells and the parental cells are shown in Table III. Both KKU-M139/GEM and KKU-M214/GEM were remarkably resistant to gemcitabine compared with their parental cells. KKU-M214/GEM was more resistant to gemcitabine with resistant ratio (RR) of 62.31 compared with KKU-M139/GEM with RR of 25.88 (Table III). In addition,

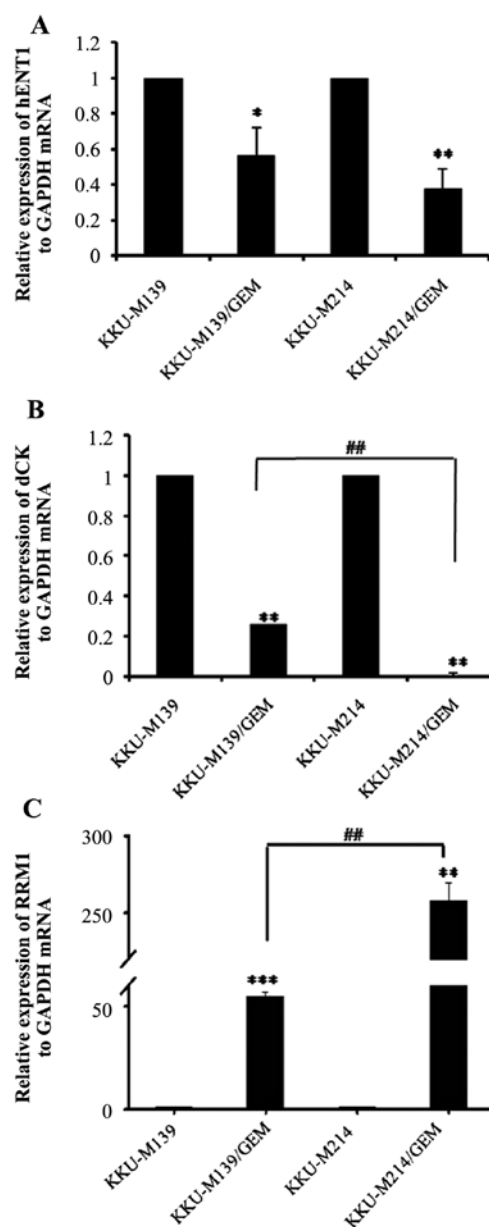


Figure 1. Decreased mRNA expression of (A) *hENTI* and (B) *dCK* while increased mRNA expression of (C) *RRM1* in KKU-M139/GEM and KKU-M214/GEM compared with the parental cells by real-time PCR. *GAPDH* was amplified as an internal control. Data are expressed as the mean ± SD of three independent experiments. Statistical analyses of parental and gemcitabine-resistant cells were performed using Student's t-test, *p<0.05, **p<0.01, ***p<0.001 indicate a significant difference. Comparison between two CCA cell lines were analyzed by Mann-Whitney test, ##p<0.01 indicates a significant difference.

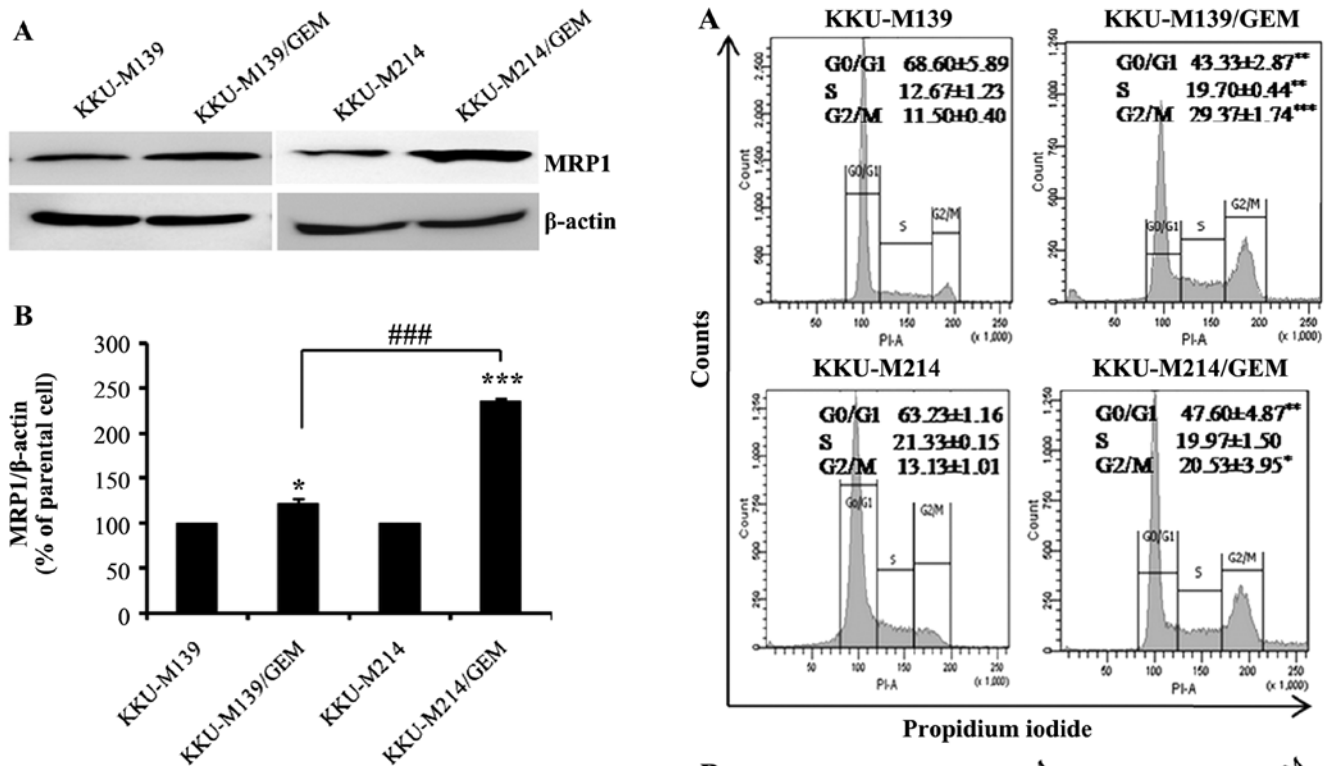


Figure 2. Expression level of MRP1 protein was higher in KKKU-M139/GEM and KKKU-M214/GEM compared with their parental cells. (A) Western blot analysis for MRP1 protein was performed in KKKU-M139, KKKU-M139/GEM, KKKU-M214, KKKU-M214/GEM. (B) The band intensity of MRP1 protein was quantified by densitometry and normalized to β-actin. Data are expressed as mean ± SD of three independent experiments. Statistical analyses of parental and gemcitabine-resistant cells were performed using Student's t-test, *p<0.05, ***p<0.001 indicate a significant difference. Comparison between two CCA cell lines were analyzed by Mann-Whitney test, ###p<0.001 indicates a significant difference.

both resistant cell lines showed cross-resistance against 5-FU, doxorubicin and paclitaxel, suggesting their acquisition of a potential multidrug-resistant (MDR) phenotype. IC₅₀ values of gemcitabine of both resistant cells remained unchanged after sequential passages in gemcitabine-free medium for 2 months with the IC₅₀ value of 10.26±0.17 μM for KKKU-M139/GEM, and that of 26.62±0.16 μM for KKKU-M214/GEM. Similar stability was also observed after storage of the cells at -80°C for 3 months with the IC₅₀ values of 10.27±0.04 μM for KKKU-M139/GEM and that of 27.08±0.41 μM for KKKU-M214/GEM.

Alteration of *hENT1*, *dCK* and *RRM1* mRNA expression in gemcitabine-resistant CCA cell lines. The expression levels of *hENT1*, *dCK* and *RRM1* mRNA in the parental and gemcitabine-resistant cells were determined using real-time RT-PCR. The expression levels of *hENT1* and *dCK* mRNA in both gemcitabine-resistant cell lines were significantly lower than that of their parental cells (p<0.05, Fig. 1). In contrast, the expression levels of *RRM1* mRNA in the gemcitabine-resistant cells were significantly higher than that of their respective parental cells (p<0.01, Fig. 1). KKKU-M214/GEM showed lower expression levels of *hENT1* and *dCK* mRNA (p<0.01, Fig. 1B) and significantly higher expression level of *RRM1* mRNA (p<0.01, Fig. 1C) than KKKU-M139/GEM.

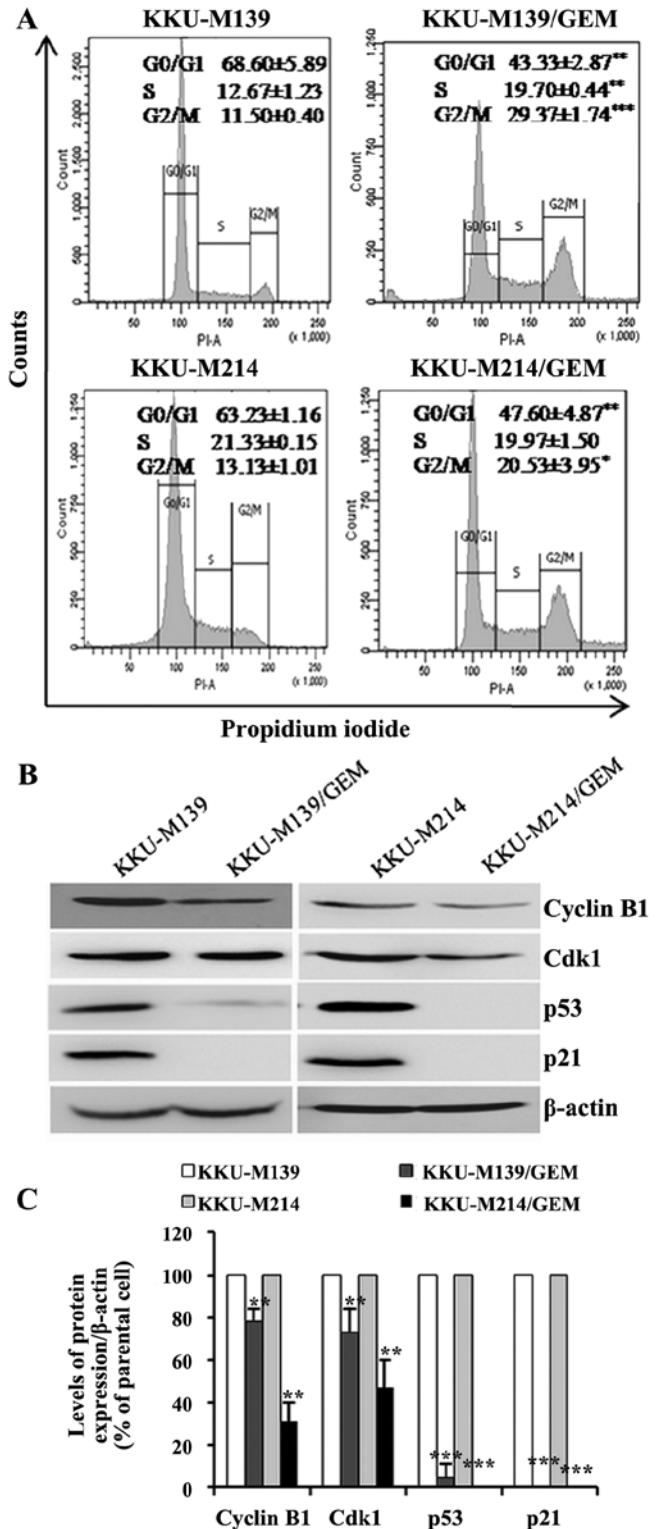


Figure 3. Change in cell cycle distribution and downregulation of G2/M phase-regulated proteins in KKKU-M139/GEM and KKKU-M214/GEM cells. (A) KKKU-M139 and KKKU-M139/GEM (top panel) and KKKU-M214 and KKKU-M214/GEM (bottom panel) were placed onto the 6-well plates in drug-free medium for 24 h and flow cytometry was performed. (B) Western blot analysis of G2/M phase regulated protein expression and subsequent densitometry are shown. The densities of cyclin B1, Cdk1, p53 and p21 were decreased in KKKU-M139/GEM and KKKU-M214/GEM compared with their parental cells. (C) Densitometric scanning of bands after normalization to β-actin demonstrated cyclin B1, Cdk1, p53 and p21 protein expressions in KKKU-M139/GEM and KKKU-M214/GEM. All data are expressed as mean ± SD of three separated experiments. Statistical analyses of parental and gemcitabine-resistant cells were performed using Student's t-test, *p<0.05, **p<0.01, ***p<0.001 indicate a significant difference.

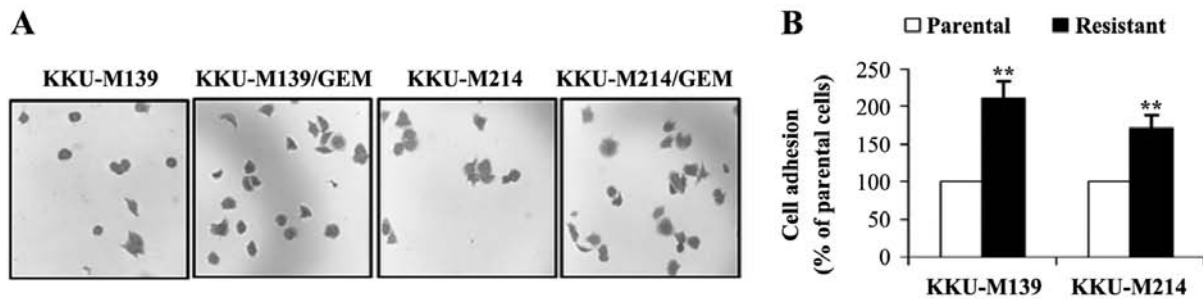


Figure 4. Cell adhesion was increased in KKU-M139/GEM and KKU-M214/GEM cells compared with their parental cells. (A) Representative images of SRB-stained adhesive cells determined by adhesion assay. (B) Percentage (%) of cell adhesion compared to the parental cells. Statistical analyses of parental and gemcitabine-resistant cells were performed using Student's t-test, ** $p < 0.01$ indicates a significant difference.

MRP1 protein expression is increased in gemcitabine-resistant CCA cell lines. The MRP1 protein expression in parental and gemcitabine-resistant cell lines were evaluated using western blot analysis. Compared to the parental cells, the MRP1 protein expression was significantly higher in gemcitabine-resistant cells ($p < 0.05$, Fig. 2).

Change in cell cycle distribution in gemcitabine-resistant CCA cell lines. Cell cycle distributions in parental and gemcitabine-resistant cells were determined using flow cytometry. The proportion of the cells in S and G2/M phases were significantly higher in drug-resistant KKU-M139/GEM (19.7 ± 0.44 and $29.37 \pm 1.74\%$) compared with those of parental cells (12.67 ± 1.23 and $11.5 \pm 0.40\%$) ($p < 0.01$, Fig. 3A). In KKU-M214/GEM cells, the cells in G2/M phase ($20.53 \pm 3.95\%$) was significantly higher than that ($13.13 \pm 1.01\%$) in KKU-M214 cells, but the proportions of the cells in G0/G1 in KKU-M139/GEM and KKU-M214/GEM cells (43.33 ± 2.87 and $47.60 \pm 4.87\%$) were significantly lower than those of KKU-M139 and KKU-M214 cells (68.60 ± 5.89 and $63.23 \pm 1.16\%$) ($p < 0.01$, Fig. 3A).

Downregulation of G2/M phase-regulated proteins in gemcitabine-resistant CCA cell lines. Western blot analyses showed that the protein expression of cyclin B1, cyclin-dependent kinase 1 (Cdk1), p53 and p21 was significantly lower in gemcitabine-resistant cells compared to the parental cells ($p < 0.01$, Fig. 3B and C), suggesting the possible role of cyclin B1, Cdk1, p53 and p21 proteins in causing G2/M phase cell cycle arrest in the drug resistant cell lines.

Enhancement of cell adhesion in gemcitabine-resistant CCA cell lines. The ability to adhere to basement membrane is an important characteristic of cancer metastasis. In the cell adhesion assay, the numbers of drug-resistant KKU-M139/GEM and KKU-M214/GEM cells adhered to Matrigel was, respectively, 2.10- and 1.72-fold higher than that of their parental cell lines ($p < 0.01$, Fig. 4).

Apoptotic evasion in gemcitabine-resistant CCA cell lines. Apoptosis induction in parental and gemcitabine-resistant CCA cell lines after treatment with gemcitabine was evaluated using Annexin V-FITC/PI staining. The percentage of apoptotic cells in KKU-M139/GEM cells after $9 \mu\text{M}$ gemcitabine treatment was 0.45-fold lower than that in parental KKU-M139 cells

($p < 0.001$, Fig. 5A and B). Similarly, the percentage of apoptotic cells in KKU-M214/GEM cells after $16 \mu\text{M}$ gemcitabine treatment was 0.39-fold lower than that in KKU-M214 cells ($p < 0.001$, Fig. 5A and B). These results indicate apoptotic evasion in KKU-M139/GEM and KKU-M214/GEM.

Aberration of apoptotic regulation in gemcitabine-resistant CCA cell lines. To gain insight into the possible mechanisms of apoptotic evasion in drug-resistant cells, the apoptotic regulated protein expressions of both intrinsic and extrinsic pathways were determined using western blot analyses. In the intrinsic pathway, p53 (Fig. 3B and C) and Bax were downregulated, while Bcl-2 was upregulated in both KKU-M139/GEM and KKU-M214/GEM compared with the parental cells ($p < 0.05$, Fig. 5C and D). In the extrinsic pathway, the FADD protein expression was not different between the parental and gemcitabine-resistant cells. Interestingly, Fas was downregulated in KKU-M214/GEM ($p < 0.001$) but not in KKU-M139/GEM cells (Fig. 5C and D). These results suggest that KKU-M214/GEM evades apoptosis via both intrinsic and extrinsic pathways, whereas KKU-M139/GEM evades apoptosis through an intrinsic pathway.

Enhancement of migration and invasion in gemcitabine-resistant CCA cell lines. The ability of CCA cells to migrate and invade the basement membrane and surrounding tissues is an important characteristics of cancer metastasis. These properties of gemcitabine-resistant cell lines and their parental cell lines were determined using a Boyden chamber assay system. Using the Transwell system, the number of migrated cells of KKU-M139/GEM was 1.89-fold higher than that of the parental cells, whereas the KKU-M214/GEM was 1.98-fold higher than those of parental cells ($p < 0.05$, Fig. 6B). Using the Matrigel invasion assay, the number of invaded cells of KKU-M139/GEM and KKU-M214/GEM were 1.82-fold and 1.72-fold higher than that in the respective parental cells ($p < 0.05$, Fig. 6D).

Increased MMP-9 and uPA activities in gemcitabine-resistant CCA cell lines. The proteolytic activity of MMP-9 of the parental and gemcitabine-resistant cell lines were measured by gelatin zymography. The MMP-9 activity in KKU-M139/GEM and KKU-M214/GEM was 1.55- and 1.21-fold higher than that in the parental cells ($p < 0.05$, Fig. 7A and B). Using western blot

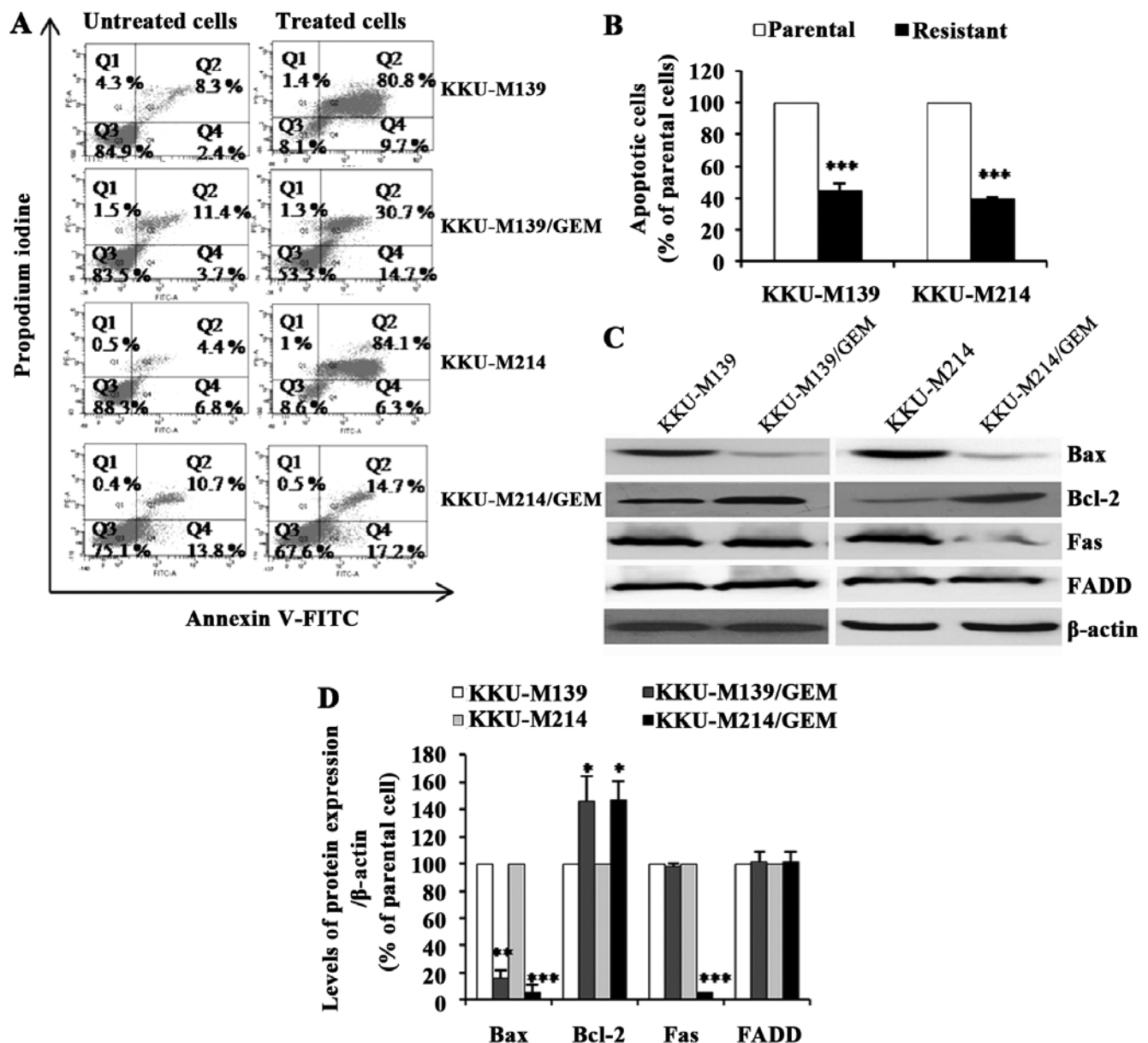


Figure 5. Apoptosis induction was decreased in KKU-M139/GEM and KKU-M214/GEM cells compared with their parental cells. KKU-M139 and KKU-M139/GEM were treated with 9 μ M gemcitabine, whereas KKU-M214 and KKU-M214/GEM were treated with 16 μ M gemcitabine. (A) Apoptotic cells in gemcitabine-treated parental and gemcitabine-resistant cells were determined by Annexin V-FITC/PI staining visualized: viable and non-apoptotic cells (Q3), early apoptotic cells (Q4), late apoptotic cells (Q2) and necrotic cells (Q1). (B) Percentages of apoptotic cells (Q2+Q4) in KKU-M139/GEM and KKU-M214/GEM were significantly lower than KKU-M139 and KKU-M214 cells. (C) Western blot analysis showed apoptosis regulated protein expression levels. Bax expression was decreased while Bcl-2 expression was increased in KKU-M139/GEM and KKU-M214/GEM compared with their parental cells. Fas expression was decreased in KKU-M214/GEM but was not changed in KKU-M139/GEM. (D) The band intensity of the proteins was quantitated by densitometry and normalized to β -actin. Data are expressed as mean \pm SD of three independent experiments. Statistical analyses of parental and gemcitabine-resistant cells were performed using Student t-test, * p <0.05, ** p <0.01, *** p <0.001 indicate a significant difference.

analyses, MMP-9 protein expression in KKU-M139/GEM and KKU-M214/GEM was 2.54- and 1.39-fold higher, respectively, than that in the parental cells (p <0.01, Fig. 7C and D). These results show that an increased enzymatic activity of MMP-9 was due to increased protein expression. The uPA activity in KKU-M139/GEM and KKU-M214/GEM is, respectively, 2.17-fold and 1.81-fold higher than that of the parental cells (p <0.01, Fig. 7E and F), as determined by casein-plasminogen zymography. These findings suggest that higher activities of MMP-9 and uPA may play a role in higher migration and inva-

sion capacities in KKU-M139/GEM and KKU-M214/GEM compared to the parental cells.

PKC signaling pathway plays an important role in apoptosis evasion, cell adhesion, migration and invasion. Cell lysates from the parental and gemcitabine-resistant cell lines were analyzed for protein expression by western blot analyses. Protein levels of all PKC fractions (cytosolic and membrane), FAK and p-FAK were significantly higher in KKU-M139/GEM and KKU-M214/GEM cells compared to those in the respec-

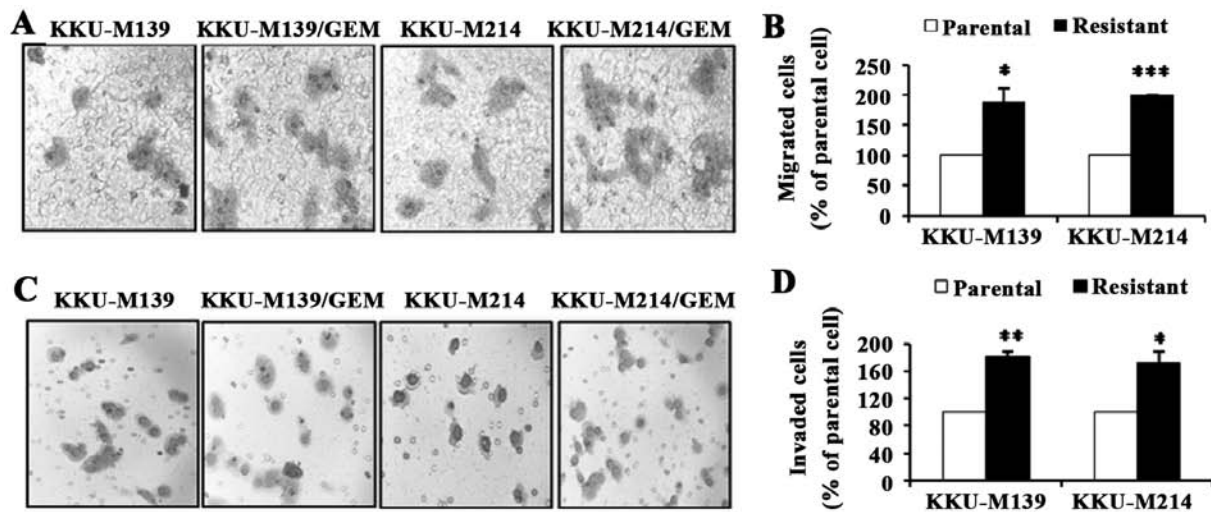


Figure 6. Migration and invasion capacities were higher in KKU-M139/GEM and KKU-M214/GEM compared with their parental cells. (A) Representative images of SRB-stained migrated cells determined by migration assay using the modified Boyden chamber assay (x200). (B) Percentage (%) of cell migration compared to parental cells. (C) Representative images of SRB-stained invaded cells determined by invasion assay using the modified Boyden chamber assay (x200). (D) Percentage (%) of cell invasion compared to parental cell. The experiments were performed in triplicate of two independent experiments. All data are expressed as mean \pm SD. Statistical analyses of parental and gemcitabine-resistant cells were performed using Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate a significant difference.

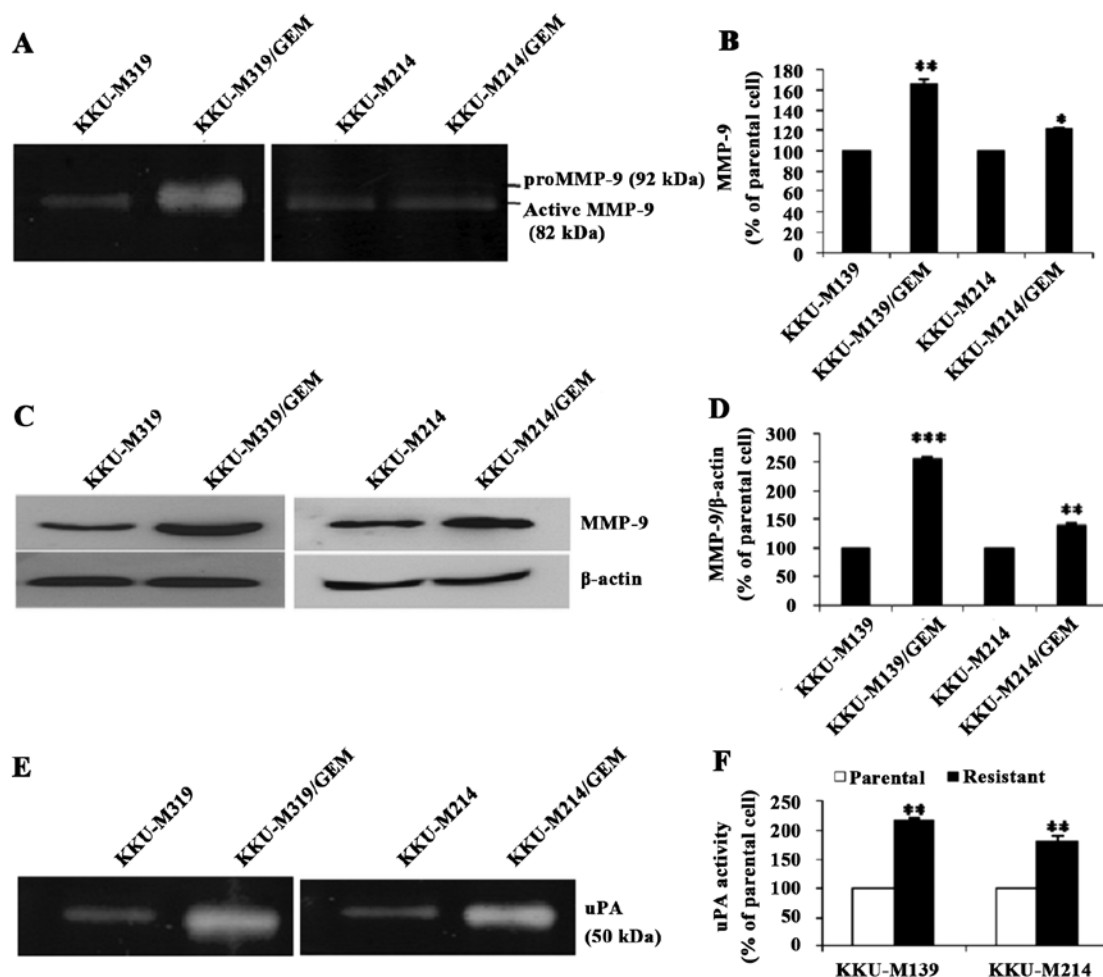


Figure 7. Activities of MMP-9 and uPA and protein expression of MMP-9 were higher in KKU-M139/GEM and KKU-M214/GEM compared with their parental cells. (A) Gelatinolytic activity of MMP-9 in the condition medium was determined by gelatin zymography. (B) The band intensities of the active MMP-9 were quantified by densitometry. (C) Protein expression of MMP-9 was determined by western blot analysis. (D) The band intensity of the MMP-9 was quantitated by densitometry and normalized to β -actin. (E) uPA activity in the conditioned medium was determined by casein-plasminogen zymography. (F) The band intensity of uPA activity was quantitated by densitometry. Data are expressed as mean \pm SD of three independent experiments. Statistical analyses of parental and gemcitabine-resistant cells were performed using Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate a significant difference.

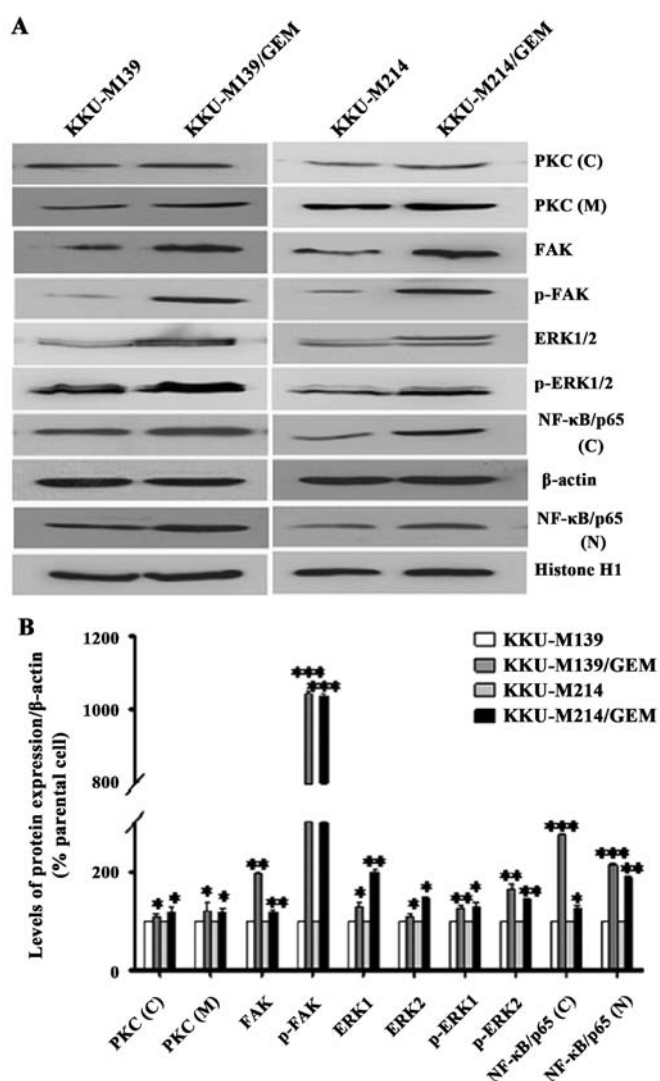


Figure 8. Activation of PKC signaling pathways in KKKU-M139/GEM and KKKU-M214/GEM. (A) Cell lysates from the parental and gemcitabine-resistant cell lines were analyzed for protein expression by western blot analyses. The protein expression levels of PKC (C, cytosolic), PKC (M, membrane), FAK, p-FAK, ERK1/2, p-ERK1/2, NF-κB/p65 (C, cytosolic) and NF-κB/p65 (N, nucleus) were higher in KKKU-M139/GEM and KKKU-M214/GEM compared with their parental cells. (B) The band intensity of the proteins was quantitated by densitometry and normalized to β-actin (total cell, cytosolic and membrane lysates) and Histone H1 (nuclear lysate). Data are expressed as mean ± SD of three independent experiments. Statistical analyses of parental and gemcitabine-resistant cells were performed using Student's t-test, *p<0.05, **p<0.01, ***p<0.001 indicate a significant difference.

tive parental cells (p<0.05, Fig. 8). In the PKC downstream signaling pathway, protein levels of ERK1/2, p-ERK1/2 and fractions of NF-κB/p65 (cytosolic and nuclear) were significantly higher in KKKU-M139/GEM and KKKU-M214/GEM cells compared to those of the respective parental cells (p<0.05, Fig. 8). These results suggest that higher expression of PKC membrane fraction, p-FAK, p-ERK1/2 and NF-κB nuclear fraction may play role in higher protein level of Bcl-2, MMP-9 and uPA in KKKU-M139/GEM and KKKU-M214/GEM cells compared to the parental cells. These findings suggest the signaling pathways involved in apoptosis evasion, cell adhesion, migration and invasion of gemcitabine-resistant CCA cell lines as proposed in Fig. 9.

Discussion

In this study, we successfully established highly gemcitabine-resistant CCA cell lines, from the respective parental CCA cell lines, KKKU-M139 and KKKU-M214. The IC₅₀ values of KKKU-M139/GEM and KKKU-M214/GEM were 10.16 and 28.04 μM, respectively, which was far higher than those of previously reported gemcitabine-resistant cell lines, HuCCT1 and YSCC, for which IC₅₀ was 0.26 and 0.04 μM, respectively (17). The gemcitabine-resistance remained stable after continuous culture in drug-free medium for 2 months, and was also stable even following storage at -80°C for 3 months.

Acquired gemcitabine resistance involved the alteration of gemcitabine transporter and disruption of gemcitabine metabolism to convert its active form. In our results, *RRM1* was upregulated and *hENT1* and *dCK* were downregulated, suggesting that *hENT1*, *dCK* and *RRM1* play an important role in gemcitabine resistance. In non-small cell lung cancer cells, expression of *hENT1* and *dCK* was decreased whereas *RRM1* was increased in association with acquired gemcitabine-resistance (8,11).

The importance of ATP-binding cassette (ABC) transporters in mediating drug efflux is well documented (26). MRP expression was associated with decreased intracellular accumulation of various chemotherapeutic agents (27). Our results demonstrated that both of the gemcitabine-resistant cells overexpressed MRP1 protein compared to their parental cells. Overexpression of MRP1-3 has been reported in CCA (18,28). Thus, upregulation of MRP1 led to an increase of drug efflux resulted in multidrug resistance. Even though these anticancer drugs are different in their structure and anticancer mechanisms, the disruption of drug transporter function contribute to an acquired MDR in CCA cells. Hence, KKKU-M139/GEM and KKKU-M214/GEM showed cross-resistance to several anticancer drugs simultaneously, for example; 5-FU, doxorubicin and paclitaxel. 5-FU and gemcitabine share similar molecular structure, mechanisms of action and metabolic characteristics (15). This might explain why gemcitabine-resistant CCA cells can become cross-resistant to 5-FU. Paclitaxel is primarily an M-phase-specific drug that stabilize microtubules, resulting in an arrest of the cell cycle in M-phase and apoptosis (29). The G2/M arrest of gemcitabine-resistant CCA cells prevents cells entering M-phase where paclitaxel is mostly active and gain an advantage for acquired paclitaxel resistance. Doxorubicin can only target cell cycling and proliferation. Therefore, doxorubicin may lose anticancer activity if the gemcitabine-resistant CCA cells are prevented from entering cell cycle by G2/M-phase arrest. The association of decreased expression of cyclin B1 with doxorubicin resistance was reported in breast cancer cells (30).

Extension of the population doubling time in chemoresistance has been reported in a variety of cancer cells (5,31). The prolonged proliferating time of the gemcitabine-resistant CCA cell lines reported here seems to have resulted from cell cycle arrest at G2/M phase because of decreased cyclin B1 and Cdk1 expression. Similar findings have been reported in adriamycin-resistant CCA cell line (QBC939/ADM) (32). Transition from G2 to M phase of the cell cycle is regulated by the Cdk1/cyclin B1 complex and CDKIs including p21 and p27 (33). P53 is a key tumor suppressor protein which is known

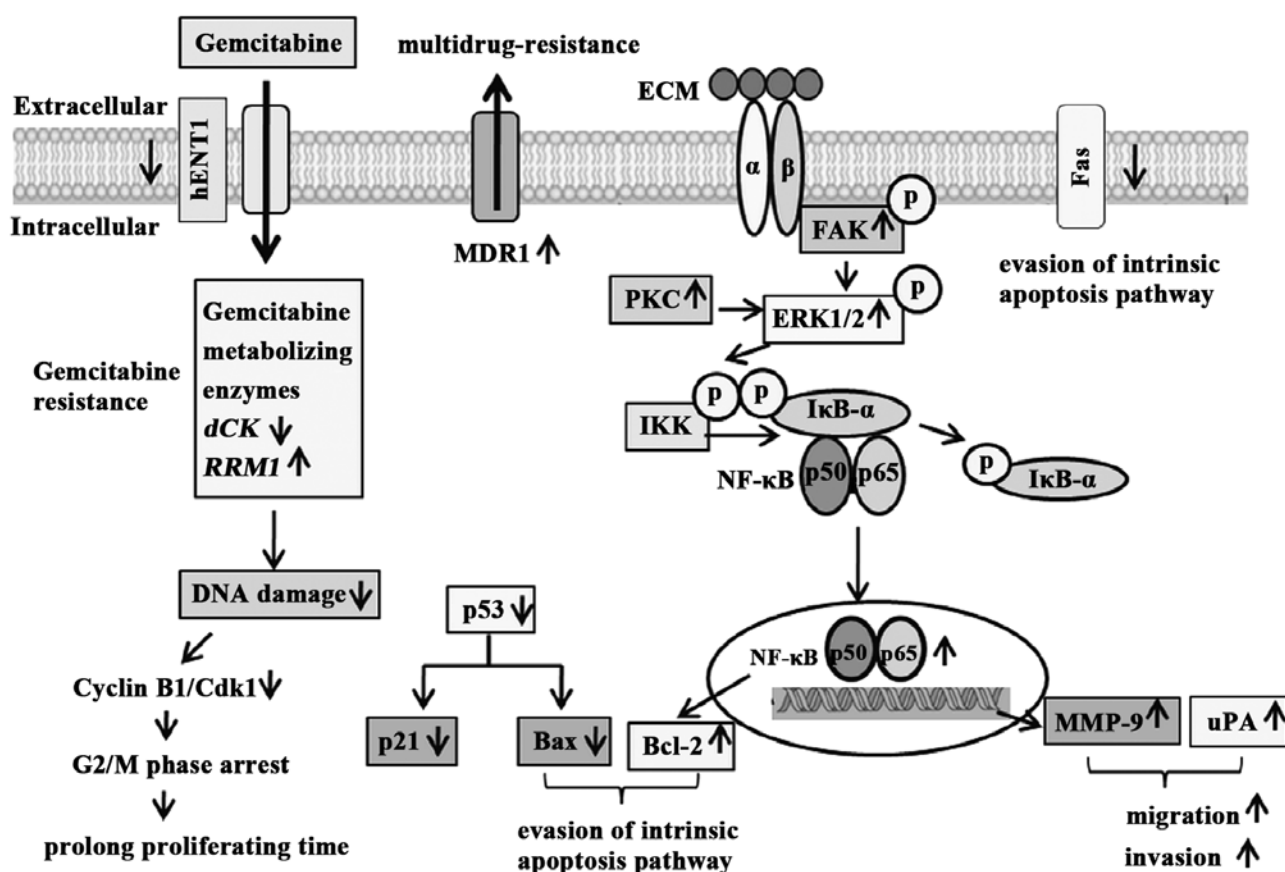


Figure 9. A possible mechanism by which CCA mediates gemcitabine resistance and invasiveness.

as a transcriptional regulator of p21 and Bax (34). Our results also suggested the downregulation of p21 via decreased p53. Loss of p21 expression after chemotherapy was observed in human resistant tumor models harboring wild-type p53 (35). Taken together, the altered cell cycle profile of gemcitabine-resistant CCA cell lines may provide some advantages from killing by anticancer drugs. G2 arrest allows damage repair and eventual resumption of the cell cycle progression, preventing cells from anticancer activity of chemotherapeutic drugs.

Evasion of apoptosis via intrinsic and extrinsic pathway has been evident as the usual mechanism for chemoresistance (13). In this study, after treatment with gemcitabine, the percentage of apoptotic cells in both of the gemcitabine-resistant CCA cells were significantly decreased compared to their parental cells. In the mitochondrial (intrinsic) apoptosis pathway, the Bcl-2 family proteins including anti-apoptotic proteins (i.e., Bcl-2, Mcl-1 and Bcl-XL) and pro-apoptotic proteins (i.e., Bax) played an important role (14). An increase in the ratio of anti- to pro-apoptotic Bcl-2 proteins has been detected in various cancers and has been correlated to tumor cell survival and apoptosis resistance (14). Acquired resistance of pancreatic cancer cell line, Capan-1, against gemcitabine was associated with upregulation of the anti-apoptotic genes bcl-XL and mcl-1 and a higher ratio of Bcl-2/Bax expression (15). In this study, downregulation of p53 expression may cause decreased Bax protein expression which is the target gene product induced by p53. Our present data indicate that

resistance to chemotherapeutic drugs of two CCA cell lines is at least partly mediated via the increase of Bcl-2 protein expression and decrease of p53 and Bax protein expression. These changes lead to increased Bcl-2/Bax ratio and evasion of the intrinsic apoptosis pathway.

In the death receptor (extrinsic) apoptosis pathway, ligation of death receptors to the tumor necrosis factor (TNF) receptor super-family (i.e., CD95 or Fas) by the corresponding ligands (i.e., CD95 ligand or Fas ligand) leads to the recruitment of proteins such as Fas-associated death domain (FADD) and caspase-8 and subsequently activation of caspase-8, caspase-3 leading to apoptotic cell death (36). In this study, Fas expression was significantly decreased in KKU-M214/GEM but was unaltered in KKU-M139/GEM compared with their parental cells. In contrast, the FADD expression was not changed in either of the gemcitabine-resistant CCA cells. These results indicate that decreased expression of Fas in KKU-M214/GEM may prevent the transmission of the death signal from the cell surface to intracellular signaling cascades leading to evasion of the extrinsic apoptosis pathway. Decreased expression of Fas has been reported in chemotherapeutic drug-resistant human renal cell carcinoma (37). It is interesting to note that KKU-M214/GEM may evade apoptosis through both intrinsic and extrinsic pathways, whereas, KKU-M139/GEM may evade apoptosis only through the intrinsic pathway. These results may explain the higher IC_{50} values of gemcitabine in KKU-M214/GEM ($28.04 \pm 0.68 \mu M$) than in KKU-M139/GEM ($10.16 \pm 0.10 \mu M$). Additionally, decrease of Fas expression

would also allow cells to evade immune responses mediated by both T and NK cells.

Invasion of cancer cells into basement membranes is a critical step in metastasis. The key markers that are involved in degradation of the ECM are the serine protease (plasmin), urokinase plasminogen activator (uPA) system, cysteine protease (cathepsin B and L) and matrix metalloproteinases (MMPs) (38). In our study, the established resistant cell lines demonstrated the relationship between drug resistance and cancer invasion. Both KKKU-M139/GEM and KKKU-M214/GEM exhibited an aggressive phenotype, i.e., higher migration and invasion capabilities with higher MMP-9 and uPA activities, similar to those in various cancer cell lines reported previously (39,40). The exposure of the human nasal carcinoma cell line RPMI-2650 to melphalan, an alkylating agent, resulted in not only an MDR phenotype but also enhancement of cell invasion *in vitro*. However, paclitaxel (Taxol) exposure promoted MDR without altered cell invasiveness (39). Osmak *et al* reported increased cathepsin D, uPA and plasminogen activator inhibitor-1 (PAI-1) in a drug-resistant cervical and laryngeal cell line, although they did not investigate the invasive/metastatic potential of these cells (40).

Environmental-mediated drug resistance (EMDR) is thought to be triggered by the adhesion of integrins from tumor cells to stromal fibroblast or to components of the surrounding extracellular matrix (ECM) (41). The signaling could be due to degradation of apoptotic proteins or enhanced stability or altered subcellular distribution of anti-apoptotic proteins and cell cycle regulators (42,43). During adhesion, ligation of integrin with ECM ligands trigger PKC translocation from cytosol to the intracellular membrane (44). PKC is a family of serine/threonine kinases associated with the regulation of cell cycle progression either during the G1/S or G2/M transition, cell adhesion, migration, invasion and drug resistance (45). Elevated levels of PKC and its downstream, FAK, have been shown in various cancer cells (44,45). FAK is a cytoplasmic tyrosine kinase that plays an important role in integrin-mediated signaling. It is the key mediator of several signaling pathways, especially ERK1/2, which is in the mitogen-activated protein kinases (MAPK) pathway (46). ERK1/2 also regulates transcription factor NF- κ B indirectly by phosphorylating inhibitor of NF- κ B kinase- α (IKK- α). IKK- α subsequently phosphorylates inhibitor of NF- κ B (I κ B), leading to ubiquitination and degradation of I κ B- α . This promotes the translocation of NF- κ B from the cytoplasm to the nucleus driving the transcription of its target genes (47,48). Our results confirm previous finding that all fractions of PKC expression, FAK phosphorylation, ERK1/2 phosphorylation, two fractions of NF- κ B/p65 expression were increased in both gemcitabine-resistant CCA cells suggesting the involvement of the PKC and MAPK signaling pathways. Additionally, ERK1/2 signaling is responsible for resistance to gemcitabine in other CCA cells (49). Also in CCA (SCK^R) cells, the activation of AKT and ERK1/2 signaling induced by cisplatin is likely to be the mechanism of cisplatin resistance, while the activation of ERK1/2 signaling is involved in 5-FU and gemcitabine resistance (50). The possible signaling pathways involved in the mediation of gemcitabine resistance of CCA are shown in Fig. 9.

In conclusion, the establishment of CCA cell lines highly resistant to gemcitabine was achieved using stepwise long-term exposure to the ascending series of gemcitabine. The established cell lines harbor multidrug resistance and invasiveness. The mechanism of enhanced drug resistance may include MRP1 expression, activation of the PKC signaling pathway, NF- κ B activation and its downstream targets resulting in prolongation of proliferation time, evasion from apoptosis, and an increase of cancer invasiveness. An increased understanding of relationship between drug resistance and cancer cell invasiveness will ultimately lead to a consideration of means by which induction of the aggressive phenotype should be avoided. Finally, molecular targets for circumvention of multidrug resistance and invasion are revealed for possible targeted therapy for CCA.

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