Regular Article

Berberine Induces Cell Cycle Arrest in Cholangiocarcinoma Cell Lines *via* Inhibition of NF-κB and STAT3 Pathways

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Berberine is a natural compound found in several herbs. Anticancer activity of berberine was reported in several cancers, however, little is known regarding the effects of berberine against cholangiocarcinoma (CCA). In this study, the growth inhibitory effects of berberine on CCA cell lines and its molecular mechanisms were explored. Cell growth and cell cycle distribution were examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry. The expression levels of cell cycle regulatory proteins were determined by Western blot analysis. Berberine significantly inhibited growth of CCA cell lines in a dose and time dependent fashion. The inhibition was largely attributed to cell cycle arrest at the G1 phase through the reduction of cyclin D1, and cyclin E. Moreover, berberine could reduce the expression and activation of signal transducers and activator of transcription 3 (STAT3) and probably nuclear factor-kappaB (NF-κB) *via* suppression of extracellular signal-regulated kinase (ERK) 1/2 action. These results highlight the potential of berberine to be a multi-target agent for CCA treatment.

Key words cholangiocarcinoma; berberine; cell cycle arrest; nuclear factor-kappaB; signal transducer and activator of transcription 3

Cholangiocarcinoma (CCA) is a malignant tumor which originates from the biliary epithelium. CCA is a rare cancer worldwide but the incidence is very high in northeastern Thailand. The epidemiology and experimental evidence indicates that the liver fluke, *Opisthorchis viverrini* (*Ov*), is a major risk factor for CCA in Thailand.[1,2] Current treatment for CCA is based on surgery, but its success is still limited due to late presentation and delayed diagnosis. Adjuvant chemotherapy to improve the therapeutic outcome for CCA has been attempted and continuously investigated.[3] The results, however, were unsatisfactory due to drug resistance. Therefore, searching for new chemotherapeutic agents for an effective CCA treatment is urgently required.

Berberine is an isoquinoline alkaloid which is abundant in several medicinal Chinese plants, such as *Berberis aquifolium*, *Berberis vulgaris* and *Berberis aristata*.[4] Recently, berberine has been used for treatments of various diseases, *e.g.*, type 2 diabetes, hypercholesterolemia and diarrhea without obvious toxicity to humans.[5] Currently, anti-cancer activity of berberine has been reported. The strong anti-tumor growth *via* induction of cell cycle arrest, apoptosis and inhibition of metastasis have been shown in several *in vitro* and *in vivo* studies.[6–8] Little, however, is known about the anti-cancer activities of berberine on CCA. The present work was aimed to determine the anti-cancer activities and potential molecular mechanisms of berberine on CCA cell lines.

MATERIALS AND METHODS

Berberine Berberine chloride powder (Sigma-Aldrich, St. Louis, MO, U.S.A.) was solubilized in warm distilled water, filtered through 0.2 micron filters and stored at −20°C until use.

Cell Culture Two human CCA cell lines, KKU-213 and -214, were established from primary tumors[3] of a Thai CCA patient and registered at the Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan. The immortalized normal cholangiocyte cell line, MMNK-1, was generated and supplied by Prof. Naoya Kobayashi.[9] These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, and 1% (v/v) antibiotic-antimycotic (Gibco, NY, U.S.A.) at 37°C in a humidified 5% CO_2 atmosphere.

Cell Proliferation The anti-proliferative activities of berberine were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Invitrogen, CA, U.S.A.). Cells (5×10^3 per well) were seeded in a 96 well plate and incubated overnight before being treated with various concentrations of berberine for 24, 48 and 72 h. Cells treated with water were used as controls. At the end of incubation time, MTT was added and incubated for 3 h. Then the formazan crystals were solubilized by acid isopropanol and the absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) reader (TECAN Sunrise, Mannedorf, Switzerland). The IC_{50} for berberine was determined from the log concentration effect curves using non-linear regression analysis in Graph Pad Prism (Graph Pad Software Inc., CA, U.S.A.).

Cell Cycle Analysis Cell cycle distributions were determined using propidium iodide (PI) (Invitrogen) staining and flow cytometry. After being treated with berberine for 24 and 48 h, adherent cells were washed with phosphate buffered saline (PBS), trypsinized, resuspended in 70% cold ethanol and stored at −20°C overnight. Cell pellets were washed

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twice with PBS, resuspended in staining solutions (5 μg/mL PI in PBS) and incubated on ice for 10 min in the dark. Cell cycle distributions were determined using flow cytometry (BD FACSCanto II, BD Bioscience, CA, U.S.A.) and data were analyzed using BD FACSDiva software (BD Bioscience).

Protein Preparation and Western Blotting After berberine treatment, cells were lysed with Nonidet P-40 (NP-40) lysis buffer. Protein lysates of 30 μg were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, U.K.). The immunoreactivity was detected by incubating with Amersham ECL Prime (GE Healthcare). Intensity of each protein band was measured using a Gel-Pro Analyzer, software version 3 (Media Cybernetics, MD, U.S.A.).

Sources of antibodies were as followed: anti-Cyclin D1 (H-295), anti-Cyclin E (C-19), anti-nuclear factor-kappaB (NF-κB) p50 (E-10), anti-NF-κB p65 (F-6), anti-NF-κB p52 (C-5), anti-signal transducer and activator of transcription 3 (STAT3) (C-20), anti-p-STAT3 (B-7), anti-p-STAT3 (serine (Ser) 727), anti-extracellular signal-regulated kinases (ERK1) (K-23), anti-p-ERK (E-4) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), and anti-β actin (AC-15) was from Sigma-Aldrich.

Statistical Analysis The results are presented as a mean±standard deviation (S.D.) from at least three separate experiments. Statistical significance was determined using Student's *t*-test. $p \leq 0.05$ was considered statistically significant.

RESULTS

Berberine Inhibited Growth of CCA Cell Lines To determine the effect of berberine on the proliferation of CCA cells, two human CCA cell lines, KKU-213 and -214, and the immortalized human cholangiocyte cell line, MMNK-1, were treated with 0–160 μM berberine for 24, 48 and 72 h and the viable cells were determined using the MTT assay. As shown in Fig. 1, berberine inhibited growth of all cell lines in a dose- and time-dependent manner. Berberine, however, was more effective in suppressing proliferation of KKU-213 and -214 than MMNK-1 cells. The IC_{50}s of berberine for KKU-213 at 48 and 72 h were 10.3±3.5 and 4.2±0.3 μM and those for KKU-214 were 9.3±1.2 and 3.0±1.0 μM, whereas those of MMNK-1 cells were >160 and 133±15 μM (Supplementary Table 1).

Berberine Induced G1 Phase Cell Cycle Arrest *via* Inhibition of Cell Cycle Regulatory Proteins To determine the effect of berberine on cell cycle distribution, CCA cell lines were treated with 5, 10 and 20 μM of berberine for 24 and 48 h, stained with propidium iodide and subjected to flow cytometry. As shown in Fig. 2A, the G1 phase cells of cells treated with berberine were gradually increased with the increase of time and dose. Compared to the control cells, the accumulations of cells at G1 phase were significantly observed in KKU-213 and -214 treated with 10 and 20 μM berberine at 24 and 48 h.

To investigate the mechanisms by which berberine induced G1 phase arrest, the expression of cell cycle regulatory proteins, cyclin D1 and cyclin E were determined. Western blot analyses showed that supplementation of berberine inhibited the expressions of cyclin D1 and cyclin E of both KKU-213 and -214 cells (Fig. 2B) in dose and time dependent fashions. These results indicated that berberine inhibited cell cycle progression from G1 to S phase by suppression of G1 phase regulatory proteins.

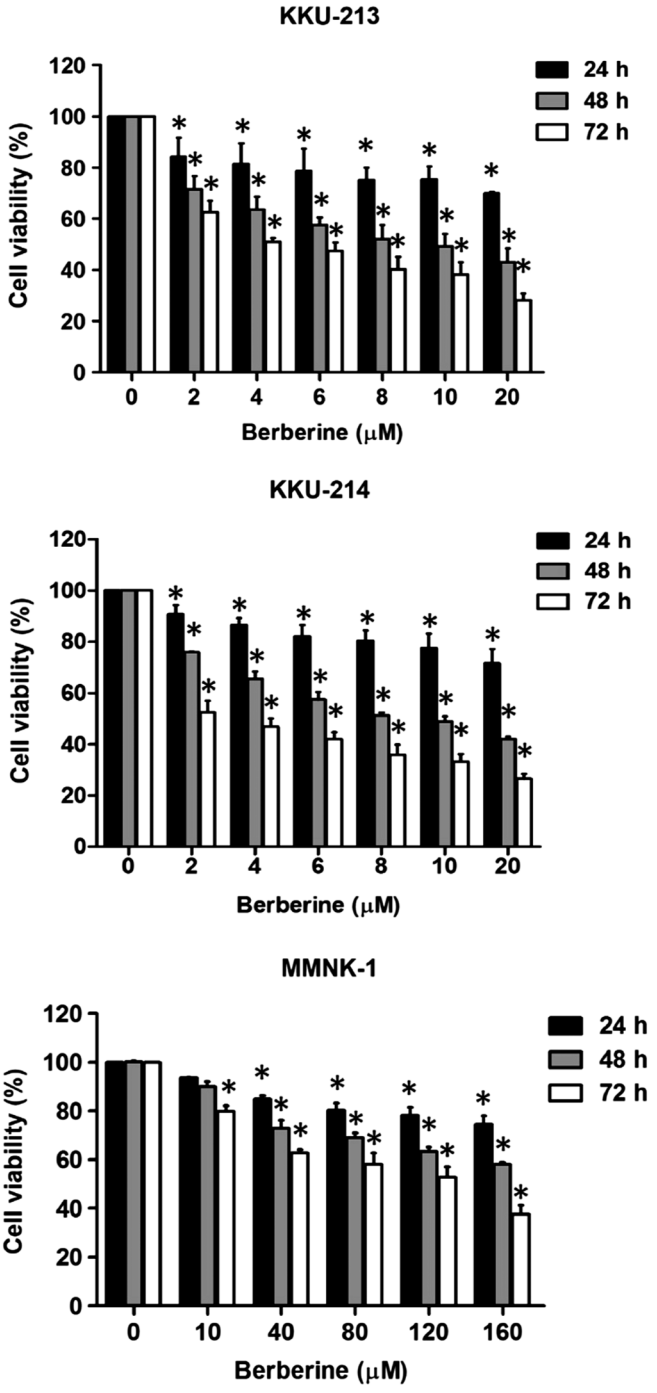

Fig. 1. Berberine Preferentially Inhibits CCA Cell Proliferation

The anti-proliferative effects were determined by the MTT assay. These results are presented as percentage of cell numbers relative to untreated cells. A bar graph represents the mean±S.D. from three independent experiments. *$p \leq 0.05$.

Berberine Suppressed the Activation of STAT3 and NF-κB Pathways NF-κB, a key transcription factor involved in cancer progression,[10,11] has been shown to be activated and overexpressed in many cancers including CCA. Berberine was then investigated as to whether it affected the action of NF-κB in CCA cells. The expression levels of NF-κB (p50/p105, p52/p100 and p65) in berberine treated cells were determined

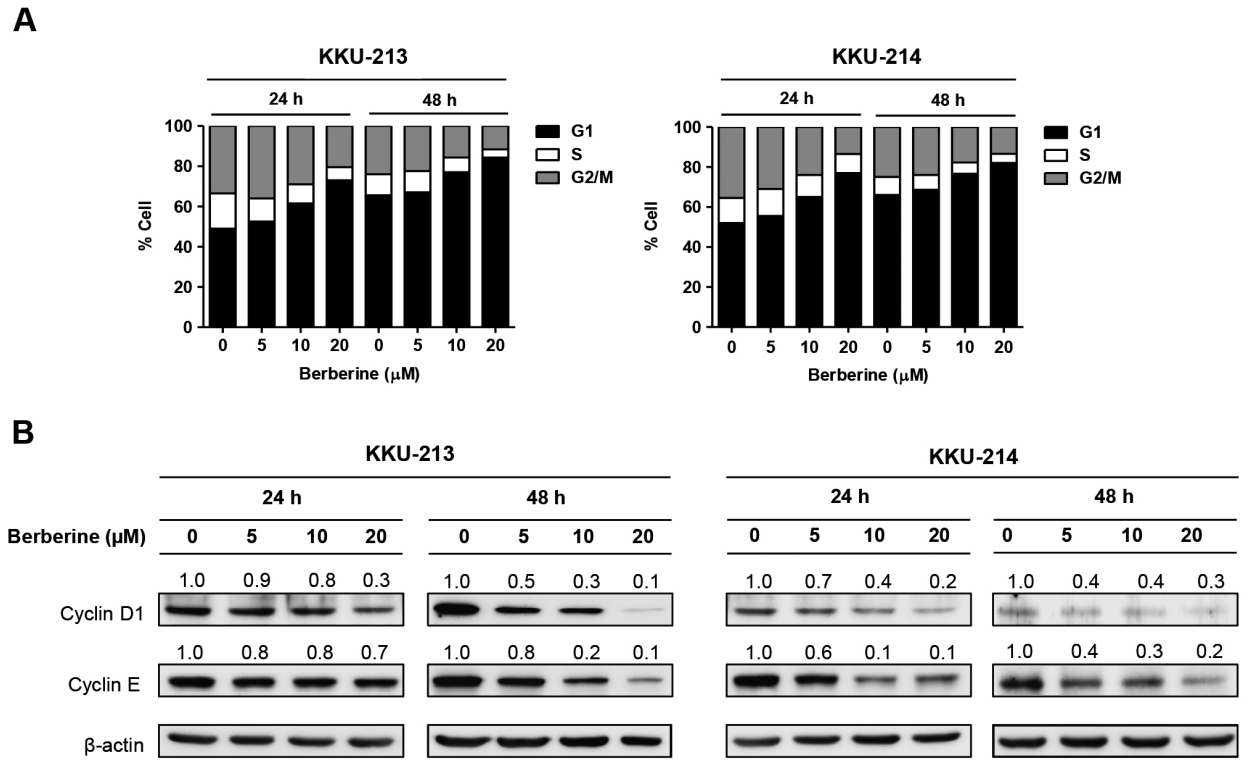

Fig. 2. Berberine Induces G1 Cell Cycle Arrest *via* Decreasing Expression of G1 Cell Cycle Regulatory Proteins in CCA Cell Lines

Cell cycle distribution is represented as mean percentage of cells in each phase. (A) Cell cycle regulatory proteins, cyclin D1 and cyclin E were determined by Western blot analysis. *β*-Actin was used to verify equal protein loading. (B) Intensity of each band was normalized with actin and compared relative to untreated cells (=1), as indicated by the number shown on the top of each band. The data are the representation of two independent experiments.

using Western blotting. Expressions of p50, p52, p65 and its precursors p105, p100 were gradually decreased in berberine treated KKU-213 cells according to the incubation times and doses of berberine (Fig. 3A). The effect of berberine on NF-*κ*B, however, was not demonstrated in KKU-214 treated cells.

As berberine is also known for its action on STAT3,[12–14] the effect of berberine on STAT3 signaling was further explored. The Western blot analyses of STAT3 and pSTAT3 (tyrosine (Tyr) 705, and Ser 727) were performed in CCA cells treated with berberine in comparison with the control cells. Berberine reduced STAT3 expression and inhibited the phosphorylation of both STAT3 at Tyr705 and Ser727 related to dose- and time-dependent manners (Fig. 3B). At 48 h of 20 μM berberine treatment, the expression levels of p-STAT3 (Tyr705) in berberine treated cells were reduced from 1.0 to 0.1–0.2 and those of p-STAT3 (Ser727) were reduced from 1.0 to 0.2–0.4.

Berberine Suppressed the Expressions and Activations of ERK For a better understanding of the molecular mechanism of berberine on NF-*κ*B and STAT3, the effect of berberine on the expression of extracellular signal-regulated kinase (ERKs), a STAT3 and NF-*κ*B regulatory protein, was explored. The results showed that berberine could reduce the expression and phosphorylation of ERK1/2 in CCA cell lines, KKU-213 and -214, as compared to the untreated cells (Fig. 4).

DISCUSSION

The intensive studies of berberine, an extracted compound from roots and stem barks of the *Berberis* species, showed the promising outcomes of berberine in inhibiting cancer cell proliferation and metastasis as well as inducing apoptosis in several cancer cell lines. The present study reported for the first time that berberine significantly suppressed growth of CCA cells *via* inhibitions of ERK1/2, NF-*κ*B and STAT3 pathways.

The anti-cancer activity effects of berberine in the non-*Ov*-associated human CCA cell line, QBC939 were shown to be *via* the induction of G1 cell cycle arrest and apoptosis.[15] Similar results were also observed in an *Ov*-associated CCA hamster cell line.[16] The underlying molecular mechanisms of berberine induced cell cycle arrest and apoptosis in CCA cells, however, have remained unrevealed.

In the present study, the anti-proliferative effects of berberine were determined in the *Ov*-associated human CCA cell lines, KKU-213 and -214 and in the immortal cholangiocytes, MMNK1. Berberine could inhibit proliferations of all cell lines in dose- and time- dependent fashions. The anti-proliferative activity of berberine, however, is preferentially active on CCA cells rather than normal cholangiocytes, as the IC_{50} of berberine to MMNK-1 was >30 times that of CCA cells at 72 h. This observation is similar to those reported for normal osteoblast cells,[16] Chang liver cells, a non-tumor liver cell line[17] and normal human keratinocytes.[18]

The anti-cancer activity effects of berberine were also reported in the non-*Ov*-associated human CCA cell line, QBC939.[12] *Ov*-associated human CCA cell lines, KKU-213 and -214, however, were more sensitive to berberine than the non *Ov*-associated CCA cell line, QBC939, as the effective doses of KKU-213, -214 were 2–20 μM *vs.* 10–80 μM of QBC939. Berberine seems preferentially active on CCA cell lines rather than on other cancer cell lines when comparing the IC_{50} of berberine on several cancer cell lines, *e.g.*,

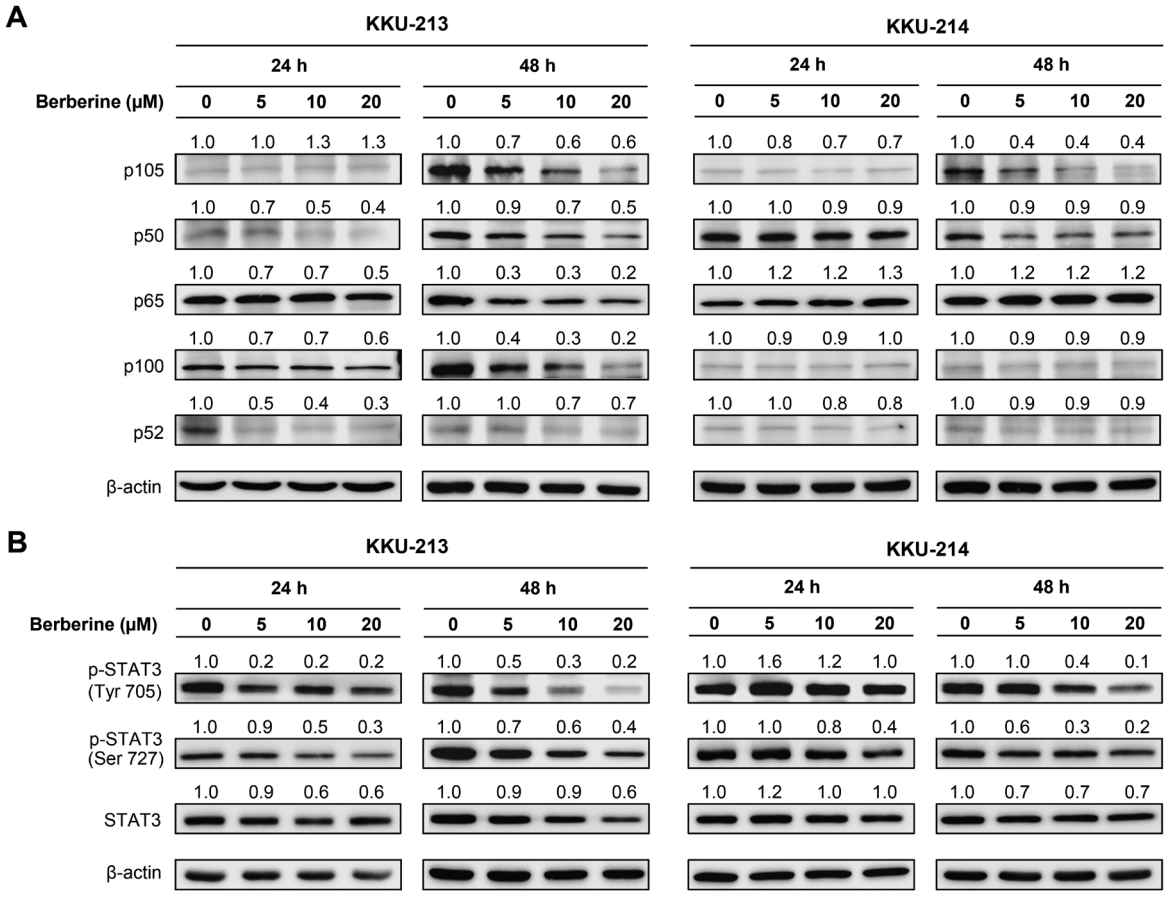

Fig. 3. Effects of Berberine on the NF-κB and STAT3 Activation and Expression in CCA Cell Lines

After treatment with or without berberine (5, 10 and 20 μM) for 24 and 48 h, proteins were collected and subjected to Western blot analysis to determine the expression level of (A) NF-κB proteins (p100, p52, p105, p50 and p65) and (B) STAT3, p-STAT3 (Tyr705) and p-STAT3 (Ser727). Normalized band intensities are presented. The data are the representation of two independent experiments.

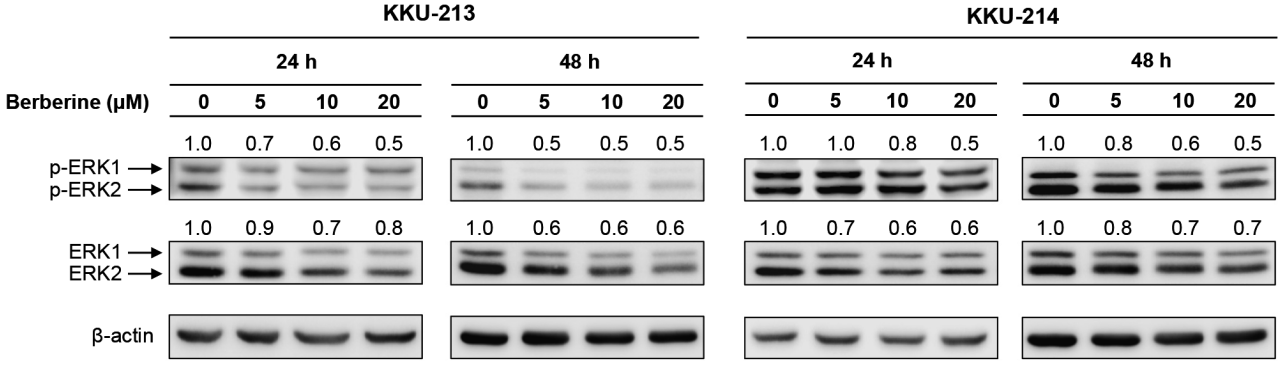

Fig. 4. Berberine Reduces ERKs Action in CCA Cell Lines

Expressions of ERK1/2 and p-ERK1/2 in KKU-213 and -214 cells were determined using Western blot analysis. Normalized expression levels are indicated. The data are the representation of two independent experiment.

epidermoid carcinoma A431 cells,[19] neuroblastoma cells,[20] and prostate carcinoma cells (LNCaP, DU145 and PC-3).[21] The sensitivity and selectivity of berberine on CCA cells demonstrated the potential use of berberine as supplementary therapy for CCA.

The growth inhibitory effects of berberine have been shown to be due to the induction of cell cycle arrest and/or promotion of cell death, depending on the cancer types and doses of berberine. In the present study, 10–20 μM of berberine significantly induced G1 arrest in CCA cells in dose and time dependent fashions. The induction of cell cycle arrest at G1 phase by low doses of berberine ($<50\,\mu$M) were demonstrated in several cancer cell lines including human bladder cancer cells,[22] a murine leukemia cell line[23] and a non-*Ov*-associated CCA cell line.[15] In addition, high doses of berberine ($>50\,\mu$M) promoted G2/M arrest or induction of apoptosis as reported in many human cancer cells, *e.g.*, gastric cancer[24] and prostate cancer.[25]

The induction of cell cycle arrest at G1 to S phase by berberine was further demonstrated in the present study to be through the suppression of cyclin D1 and cyclin E expressions. This observation is in accordance with the previous report

in QBC939 CCA cells, however, only suppression of cyclin D1 but not cyclin E expression was shown in QBC939 CCA cells.[15] It is well known that cyclin E acts after cyclin D1 in regulation of the G1/S phase transition, and is the critical and essential step for progressing cells into the S phase.[26] The present results suggest that berberine inhibited the initiation and progression of the G1 phase and effectively halted CCA cells at the G1 phase. This is possibly the reason for the high sensitivity of CCA cells to berberine compared to other cancer cells.

Berberine affected STAT3, a major pathway that plays vital roles in pathogenesis of many cancers including CCA.[11] The present study demonstrated that berberine inhibited the expression of STAT3 and phosphorylation of STAT3 at both Tyr705 and Ser727 in both CCA cell lines. A similar effect was reported in nasopharyngeal carcinoma cells.[12]

NF-κB, a transcription factor that controls expression of many genes involved in carcinogenesis and cancer progression, is constitutively activated in many tumor cells including CCA.[10] Suppression of NF-κB significantly reduced growth, cell motility and induced apoptosis of cancer cells. Berberine can inhibit NF-κB in many ways. It was shown in a primary effusion lymphoma cell line that berberine suppressed the phosphorylation of inhibitor of kappa B kinase (IKK) and inhibitor of κB (IκB), and degradation of IκB.[27] In human lung cells, berberine was shown to directly inhibit NF-κB activity by modification of cysteine-179 of the IKK-β subunit, resulting in the inhibition of transcriptions of several NF-κB targeted genes including cyclin D1.[28]

The present study demonstrated that berberine exhibited similar anti-tumor activity against 2 CCA cell lines, KKU-213 and -214. Berberine suppressed the expression and activation of STAT3 and ERK in both cell lines at a comparable level, however, berberine had a stronger effect on the expression and activation of NF-κB in KKU-213 than KKU-214. This discrepancy may due to: firstly, KKU-213 is more dependent on NF-κB action than KKU-214. KKU-213 possessed higher basal levels of NF-κB precursors (p100 and p105) than KKU-214 cells.[10,29] As a result, KKU-213 was more strongly affected than KKU-214 when NF-κB activation was inhibited. This assumption was supported by the fact that DHMEQ, a specific NF-κB inhibitor, had a stronger effect on KKU-213 than KKU-214, *in vitro* and *in vivo*.[10] Similar findings were also confirmed in Seubwai *et al.*[29] Secondly, berberine possibly affected KKU-214 *via* signaling pathways other than NF-κB. It was shown recently that berberine could down-regulate cyclooxygenase (COX)-2/prostaglandin E2 (PGE2),[13] miR-93/PTEN/Akt,[30] and epidermal growth factor receptor (EGFR),[31] *etc*. These candidates also affected cell proliferation and are probably responsible for the action of berberine in KKU-M214. The increased NF-κB expression in the control cells at 48 h when compared to those at 24 h was evident in both CCA cell lines. This observation is probably due to the activation of interleukin 6 (IL-6) produced from CCA cells. As a large amount of biologically active IL-6 was found in the culture medium of the CCA cell line,[32] therefore the increase of IL-6 could activate NF-κB expression in cancer cells.[33]

Inhibition of STAT3 and possibly NF-κB and pathways in CCA cell lines might be due to the modulation of ERK1/2 by decreasing of ERK1/2 expression and phosphorylation. ERK1/2 plays important roles in the regulation of cell proliferation, motility and cell survival of many cancer cells. Overexpression of pERK1/2 protein was seen in 49.2% of CCA cases.[34] ERKs can be activated by several stimuli including growth factors, cytokines and carcinogens. The ERK phosphorylation cascade is linked to cell surface receptor tyrosine kinases (RTKs) and other upstream signaling proteins with known oncogenic potential such as EGFR and human EGF receptor 2 (HER2) which are overexpressed in CCA.[35] Suppression of the EGFR signaling pathway by berberine has been reported by several groups.[31,36,37] Berberine inhibited growth and promoted apoptosis of breast cancer cells[38] and the mechanism was shown to be *via* inhibition of the HER2 signaling pathway. This evidence suggests the inhibition of ERK upstream stimuli, EGFR and HER2 being the mechanism by which berberine affected the ERK pathway as observed in the current study.

ERK activation activates NF-κB signaling. In contrast, the NF-κB pathway was suppressed by PD98059, a specific inhibitor of ERK.[39] ERK phosphorylates STAT3 on serine 727 site of STAT3[40] while phosphorylation on tyrosine 705 residue of STAT3 is through JAKs and Src kinase. Several reports demonstrated that STAT3 phosphorylation at both sites is required for full activation of STAT3. Therefore, the inhibition of NF-κB and STAT3 phosphorylation was partly due to the inhibition of ERK1/2 expression and phosphorylation. The direct linkage between ERK/NF-κB/STAT3 was shown by using PD98059, a potent and selective inhibitor of MEK/ERK phosphorylation. Inhibition of ERK phosphorylation could reduce expression and/or phosphorylation of NF-κB pathway (p50, p52, p65) and STAT3 in KKU-213 and -214 cells treated with PD98059 (data not shown).

In summary, the anti-cancer activity of berberine on an *Ov*-associated CCA cell lines was demonstrated *in vitro*. Berberine inhibits growth of CCA cell lines by induction of cell cycle arrest at G1 phase. In addition, the current study provides first evidence that berberine potently inhibits the growth of CCA cells by inhibition of ERK1/2 which resulted in suppression of the NF-κB and STAT3 pathways. These findings suggest berberine to be a good candidate as adjunct therapy for CCA.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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