

13-*cis*-retinoic acid inhibits the self-renewal, migration, invasion and adhesion of cholangiocarcinoma cells

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Abstract. 13-*cis*-retinoic acid (13CRA), a Food and Drug Administration-approved drug for severe acne, is currently being investigated for its potential use in skin cancer prevention. 13CRA has been reported to exhibit antitumor effects against various types of cancer cells, both *in vitro* and *in vivo*. However, to the best of our knowledge, no information is yet available regarding the effects of 13CRA on cholangiocarcinoma (CCA), a malignancy of the bile duct epithelia. Currently, there are no reliably effective therapeutic options for metastatic CCA. The present study thus aimed to evaluate the effects of 13CRA on the self-renewal, migration, invasion and adhesion of CCA cells, and also investigated the underlying mechanisms. The results revealed that 13CRA suppressed cell proliferation via the inhibition of the self-renewal ability of CCA cells. 13CRA induced cell cycle arrest at the G₂/M phase in KKU-100 and KKU-213B CCA cells through the regulation of cell cycle-regulatory genes and proteins. 13CRA reduced the cell migratory ability of both cell lines via the modulation of the genes and proteins associated with epithelial-mesenchymal transition. 13CRA also inhibited the invasive and adhesive abilities of CCA cells via the suppression of genes and proteins associated with the invasion and adhesion of CCA cells. On the whole, these results suggested that 13CRA exerts suppressive effects on CCA cell proliferation, migration, adhesion and invasion.

Introduction

13-*cis*-retinoic acid (13CRA), a member of the family of vitamin A compounds (1), plays a role in several cellular processes,

such as cell proliferation, differentiation and apoptosis (2). At present, 13CRA, a Food and Drug Administration-approved drug for severe acne, is a readily available and well-tolerated agent that has been reported to exhibit antitumor potential both *in vitro* and *in vivo* in various types of cancer (3-8). Previous preclinical studies have demonstrated that 13CRA inhibits the proliferation of cancer cells, including small-cell lung, gastric and breast cancer cells (4-6). In addition, 13CRA induces apoptosis and exerts a suppressive effect on the metastasis of melanoma and colon cancer (7,8). 13CRA has been investigated for its anticancer activity for several years, and its potential mechanisms of action have been explored in various cancer types; however, to the best of our knowledge, there is no report available to date on the anticancer activity and potential use of 13CRA in cholangiocarcinoma (CCA). Thus, information on the roles of 13CRA in CCA cells may provide critical, useful evidence for further studies.

CCA is an aggressive tumor with a poor prognosis due to its late clinical presentation and lack of effective non-surgical therapies (9). Several risk factors for CCA have been identified, including primary sclerosing cholangitis, hepatobiliary parasites, hepatolithiasis, Caroli disease, choledochal cysts and Thorotrast (10). In Asia, CCA is associated with chronic infection with the liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis*, which is a main risk factor for CCA development (11). The majority of patients with CCA are diagnosed at a late state; at this stage, the metastasis of CCA has already occurred. Surgical treatment is not suitable for these patients, and only palliative treatment is possible (9). For patients with advanced and metastatic CCA, the therapeutic option is adjuvant chemotherapy, and the standard regimen with gemcitabine/cisplatin combination therapy leads to an overall survival time of only ~1 year (12). Thus, there is a critical need for the identification of novel and effective therapies for patients with CCA at advanced and metastatic phases of the disease. Among numerous efforts conducted to date aimed at improving the efficacy of CCA treatment, targeting hallmarks of CCA carcinogenesis, such as sustaining proliferative ability, epithelial-mesenchymal transition (EMT), stemness and plasticity properties have particularly attracted attention.

Cell proliferation is a key aspect of cancer development and progression, and the self-renewal capacity of cancer cells has been linked to cancer recurrence (13). Sustained

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proliferative signaling via c-Myc, an oncogenic molecule, has been shown to result in the altered expression or activity of cell cycle-related genes or proteins, which can stimulate growth abnormality and cancer progression (13,14). Previous studies have suggested the anti-proliferative effects of 13CRA on cancer cells, including breast and gastric cancer cells (5,6). Furthermore, 13CRA has been shown to induce the cell cycle arrest of the immortalized sebocyte cell line, SEB-1, at the G₁ phase via the upregulation of p21 and the downregulation of cyclin D1 proteins (15).

EMT is a cell plasticity-promoting phenomenon that enables cancer cells (of epithelial origin) to acquire mesenchymal features with invasive properties, which leads to metastatic colonization (16,17). In CCA, the expression of EMT-related transcription factors, such as Snail, a zinc finger E-box-binding homeobox and Twist, is associated with a poor prognosis (18). CCA exhibits several mesenchymal phenotypic features, known to be associated with an increased motility and invasiveness; these features are also associated with mechanisms involving the downregulation of the epithelial phenotypic molecules, E-cadherin (E-cad) and β -catenin, and the upregulation of the EMT-related transcription factors, Snail1, Twist and S100 calcium binding protein A4 (19). The downregulation of E-cad in CCA cells also increases the expression of vimentin, a mesenchymal marker, which subsequently leads to an increased migration (20). Upon detachment from the primary tumor through EMT, cancer cells leave their primary site and initiate the metastatic process of cancer progression (16). The metastasis cascade is generally a complex process involving five key steps of metastasis: Migration/invasion, intravasation, survival/circulation, extravasation and colonization/proliferation (21). The process of metastasis requires multiple steps, such as the degradation of the extracellular matrix by matrix metalloproteinases (MMPs), particularly MMP-1, MMP-2, MMP-3 and MMP-9, which are abundantly released in malignant cholangiocytes (9). Furthermore, cell surface adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) also participate in the binding, recognition and adhesion of cells, which are involved in the metastatic process (16,22). The expression of the inflammatory molecule, cyclooxygenase-2 (COX-2), is induced in response to various stimuli, including inflammatory cytokines and MMPs, which are associated with cancer metastasis (23-25). Previously, CCA has been reported to exhibit an aggressive behavior, and to be associated with MMP-9, ICAM-1 and COX-2, suggesting the suppression of these molecules as targets for CCA treatment (26,27).

The present study aimed to evaluate the effects of 13CRA on the proliferation, migration, adhesion and invasion of CCA cells. In addition, the potential mechanisms underlying the cancer-suppressive effects of 13CRA on CCA cells were investigated.

Materials and methods

CCA cell lines and culture. For the present study, two human CCA cell lines, K KU-100 and K KU-213B, were kindly provided by Professor Banchob Sripana (Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand). Cell line authentication was performed by short tandem repeat (STR) analysis. The DNA markers of

23 STR loci and the sex marker, amelogenin, were analyzed using the AmpFLSTR Identifier PCR Amplification kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). DNA STR analysis of the K KU-213B and K KU-100 cells has been previously described (28-30) and the profile was similar to the STR profile of K KU-213 cells deposited in JCRB Cell Bank, Japan. The K KU-213B cells are tumor cell variants similar to the K KU-213 cells; Sripana *et al.* (29) demonstrated that the K KU-213B cells exhibited a greater motility than the K KU-213 cells, as evidenced by Boyden chamber assays. The cells were routinely cultured in Ham's F12 medium (Gibco; Thermo Fisher Scientific, Inc.; pH 7.4) supplemented with 1 mM sodium bicarbonate, 10 mM HEPES, 100 U/ml penicillin G, 50 μ g/ml gentamicin sulfate and 10% (v/v) fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.), and were maintained under 5% CO₂ in a humidified incubator at 37°C. Cells were sub-cultured every 2-3 days at 80% confluency using 0.25% (v/v) trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.).

Sulforhodamine B (SRB) assay. The CCA cells were seeded into 96-wells at 7.5x10³ cells/well, incubated overnight and then treated with 0.00, 1.25, 2.50, 5.00, 10.00 and 20.00 μ M 13CRA (R6256; MilliporeSigma) in serum-free medium (Ham's F12 medium without 10% FBS) for 12, 24 and 48 h under 5% CO₂ in a humidified incubator at 37°C. Following treatment, the cells were incubated with ice-cold 10% trichloroacetic acid (T6399; MilliporeSigma) at 4°C for 1 h, followed by washing with deionized (DI) water and staining with 0.4% SRB in 1% acetic acid at room temperature for 30 min. The SRB dye inside the cells was solubilized with 10 mM Tris-base (pH 10.5) solution, and the absorbance was then measured at 540 nm using a microplate reader (Sunrise™ Elisa Plate Reader; Tecan Group, Ltd.). The experiments were independently performed \geq 3 times. Cell viability was expressed as a percentage of cell cytotoxicity relative to the untreated control, which was considered as no having cytotoxicity. The half-maximal inhibitory concentration (IC₅₀) was calculated by fitting the dose-response curve using SigmaPlot v12 Software (SigmaPlot for Windows; Systat Software, Inc.).

Clonogenic survival assay. The CCA cells were seeded into a six-well plate at a density of 2.5x10⁵ cells/well overnight and treated with 0.00, 0.312, 0.625, 1.25, 2.50 and 5.00 μ M 13CRA in serum-free medium for 48 h. Following treatment, the CCA cells were trypsinized, and the viable-treated cells were seeded into a new six-well plate at a density of 600 cells/well in serum-containing medium. The cells were incubated in 5% CO₂ in a humidified incubator at 37°C for an additional 8 days, and the culture medium was freshly renewed every 3 days. Finally, the colonies were fixed with absolute methanol for 30 min at 4°C and stained with 0.25% crystal violet (61135, Sigma-Aldrich; Merck KGaA) in 2% ethanol at room temperature for 30 min. Images of the colonies were captured using the ChemiDoc™ MP Imaging system (Bio-Rad, Laboratories, Inc.) and the number of colonies was then counted using Image-Pro Plus v4.5.0.29 software (Media Cybernetics). The colony forming ability was calculated as a percentage by comparison with the untreated control cells. The experiments were performed independently three times.

Cell cycle distribution analysis by propidium iodide (PI) staining. The CCA cells were seeded in a six-well plate at a density of 2.5×10^5 cells/well overnight. The following day, the cells were treated with 0.00, 1.25 and $2.50 \mu\text{M}$ 13CRA in serum-free medium for 48 h. At the end of treatment, the cells were collected, washed twice with PBS and fixed with 70% ethanol at -80°C for 4 h. The cell suspension was then maintained at -20°C for 3 h before being subjected to staining with a PI solution containing 0.5% Triton-X in PBS, 0.02 mg/ml PI and 0.02 mg/ml RNase A. The cell suspension was incubated for 1 h at 4°C in the dark. The percentage of cells in the different phases of the cell cycle (cell cycle distribution) was analyzed using a BD FACS Canto™ II flow cytometer and FACSDiva™ software version V6.1.3 (both from BD Biosciences). Three independent experiments were performed.

Cell adhesion assay. The CCA cells were seeded in a six-well plate at a density of 2×10^5 cells/well overnight and treated with 0.00, 1.25 and $2.50 \mu\text{M}$ 13CRA in serum-free medium for 48 h. Following treatment, the CCA cells were harvested and re-plated into a fibronectin-coated 96-well plate at a density of 2×10^4 cells/well. Following 30 min of incubation at 37°C , the non-adherent cells were removed. The adhered cells were washed gently with PBS, fixed with ice-cold absolute methanol and washed with DI water prior to staining with 0.25% crystal violet at room temperature for 30 min. The stained cells were captured under an Eclipse TS100 microscope (Nikon Corporation). Finally, the crystal violet was solubilized with 10% methanol in 5% glacial acetic acid solution, and the absorbance was measured at 540 nm using a microplate reader (Sunrise™ Elisa Plate Reader; Tecan Group, Ltd.). The percentage of cell adhesion was calculated by comparison with the untreated control cells.

Transwell migration assay. The CCA cells were seeded into the upper chamber of a Transwell plate (8.0 μm Pore Polycarbonate Membrane Insert, 3422, Corning, Inc.) at a density of 2×10^4 cells/well in serum-free medium and were allowed to attach to the membrane overnight. On the following day, the cells were treated with 0.00, 1.25 and $2.50 \mu\text{M}$ 13CRA in serum-free medium, while the bottom chamber was filled with serum-containing medium without 13CRA. Following 24 h of incubation at 37°C , the cells were fixed with absolute methanol and stained with 0.25% crystal violet in 2% ethanol at room temperature for 30 min. The non-migrated cells were gently removed using a cotton swab, and the number of migrated cells on the bottom side of the membrane of the Transwell plate was quantified. The image of migrated cells was captured under an Eclipse TS100 microscope (Nikon Corporation), the number of cells was then counted using Image-Pro Plus v4.5.0.29 program (Media Cybernetics, Inc.) and the percentage of migrated cells was calculated by comparison with the untreated control cells. A total of three independent experiments were performed.

Matrigel invasion assay. The CCA cells were seeded into the upper chamber of a Transwell plate coated with Matrigel at a density of 2×10^4 cells/well in serum-free medium, and were allowed to attach to the thin layer of Matrigel overnight. The cells were treated with 0.00, 1.25 and $2.50 \mu\text{M}$ 13CRA in serum-free

medium, while the lower part of the chamber was filled with serum-containing medium without 13CRA. Following incubation for 48 h at 37°C , the invaded cells were fixed with absolute methanol and stained with 0.5% crystal violet in 2% ethanol at room temperature for 30 min, while the non-invaded cells and the thin layer of Matrigel on the upper Transwell chamber were gently removed using a cotton swab. The number of invaded cells on the bottom side of the Transwell plate was quantified under a light microscope. The number of invaded cells was counted, and the percentage of invaded cells was calculated by comparison with the untreated control cells. A total of three independent experiments were performed.

Reverse transcription-quantitative PCR (RT-qPCR). The CCA cells were seeded in a six-well plate at a density of 2.5×10^5 cells/well overnight and then treated with 0.00, 1.25 and $2.50 \mu\text{M}$ 13CRA in serum-free medium for 24 h. Following treatment, total RNA was isolated using TRIzol™ reagent (15596026, Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. cDNA synthesis was performed in a C1000™ thermal cycler (Bio-Rad Laboratories, Inc.) using 5X iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Inc.) following the manufacturer's instructions. cDNA served as a template for qPCR amplification. RT-qPCR was performed using Light Cycler® 480II/384 (Roche Applied Science). The thermocycling conditions were as follows: 95°C for 3 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 31 sec, 1 cycle of melting curve (95°C for 5 sec, 72°C for 5 sec, and 97°C continuous), and a cooling cycle (40°C for 10 min). To verify the purity of the products, a melting curve analysis was performed after each run. The specific primers used are listed in Table SI. The expression of target genes was calculated and represented as a ratio to the expression of the housekeeping reference gene, β -actin. The experiment was performed independently three times.

Western blot analysis. The CCA cells were seeded in a six-well plate at a density of 2.5×10^5 cells/well overnight and treated with 0.00, 1.25 and $2.50 \mu\text{M}$ 13CRA in serum-free medium. Following treatment, the cells were lysed with RIPA cell lysis buffer containing 1X Halt™ Protease Inhibitor Cocktail (87786; Thermo Fisher Scientific, Inc.). The samples were then centrifuged at $13,000 \times g$, 4°C for 30 min and supernatants were collected and stored at -80°C until use. The protein concentration of the cell lysate solutions was determined using Bradford protein assay (Bio-Rad Laboratories, Inc.) following the manufacturer's instructions. A total of 30 μg of protein from whole cell lysates was loaded onto 10% SDS-PAGE and separated using SE 260 mini-vertical gel electrophoresis (Hoefer, Inc.). For western blotting, proteins in the gel were transferred onto PVDF membranes using Owl™ HEP-1 Semidry Electrobloetter (HEP-1; Thermo Fisher Scientific, Inc.). The membranes were incubated with the primary antibodies. Following incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies (all primary and secondary antibodies, and conditions are presented in Table SII), the protein bands were developed and detected using Luminata™ Forte Western HRP Substrate (MilliporeSigma), and imaged using the ChemiDoc™ MP Imaging system (Bio-Rad, Laboratories, Inc.). The intensity of the protein bands was analyzed using

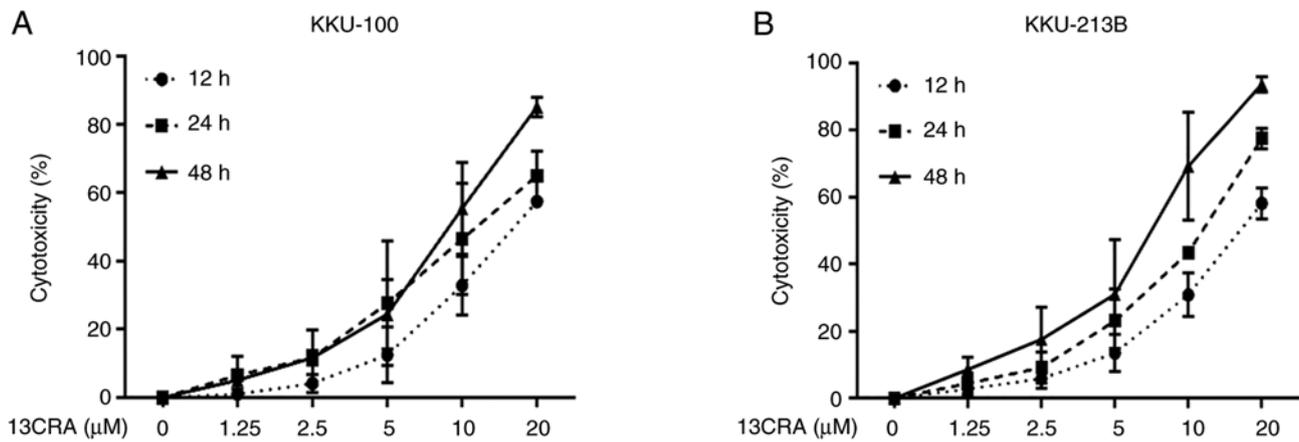


Figure 1. Cytotoxicity of 13CRA on cholangiocarcinoma cells. (A) KKU-100 and (B) KKU-213B cells were treated with various concentrations of 13CRA for 12, 24 and 48 h. Cell viability was measured following treatment using sulforhodamine B assay, and the percentage of cytotoxicity was calculated. Data are presented as the mean \pm SD from three independent experiments. 13CRA, 13-*cis*-retinoic acid.

Image Lab software version 6.0 (Bio-Rad Laboratories, Inc.) and expressed as a ratio to the housekeeping reference protein, β -actin. The experiments were independently performed ≥ 3 times.

Statistical analysis. Data are expressed as the mean \pm standard deviation of ≥ 3 independent experiments. Comparisons between the untreated control and the treatment groups were performed using one-way ANOVA followed by Tukey's post hoc test using SigmaStat v2.0 software (Systat Software Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of 13CRA on the viability of CCA cells. In the present study, two CCA cell lines (KKU-100 and KKU-213B) were treated with increasing concentrations of 13CRA ranging from 1.25 to 20 μ M for 12, 24 and 48 h. The results revealed that 13CRA suppressed the viability of both CCA cell lines in a concentration- and time-dependent manner (Fig. 1). The half-maximal inhibitory concentrations (IC_{50}) of 13CRA for the viability of the KKU-100 cells at 12, 24 and 48 h were 20.48 ± 8.27 , 12.35 ± 3.60 and 9.33 ± 7.53 μ M, respectively. The IC_{50} values of 13CRA for the viability of the KKU-213B cells at 12, 24 and 48 h were 20.27 ± 16.34 , 11.89 ± 9.20 and 6.66 ± 5.11 μ M, respectively. 13CRA is a well-tolerated agent for severe acne; however, the absence of experiments on non-cancerous cells used as a negative control is a limitation of the present study. To explore the tumor-suppressive activity of 13CRA at concentrations lower than the IC_{50} values, concentrations of 1.25 and 2.5 μ M 13CRA were used in the subsequent experiments.

Effects of 13CRA on the clonogenic self-renewal ability of CCA cells. To identify and quantify the effects of 13CRA on the self-renewal ability of CCA cells, a clonogenic survival assay was performed. Following treatment with 13CRA for 48 h, only the viable 13CRA-treated cells were cultured for an additional 8 days to allow colony formation. The results

revealed that 13CRA significantly reduced the colony formation ability of both CCA cell lines in a concentration-dependent manner. At a concentration of 2.5 μ M, which induced minimal cytotoxicity (10-15%) in the KKU-100 and KKU-213B cells, 13CRA markedly suppressed colony formation compared with that of the untreated control cells ($>55\%$; Fig. 2). These results indicated that 13CRA exerted a potent inhibitory effect on the self-renewal ability of CCA cells.

Effect of 13CRA on the cell cycle progression of CCA cells. Since the present study demonstrated that 13CRA exerted an anti-proliferative effect on CCA cells by inhibiting their self-renewal ability, it was further investigated whether this effect of 13CRA was caused by alterations of the cell cycle. Thus, the cell cycle of 13CRA-treated and untreated CCA cells was analyzed using flow cytometry. The results revealed that 1.25 and 2.5 μ M 13CRA induced significant cell cycle arrest at the G_2/M phase, and decreased the number of cells at the G_1 phase in both CCA cell lines (Fig. 3) compared with the untreated control cells. These findings suggested that the 13CRA-induced cell cycle arrest at the G_2/M phase resulted in the inhibition of the proliferation of CCA cells.

Effects of 13CRA on the expression of cell cycle-regulatory genes in CCA cells. Since the present study demonstrated the inhibitory effects of 13CRA on the cell cycle progression of CCA cells, the effects of 13CRA on the mRNA expression of cell cycle-regulatory genes, including *p21*, *c-Myc* and *cyclin B1*, were further investigated using RT-qPCR. The results demonstrated that, following treatment with 13CRA for 48 h, the expression of *p21*, a cyclin-dependent kinase inhibitor, was upregulated, while the expression of the cell cycle activator genes *c-Myc* and *cyclin B1* was downregulated ($P < 0.05$; Fig. 4). These results suggested that cell cycle arrest upon incubation with 13CRA was partly mediated by the altered expression of cell-cycle regulatory genes.

Effects of 13CRA on the expression of cell cycle-regulatory proteins in CCA cells. To further confirm the effect of 13CRA on cell cycle regulation, the expression of the cell cycle-related

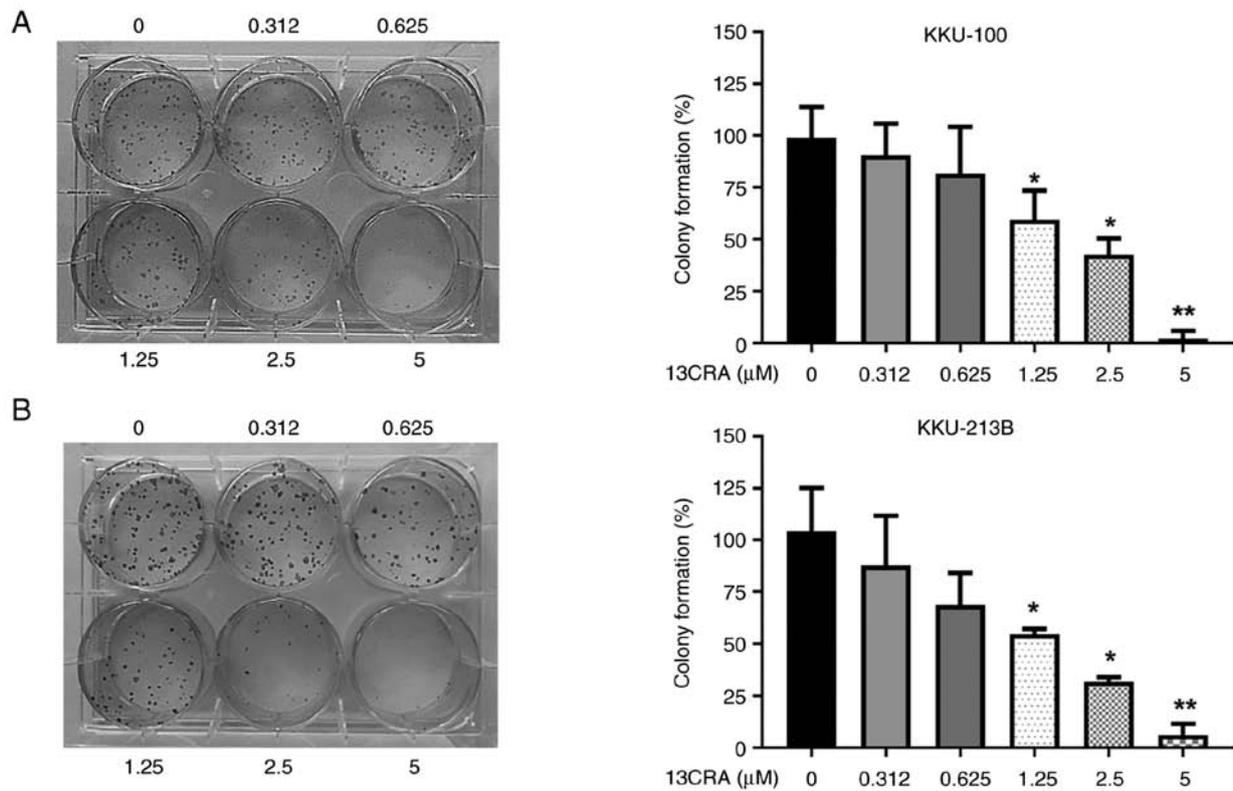


Figure 2. 13CRA inhibits the self-renewal ability of cholangiocarcinoma cells. (A) KKU-100 and (B) KKU-213B cells were treated with various concentrations of 13CRA for 48 h. The viable-treated cells were harvested, and only 600 cells were cultured into each well for an additional 8 days to allow colony formation. The number of colonies was counted and calculated as the percentage of colonies formed compared to the untreated control. Data are presented as the mean \pm SD from three independent experiments. * $P<0.05$ and ** $P<0.01$ vs. the untreated control. 13CRA, 13-*cis*-retinoic acid.

proteins, including p53, p21, cyclin B1 and cyclin D1, was evaluated using western blot analysis. The levels of p53 (the upstream regulator of the cell cycle) and p21 (the cell cycle inhibitor) were significantly increased ($P<0.05$) in both the KKU-100 (Fig. 5A-C) and KKU-213B (Fig. 5A, E and F) cells. Conversely, the levels of cyclin B1 (the G_2/M cell cycle driver) were significantly decreased compared with those of the untreated control cells (Fig. 5A, D and G). Thus, in addition to the effects of 13CRA on the expression of the *p21*, *c-Myc* and *cyclin B1* genes, 13CRA also induced an increase in the expression of the cell cycle inhibitors, p53 and p21, as well as a decrease in the G_2/M driver cyclin B1, which eventually led to cell cycle arrest in the G_2/M phase. Since 13CRA induced a decrease in the number of cells at the G_1 phase of the cell cycle, the present study investigated the expression of cyclin D1, which is the regulatory protein of the G_1 phase of the cell cycle. Following treatment with 13CRA for 48 h, the protein level of cyclin D1 significantly decreased in both CCA cell lines ($P<0.05$) compared with that of the untreated control cells (Fig. 5H-J).

Effects of 13CRA on the migration of CCA cells. The present study then evaluated the effects of 13CRA on the migratory ability of CCA cells using Transwell migration assay. The results revealed that 13CRA significantly suppressed the migration of both KKU-100 and KKU-213B CCA cells compared with that of the untreated control cells (Fig. 6). At a concentration of 1.25 μ M, which exerted markedly low cytotoxicity at 48 h of treatment, 13CRA inhibited almost

50% of the cells migrating through the Transwell membrane. These results indicated that 13CRA potentially suppressed the migratory ability of CCA cells.

Effects of 13CRA on the expression of EMT-related genes and proteins in CCA cells. EMT is a cell plasticity-promoting phenomenon that allows cancer cells to migrate (16,17). Since the present study demonstrated the inhibitory effects of 13CRA on the migratory ability of CCA cells, it then investigated the effects of 13CRA on the mRNA expression of EMT-related genes, including the epithelial marker, *E-cad*, and the mesenchymal markers, *Snail* and *vimentin*, using RT-qPCR. Treatment with 13CRA significantly upregulated the expression of the epithelial marker gene, *E-cad*, while it downregulated the expression of the mesenchymal marker genes, *Snail* and *vimentin*, in both the KKU-100 (Fig. 7A-C) and KKU-213B (Fig. 7D-F) cells ($P<0.05$). Furthermore, the effects of 13CRA on the expression of two major proteins regulating EMT transition (*E-cad* and *vimentin*) were investigated using western blot analysis. The results revealed that 13CRA increased the expression of the epithelial marker protein, *E-cad*, and decreased the expression of the mesenchymal marker protein, *vimentin*, in both CCA cell lines (Fig. 7G). These results indicated that 13CRA modulated the expression of EMT-related genes and proteins in CCA cells.

Effects of 13CRA on the invasion and adhesion of CCA cells. Since cell invasion and adhesion are critical steps contributing to the metastatic phenotype (16), the present study further

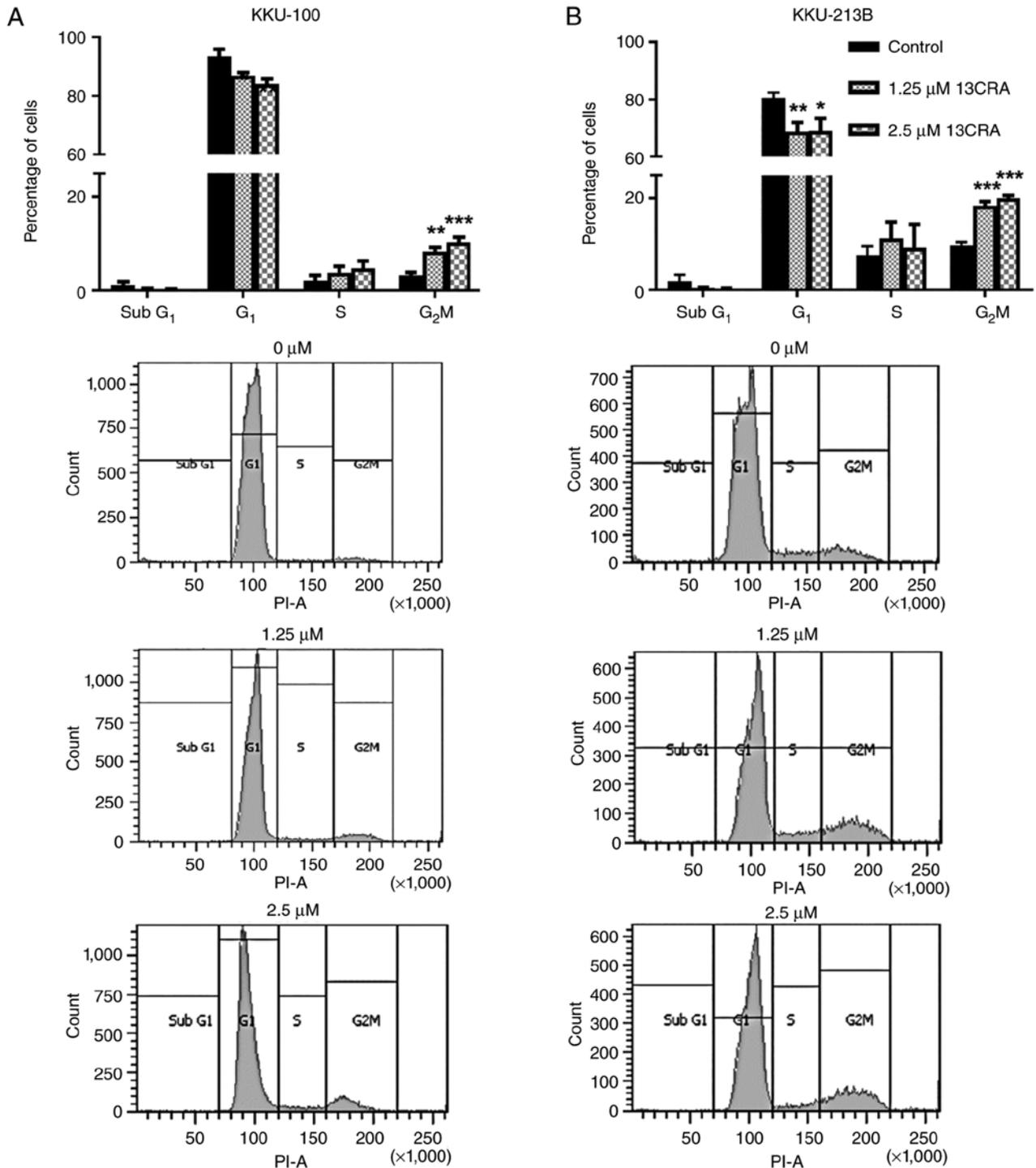


Figure 3. 13CRA arrests the cell cycle at the G₂/M phase in cholangiocarcinoma cells. (A) KKKU-100 and (B) KKKU-213B cells were treated with 1.25 or 2.5 μM 13CRA for 48 h, and the cell cycle distribution was then examined using flow cytometry with propidium iodide DNA staining. Data are presented as the mean ± SD from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. the untreated control. 13CRA, 13-*cis*-retinoic acid.

examined the effects of 13CRA on these two properties in CCA cells using Matrigel invasion and cell adhesion assays. When both CCA cell lines (KKU-100 and KKKU-213B) were treated with 1.25 and 2.5 μM 13CRA for 48 h, the number of invaded cells through the Transwell membrane coated with Matrigel was significantly (P<0.05) reduced compared with that of the untreated controls (Fig. 8A and B). Similarly, 13CRA treatment induced a significant decrease in the percentage of adherent cells on the surface of the culture plate in both CCA

cell lines compared with that of the untreated control cells (Fig. 8C and D).

Effects of 13CRA on the expression of invasion- and adhesion-related genes and proteins in CCA cells. Since 13CRA treatment induced a significant inhibition of the invasion and adhesion properties of CCA cells, RT-qPCR was performed to explore the effects of 13CRA on the mRNA expression of adhesion- and invasion-related genes, including

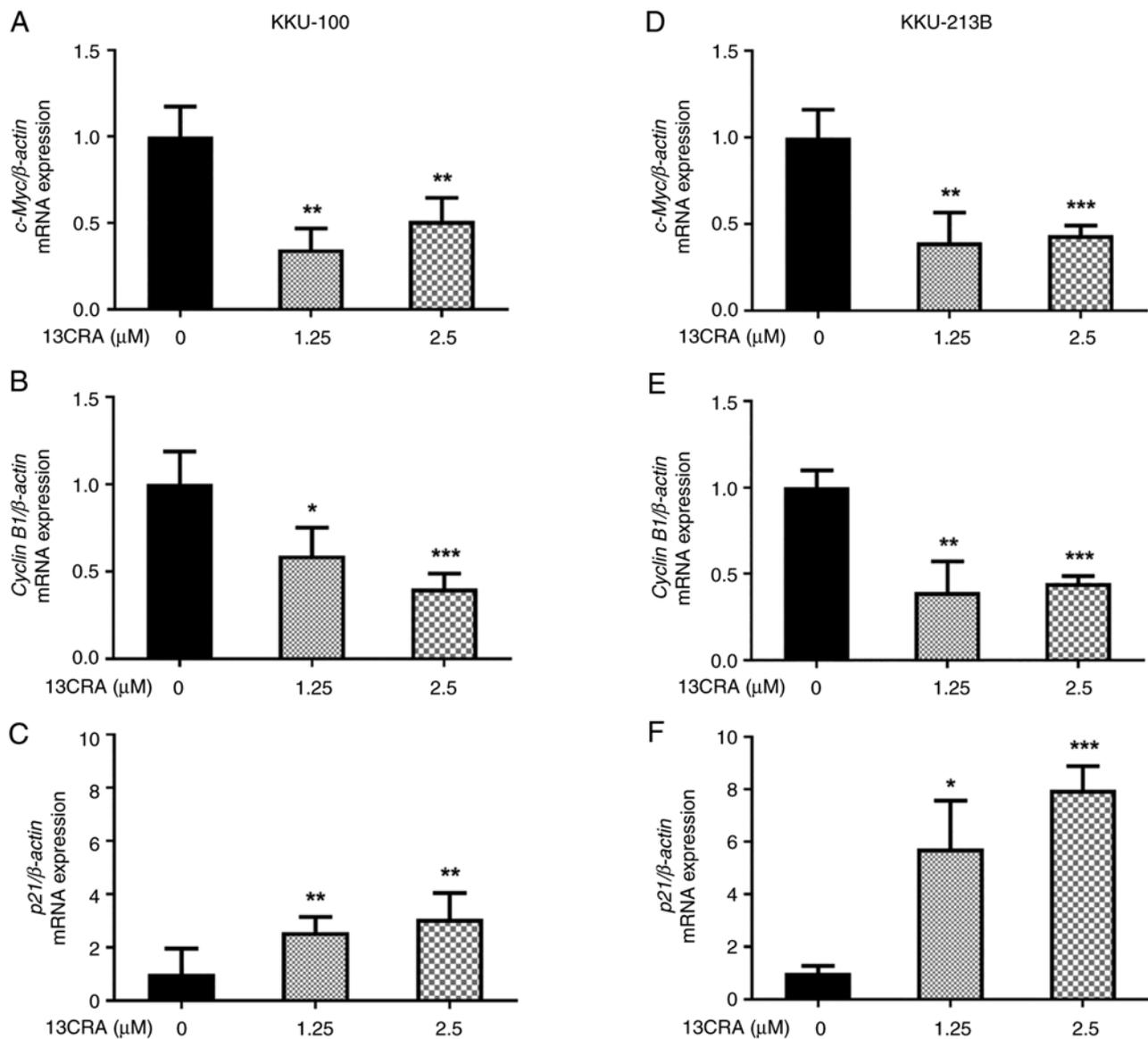


Figure 4. Effects of 13CRA on the expression of cell cycle regulatory genes in cholangiocarcinoma cells. KKKU-100 and KKKU-213B cells were treated with 1.25 or 2.5 μM 13CRA for 48 h, and the expression levels of *c-Myc*, *p21* and *cyclin B1* genes were determined using reverse transcription-quantitative PCR. Quantification of the mRNA levels of (A) *c-Myc*, (B) *cyclin B1* and (C) *p21* in KKKU-100 and (D) *c-Myc*, (E) *cyclin B1* and (F) *p21* in KKKU-213B cells, which were normalized to those of β -actin, which acted as an internal control. Data are presented as the mean \pm SD from three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. the untreated control. 13CRA, 13-*cis*-retinoic acid.

ICAM-1, *COX-2*, *MMP-2* and *MMP-9*. Following treatment for 48 h, treatment with 1.25 and 2.5 μM 13CRA significantly downregulated the mRNA expression of *ICAM-1*, *COX-2* and *MMP-2* in KKKU-100 (Fig. 9A-C) and KKKU-213B (Fig. 9E-G) cells ($P < 0.05$). Furthermore, there was a significant decrease in *MMP-9* mRNA expression in the 13CRA-treated KKKU-213B cells (Fig. 9H) compared with that of the untreated control cells.

In addition, the present study further investigated the effects of 13CRA on the expression of adhesion- and invasion-related proteins using western blot analysis. The results revealed that 13CRA decreased the expression of the adhesion-related protein, *ICAM-1*, in both CCA cell lines (Fig. 9I). 13CRA decreased the expression of the invasion-related protein, *MMP-9*, in KKKU-213B cells, and a decreasing trend in *MMP-9* protein expression was also observed in the KKKU-100

cells treated with 13CRA (Fig. 9I). This suppressive effect of 13CRA on the expression of these major adhesion- and invasion-related genes and proteins in CCA cells supported the anti-invasion and anti-adhesion activities of 13CRA in CCA cells.

Discussion

The findings of the present study revealed the inhibitory effects of 13CRA on the self-renewal, migration, invasion and adhesion of CCA cells. 13CRA suppressed cell proliferation by inducing cell cycle arrest at the G_2/M phase and decreased the number of cells in the G_1 phase. Protein analyses using western blotting demonstrated that treatment of the CCA cells with 13CRA induced a significant increase in the expression of the cell cycle inhibitor proteins, p53 and p21, and decreased

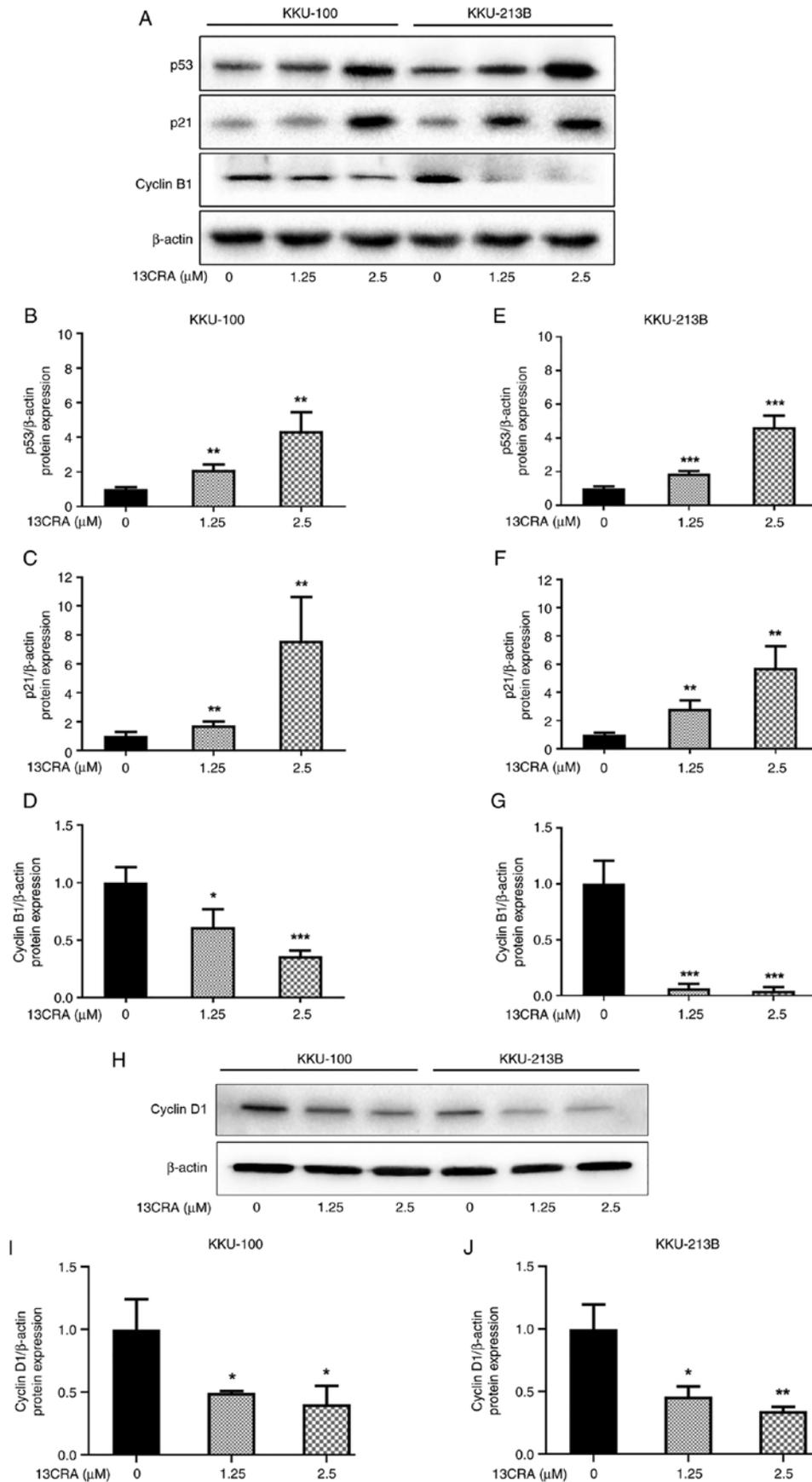


Figure 5. Effects of 13CRA on the expression of cell cycle-regulatory proteins in cholangiocarcinoma cells. (A) KKU-100 and KKU-213B cells were treated with 1.25 or 2.5 μM 13CRA for 48 h, and the expression levels of p53, p21 and cyclin B1 proteins were assessed using western blot analysis. The intensity of the (B) p53, (C) p21 and (D) cyclin B1 protein bands in the KKU-100 and (E) p53, (F) p21 and (G) cyclin B1 in the KKU-213B cell lysates was quantified by normalization to β -actin. (H) The protein level of cyclin D1 and the intensity of the cyclin D1 bands in (I) KKU-100 and (J) KKU-213B cell lysates was quantified by normalization to β -actin. Data are presented as the mean \pm SD from three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. the untreated control. 13CRA, 13-*cis*-retinoic acid.

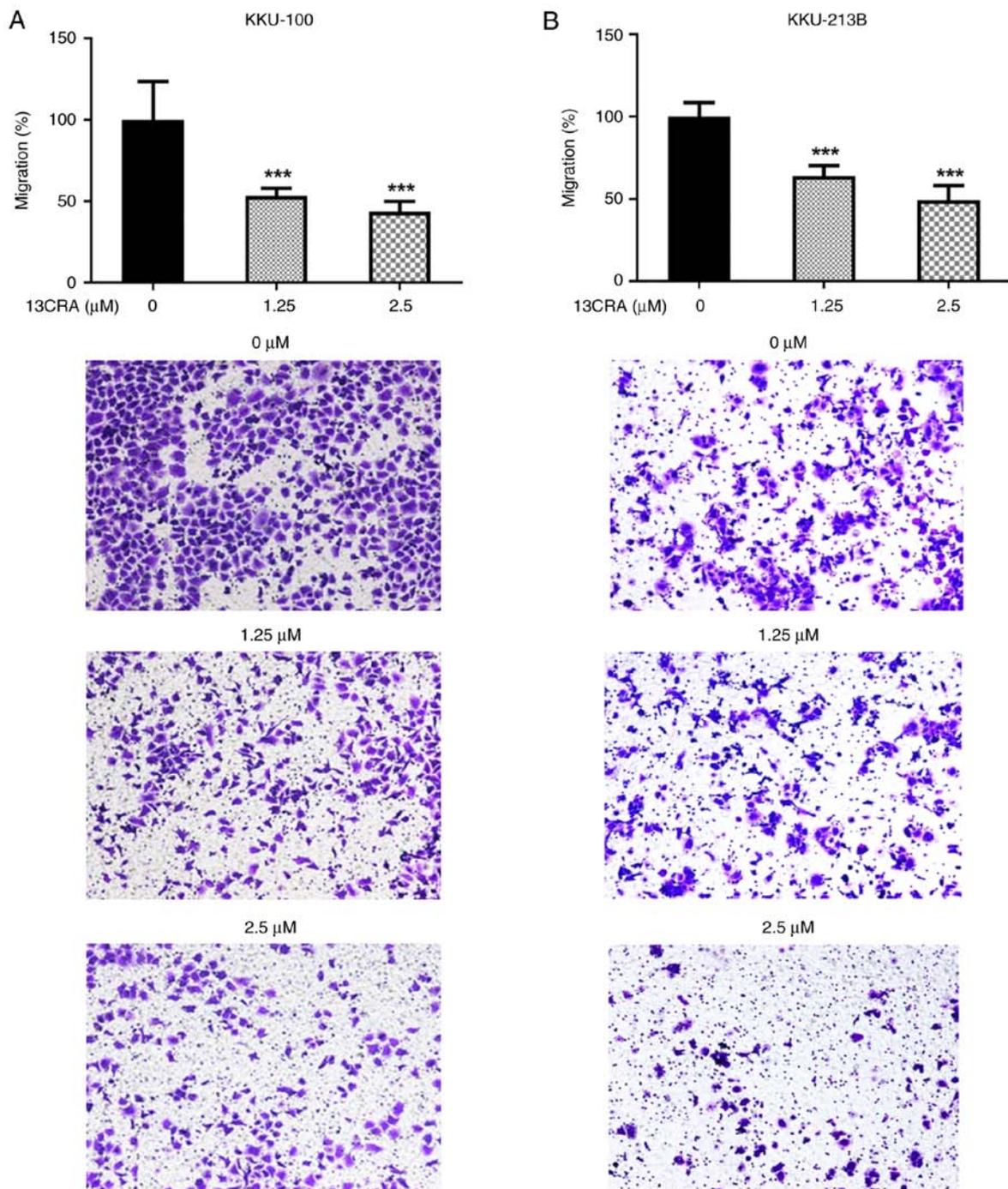


Figure 6. Effects of 13CRA on the migration of cholangiocarcinoma cells. (A) KKU-100 and (B) KKU-213B cells were seeded into a Transwell plate, and treated with 1.25 and 2.5 μM 13CRA for 24 h. The number of migrated cells was counted and calculated as a percentage of the migrated cells of the untreated control. Representative images of cell migration were captured under a light microscope (magnification, $\times 10$). Data are presented as the mean \pm SD from three independent experiments. *** $P < 0.001$ vs. the untreated control. 13CRA, 13-*cis*-retinoic acid.

the expression of cyclin B1 (a protein that regulates the G₂/M transition of the cell cycle) and cyclin D1 (a regulatory protein of the G₁ transition the cell cycle). RT-qPCR analyses of mRNA expression revealed that 13CRA induced the concurrent upregulation of *p21* expression, and the downregulation of *c-Myc* and *cyclin B1* expression. In addition, 13CRA markedly inhibited CCA cell migration by controlling the expression of EMT-related genes (*E-cad*, *Snail* and *vimentin*) and proteins (E-cad and vimentin). Furthermore, 13CRA significantly reduced the invasion and adhesion of CCA cells, and decreased

the expression of adhesion- and invasion-related genes (*ICAM-1*, *COX-2*, *MMP-2* and *MMP-9*) and proteins (ICAM-1 and MMP-9). However, even though the anticancer activity of 13CRA in CCA cells has been demonstrated in the present study, the absence of results using cholangiocytes (cells used as a control) is a limitation of the study.

13CRA has been extensively investigated for its potential use in cancer treatment and prevention. Previous preclinical studies have demonstrated that 13CRA inhibits the proliferation, migration and invasion of several cancer

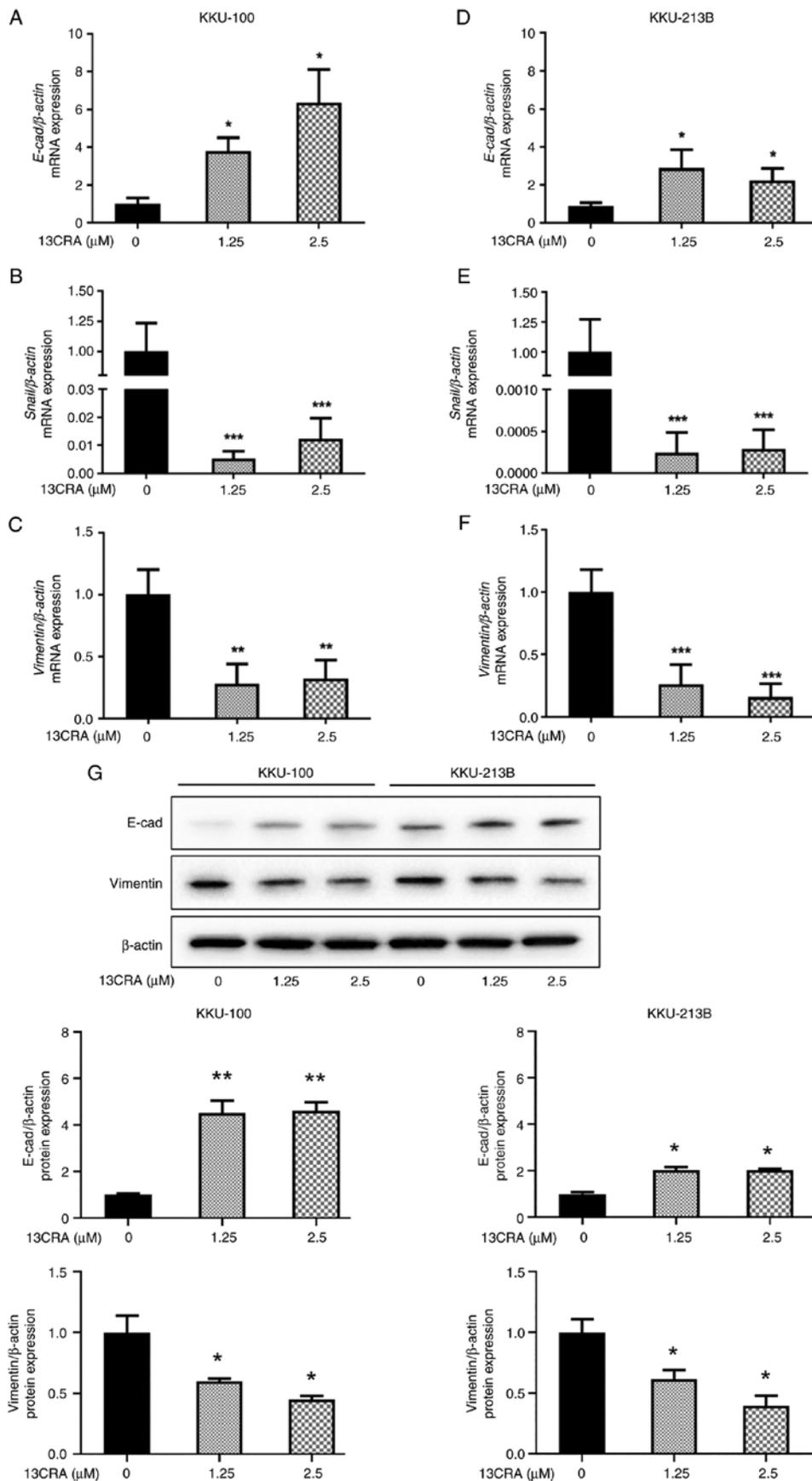


Figure 7. Effects of 13CRA on the expression of epithelial-mesenchymal transition-related genes and proteins in cholangiocarcinoma cells. KKU-100 and KKU-213B cells were treated with 1.25 or 2.5 μ M 13CRA for 24 h, and expression levels of *E-cad*, *Snail* and *vimentin* genes were determined using reverse transcription-quantitative PCR. The mRNA levels of *E-cad*, *Snail* and *vimentin* in (A-C) KKU-100 and (D-F) KKU-213B cells were quantified by normalization to β -actin. (G) The expression levels of E-cad and vimentin proteins were assessed using western blot analysis and the intensity of the E-cad and vimentin bands was quantified by normalization to β -actin. Data are presented as the mean \pm SD from three independent experiments. * P <0.05, ** P <0.01 and *** P <0.001 vs. the untreated control. 13CRA, 13-*cis*-retinoic acid; *E-cad*, *E-cadherin*.

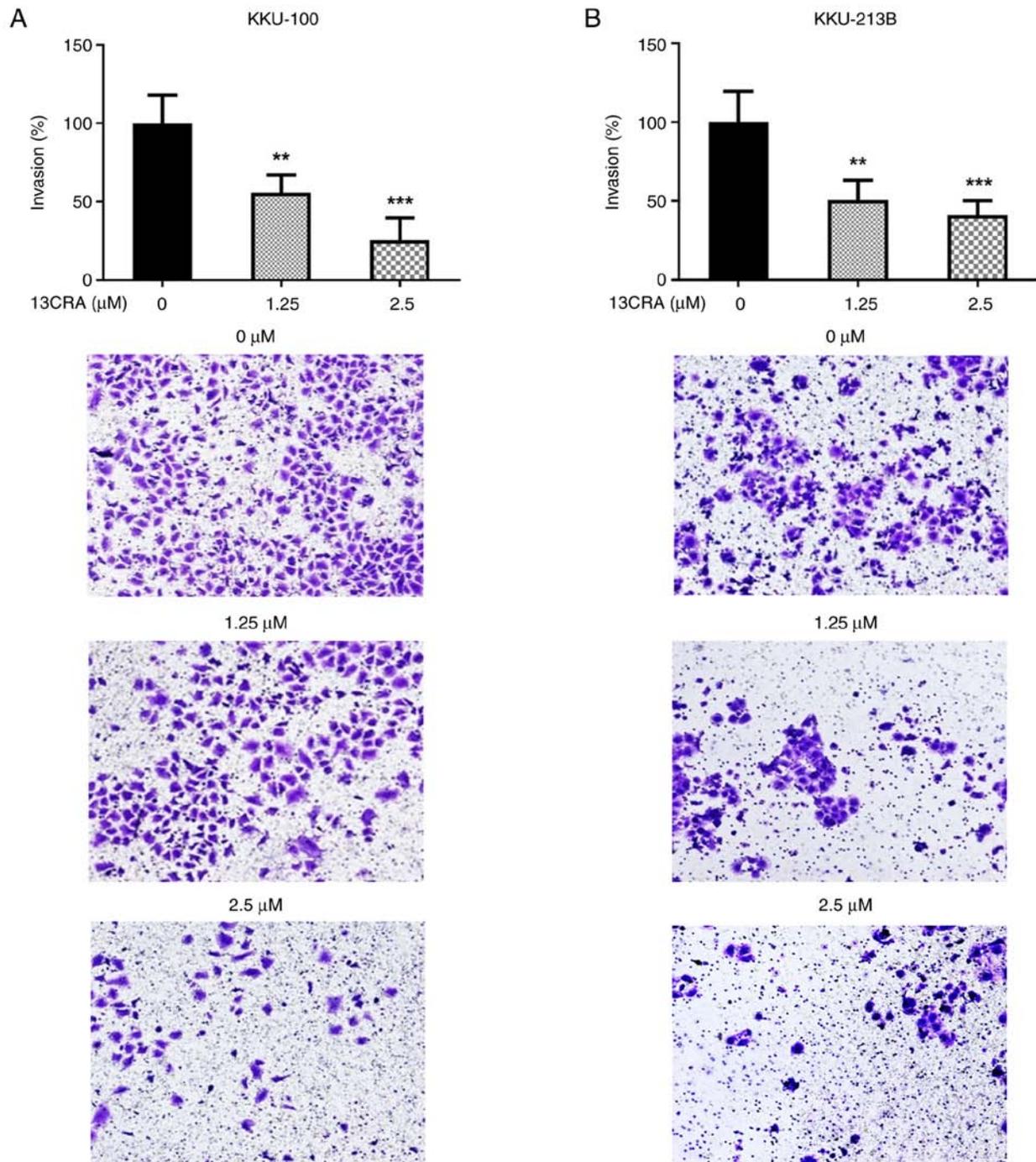


Figure 8. Continued.

types (4-6,31,32). Previously, all-*trans*-retinoic acid (ATRA), a retinoid used for the treatment of acne and acute promyelocytic leukemia, has been shown to exert apoptosis-inducing effects on CCA cells (30,33); the present study demonstrated the cytotoxicity of 13CRA against KKU-100 and KKU-213B cells. Thus, these results suggest the cytotoxicity of retinoid drugs towards CCA cells. Considering the IC_{50} values, the magnitude of the sensitivity to 13CRA between CCA cells (KKU-100 and KKU-213B) used herein and breast cancer cells (triple-negative MDA-MB-231 cells) previously used was in the same micromolar range (6). Moreover, the blood levels in the micromolar range of 13CRA in humans can be achieved by

the oral administration of 80 mg 13CRA twice daily (34). The present study demonstrated the inhibitory effects of 13CRA on CCA; however, the absence of experiments on non-cancerous cells used as a negative control is a limitation of the study.

In the present study, 13CRA exerted a direct cytotoxic effect and inhibited the self-renewal ability of two CCA cell lines. It has previously been reported that 13CRA inhibits the proliferation of breast and gastric cancer cells (5,6). Considering the concentrations of 13CRA required for an anti-proliferative effect, the CCA cells were more sensitive to 13CRA treatment than breast and gastric cancer cells (5,6). Thus, to elucidate the possible underlying mechanisms of the

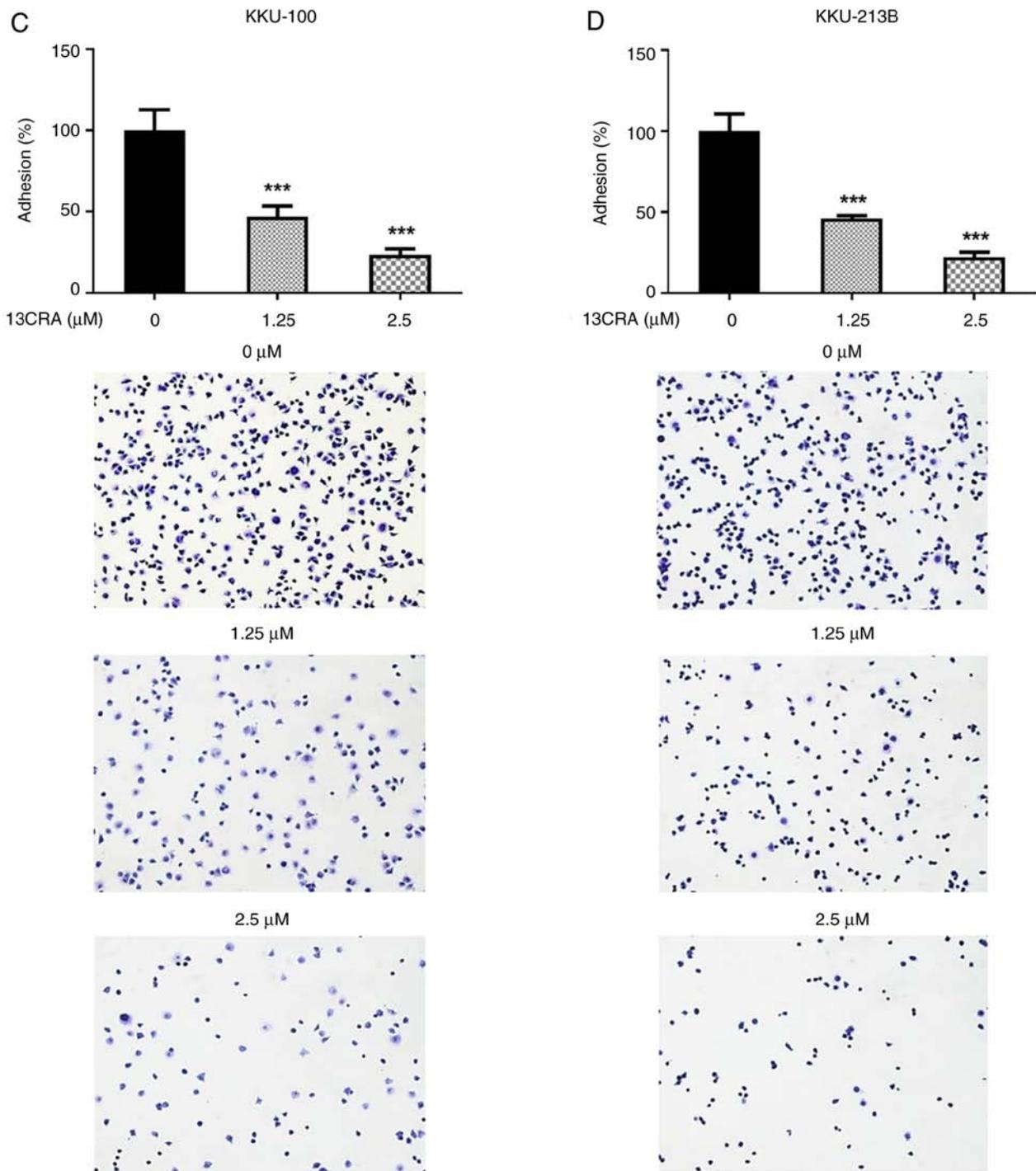


Figure 8. 13CRA inhibits the invasion and adhesion of cholangiocarcinoma cells. To examine cell invasion, the (A) KKKU-100 and (B) KKKU-213B cells were seeded into a Transwell plated coated with Matrigel, and treated with 1.25 or 2.5 μM 13CRA for 48 h. The numbers of invaded cells were counted and calculated as percentages of cell invasion vs. the untreated control. To examine cell adhesion, following treatment with 13CRA for 48 h, the (C) KKKU-100 and (D) KKKU-213B cells were harvested and seeded into a 96-well plate coated with fibronectin. Following 30 min of incubation, the cells were stained with crystal violet, their absorbance at 540 nm was read, and the percentage of cell adhesion was calculated. Representative images of cell adhesion and invasion were captured under a light microscope (magnification, $\times 10$). Data are presented as the mean \pm SD from three independent experiments. ** $P < 0.01$ and *** $P < 0.001$ vs. the untreated control. 13CRA, 13-*cis*-retinoic acid.

anti-proliferative effects of 13CRA, the present study analyzed the cell cycle distribution patterns of 13CRA-treated cells using flow cytometry. At the concentrations which slightly inhibited cell proliferation, i.e., those lower than the IC_{50} value of 13CRA at 48 h, 13CRA inhibited cell cycle progression at the G_2/M phase and decreased the number of cells in the G_1 phase. Retinoid medications are known to induce cell cycle

arrest via the altered expression of genes and proteins of cell cycle regulators in several cancer types, such as gastric cancer, melanoma and leukemia cells (35-38). Previously, 13CRA was shown to induce cell cycle arrest by decreasing DNA synthesis, increasing p21 protein levels and decreasing cyclin D1 expression in human sebocytes (15). In the present study, 13CRA induced cell cycle arrest at the G_2/M phase, and

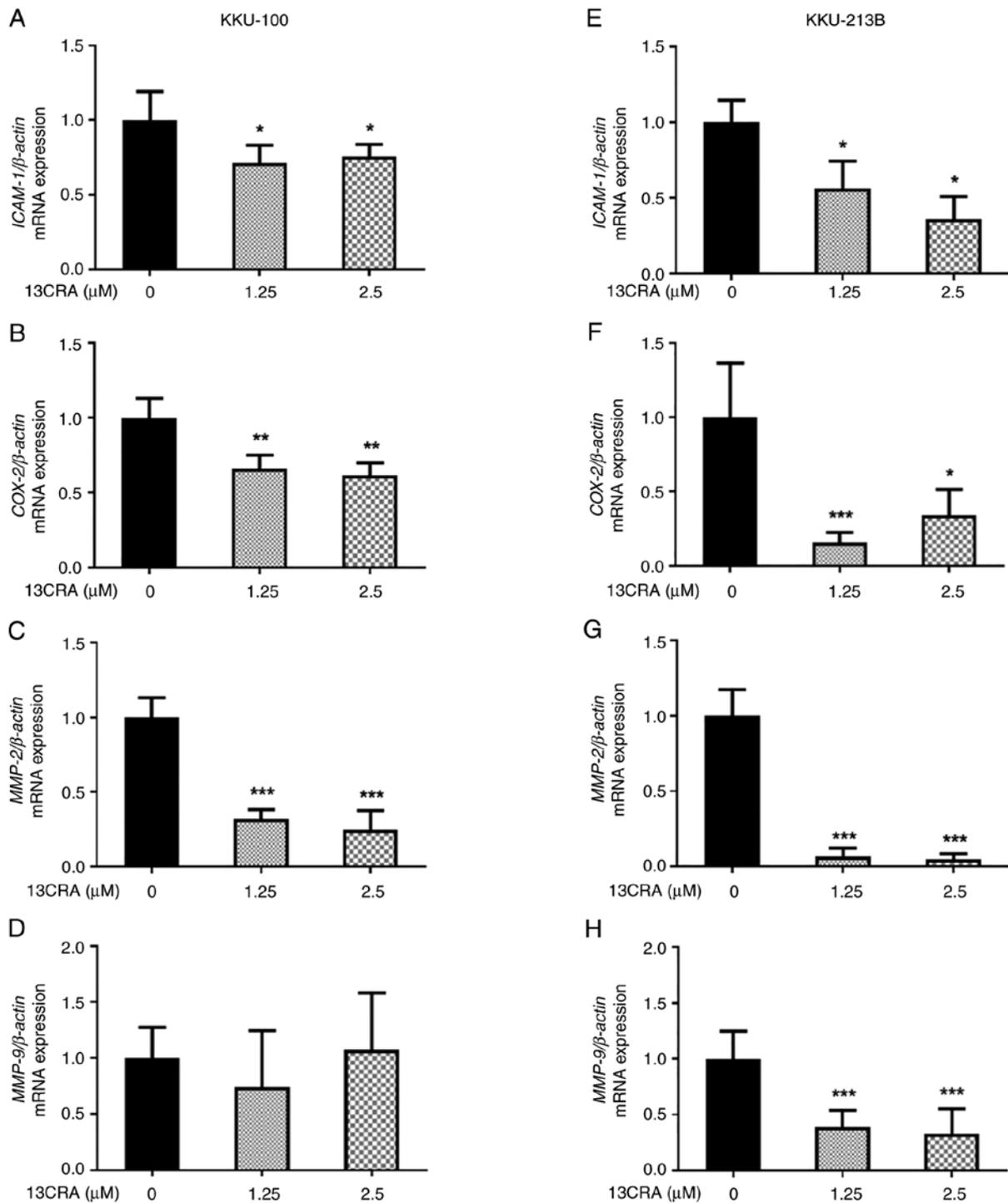


Figure 9. Continued.

altered the expression of the cell cycle regulatory molecules, p21 and cyclin B1, at the mRNA and protein levels. In addition, 13CRA altered the expression of upstream molecules involved in cell cycle progression by upregulating p53 protein expression. 13CRA also decreased the expression of cyclin D1, the regulatory protein of the G₁ transition of the cell cycle. Similarly, ATRA was previously reported to induce cell cycle arrest at the G₀/G₁ phase in a human monoblastic cell line, which was associated with the marked downregulation of c-Myc and cyclin E levels, and increased p21 expression (39). In the present study, 13CRA inhibited the self-renewal ability

of CCA cells through G₂/M arrest via the alteration of the expression of cell cycle regulatory genes and proteins.

Targeting the processes indicative of hallmarks of cholangiocarcinogenesis, such as EMT, stemness and plasticity properties, has attracted particular attention for chemotherapy (16). It has been previously suggested that ATRA reverses the EMT process by upregulating the levels of epithelial markers and downregulating those of mesenchymal markers of colorectal cancer and hepatocellular carcinoma cells (40,41). A previous study demonstrated that 13CRA inhibited vascular endothelial cell migration through the suppression

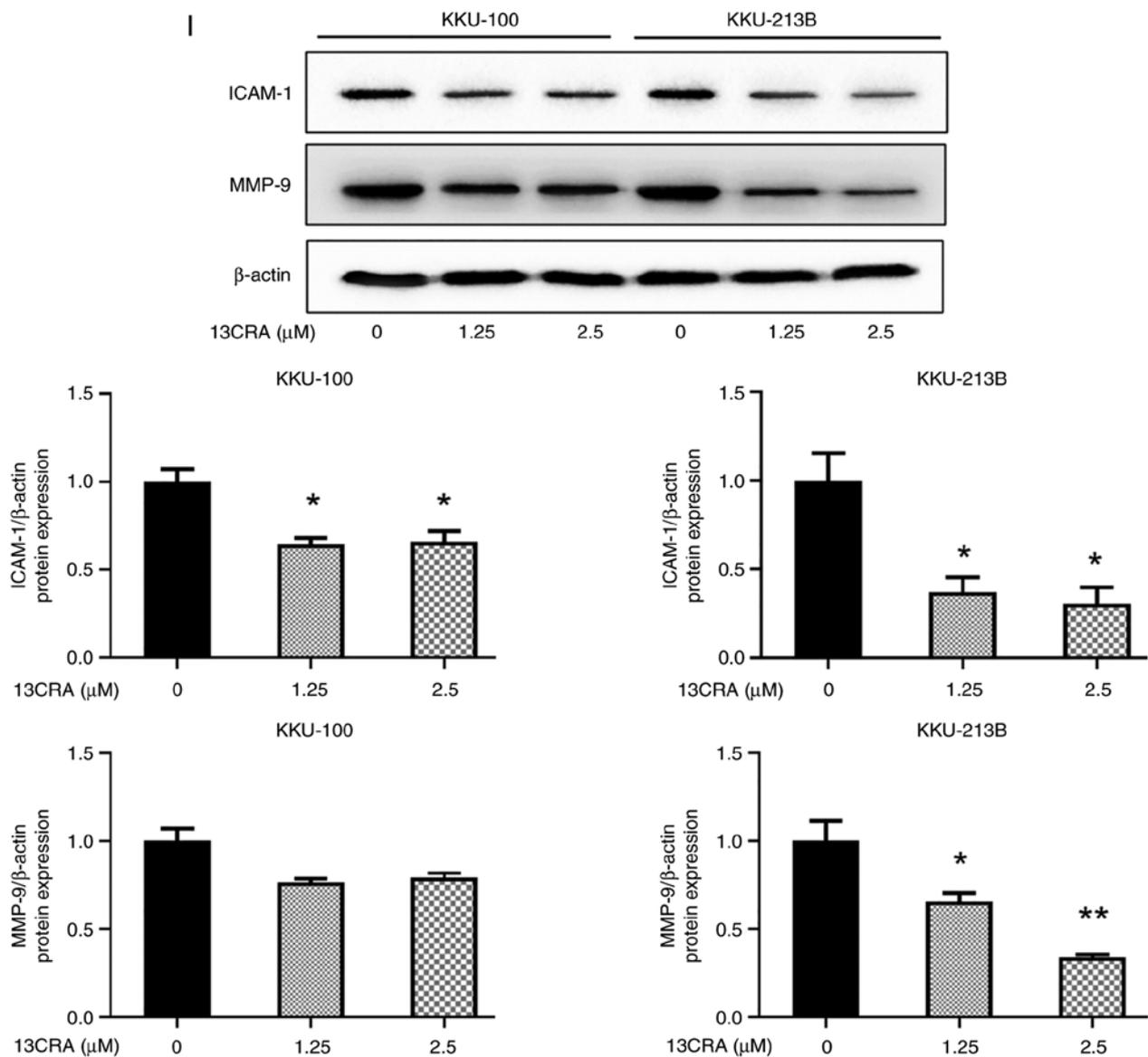


Figure 9. 13CRA downregulates the expression of adhesion- and invasion-related genes and proteins in cholangiocarcinoma cells. (A-D) KKKU-100 and (E-H) KKKU-213B cells were treated with 1.25 or 2.5 μ M 13CRA for 48 h. The expression levels of *ICAM-1*, *COX-2*, *MMP-2* and *MMP-9* genes were determined using reverse transcription-quantitative PCR, and the quantification of the mRNA levels of *ICAM-1*, *COX-2*, *MMP-2* and *MMP-9* was performed via normalization to β -actin. (I) The expression levels of ICAM-1 and MMP9 proteins were assessed using western blot analysis and the intensity of the ICAM-1 and MMP9 bands was quantified by normalization to β -actin. Data are presented as the mean \pm SD from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. the untreated control. 13CRA, 13-*cis*-retinoic acid; *MMP*, matrix metalloproteinase; *ICAM-1*, intercellular adhesion molecule-1; *COX-2*, cyclooxygenase-2.

of NF- κ B, a transcription factor that plays a major role in mediating cancer metastasis (31). The present study revealed the effects of 13CRA on EMT-related genes and proteins. Treatment of the CCA cells with 13CRA suppressed vertical cell migration in association with the reversal of EMT-related markers, i.e., the upregulation of *E-cad* (an epithelial marker) and the downregulation of *Snail* and *vimentin* (mesenchymal markers), and it also increased the protein expression level of *E-cad* and decreased the protein expression level of *vimentin*. Notably, a previous study reported the markedly low expression of *E-cad* in KKKU-100 cells, while treatment with 13CRA led to the upregulation of *E-cad* expression at both the mRNA and protein levels (41).

Furthermore, the results of the present study demonstrated the potent inhibitory effects of 13CRA on CCA cell invasion

and adhesion. At a concentration of 1.25 μ M, which exerted only minimal cytotoxicity, 13CRA inhibited >50% of cell invasion and adhesion. Previous studies have also reported that 13CRA inhibits the adhesion of squamous cell carcinoma and reduces the invasion of colon cancer cells; at 1 μ M, 13CRA was shown to exert ~50% inhibition (7,42). Previous studies have revealed that 13CRA inhibits the invasion of colon cancer by suppressing the expression of *MMP-7* and *COX-2* (7,43). The present study demonstrated that the inhibitory effects of 13CRA on the invasion and adhesion of CCA cells were possibly associated with the decreased expression of adhesion- and invasion-related genes and proteins. Notably, the lack of a change in *MMP-9* mRNA expression in KKKU-100 cells may be caused by differences in genetic alterations and backgrounds between the KKKU-100 and

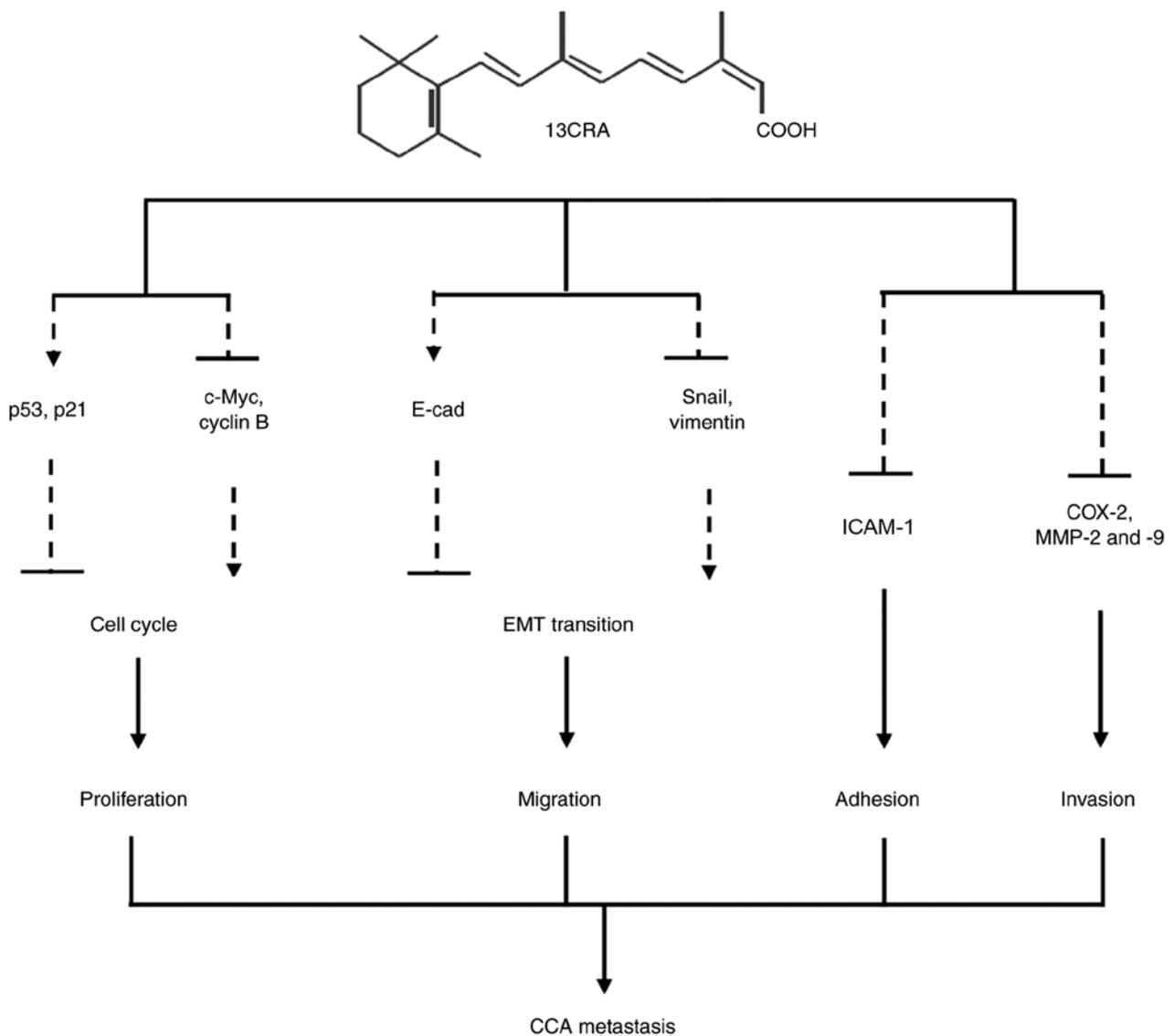


Figure 10. Inhibitory effects of 13CRA on the proliferation, migration, invasion and adhesion of CCA cells, and the possible underlying mechanisms. 13CRA, 13-*cis*-retinoic acid. MMP, metalloproteinase; ICAM-1, intercellular adhesion molecule-1; COX-2, cyclooxygenase-2; CCA, cholangiocarcinoma.

KKU-213B CCA cells (28-30,44). Nevertheless, the expression of MMP-9 exhibited a decreasing trend in KKU-100 cells, which suggested that 13CRA may modulate other molecules associated with the post-translational modification of MMP-9 that affect the stability and activity of the MMP family (45). The inhibitory effects of 13CRA on the proliferation, migration, invasion and adhesion of CCA cells and the possible underlying mechanisms are summarized in Fig. 10. 13CRA can regulate the expression of several key genes and proteins that control cancer cell behavior including cell cycle progression, EMT and cell invasion and adhesion. 13CRA modulates the expression of p53, p21, c-Myc and cyclin B1, leading to the inhibition of the self-renewal ability of CCA cells. 13CRA also controls the expression of E-cad, Snail and vimentin, which subsequently alter EMT and cell migration. In addition, 13CRA can modify the expression of ICAM-1, COX-2, MMP-2 and MMP-9, impairing the invasion and adhesion of CCA cells.

In conclusion, the present study demonstrated that 13CRA reduced cell proliferation by leading to cell cycle arrest at

the G₂/M phase. 13CRA suppressed cell migration possibly through the reversal of the expression of EMT-related genes and proteins. 13CRA also inhibited cell invasion and adhesion by suppressing the expression of adhesion- and invasion-related genes and proteins. Thus, these results indicated that 13CRA may be useful for CCA therapy; however, the anticancer activity of 13CRA both *in vitro* and *in vivo* models warrants further investigations to demonstrate the beneficial effects of 13CRA for the treatment of CCA.

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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

SB participated in performing experiments, analyzing the results and writing the manuscript. VK, LS and SK collaborated in analyzing the data and providing critical comments. AP participated in designing the study, planning the experiments, analyzing the results and writing the manuscript. SB and AP 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.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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