All-*trans*-retinoic acid induces RARB-dependent apoptosis via ROS induction and enhances cisplatin sensitivity by NRF2 downregulation in cholangiocarcinoma cells

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Abstract. All-*trans*-retinoic acid (ATRA) has been clinically used to treat acute promyelocytic leukemia and is being studied to treat other types of cancer; however, the therapeutic role and mechanism of ATRA against cholangiocarcinoma (CCA) remain unclear. The present study investigated the cytotoxic effect and underlying mechanisms of ATRA on CCA cell lines. Cell viability was evaluated by sulforhodamine B assay. Intracellular reactive oxygen species (ROS) levels were assessed by dihydroethidium assay. Apoptosis analysis was performed by flow cytometry. The pathways of apoptotic cell death induction were examined using enzymatic caspase activity assay. Proteins associated with apoptosis were evaluated by western blotting. The effects on gene expression were analyzed by reverse transcription-quantitative PCR analysis. ATRA induced a concentration- and time-dependent toxicity in CCA cells. Furthermore, when the cytotoxicity of ATRA against retinoic acid receptor (RAR)-deficient cells was assessed, it was revealed that ATRA cytotoxicity was RARB-dependent. Following ATRA treatment, there was a significant accumulation of cellular ROS and ATRA-induced ROS generation led to an increase in the expression levels of apoptosis-inducing proteins and intrinsic apoptosis. Pre-treatment with ROS scavengers could diminish the apoptotic effect of ATRA, suggesting that ROS and mitochondria may have an essential role in the induction of apoptosis. Furthermore, following ATRA treatment, an increase in cellular ROS content was associated with suppressing nuclear factor erythroid 2-related factor 2 *(NFE2L2* or *NRF2)* and *NRF2*-downstream active genes. ATRA also suppressed cisplatin-induced *NRF2* expression, suggesting that the enhancement of cisplatin cytotoxicity by ATRA may be associated with the downregulation of NRF2 signaling. In conclusion, the results of the present study demonstrated that ATRA could be repurposed as an alternative drug for CCA therapy.

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Introduction

Cholangiocarcinoma (CCA) is a rare and aggressive type of cancer arising from cholangiocytes of the biliary tract. It is the second most common primary liver malignancy and its incidence is increasing worldwide, with the highest CCA incidence rates reported in countries in Southeast Asia (1-3). CCA is characterized as asymptomatic until it is diagnosed having progressed to the advanced stage (4,5). Despite advances in surgical, medical and molecular-targeted therapy the 5-year survival rates of patients with CCA have remained low in recent decades (6). Therefore, it is important to identify more effective therapeutic strategies against CCA, especially those that overcome drug resistance and improve clinical outcomes.

All-*trans*-retinoic acid (ATRA) is a natural active metabolite of vitamin A, which serves an essential role in several physiological processes, and aids cell growth and development, particularly in early embryogenesis (7-9). ATRA acts as a pan-agonist of retinoic acid receptors (RARs), which consist of RARA, RARB and RARG subtypes (10). ATRA activates RARs and forms heterodimers with retinoid-X receptors (RXRs), which bind to the retinoic acid response elements and initiate the transcription of retinoic acid-targeted genes. Numerous target genes are associated with cell differentiation and have particular relevance to the retinoid-mediated regulation of myelopoiesis (7,8).

Currently, ATRA is clinically used to treat acute promyelocytic leukemia by promoting terminal differentiation of hematopoietic progression (11-13). ATRA has also been studied as a preventative and therapeutic agent against several types of cancer, including breast cancer, hepatocellular carcinoma, esophageal cancer and thyroid cancer (14-17),

and as an adjunct medication to increase chemotherapeutic response (18-21). Previously, the effects of ATRA on apoptosis, proliferation, migration and invasion have been reported in CCA (22). In addition, a previous report demonstrated that upregulating RARB in CCA tissue enhanced apoptosis in CCA and thus improved CCA chemotherapeutic sensitivity (23). Therefore, it may be suggested that, in CCA, RARB has a tumor-suppressive role.

Previous studies have indicated that retinoids and vitamin A derivatives cause mitochondrial dysfunction, and trigger reactive oxygen species (ROS) production to induce cellular damage and apoptosis (24,25). Additionally, ATRA has been reported to induce ROS production in Sertoli cells and NB4 cells (26,27). Furthermore, ROS has been shown to be associated with the activation of apoptosis in CCA cells (28). In CCA, the high expression of nuclear factor erythroid 2-related factor 2 (NFE2L2 or NRF2), which is the master regulator of cytoprotective and antioxidant enzymes, may contribute to promote cancer cell growth, anti-apoptosis and chemotherapeutic resistance (29,30). Moreover, it has been suggested that ATRA may act as a potential inhibitor of NRF2 activation (31).

Based on these aforementioned studies, the therapeutic potential and underlying mechanisms of ATRA against CCA should be elucidated. Therefore, the present study aimed to explore the apoptotic effect and underlying molecular mechanisms of ATRA in CCA cells. In addition, the potential use of ATRA for enhancing the sensitivity of anticancer agents was investigated.

Materials and methods

Cell lines and cell culture. In the present study, two human CCA cell lines, KKU-100 and KKU-213B, were established and generously provided by Professor Banchob Sripa (Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand). Cell line authentication was verified by highly polymorphic short tandem repeat (STR) analysis; DNA markers of 23 STR loci and the sex marker (amelogenin) were analyzed using the AmpFLSTR Identifiler PCR Amplification kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Based on the STR analysis, the cell lines used in the present study, KKU-213B and KKU-100, shared similar markers and matched well with the partial STR profile of cell line identity, as described previously (32,33).

Both cell lines were routinely grown in monolayer cultures in Ham's F12 medium (Gibco; Thermo Fisher Scientific, Inc.) pH 7.4, supplemented with 10% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin G and 50 μg/ml gentamicin sulfate. Cells were maintained under an atmosphere containing 5% CO_2 at 37˚C and were sub-passaged every 3 days using 0.5% trypsin-EDTA. Cell numbers were counted using a hemocytometer.

Sulforhodamine B (SRB) assay. Cell viability was detected using the SRB assay. Briefly, KKU-100 and KKU-213B cells were treated with 0.00, 1.25, 2.50, 5.00, 10.00 and 20.00 μM ATRA (R2625; MilliporeSigma) in serum-free Ham's F12 medium for 12, 24, and 48 h under an atmosphere containing 5% CO_2 at 37˚C. The cells were then incubated with 100 μl ice-cold 10% of trichloroacetic acid at 4˚C for 1 h and washed with deionized water. Subsequently, the cells were stained with 50 μl 0.4% SRB (MilliporeSigma) in 1% acetic acid at room temperature for 30 min. The cells were solubilized in 10 mM Tris-base (pH 10.5) solution, and the absorbance was measured at 540 nm using a microplate reader. Cell cytotoxicity was expressed in terms of a percentage compared with the untreated control group; half-maximal inhibitory concentration (IC_{50}) was calculated from the dose-response curves. To study the effects of ATRA on the sensitivity of anticancer drugs, cells were treated with ATRA or anticancer drugs or the combined treatment of ATRA and anticancer drugs for 48 h at 37˚C before SRB assay. The concentrations of drugs were as follows: 2.5 μM ATRA; 30, 100, 300 μM 5-fluorouracil (5-FU); 0.001, 0.010, 0.100 μM gemcitabine (Gem); 2.5, 5.0, 10.0 μM cisplatin (Cis); and 0.01, 0.10 and 1.00 μM doxorubicin (Doxo). Doxo, Cis and 5-FU were from Boryung pharmceutical and Gem was from Eli Lily.

Annexin V-PE/7-AAD cell apoptosis analysis. For the apoptosis assay, KKU-100 and KKU-213B cells were treated with 0.00, 1.25, and 5.00 μM ATRA in serum-free Ham's F12 medium for 48 h at 37˚C. Subsequently, the cells were collected, washed with PBS twice and resuspended in 1X Annexin V binding buffer included in the kit at a concentration of $1x10^6$ cells/ml. The cell suspension was then incubated with Annexin V-PE and 7-ADD (BD Pharmingen™ PE Annexin V Apoptosis Detection Kit I; BD Biosciences) for 15 min at room temperature in the dark, after which flow cytometry was performed using BD FACS Canto™ II and FACSDiva™ software v6.1.3 (both from BD Biosciences). To study the role of ROS in apoptosis induction by ATRA, cells were pre-treated with 2.0 mM NAC (A7250, Sigma Chemical) or 0.5 mM TEMPOL (176141, Sigma Chemical) for 3 h and then incubated with 0, 1.25 and 2.5 μM ATRA for 48 h at 37˚C before flow cytometry analysis.

Dihydroethidium (DHE) staining analysis of ROS. For cellular ROS detection, KKU-100 and KKU-213B cells were treated with 0.00, 1.25 and 2.50 μM ATRA combined with 25 μM DHE (Calbiochem; Merck KGaA) in serum-free Ham's F12 medium; the cells were incubated in a humidified atmosphere containing 5% CO_2 at 37˚C for 90 min. Subsequently, the cells were measured for the intensity of fluorescent signals of ethidium using a Gemini XPS fluorescent plate reader (Molecular Devices, LLC.), with excitation and emission wavelengths of 518 and 605 nm, respectively. Furthermore, the fluorescence signal of ethidium was captured under a 4x magnification power florescence microscope with a G-2A filter.

Caspase activity assays. To assess the protease activities of caspase-3, -8 and -9, KKU-100 and KKU-213B cells were treated with 0.00, 1.25 and 2.50 μM ATRA for 12 h at 37˚C. The cells were then trypsinized and the cell pellets were lysed on ice for 30 min with 50 μl lysis buffer included in the kit per $1x10^6$ cells. Subsequently, the supernatant was transferred into a 96-black well clear-bottom plate. To measure caspase-3 activity, the supernatant was incubated with the caspase-3 substrate (Z-DEVD-AMC, cat. no. E13183) according to the manufacturer's instructions (Molecular Probes; Thermo Fisher

Scientific, Inc.). The fluorescent signals were detected at excitation and emission wavelengths of 340 and 440 nm, respectively. To assess the activities of caspase-8 and -9, the supernatant was incubated with the caspase-8 substrate (IETD-AFC; cat. no. ab39534; Abcam) or caspase-9 substrate (Ac-LEHD-AFC, cat. no. 218765; Calbiochem) according to the manufacturer's instructions. The fluorescent signals were detected at excitation and emission wavelengths of 400 and 440 nm, respectively.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following treatment with 1.25 and 2.50 μM ATRA for 48 h at 37˚C, total RNA was isolated using TRIzol® LS reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For RT, a mixture of total RNA and 5X iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories Inc.) was mixed with RNase-free water, and the reaction was performed in a C1000™ Thermal Cycler (Bio-Rad Laboratories, Inc.). The conditions for cDNA synthesis included priming for 5 min at 25˚C followed by RT for 30 min at 42˚C; the reaction was terminated by incubation for 15 min at 70˚C. A mixture consisting of specific primers, 2X QPCR Green Master Mix (biotechrabbit GmbH), cDNA and sterile water underwent qPCR using a Light Cycler® 480 II/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 s, 72˚C for 5 s, 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. To quantify the relative expression levels of genes, relative quantitation using the standard curve method was performed (29). The expression levels of target mRNA were expressed as a ratio to *ACTB* mRNA. Primers used are listed in Table SI.

Western blot analysis. After treatment with 1.25 and 2.50 μM ATRA for 6 h at 37˚C, cells were lysed using RIPA cell lysis buffer (Amresco, LLC), and protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories Inc.) according to the manufacturer's instructions. Whole-cell protein extracts were separated by SDS-PAGE on 10% gels using an SE 260 mini-vertical gel electrophoresis unit (Hoefer, Inc.). The proteins were then transferred onto a PVDF membrane (Immobilon®-P; cat. no. IPVH00010; MerckMillipore) using Owl™ HEP-1 Semidry Electroblotter (cat. no. HEP1; Thermo Fisher Scientific, Inc.) and blocked with 5% (w/v) skimmed milk at room temperature for 1 h. Subsequently, the membranes were incubated with the following primary antibodies: Anti-AIF, anti-Bax, anti-cytochrome *c*, anti-RARA, anti-RARB, anti-ACTB (all from Santa Cruz Biotechnology, Inc.; antibodies are described in Table SII) and anti-RARG (Cell Signaling Technology Inc.). The membranes were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. The antibodies used are listed in Table SII. The protein bands were detected using a Luminata™ Forte Western HRP Substrate (Merck Millipore Corporation) under a ChemiDoc™ MP Imaging system (Bio-Rad Laboratories, Inc.). To semi-quantify the target protein expression levels, the intensity of the target protein bands was analyzed using Image Lab 6.0 software (Bio-Rad, Hercules) and normalized to that of ACTB.

CRISPR/Cas9-mediated RARγ knockout. To assess the role of RAR in the response of CCA cells to ATRA, CRISPR/Cas9-mediated knockout was performed. The RARG CRISPR cloning vector pLentiCRISPR v2 was purchased from GenScript® (RARG CRISPR guide RNA 2; Cat. no. SC1805), sequence of the gRNA is shown in Table SIII. Transfection of RARG CRISPR into the cells was carried out using Lipofectamine® LTX with Plus Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. KKU-213B cells were transfected with 0.5 μg RARG CRISPR in a liposome complex for 24 h at 37˚C and maintained for cell growth. After that, the transfected cells were selected using 0.5 μg/ml puromycin followed by single clone selection. The single clones were assessed for RARG protein expression using western blot analysis and DNA sequencing was performed to confirm RARA knockout. PCR amplification was performed using C1000™ Thermal Cycler (BioRad) and the conditions were as follows: an initial denaturation at 94˚C for 2 min followed by 35 amplification cycles of 30 sec denaturation at 94˚C, 30 sec annealing at 60˚C, 45 sec elongation at 72˚C and one cycle of final elongation at 72˚C for 5 min. The purified PCR products were subjected to nucleotide sequencing by Apical Scientific Sdn. Bhd. The Sanger sequences of KKU-M213B cells with detected CRISPR-mediated mutation in exon 1 of *RARG* (NM_000966.6) are shown in Data S1 and the sequencing traces of RARG CRISPR is shown in Fig. S1. RARG CRISPR mediated indel which encodes a truncated protein with a 133 amino acid sequence, as predicted using web.expasy.org/translate/(Data S1).

Small interfering RNA (siRNA). siRNAs are a frequently used tool to study the role of proteins of interest on cell function. siRNA transfection is a knockdown technique that has a transient effect on the expression of the targeted protein; by contrast, CRISPR/Cas9 is a knockout technique that has permanent effect. However, the CRISPR/Cas9 method is a complex and time-consuming process with a high failure rate. In the present study, knockout cells were developed using the CRISPR/Cas9 technique for all three RARs (data not shown); however, only CRISPR/Cas9-mediated RARG knockout cells were successfully created. Therefore, the siRNA technique was used to evaluate the role of the other RARs on the response of CCA cells to ATRA. Notably, the use of different techniques for knockdown/knockout may be a limitation of the present study.

Since the CRISPR/Cas9 transfection of at least two different gRNA target sequences for RARA or RARB failed (data not shown), RARA and RARB siRNA transfection was performed. RARA, RARB and non-targeting siRNA were purchased from GE Healthcare Dharmacon, Inc., and a detailed list of siRNA sequences is provided in Table SIII. Transfection of siRNA into cells was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, KKU-213B cells at a density of $1.5x10^5$ cells/well were seeded into a 6-well plate, and cells were transfected with 100 pmol RARA siRNA, RARB siRNA or non-targeting (NT) siRNA. The liposome-siRNA

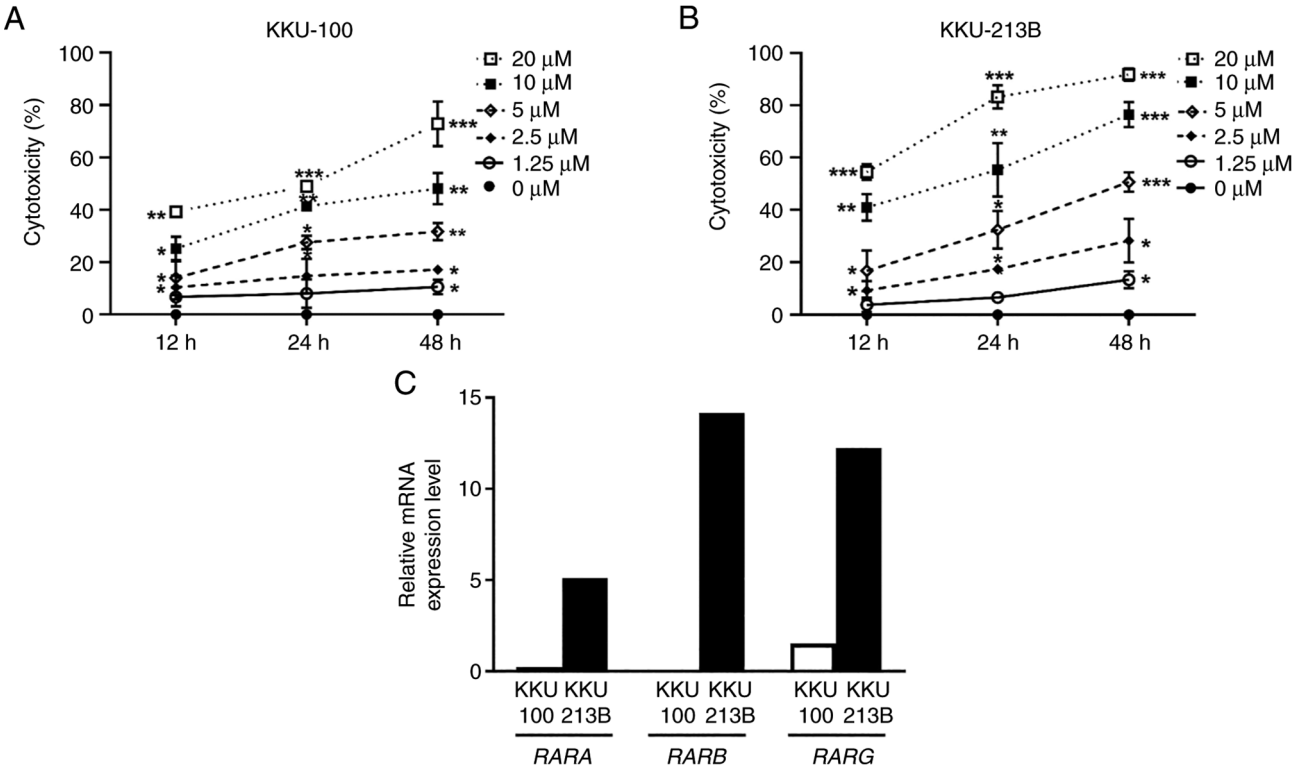

Figure 1. mRNA expression levels of RARs in cholangiocarcinoma cells and effect of ATRA on cell viability. (A) KKU-100 and (B) KKU-213B cells were treated with increasing concentrations of ATRA for 12, 24, and 48 h. SRB staining was performed to detect cell viability. Data are presented as the mean ± SD from three independent experiments. (C) mRNA expression levels of *RARA*, *RARB* and *RARG* in KKU-100 and KKU-213B cells were quantified using RT-qPCR and normalized to *ACTB*. Data from two independent experiments are presented. *P<0.05, **P<0.01 and ***P<0.001 vs. untreated control. ATRA, all-*trans*-retinoic acid; RAR, retinoic acid receptor.

complex was added to cells in serum-free medium without antibiotics for 6 h at 37˚C. Subsequently, the transfected cells were incubated further in culture medium for 48 h at 37˚C; the control group transfected with non-targeting siRNA was cultured under the same conditions. The efficiency of siRNAs was determined by western blotting of RARA and RARB proteins. siRNA-transfected cells were used to assess effects of ATRA on cell viability.

Statistical analysis. Data are presented as the mean ± SD from three independent experiments. Statistical comparisons between the control and treatment groups were performed by one-way ANOVA followed by Tukey's post hoc test using GraphPad Prism v8.0 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

ATRA reduces CCA cell viability. The present study evaluated the effect of ATRA on the viability of KKU-100 and KKU-213B cells. The SRB assay revealed that ATRA significantly reduced the viability of both CCA cell lines in a dose- and time-dependent manner (Fig. 1A and B). The IC_{50} values at 48 h were 10.29±3.86 μM for KKU-100 cells and 4.58±1.90 μM for KKU-213B cells. Subsequently, RT-qPCR analysis was used to assess the basal mRNA expression levels of RARs; KKU-100 expressed low levels of *RARA*, *RARB* and *RARG*, whereas KKU-213B cells expressed higher levels of these receptors (Fig. 1C). These findings indicated that KKU-213B cells were more sensitive to ATRA cytotoxicity than KKU-100 cells.

ATRA cytotoxicity in CCA cells is RARB-dependent. ATRA is a pan-agonist of RARs (RARA, RARB and RARG). ATRA binding to RARs has been reported to be sufficient for RAR-RXR heterodimers to confer ligand-dependent activation of target gene transcription (10), which can affect cell growth and development, and responses to toxicant exposure. To examine whether RARA, RARB and RARG contributed to ATRA cytotoxicity in CCA cells, a loss-of-function approach was used. KKU-213B cells with high RAR expression were transfected with siRARA, siRARB, non-targeting control siRNA or RARG CRISPR, and the expression levels of RARs were validated by western blotting. Knockdown of RARA and RARB using siRNA efficiently decreased the protein expression levels of RARA and RARB (Fig. 2A and C). Compared with in parental KKU-213B cells, CRISPR/Cas9-mediated RARG knockout cells exhibited a clear loss of RARG expression (Fig. 2E). Both siRNA transfection and CRISPR/Cas9 are very useful techniques to study the role of the proteins of interest on cell function. Notably, the effects of siRNA are transient, whereas those of CRISPR/Cas9 are permanent; however, both techniques perform a similar function and effectively suppressed the expression levels of RAR subtypes by >80%.

When the RAR-deficient cells were tested for ATRA cytotoxicity, the results revealed that the IC_{50} values of 48-h ATRA

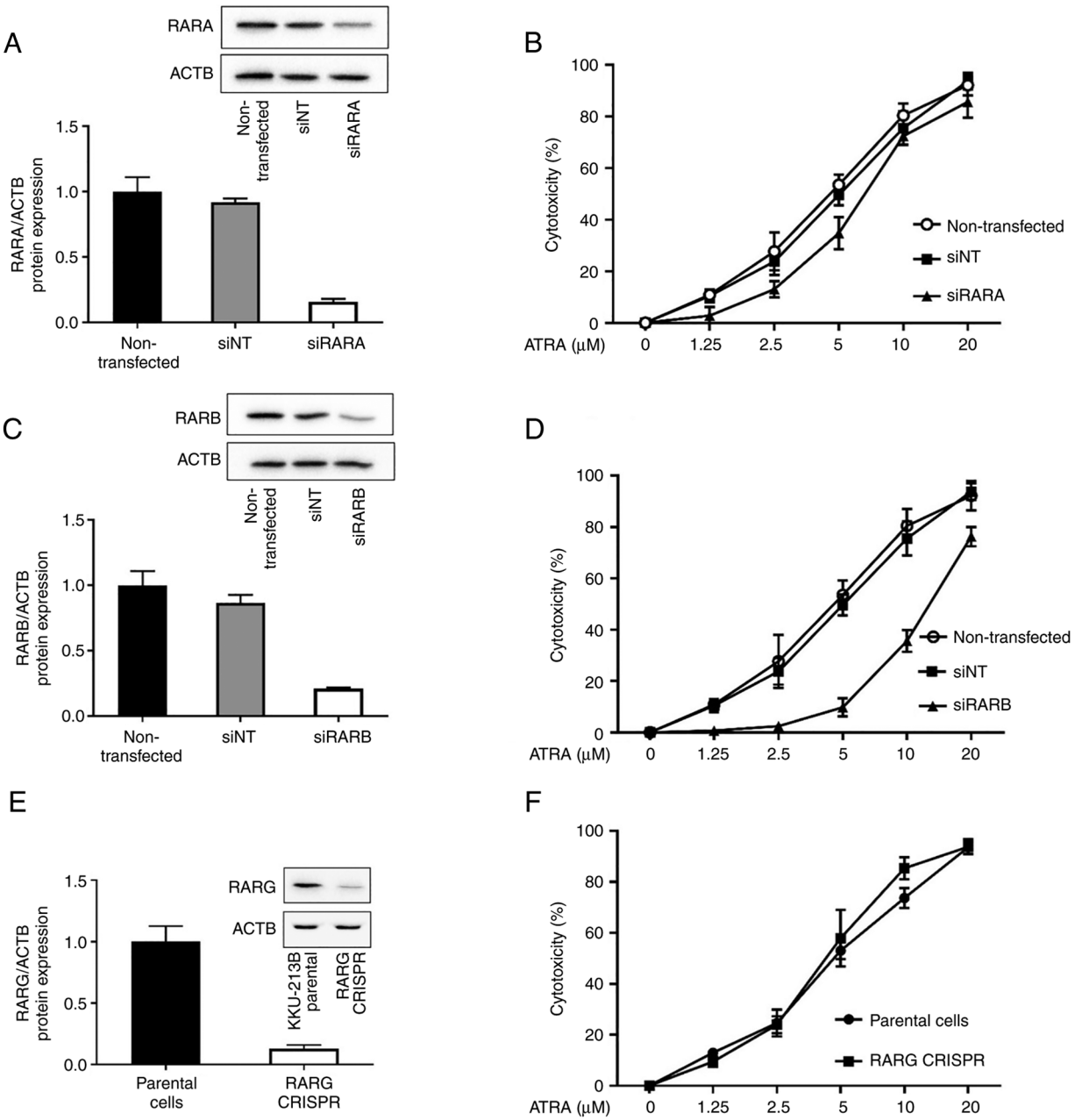

Figure 2. ATRA cytotoxicity in cholangiocarcinoma cells is partly RAR-dependent. KKU-213B cells were transfected with siRARA, siRARB, siNT or RARG CRISPR. The RAR expression and cell viability of cells were transfected with (A and B) siRARA, (C and D) siRARB and (E and F) RARG CRISPR are shown. Protein expression level was assessed by western blot analysis and cell viability was measured following treatment with increasing concentrations of ATRA for 48 h. Data are presented as the mean ± SD from two independent experiments. ATRA, all-*trans*-retinoic acid; RAR, retinoic acid receptor; NT, non-targeting; si, small interfering.

treatment were not altered by siRARA or RARG CRISPR compared with in cells transfected with the non-targeting control siRNA or in KKU-213B parental cells, respectively (Fig. 2B and F; Table I). Notably, knockdown of RARβ by siRNA caused a 3-fold increase in the IC_{50} value of ATRA (14.7±2.4 μM) when compared with the IC_{50} value observed in cells transfected with the non-targeting control siRNA (4.8±2.5 μM) (Fig. 2D; Table I). These results suggested that RARβ was, at least in part, required for ATRA cytotoxicity in CCA cells.

ATRA dose-dependently induces CCA cell apoptosis. To further examine the cell death mechanism induced by ATRA treatment in CCA cells, apoptosis was evaluated by flow cytometry. We selected 1.25 and 2.5 μM ATRA, which induced cytotoxicity in both CCA cell lines, to explore the time sequencing of the apoptosis cascade. The results revealed that ATRA significantly increased the number of Annexin V-PE-positive cells in Q2 and Q4, which is an index of apoptotic cells, compared with in the untreated control group (Fig. 3). Following treatment with 1.25 and 2.5 μM ATRA for 48 h, the apoptotic rates of KKU-100 cells were 22 and 38%, whereas the apoptotic rates of KKU-213B cells were 22 and 41%, respectively (Fig. 3A and B). The representative images from one experiment of flow cytometry were shown in Fig. 3C for KKU-100 and Fig. 3D for KKU-213B cells. These

Table I. IC_{50} values of ATRA in KKU-213B cells following RAR knockdown/knockout.

Condition	IC_{50} of 48-h ATRA, μM
siNT	4.8±2.5
siRARA	6.3±4.8
siRARB	14.7±2.4
Parental cells	4.6±2.2
RARG CRISPR	4.1±2.6

ATRA, all-*trans*-retinoic acid; IC_{50}, half maximal inhibitory concentration; NT, non-targeting; RAR, retinoic acid receptor; si, small interfering.

results indicated that ATRA induced cytotoxicity in CCA cells via the induction of apoptosis.

ATRA-induced CCA cell apoptosis is mediated through activation of caspase-3 and -9. Since ATRA was revealed to induce apoptosis, the present study further explored the mechanisms underlying its effects. The pathways of apoptotic induction following ATRA treatment were examined using enzymatic caspase activity assays. Following treatment with 1.25 and 2.5 μM ATRA for 12 h, there was an increase in caspase-9 and -3 enzyme activity in both CCA cell lines compared with that in the untreated control cells ($P<0.05$; Fig. 4A, B, E and F). These results indicated that the mitochondrial apoptotic pathway was activated. Notably, ATRA at a concentration of 2.5 μM significantly increased caspase-8 activity in KKU-100 cells (Fig. 4C), but not in KKU-213B (Fig. 4D); suggesting ATRA-induced activation of the extrinsic pathway may be dependent on concentration and cell type.

ATRA increases the expression levels of apoptosis-associated proteins, Bax, AIF and cytochrome c, in CCA cells. The mechanism underlying ATRA-induced apoptosis was further investigated. The effect of ATRA on the expression levels of pro-apoptotic proteins, including Bax, cytochrome *c* and AIF, were determined by western blot analysis. Following treatment with ATRA (1.25 and 2.5 μM) for 6 h, the protein expression levels of Bax, cytochrome *c* and AIF were significantly increased in KKU-100 (Fig. 5A, C, E and G) and KKU-213B (Fig. 5B, D, F and -H) cells. These results revealed that ATRA upregulated the expression levels of apoptosis-inducing proteins in CCA cells, resulting in the initiation of apoptosis.

ATRA increases the cellular content of ROS in CCA cells. Several studies have reported that increased ROS are associated with the initiation and activation of apoptosis in various types of cancer, including CCA (28,34,35). To investigate the effects of ATRA on intracellular ROS content, a DHE assay was performed. Following treatment with ATRA (1.25 and 2.5 μM) for 90 min, the intracellular ROS levels were significantly increased in KKU-100 (Fig. 6A) and KKU-213B cells (Fig. 6B). Phase-contrast microscopy revealed that ATRA-treated and untreated control cells maintained their original morphology and cell density and the representative images of ethidium staining in the nucleus are shown in Fig. 6C.

ATRA-induced cellular ROS accumulation contributes to CCA cell apoptosis. Previous observations revealed that ATRA increased cellular ROS levels in CCA cells. Subsequently, the causal relationship between ROS and apoptosis induction by ATRA was further investigated. KKU-100 and KKU-213B cells were pre-treated with ROS scavengers NAC and TEMPOL, and were then exposed to 1.25 and 2.5 μM ATRA prior to apoptosis analysis by flow cytometry. When cells were pre-treated with NAC or TEMPOL, these ROS scavengers completely blocked the apoptotic effect of ATRA (KKU-100, Fig. 7A; KKU-213B, Fig. 7B). These results supported that ATRA-induced ROS accumulation was essential for the apoptosis of CCA cells. Moreover, in cells treated with a high dose of ATRA (5 μM), NAC and TEM also partially suppressed ATRA-induced apoptosis (Figs. S2 and S3). NAC or TEMPOL partly reduce the percentage of cell death by high dose of ATRA, suggesting non-ROS mechanisms involving in ATRA cytotoxicity. These findings indicated that ATRA induced the apoptosis of CCA cells by both ROS-dependent and ROS-independent mechanisms.

ATRA downregulates the expression levels of NRF2 and NRF2 target antioxidant genes in CCA cells. Since NRF2 signaling is the primary regulator for the balance of cellular ROS levels and ROS accumulation was essential for the ATRA-induced apoptosis of CCA cells, the present study investigated the effects of ATRA on the expression levels of *NRF2* and *NRF2* target genes, which encode for cellular antioxidant proteins. The results revealed that the expression levels of *NRF2* and *NRF2* target genes, including *NQO1*, *GCLC* and *GSTP1*, were decreased in both KKU-100 (Fig. 8A, C, E and G) and KKU-213B (Fig. 8B, D, F and H) cells following ATRA treatment. These results suggested that ROS accumulation after ATRA treatment may be caused by downregulation of the *NRF2* pathway, leading to the induction of CCA cell apoptosis.

ATRA enhances the cytotoxicity of anticancer drugs partly through NRF2 downregulation. To evaluate whether ATRA could enhance the cytotoxic effect of anticancer drugs, an SRB assay was performed. Cells were treated with 2.5 μM ATRA or anticancer drugs, or a combination of ATRA and anticancer drugs, including 5-fluorouracil, gemcitabine, cisplatin and doxorubicin, for 48 h. In KKU-100 cells, ATRA significantly increased the cytotoxicity of 10 μM cisplatin (Fig. 9E); however, it had no effects on the cytotoxicity of other drugs used in the present study (Fig. 9A, B and D). In KKU-213B cells, ATRA significantly enhanced the cytotoxicity of 100 and 300 μM 5-fluorouracil and 2.5, 5, 10 μM cisplatin (Fig. 9B and F). ATRA treatment resulted in markedly improved cisplatin sensitivity in KKU-213B cells compared with in KKU-100 cells. ATRA did not change the sensitivity of KKU-213B cells to gemcitabine and doxorubicin (Fig. 9D and H).

In CCA, cisplatin has been reported to induce the expression of NRF2 target antioxidant genes to promote chemoresistance (30). Since NRF2 is the primary regulator of cellular antioxidant defense, the present study explored

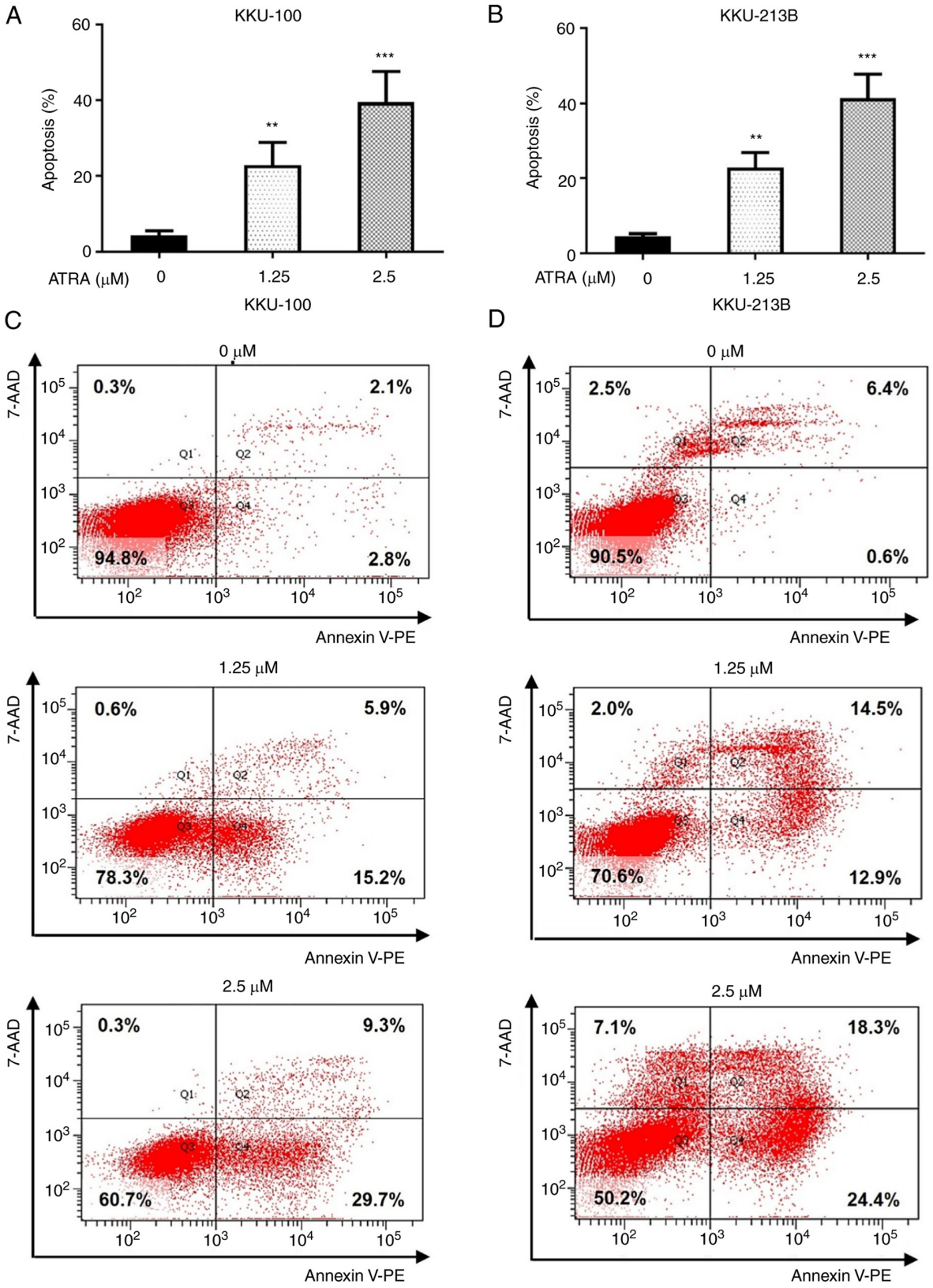

Figure 3. Apoptosis is induced by ATRA in cholangiocarcinoma cells. (A) KKU-100 and (B) KKU-213B cells were treated with 1.25 and 2.5 μM ATRA for 48 h. Annexin V-PE/7-AAD staining and flow cytometry was performed to detect the percentage of apoptotic cells. The representative images from one experiment of flow cytometry in (C) KKU-100 and (D) KKU-213B cells are shown. Data are presented as the mean ± SD from three independent experiments. **P<0.01 and ***P<0.001 compared with the untreated control. ATRA, all-*trans*-retinoic acid.

whether ATRA enhanced cisplatin cytotoxicity in CCA cells by suppressing *NRF2*. Firstly, it was confirmed that *NRF2* expression was upregulated following cisplatin treatment, whereas ATRA alone significantly reduced the mRNA expression levels of *NRF2* in KKU-100 and KKU-213B cells compared with untreated control cells (Fig. 9I and J). Both

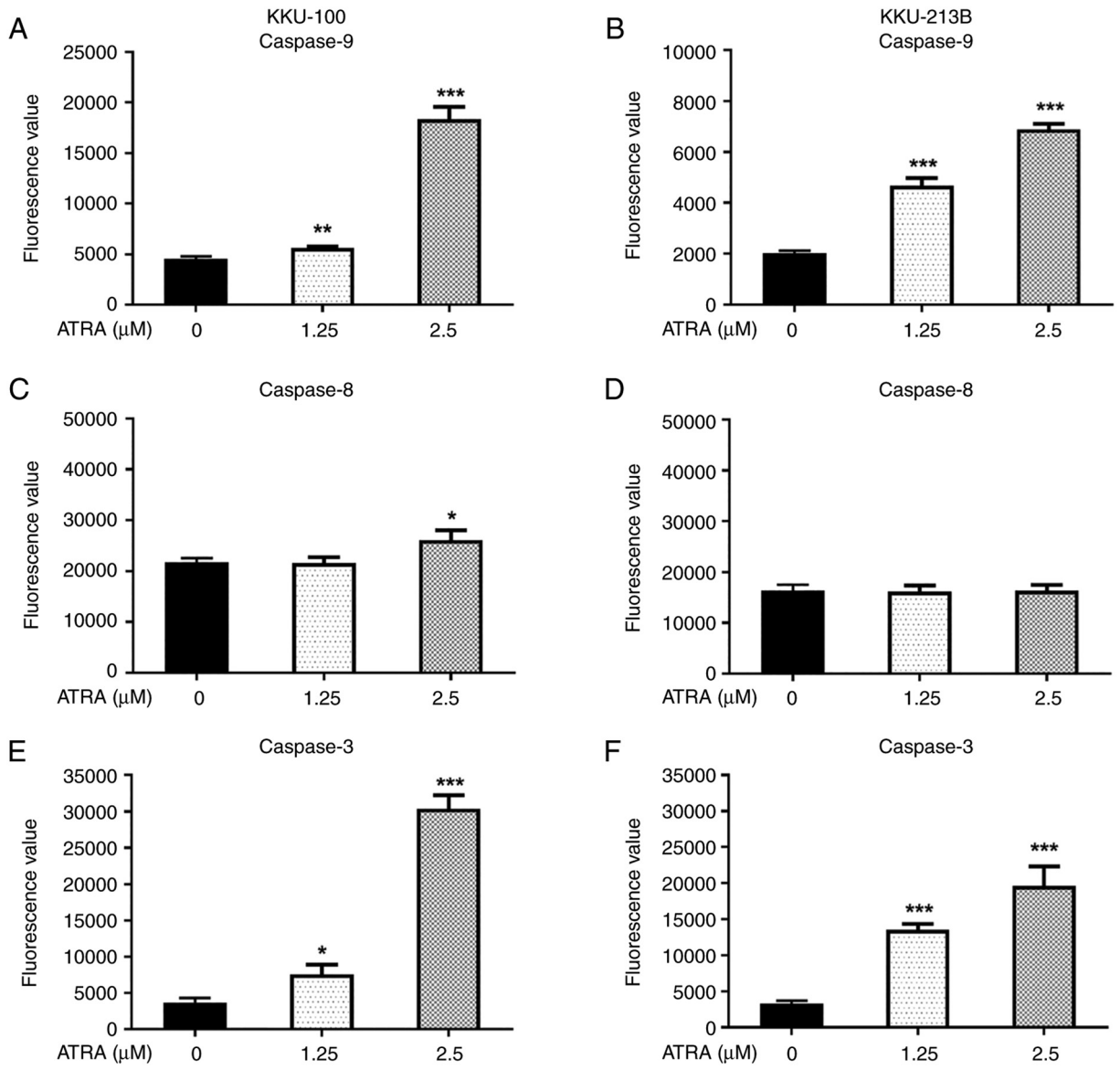

Figure 4. Activation of caspase-3 and caspase-9 by ATRA in cholangiocarcinoma cells. KKU-100 and KKU-213B cells were treated with 1.25 and 2.5 μM ATRA for 12 h. The fluorescence signals were assessed and (A and B) caspase-9, (C and D) caspase-8 and (E and F) caspase-3 activities were determined in KKU-100 cells and KKU-213B cells. Data are presented as the mean ± SD from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared with the untreated control. ATRA, all-*trans*-retinoic acid.

CCA cell lines, co-treatment with ATRA and cisplatin showed decreased mRNA expression levels of *NRF2* compared with cells treated with cisplatin alone (Fig. 9I, J). These results suggested that ATRA suppressed cisplatin-induced *NRF2*, which may increase the cisplatin sensitivity of CCA cells.

Discussion

The present study assessed the effects of ATRA treatment on CCA cell apoptosis at different timepoints, and on the underlying mechanism and cascade. ROS are important factors that induce intracellular stress and trigger apoptosis; notably, several studies have suggested that 90 min is sufficient to monitor the changes in intracellular ROS following exposure to stimuli (29,30). Similarly, in the present study, ATRA-induced ROS production was observed at 90 min and alterations in the expression levels of pro-apoptotic proteins were detected 6 h after ATRA-induced ROS production. According to apoptotic signaling cascades, the increase in pro-apoptotic proteins can trigger caspase activation; in the present study, the activity of caspases was elevated at 12 h. Following ATRA-induced caspase activation, it was further confirmed that apoptosis was induced at 48 h by flow cytometry. Previously, decreases in *NRF2* and *NRF2* target genes have been reported to be associated with apoptosis induction (29,30); therefore, the effect of ATRA on the expression levels of *NRF2* and *NRF2* target genes were assessed at 48 h, which is the time at which apoptosis induction was detected.

The results of the present study revealed that ATRA-induced cytotoxicity in CCA cells, at least in part, depended on the specific receptor RARB. Treatment with ATRA promoted apoptosis in CCA cells by activating the intrinsic pathway via induction of Bax, AIF, cytochrome *c* and caspase-9 enzyme. ATRA caused an increase in intracellular ROS content, leading

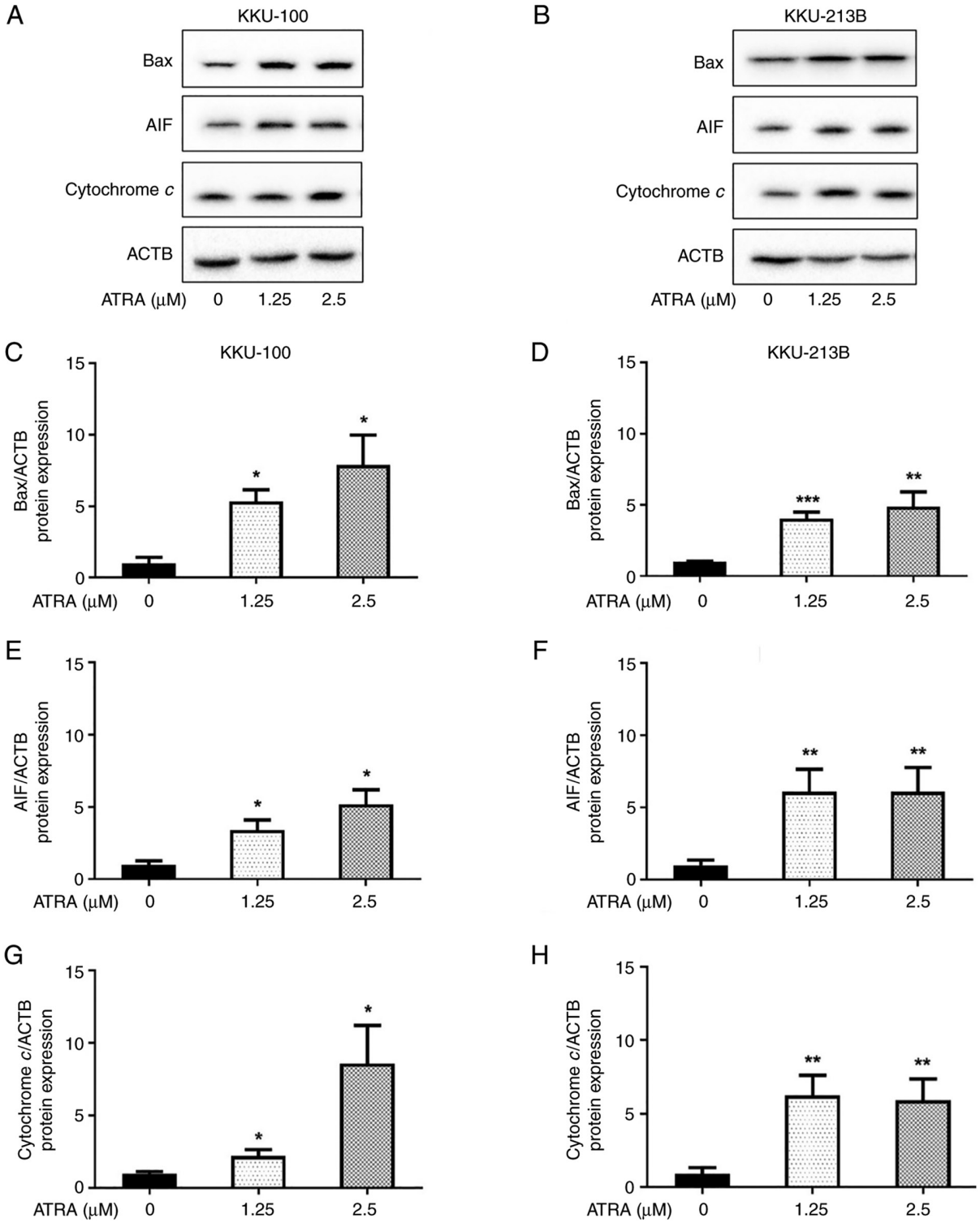

Figure 5. ATRA induces the expression of pro-apoptotic proteins in cholangiocarcinoma cells. (A and B) KKU-100 and KKU-213B cells were treated with 1.25 and 2.5 μM ATRA for 6 h, and the expression levels of Bax, AIF and cytochrome *c* proteins were assessed by western blot analysis. The intensity of (C and D) Bax, (E and F) AIF and (G and H) cytochrome *c* bands in KKU-100 and KKU-213B cells were semi-quantified. Data are presented as the mean ± SD from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared with the untreated control. ATRA, all-*trans*-retinoic acid.

to the observed cytotoxicity of ATRA. By contrast, pre-treatment with ROS scavengers NAC and TEMPOL diminished the apoptosis-inducing effect of ATRA; however, NAC and TEMPOL also have other anti-apoptotic mechanisms and can act as direct antioxidants to scavenge radical molecules or as indirect antioxidants by restoring redox cycling system in cells (29). NAC and TEMPOL also control the redox balance of redox-sensitive proteins, such as PI3K, NRF2 and p53, thus they may exert anti-apoptotic effects via regulation of these proteins (36-38).

ATRA could suppress the expression of *NRF2* and *NRF2* target antioxidant genes; ATRA also caused cellular ROS

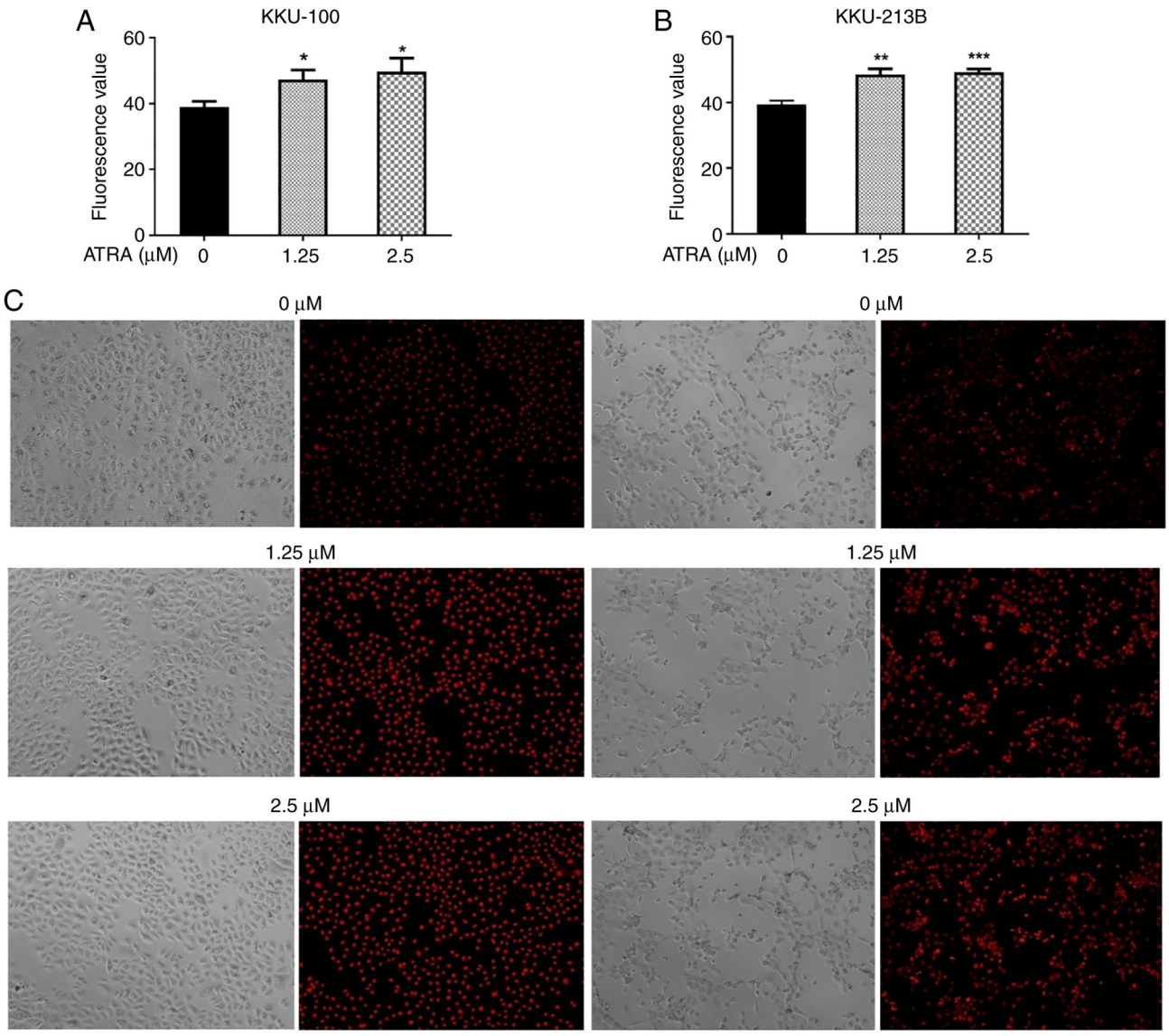

Figure 6. Increased intracellular ROS levels were induced by ATRA in cholangiocarcinoma cells. KKU-100 and KKU-213B cells were treated with 1.25 and 2.5 μM ATRA combined with dihydroethidium for 90 min. The ROS levels in (A) KKU-100 and (B) KKU-213B cells were determined using a fluorescence plate reader. (C) Representative images of phase contrast and ethidium staining in the nucleus were captured under a 4X magnification power florescence microscope. Data are presented as the mean ± SD from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared with the untreated control. ATRA, all-*trans*-retinoic acid; ROS, reactive oxygen species.

accumulation. In addition, ATRA significantly enhanced the sensitivity of CCA cells to cisplatin. Notably, the improvement of the cisplatin response in ATRA-treated CCA cells may be related to the ATRA-downregulated *NRF2* gene, which is considered the master regulator for cellular antioxidant defense.

Previous studies reported the cytotoxic effects of ATRA on several types of cancer cells, such as breast cancer, non-small cell lung carcinoma, gastric cancer and CCA (22,39-41). The present study confirmed that ATRA had a potent cytotoxic effect on CCA with IC_{50} values at 48 h as 4.58 μM in KKU-213B and 10.29 μM in KKU-100 cells. It is well known that ATRA acts as a pan-agonist of the RARs to regulate several physiological processes, and control growth and development. Two CCA cell lines with different expression levels of RARs were used in the present study. KKU-213B cells possessed high expression levels of RARs, whereas KKU-100 cells exhibited lower RAR expression. In the current study, the IC_{50} of ATRA in KKU-213B cells was 2-fold lower than that in KKU-100, thus suggesting that cells with higher RAR expression were more sensitive to ATRA cytotoxicity. To prove that ATRA sensitivity was dependent on RARs, cells deficient in different types of RAR were created by transfecting KKU-213B cells with siRARA, siRARB, non-targeting control siRNA or RARG CRISPR. Knockdown/knockout of RARA or RARG had no effect on ATRA sensitivity compared with in the control groups. Notably, cells with a loss in RARB expression via siRNA were less sensitive to ATRA, suggesting that ATRA cytotoxicity was partly mediated through RARB. Previous reports have identified RARB-mediated cell cytotoxicity through alterations in histone acetyltransferase, apoptosis-associated proteins and cell cycle-associated proteins in oral cancer and breast cancer cells (42,43). Furthermore, the association between

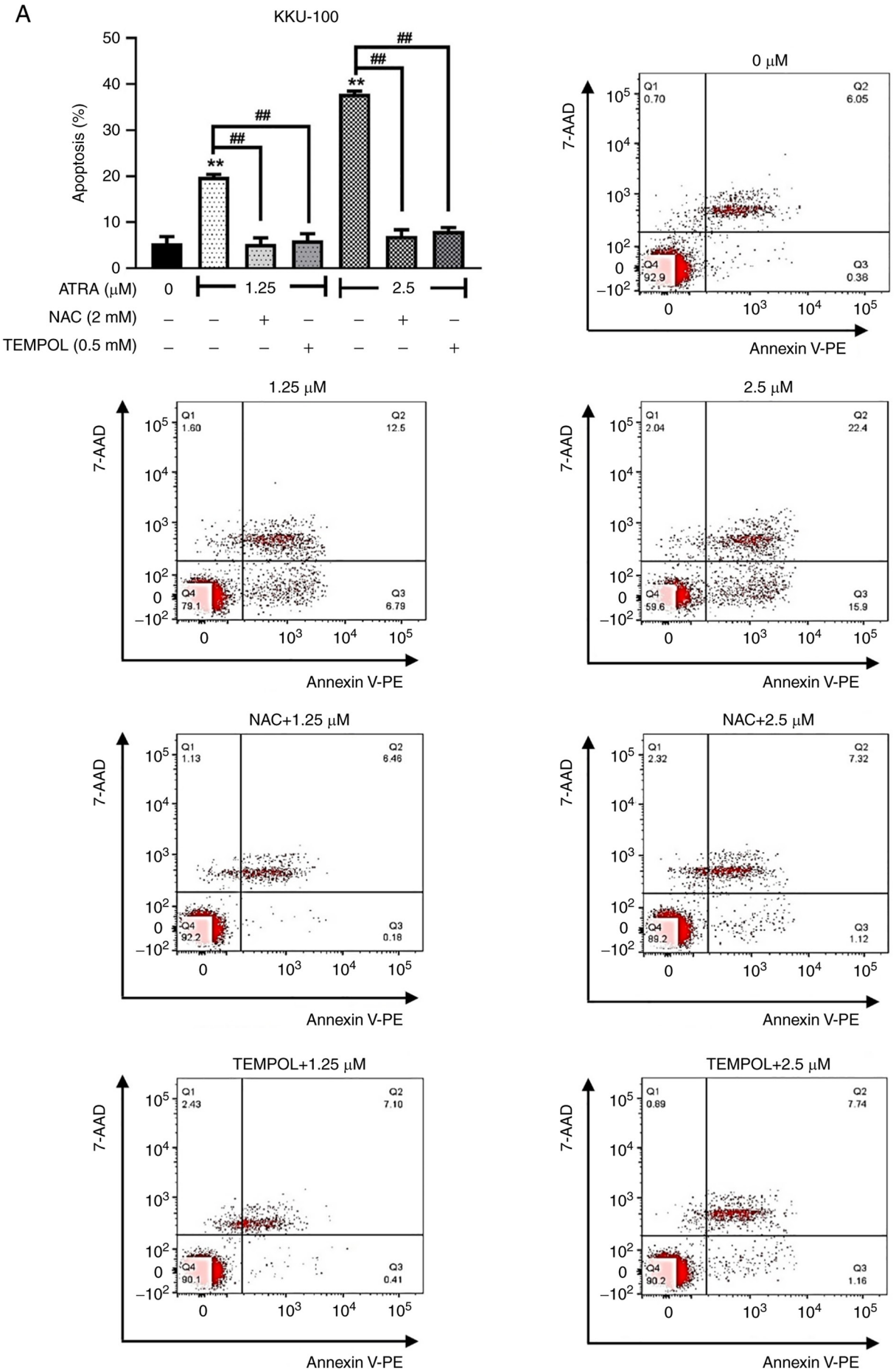

Figure 7. Continued.

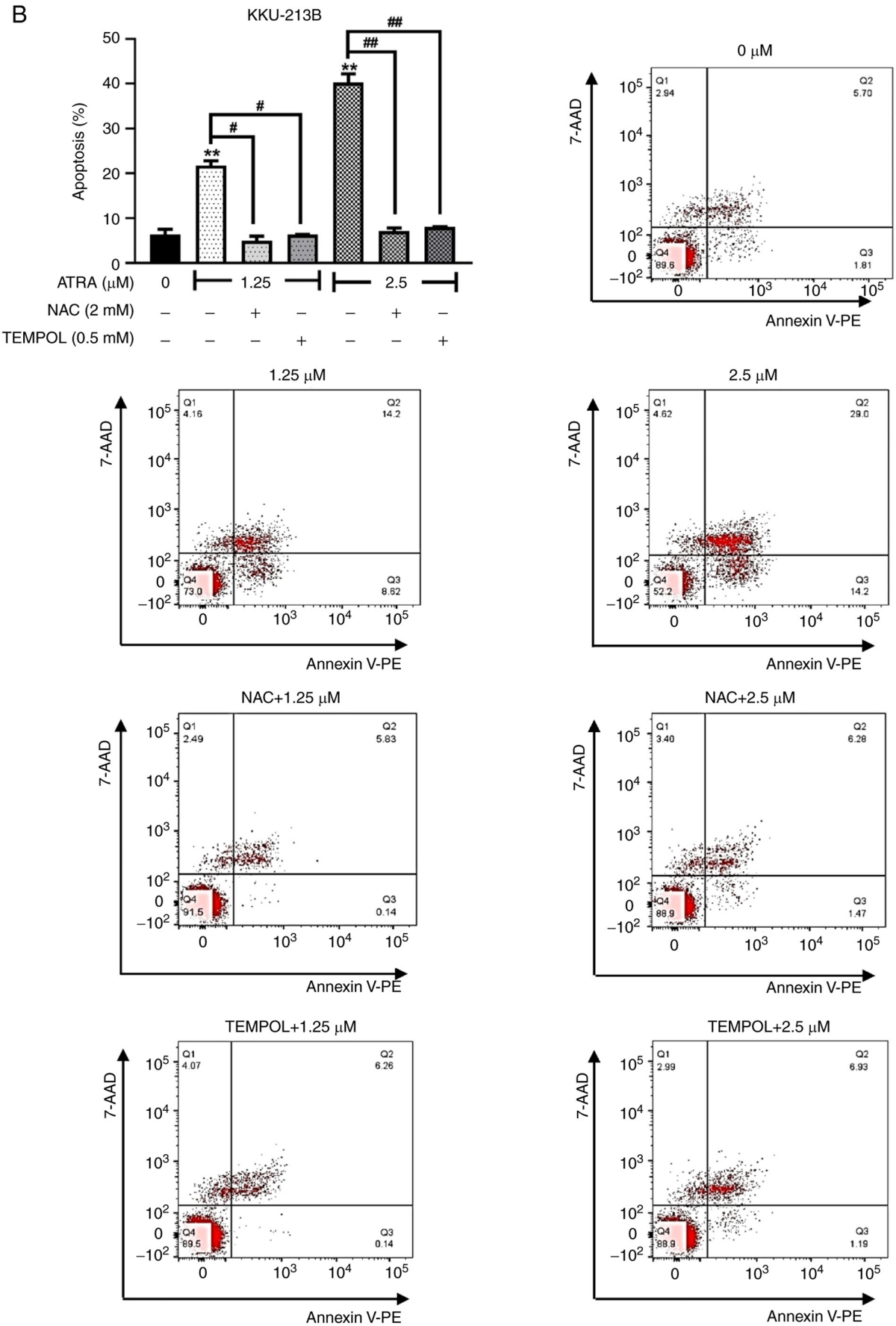

Figure 7. ATRA-induced apoptosis is causally related to ROS formation. (A) KKU-100 and (B) KKU-213B cells were 3-h pre-treated with ROS scavengers (NAC or TEMPOL) and further incubated with 1.25 and 2.5 μM ATRA for 48 h. Annexin V-PE/7-AAD staining and flow cytometry was performed to detect apoptotic cell death. Data are presented as the mean ± SD from three independent experiments. **P<0.01 compared with the untreated control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 compared with ATRA alone. ATRA, all-*trans*-retinoic acid; ROS, reactive oxygen species.

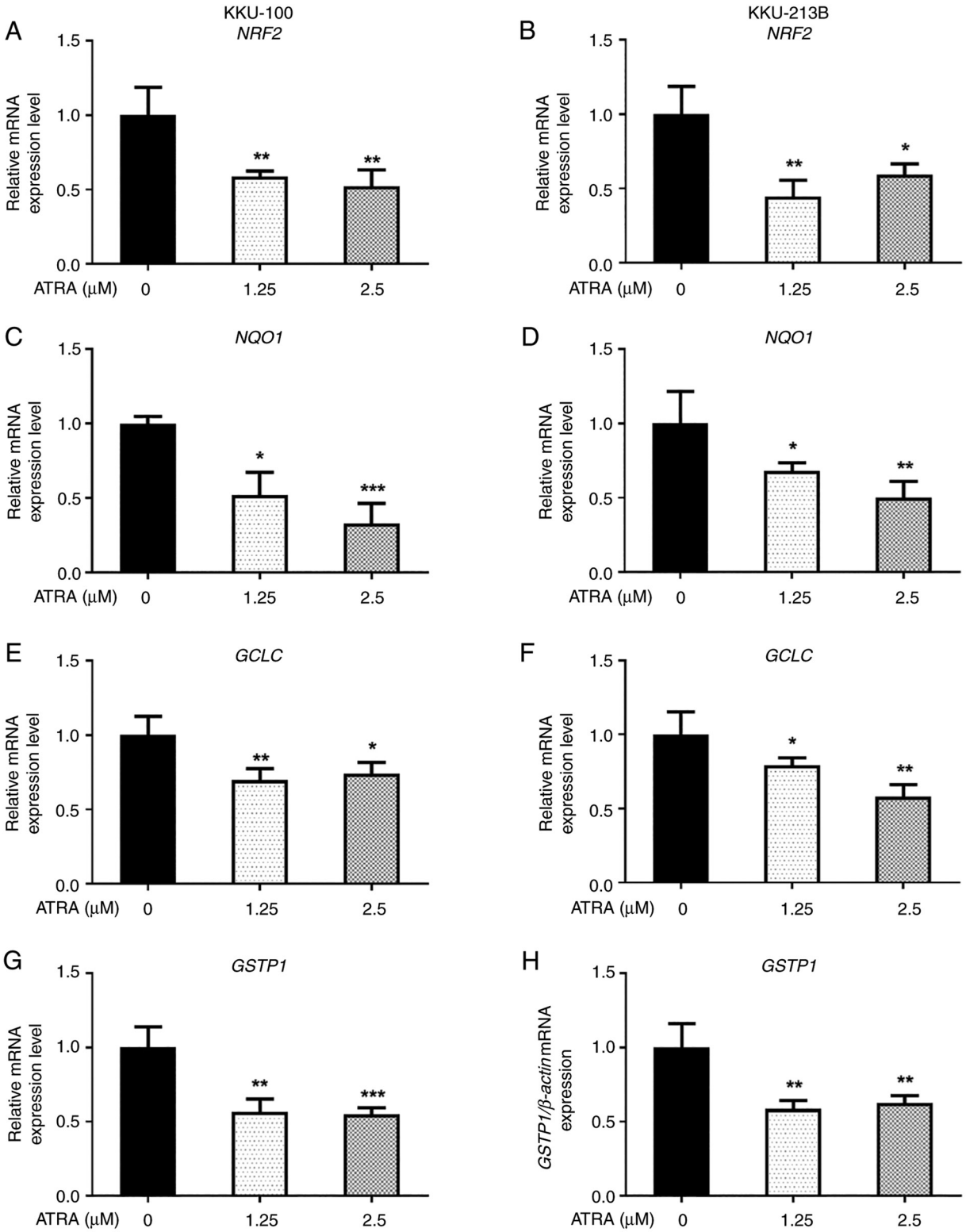

Figure 8. ATRA downregulates the expression levels of *NRF2* and *NRF2* target antioxidant genes. KKU-100 and KKU-213B cells were treated with 1.25 and 2.5 μM ATRA for 48 h, and the mRNA expression levels of (A and B) *NRF2*, (C and D) *NQO1*, (E and F) *GCLC* and (G and H) *GSTP1* were determined in KKU-100 and KKU-213B cells by RT-qPCR and normalized to *ACTB*. Data are presented as the mean ± SD from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared with the untreated control. ATRA, all-*trans*-retinoic acid; *NRF2*, nuclear factor erythroid 2-related factor 2.

altered expression of RARs or dysfunctional RARs with the malignant transformation of human cells has previously been presented. In CCA, Ren *et al* (23) proposed the role of RARB as a tumor suppressor and reported that upregulation of RARβ reversed drug resistance by enhancing apoptotic susceptibility. Similarly, the present study demonstrated that RARB expression was one of the required factors for ATRA sensitivity, supporting a tumor-suppressor role of RARB in CCA.

The present study demonstrated that ATRA induced cell death by apoptosis induction in CCA cells. Previous studies

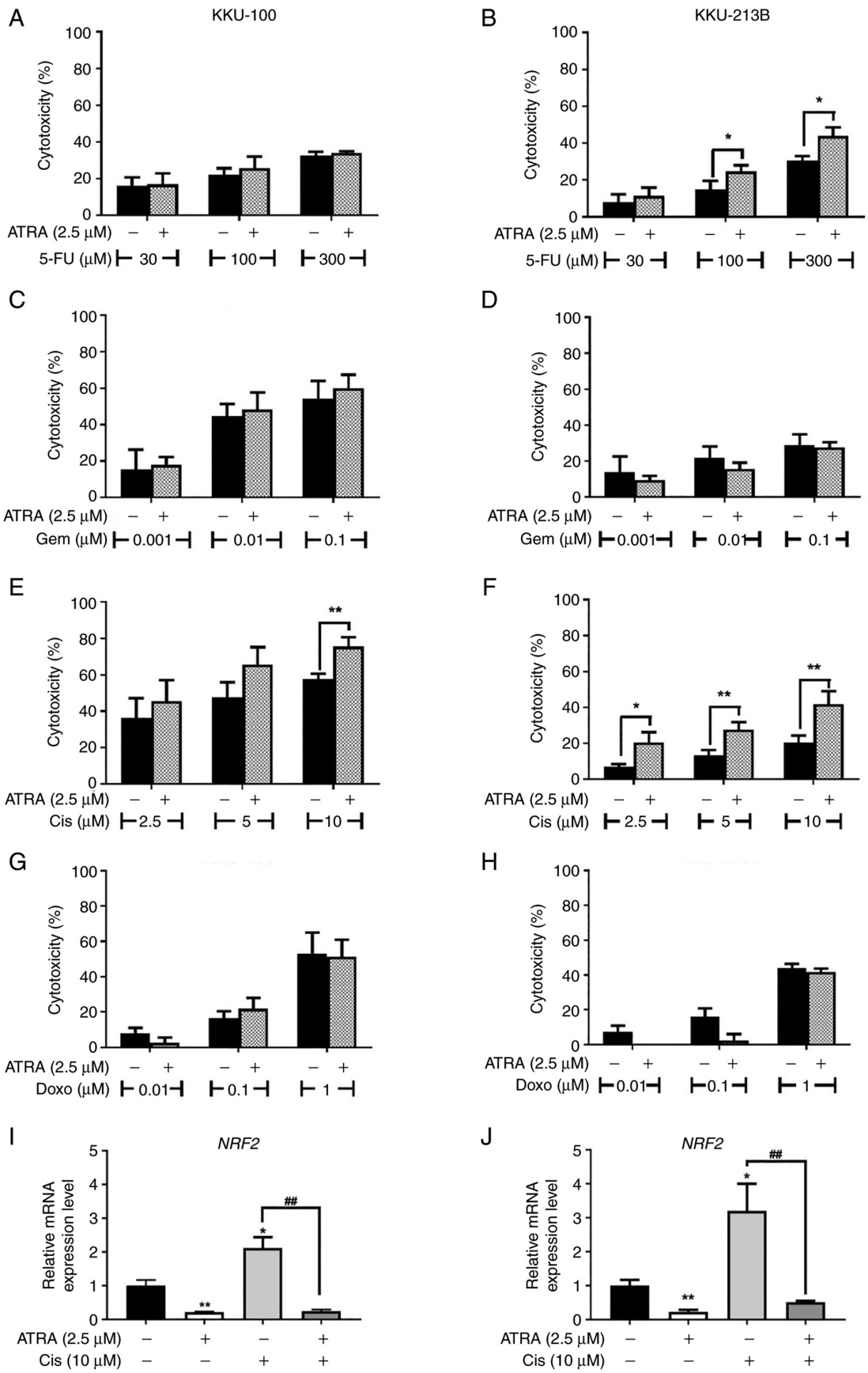

Figure 9. Cytotoxicity of anticancer drugs is enhanced by ATRA co-treatment. (A, C, F and G) KKU-100 and (B, D, E and H) KKU-213B cells were treated with ATRA or anticancer drugs, or were co-treated with ATRA and (A and B) 5-FU, (C and D) Gem, (E and F) Cis and (G and H) Doxo anticancer drugs. Data are presented as the mean ± SD from three independent experiments. *P<0.05 and **P<0.01. mRNA expression levels of *NRF2* in (I) KKU-100 and (J) KKU-213B cells were determined by reverse transcription-quantitative PCR and normalized to *ACTB*. Data are presented as the mean ± SD from three independent experiments. *P<0.05 and **P<0.01 compared with the untreated control; $^{\#\#}$P<0.01 compared with Cis alone. 5-FU, 5-fluorouracil; ATRA, all-*trans*-retinoic acid; Cis, cisplatin; Doxo, doxorubicin; Gem, gemcitabine; *NRF2*, nuclear factor erythroid 2-related factor 2.

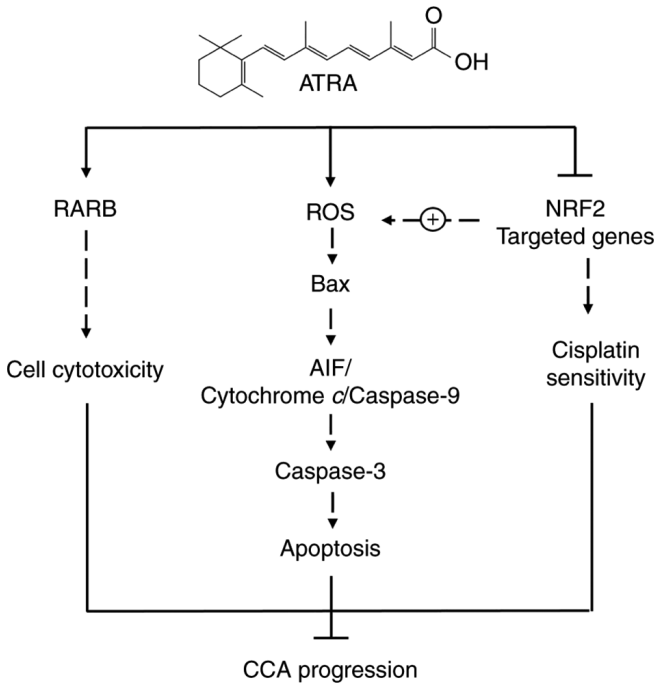

Figure 10. Possible mechanism underlying the induction of apoptosis and enhancement of chemosensitivity mediated by ATRA in CCA cells. ATRA, all-*trans*-retinoic acid; CCA, cholangiocarcinoma; RAR, retinoic acid receptor; ROS, reactive oxygen species; NRF2, nuclear factor erythroid 2-related factor 2; The dash lines indicate undetermined processes.

have reported that ATRA can promote the apoptosis of several types of cancer cells, such as pancreatic cancer, breast cancer and medulloblastoma (15-20). Apoptotic cell death occurs when cells have a loss of mitochondria membrane potential, upregulation of caspase-3 and an increase in DNA damage (44-46). It is well accepted that ROS accumulation is one of the initial events that trigger apoptosis in cancer cells, including in CCA (28,34,35). The present study revealed that ATRA enhanced ROS production, consequently inducing intracellular stress and upregulating the expression levels of pro-apoptotic proteins. Upregulated Bax protein may form pores in mitochondria leading to loss of membrane potential and leakage of apoptotic inducer components, such as AIF and cytochrome *c*, into cytosol. When translocated into the nucleus, AIF can trigger chromatin condensation and DNA fragmentation, whereas cytochrome *c* forms the apoptosome to activate caspase-9 and -3 activities leading to apoptosis (28). Although the extrinsic (caspase-8 mediated) pathway was unlikely affected by ATRA, the present study revealed that, at a high dose, ATRA could increase the extrinsic pathway in KKU-100 cells by increasing caspase-8 enzymatic activity (Fig. 4C). Dhandapani *et al* (47) previously showed that ATRA sensitized cancer cells to TRAIL-induced apoptosis by upregulating the expression of TRAIL-R1. However, in the present study, whether ATRA increased caspase-8 activity and induced the extrinsic pathway through the TRAIL mechanism in CCA has not been confirmed. In addition, the ATRA-induced extrinsic pathway was only observed in KKU-100 cells; thus, ATRA-induced activation of the extrinsic pathway may be concentration-dependent and cell type-specific.

The present study demonstrated that ATRA-induced ROS production mediated CCA cell apoptosis. Human cells use NRF2 signaling as the primary regulator for controlling the balance of cellular ROS levels. Loss or dysfunction of NRF2 signaling can lead to abnormal cell survival and death (29,30). The present study identified a suppressive effect of ATRA on the expression levels of *NRF2* and *NRF2* target genes in CCA cells, which may explain the increasing cellular ROS content induced by ATRA treatment.

CCA is a type of cancer that can exhibit chemoresistance, thus identifying more effective therapeutics against CCA resistance is essential to research. Previous studies have revealed that ATRA can enhance cisplatin sensitivity in liver cancer cells, 5-fluorouracil sensitivity in breast cancer cells and gemcitabine sensitivity in pancreatic cancer cells (18-20). In the present study, the effects of ATRA on the sensitivity of the common four chemotherapeutic agents used in CCA treatment, 5-fluorouracil, gemcitabine, cisplatin and doxorubicin, were tested. The results revealed that ATRA could increase the sensitivity of cisplatin in both CCA cell lines and 5-FU in KKU-213B cells. Notably, the improved sensitivity of cisplatin and 5-fluorouracil was more marked in KKU-213B cells than in KKU-100 cells. Cisplatin sensitivity may depend on the type of cancer cells; differences in genetic background between KKU-213B and KKU-100 CCA cells may be the cause of the different anticancer response (30). Therefore, RARs may not be the only factor driving cisplatin sensitivity in CCA cells, and it was hypothesized that the cytotoxic effect of ATRA in CCA cells may be both RAR-dependent in KKU-213B cells and RAR-independent in KKU-100 cells. There was no significant effect of ATRA on doxorubicin sensitivity in both CCA cells. A previous report demonstrated that ATRA reduced doxorubicin cytotoxicity through suppressing ROS generation by restoring the mRNA and protein expression levels of phase II detoxifying enzyme and via ERK2 activation in cardiomyocytes, without compromising doxorubicin cytotoxicity in gastric cancer cells (48). However, the combination effect of ATRA and doxorubicin in CCA treatment need to be confirmed in a further study.

In most types of cancer, drug resistance after long-term chemotherapy is an important event associated with poor clinical outcomes. Impaired anticancer drug response can be caused by increasing cellular protection and antioxidant defense via NRF2 activation. Increased *NRF2* and *NRF2* target genes after anticancer drug treatment has been presented in several types of cancer. Furthermore, suppressing NRF2 has been proposed as a strategy to improve the sensitivity of chemotherapeutic agents in CCA (29,30). In the present study, ATRA suppressed the expression levels of *NRF2* and *NRF2* target genes, and prevented cisplatin-induced *NRF2* expression; therefore, the effects of ATRA on enhancing the sensitivity of CCA cells to anticancer agents may be partly mediated via suppressing NRF2 signaling. The expression levels of *NRF2* and *NRF2*-related genes were detected at 48 h after ATRA treatment; therefore, the differences in these expression levels may not be a direct cause of ROS production at 90 min. However, the downregulation of *NRF2* and *NRF2*-related genes, which may be associated with increased cellular oxidative stress, and decreased protection and survival of cells, could be one important factor driving cell death

and impaired cellular defensive mechanism against noxious stimuli and anticancer drug toxicity (29,30). The possible mechanism of action of ATRA in the induction of apoptosis and enhancement of chemosensitivity in CCA cells has been summarized in Fig. 10.

In conclusion, the present study demonstrated that ATRA had an anticancer effect on CCA cells. ATRA promoted cytotoxicity partly via RARB, and induced the intrinsic pathway of apoptosis by enhancing ROS accumulation. Furthermore, ATRA suppressed NRF2 signaling, which in turn may cause impaired ROS balance and enhance the sensitivity of anticancer drugs. Moreover, ROS induction may be mediated via RARB; however, the results of the present study did not prove this concept and further studies are required. Notably, the present study primarily evaluated the effect of ATRA on the induction of apoptosis, its potential use for improvements in the response to anticancer drugs, and explored the anticancer actions and underlying mechanisms of ATRA in CCA cells. However, as the present study used a cell culture model to demonstrate the effect of ATRA on CCA, this may be a limitation of this study and the findings may differ from the real-world situation. Therefore, using *in vivo* animal models and patient-derived materials is required to assess the role of ATRA. Notably, the results of the present study indicated that ATRA may be of use in CCA therapy; however, further *in vivo* studies are warranted to approve the potential use of ATRA for CCA therapy.

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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, AP, VK, LS and SK designed the study. SB and AP performed the experiments. SB, AP, VK, LS and SK analyzed the data and interpreted the results. AP, SB and SK wrote 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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