Inhibition of NF-κB Activity Enhances Sensitivity to Anticancer Drugs in Cholangiocarcinoma Cells

Wunchana Seubwai,*†‡ Kulthida Vaeteewoottacharn,‡§ Ratthaphol Kraiklang,¶
Kazuo Umezawa,# Seiji Okada,** and Sopit Wongkham‡§

*Department of Forensic Medicine, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
†Comprehensive Cancer Research Group, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
‡Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
§Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
¶Department of Nutrition, Faculty of Public Health, Khon Kaen University, Khon Kaen, Thailand
#Department of Molecular Target Medicine, Aichi Medical University, Nagakute, Japan
**Division of Hematopoiesis, Center for AIDS Research, Kumamoto University, Honjo, Kumamoto, Japan

Cholangiocarcinoma (CCA) is a dismal cancer. At present, there is no effective chemotherapeutic regimen for CCA. This may be due to the marked resistance of CCA to chemotherapy drugs, for which a mechanism remains unknown. Nuclear factor-κB (NF-κB) is constitutively activated in a variety of cancer cells, including CCA. It has been shown to play roles in growth, metastasis, and chemoresistance of cancer. In the present study, we examined whether NF-κB is involved in the chemoresistance of CCA and whether dehydroxymethylepoxy-quinomicin (DHMEQ), an effective NF-κB inhibitor, can overcome the drug resistance of CCA. Two CCA cell lines, KKU-M213 and KKU-M214, were treated with DHMEQ and/or chemotherapeutic drugs. Cell viability, apoptosis, and the expressions of the ATP-binding cassette (ABC) transporters were compared. The combination of chemotherapy drugs, 5-fluorouracil, cisplatin, and doxorubicin, with DHMEQ significantly enhanced the cytotoxicity of all chemotherapeutic drugs compared to DHMEQ or drug alone. Furthermore, the mRNA level of ABCB1, a multidrug-resistant protein, was significantly decreased in the 5-fluorouracil combined with DHMEQ-treated cells. These findings suggest that the inhibition of NF-κB by DHMEQ enhanced the chemo-responsiveness of CCA cells, possibly by reducing the expression of ABC transporter. Inhibition of NF-κB may be a potential chemodrug-sensitizing strategy for chemoresistant cancer such as CCA.

Key word: Dehydroxymethylepoxyquinomicin (DHMEQ); Nuclear factor-κB (NF-κB); Cholangiocarcinoma (CCA); ATP-binding cassette family (ABC) transporters; Chemotherapeutic drugs

INTRODUCTION

The incidence of cholangiocarcinoma (CCA), a cancer of biliary epithelium, is increasing worldwide. Chronic inflammation of the bile duct epithelium seems to be the common risk factor of CCA around the world. In northeast Thailand, where the world incidence of CCA is high (1), epidemiology and animal studies demonstrated the association of liver fluke (*Opisthorchis viverrini*) infection and CCA in this area (2). Owing to delayed diagnosis, an operational cure is applicable for only a few patients, and most of the CCA patients are untreated or received only palliative treatment. Many chemotherapeutic drugs such as 5-fluorouracil (5-FU), gemcitabine (GEM), cisplatin (CIS), and doxorubicin (DOX) have been used for the treatment of CCA patients with a low response rate and

short median survival time (3,4). The frequent acquisition of drug-resistant phenotypes and the occurrence of secondary malignancies associated with chemotherapy are serious problems at present. The toxic effect of chemotherapy is the additional major drawback in the treatment of CCA patients. Thus, searching for new or alternative approaches of an effective treatment for CCA is needed.

Nuclear factor κB (NF-κB) has recently emerged as a potential molecular target for the treatment of several malignancies (5–7). NF-κB is activated by various stimuli including cytokines, UV radiation, chemical carcinogens, tumor necrosis factor-α, radiotherapy (8,9), and chemotherapeutic agents (10). Activated NF-κB promotes over 150 target transcripts, which include various genes involved in cell proliferation (11), angiogenesis

(11), metastasis (12), suppression of apoptosis (13), and chemotherapeutic drug resistance (14).

ATP-binding cassette family transporters (ABC transporters) play a role in the resistance of malignant cells to anticancer agents such as 5-FU, CIS, and DOX. Inhibitors for the major ABC transporter proteins contributing to multidrug resistance (MDR) have been developed. Extensive preclinical and clinical research has been carried out aimed at blocking the ABC transporters to prevent the development of drug resistance during chemotherapy (15). ABC transporters are activated through NF-kB pathway (16,17). Therefore, NF-κB would be a valid therapeutic target for an effective cancer treatment, especially for a multidrug-resistant cancer like CCA.

Our previous study showed that NF-κB proteins were overexpressed in almost all CCA patient tissues, and the NF-kB inhibitors [cepharanthene and dehydroxymethylepoxyquinomicin (DHMEQ)] could significantly reduce cell growth and enhance cell apoptosis of CCA cell lines both in vitro and xenografted mouse model (18,19). These findings suggested NF-κB as an attractive molecular target for CCA therapy. In the present study, we investigate whether inhibition of NF-kB activation by DHMEQ, a novel NF-κB inhibitor, can enhance the chemosensitivity of CCA cell lines to 5-FU, CIS, and DOX. The effect of DHMEQ on the expression of ABC transporters was also investigated.

MATERIALS AND METHODS

Cell Lines

Human CCA cell lines derived from primary CCA patient tumors, namely KKU-M213 and KKU-M214, were established (20) and registered at the Japanese Collection of Research Bioresources (JCBR) Cell Bank, Osaka, Japan. The two cell lines were used in the present study as they had high expression of all NF-κB subunits (p50, p52, and p65) (18). CCA cell lines were cultured in DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, and 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO_2.

Chemicals

DHMEQ was synthesized as described previously (21). All chemotherapeutic drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Viability Test

Cell viability was determined by MTT assay. In brief, 3×10^3 cells per well were seeded in a 96-well plate and incubated with 5 µg/ml DHMEQ alone or DHMEQ plus various concentrations of 5-FU, CIS, and DOX for 48 h at 37°C in 5% CO_2. Cells treated with 0.001% DMSO were used as a control. Subsequently, 10 µl of MTT (Sigma-Aldrich) was added to yield the final concentration of 0.5 mg/ml. After 4-h incubation, absorption at 570 nm

was determined with an automatic ELISA plate reader (Multiskan; Thermo Electron, Vantaa, Finland).

Cell Death Assay Using Annexin V/Propidium Iodide Staining

Cytotoxicity of DHMEQ on CCA cells was examined using annexin V/propidium iodide (PI) staining. KKU-M213 and KKU-M214 cells were seeded in a 24-well cell culture plate at a density of 1×10^4 cells for 24 h to adhere and subsequently treated with DHMEQ or DHMEQ plus various concentrations of 5-FU. Cells were incubated further at 37°C, 5% CO_2 for 48 h, and stained with H33342, PI, and annexin V (Molecular Probes, Eugene, OR, USA) diluted in culture medium for 30 min before image acquisition in an IN Cell Analyzer 2000 (GE Healthcare, UK). The 20× objective was used to collect images for all fluorescence channels, and five fields of view per well were monitored. Image analysis for the multiplex assay was performed using the IN Cell Analyzer Workstation ver.3.7 (GE Healthcare, UK).

RNA Extraction and Reverse Transcription

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Briefly, cells were lysed in TRIzol reagent, and chloroform was added. After centrifugation at $12,000 \times g$ for 15 min at 4°C, the RNA containing upper phase was collected, mixed with isopropanol, and centrifuged to precipitate RNA. RNA pellet was washed with 70% ethanol, air dried, dissolved in 20–30 µl of DEPC-treated water, and stored at −80°C until use. Two micrograms of total RNA was reverse transcribed to cDNA using RT-PCR according to the manufacturer's protocol (High Capacity cDNA Reverse Transcription Kits; Applied Biosystems, Foster City, CA, USA).

Real-Time PCR

The expression of ABC transporters (ABCB1, ABCC1, ABCC6, ABCC11, and ABCG2) in CCA cell lines, KKU-M213 and KKU-M214, were examined by real-time PCR with 2× SYBR Green PCR Master Mix (Roche, Mannhiem, Germany) in a LightCycle® 480 Real time PCR System (Roche Diagnostics, Mannheim, Germany). Thermal profile was 50 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 3 s. After PCR, a melting curve was constructed at the range of 50°C to 99°C. All data were analyzed using LightCycle® 480. The expressions of ABC transporters were normalized with β-actin. All primers used were the same as those described previously (22). The specificity of the primers was tested using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), Electronic PCR (http://www.ncbi.nlm.nih.gov/tools/epcr/), melting curve analysis, and the conventional PCR for a single PCR product verification.
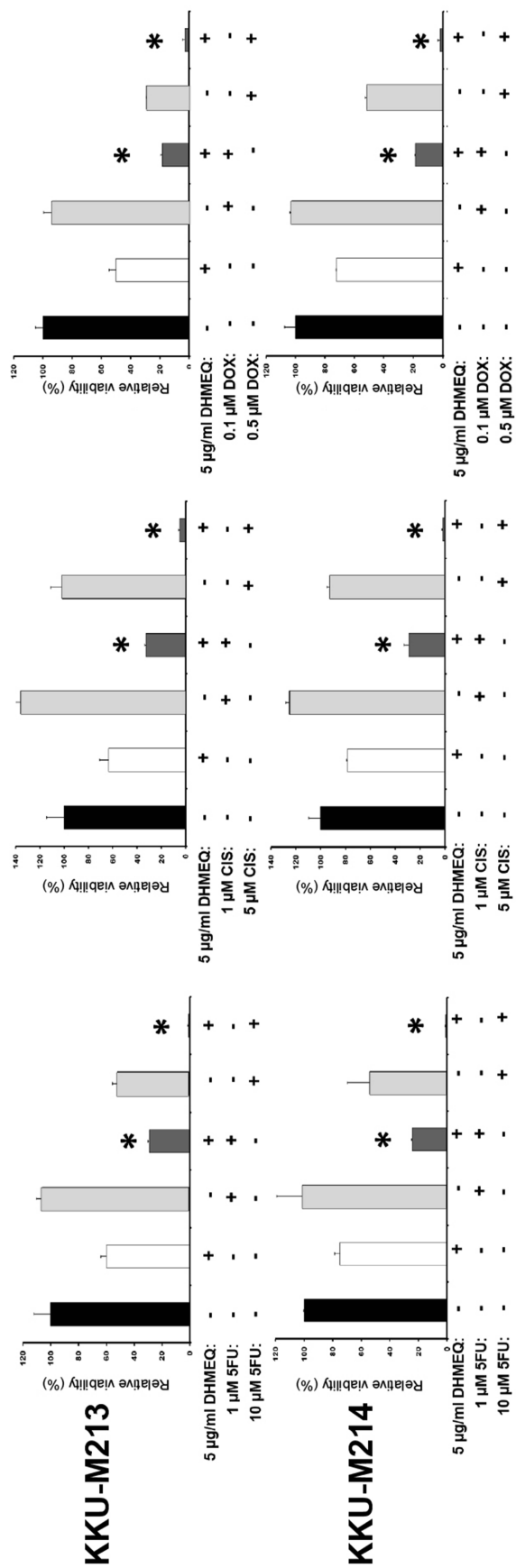
Figure 1. Cytotoxic effect of anticancer drugs with or without DHMEQ on CCA cell lines. Viabilities of KKU-M213 and KKU-M214 cells exposed to the indicated concentrations of 5-FU, CIS, and DOX with or without DHMEQ for 48 h were determined by MTT assay. Bars represent the mean and SD of triplicates. The data are a representative from two independent experiments. *p < 0.05 versus anticancer drugs alone.

Statistical Analysis

The results were presented as the mean $\pm$ SD of at least two triplicates from two separated experiments. Statistical significance was determined using the Student's *t*-test, and $p < 0.05$ was required for statistical significance.

RESULTS

Blocking of NF-κB by DHMEQ Sensitizes Human CCA Cell Lines to Anticancer Drugs

To examine whether DHMEQ could sensitize the CCA cells to certain anticancer drugs when used in combination, KKU-M213 and KKU-M214 cells were treated with the fixed dose of 5 μg/ml DHMEQ with or without various concentrations of anticancer drugs (1 and 10 μM 5-FU; 1 and 5 μM CIS; 0.1 and 0.5 μM DOX), and the viability of the cells were determined by MTT assay. DHMEQ was fixed at 5 μg/ml because it is the IC$_{50}$ for KKU-M213 and IC$_{30}$ for KKU-M214 as determined in our previous experiments (18). In addition, anticancer drugs at low concentration (1 μM 5-FU; 1 μM CIS; 0.1 μM DOX) did not show any antitumor activity in CCA cell lines. However, the combination of 5 μg/ml DHMEQ and chemodrugs significantly enhanced antitumor activity of all the chemotherapeutic drugs tested against two CCA cell lines (KKU-M213 and KKU-M214) compared to the single-agent treatment (Fig. 1).

DHMEQ Enhanced 5-FU-Induced Cell Death

As 5-FU is the widely used chemotherapeutic drug for CCA (23), the mechanism by which DHMEQ affected cell death in combination with 5-FU was further investigated. The number of dead cells was examined by staining with annexin V and PI. Nuclei were labeled with Hoechst 33258, and the fluorescent signals were imaged using IN Cell Analyzer 2000. Increase in DNA condensation (Hoechst 33258 intensity in Fig. 2A) and cell loss were clearly observed in the cells treated with the combination of DHMEQ (5 μg/ml) and 5-FU (1 and 10 μM) compared to those treated with a single agent or control (Fig. 2A). The proportion of dead cells (annexin V-positive and/or PI-positive cells) was significantly increased in both CCA cell lines after treatment with the combination of DHMEQ and 5-FU compared with a single agent or control (Fig. 2B).
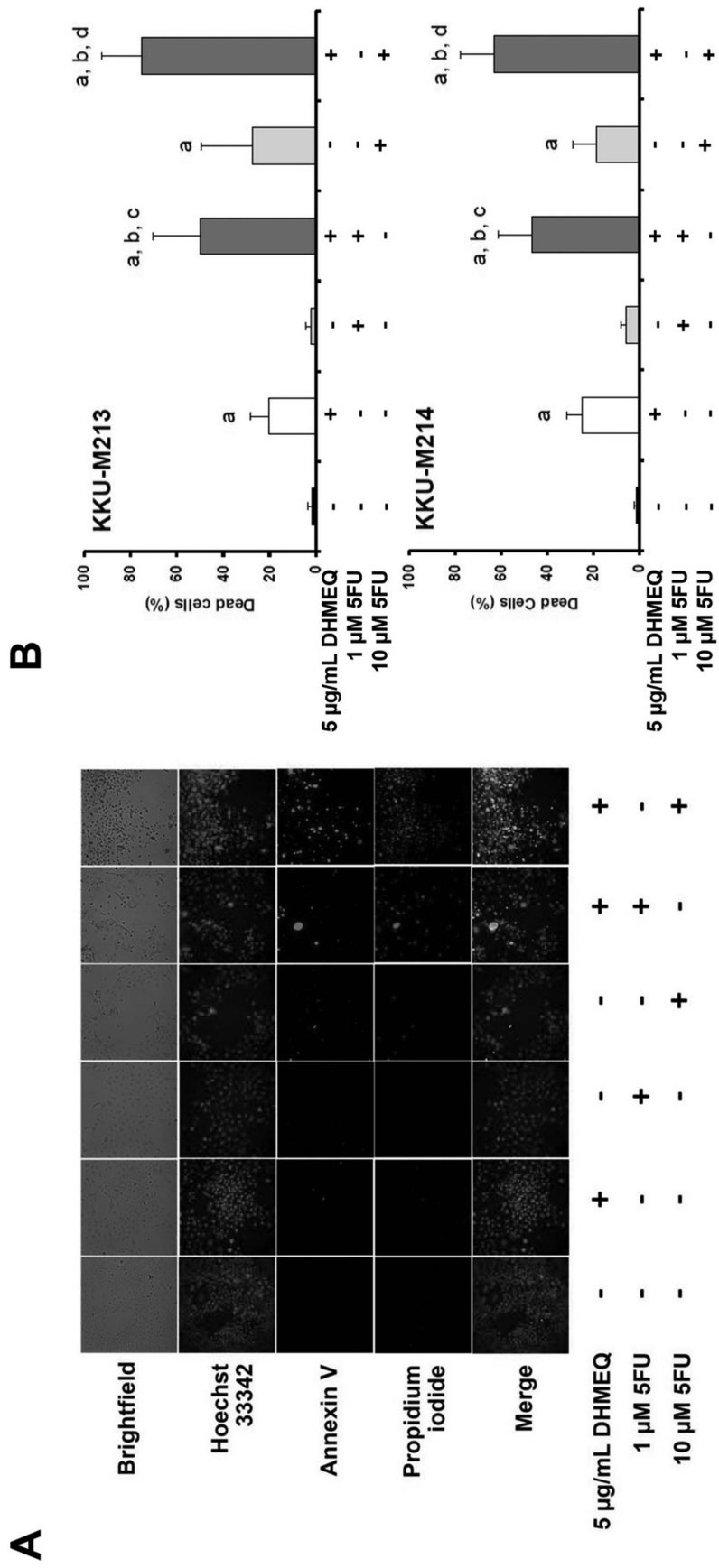
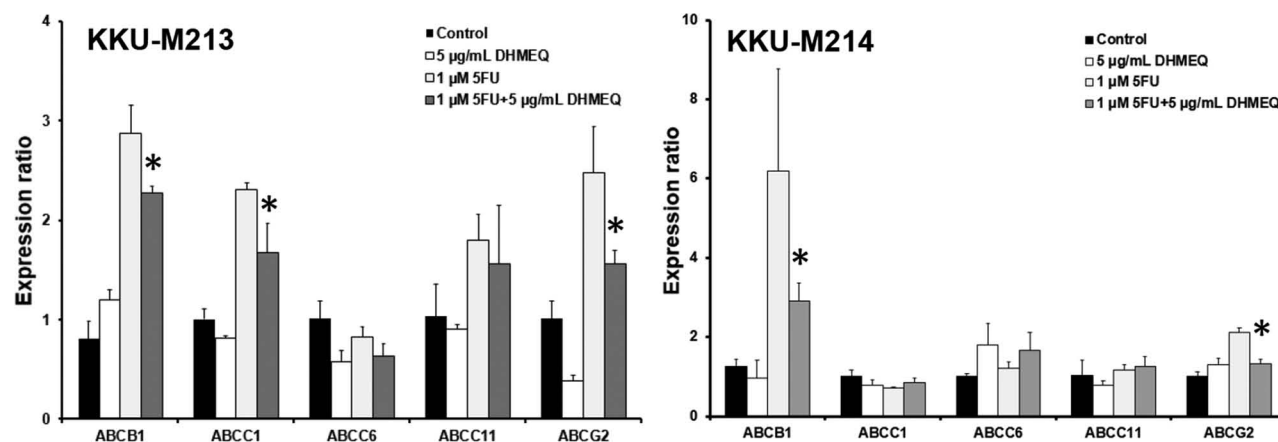### *Combination of DHMEQ and 5-FU Reduced the Expression of ABC Transporters*

Overexpression of ABC transporter mRNAs and proteins after chemotherapy are associated with the drug-resistance phenotype in various cancers (24,25). To determine whether ABC transporters are involved in 5-FU resistance of CCA cells and whether DHMEQ could reverse this association, the expression levels of ABC transporters of CCA cells after treatment with the combination of DHMEQ and 5-FU were measured by real-time PCR. Compared to the vehicle-treated cells, expression of ABCB1 in KKU-M213 and ABCC6 in KKU-M214 was slightly increased in DHMEQ-treated cells. On the other hand, the expression of ABCC1, ABCC6, and ABCG2 in KKU-M213, and ABCG2 in KKU-M214, was decreased in DHMEQ-treated cells. As expected, 5-FU treatment caused significant elevation of ABCB1, ABCC1, ABCC11, and ABCG2 transporters in KKU-M213, and ABCB1 and ABCG2 in KKU-M214 (Fig. 3). Interestingly, when these cell lines were treated with the combination of 5 μg/ml DHMEQ and 1 μM 5-FU, the enhanced expression of the ABC transporter mRNAs by 5-FU was reversed to a certain extent (Fig. 3).

DISCUSSION

NF-κB is constitutively activated in many tumor cells including CCA (5,7,18). The activation of NF-κB has been shown to play a role in carcinogenesis and progression of cancer cells by stimulating cell growth, inhibiting apoptosis, and providing a survival disadvantage (26,27). Because apoptosis is a major antitumor pathway for chemotherapy and radiation-induced cell death, it is currently believed that NF-κB might be involved in the resistance of tumor cells to chemotherapy and radiation (13,14). Furthermore, NF-κB in cancer cells can be activated by many chemotherapeutic agents, including 5-FU, paclitaxel, doxorubicin, etoposide, vincristine, vinblastine, cisplatin, tamoxifen, and camptothecin (14). In the present study, we demonstrated for the first time that combination of chemotherapeutic drug and sublethal concentration of DHMEQ (5.0 μg/ml) significantly enhanced the cytotoxicity of 5-FU, CIS, and DOX on CCA cell lines, KKU-M213 and KKU-M214. The chemosensitizing activities of DHMEQ have been reported in various cancers such as cancers of the head and neck (28) and thyroid (29). In addition, DHMEQ significantly increased cell apoptosis in CCA cell lines, KKU-M213 and KKU-M214. We also demonstrated that DHMEQ could enhance apoptotic action of chemotherapeutic drugs, possibly via suppressing the enhancement of ABC transporter expression by anticancer drugs. Increased efflux of chemotherapeutic drugs from cells is largely mediated by MDR proteins such as ABCB1 and ABCG2. A number of studies have shown that ABC transporter expression was induced by anticancer drugs, leading to an unfavorable outcome in many patients (30). The expression of ABC transporter has been reported to correlate with NF-κB activation (31). Therefore, the combination of conventional chemotherapeutics with NF-κB inhibitors has been considered as an adjunct approach to sensitize cancer cells to chemotherapy (29,32). The increase in ABC transporters, especially

Figure 2. Effect of DHMEQ on 5-FU-induced cell death of CCA cell lines. Cells were treated with 5-FU with or without DHMEQ for 48 h. Cells were stained with various markers, and the image acquisitions were taken in an IN Cell Analyzer 2000. (A) Cells with nuclear fragmentation were identified by the nuclei marker, Hoechst 33342. Dead cells were identified by the presence of FITC-annexin V and/or propidium iodide. (B) Quantitative analysis of dead cell number. Bars represent the mean and SD of triplicates. The data are a representative from two independent experiments. *$p < 0.05$: (a) versus control, (b) versus DHMEQ, (c) versus 1 μM 5-FU, (d) versus 10 μM 5-FU.

Figure 3. Effect of DHMEQ on mRNA levels of ABC transporter genes in CCA cell lines treated with 5-FU. KKU-M213 and KKU-M214 cells were exposed to indicated concentrations of 5-FU with or without DHMEQ for 48 h. Total cellular RNA was isolated, and transcript levels of the ABCB1, ABCC1, ABCC6, ABCC11, and ABCG2 genes were verified by real time-PCR. The graphs depict the ratios between amplification products of the ABC transporter genes in each treatment condition compared with the control. Bars represent the mean and SD of triplicates. The data are a representative from two independent experiments. *$p < 0.05$ versus DHMEQ.

ABCB1 and ABCG2, was obviously observed in 5-FU-treated CCA cell lines, and this probably is the offense mechanism of CCA cells to chemotherapy. However, this effect of 5-FU was reduced when cells were treated with the combination of 5-FU and DHMEQ. Inhibition of ABC transporter expression by small compounds that increase intracellular accumulation of chemotherapeutic drugs was demonstrated in several cancer cells such as the colon (33) and hepatic cancer cells (34). This may be one of the mechanisms by which NF-κB inhibitor enhances the chemosensitivity of cancer cells. A similar finding was reported with other compounds that affect NF-κB actions. For example, imatinib reverses the acquired resistance to anthracycline, such as doxorubicin, by inhibiting upregulation of the ABC transporter, ABCB1, via the inhibition of NF-κB/p65 nuclear localization (35). Clitocine, an inhibitor of adenosine kinase, was demonstrated to have a chemosensitizing effect on human hepatoma by reversing ABCB1 via downregulation of NF-κB (36).

These actions give at least two advantages for using NF-κB inhibitor to improve the outcome of CCA treatment. First, suppression of NF-κB significantly reduced the growth, cell motility, and invasion activity of CCA cells (18,19,37). Second, blocking of NF-κB action could sensitize the tumor cells to chemotherapeutic drugs via suppressing the expressions of ABC transporters leading to an increase in the intracellular accumulation of chemotherapeutic drugs in CCA cells.

In conclusion, our results provide strong evidence that the blocking action of NF-κB in CCA cell lines can enhance the antitumor activity of anticancer drugs. Suppression of ABC transporter expression, which was upregulated in response to anticancer drug treatment, could be one of the possible mechanisms by which DHMEQ enhanced the sensitivity of CCA cells to chemotherapeutic drugs. Using NF-κB inhibitor in combination with chemotherapeutic drugs may be an interesting strategy to increase the efficacy of the drug treatment in CCA patients.

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