RESEARCH ARTICLE

Diethyldithiocarbamate Suppresses an NF-κB Dependent Metastatic Pathway in Cholangiocarcinoma Cells

Pattaravadee Srikoon[1], Ryusho Kariya[1], Eriko Kudo[1], Hiroki Goto[1], Kulthida Vaeteewoottacharn[1,2], Manabu Taura[1], Sopit Wongkham[2], Seiji Okada[1]*

Abstract

Cholangiocarcinoma (CCA) is a tumor of biliary ducts, which has a high mortality rate and dismal prognosis. Constitutively activation of the transcription factor nuclear factor kappa-B (NF-κB) has been previously demonstrated in CCA. It is therefore a potential target for CCA treatment. Effects of diethyldithiocarbamate (DDTC) on NF-κB-dependent apoptosis induction in cancer have been reported; however, anti-metastasis has never been addressed. Therefore, here the focus was on DDTC effects on CCA migration and adhesiond. Anti-proliferation, anti-migration and anti-adhesion activities were determined in CCA cell lines, along with p65 protein levels and function. NF-κB target gene expression was determined by quantitative RT-PCR. DDTC inhibited CCA cell proliferation. Suppression of migration and adhesion were observed prior to anti-CCA proliferation. These effects were related to decreased p65, reduction in NF-κB DNA binding, and impaired activity. Moreover, suppression of ICAM-1 expression supported NF-κB-dependent anti-metastatic effects of DDTC. Taken together, DDTC suppression of CCA migration and adhesion through inhibition of NF-κB signaling pathway is suggested from the current study. This might be a promising treatment choice against CCA metastasis.

Keywords: Diethyldithiocarbamate - cholangiocarcinoma - NF-κB - metastasis - migration - adhesion

Introduction

Cholangiocarcinoma (CCA) is a malignant bile duct tumor with a high mortality rates (Boberg and Schrumpf, 2004). Because of lacking a precise early diagnosis of CCA, patients with CCA are usually diagnosed at advance stages (Blechacz et al., 2011). The gold standard treatment of CCA is complete cancer removal (Charbel and Al-Kawas, 2012), which is inapplicable in advance CCA. Newly developed drugs based on the mechanism of cancer metastasis are available (Dasanu et al., 2011); however, no standard regimens for advanced CCA treatment have been approved. Since CCA lacks an effective treatment, an approach to find drugs targeting tumor metastasis would be profitable to ameliorate effective treatment in CCA patients.

Cancer metastasis is a dynamic multifaceted process in which cancer cells leave the primary site and migrate to other parts of the body (Chiang and Massague, 2008). A nuclear factor kappa-B (NF-κB) signaling pathway plays roles in different aspects of oncogenesis, including metastasis (Aggarwal, 2004; Basseres and Baldwin, 2006). NF-κB is constitutively active in most cancer cell lines, including CCA cells (Aggarwal, 2004; Seubwai et al., 2010). The activation of NF-κB regulates the expression of target genes involved in cell metastasis, such as ICAM-1, *VCAM-1*, *IL-6*, *MMP-2*, and *MMP-9* (Shian et al., 2003; Schurigt et al., 2005). Numerous anti-cancer compounds targeting NF-κB have been reported (Pande and Ramos, 2005; Goto et al., 2012; Matsuno et al., 2012), most of which were developed based on the mechanism of the suppression of cell proliferation and induction of apoptosis. Recently, several metastasis-suppressing anti-cancer compounds for CCA were studied (Tang et al., 2006).

Diethyldithiocarbamate (DDTC) is a major metabolite of disulfiram which has been known as a potential treatment agent of alcoholism (Eneanya et al., 1981; Langeland and McKinley-McKee, 1996; Suh et al., 2006). DDTC was studied in many clinical aspects such as treatment of metal toxicity, AIDS, and cancers (Pang et al., 2007; Matsuno et al., 2012). Disulfiram and disulfiram derivatives; DDTC and pyrrolidine dithiocarbamate (PDTC), are well-known as NF-κB inhibitors. There interfere NF-κB pathway by inhibiting NF-κB nuclear translocation (Lovborg et al., 2006; Matsuno et al., 2012), inhibition of IκB phosphorylation (Cvek and Dvorak, 2007), and proteasome degradation (Cvek and Dvorak, 2007; Matsuno et al., 2012). Moreover, this compound induces cancer cell apoptosis and exhibits

[1]Division of Hematopoiesis, Center for AIDS Research, Kumamoto University, Kumamoto, Japan, [2]Department of Biochemistry and Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kean, Thailand *For correspondence: okadas@kumamoto-u.ac.jp

proteasome inhibitory activity in some cancer cell lines (Cvek and Dvorak, 2007; Matsuno et al., 2012). Potent anti-metastatic potentials of disulfiram and PDTC were previously revealed (Shian et al., 2003; Cho et al., 2007; Murai et al., 2010; Zhang et al., 2011); however, the anti-metastatic effects of DDTC particularly on CCA have never been reported.

Here, we verified the effects of DDTC on the metastatic capability of CCA cell lines. DDTC exhibited high potency to inhibit CCA metastasis, at least in cell migration and adhesion, via suppression of the NF-κB pathway.

Materials and Methods

Cell culture and reagents

CCA cell lines, KKU-M213, KKU-M214, and KKU-OCA17 (Seubwai et al., 2010), used in this study were kindly provided by Dr. Banchop Sripa (Khon Kaen University, Thailand). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Trace, Melbourne, Australia), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator at 37°C and 5%CO_2. Dethyldithiocarbamate (DDTC) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Tetrazolium dye methylthiotetrazole (MTT) assay

The effects of DDTC on CCA cell viability were determined by the MTT assay as previously described (Seubwai et al., 2010). In brief, cells were treated with DDTC for 24 h at 37°C. Subsequently, MTT (Sigma-Aldrich) (0.5 mg/ml final concentration) was added to each well. After 3 h, 0.04 N HCl in isopropanol was added to dissolve the crystal. The absorption at 595 nm was determined with an ELISA plate reader (Multiskan; Thermo Electron, Vantaa, Finland). OD_{595} were normalized by untreated (control) samples. DDTC affected CCA cell viability in media containing 1% FBS were determined at 12 h or 16 h.

Wound healing assay

Monolayer cell migration was measured by the methods modified as previously described (Liang et al., 2007). Briefly, cells were cultured for 12-14 h to create a confluent monolayer. Subsequently, the monolayer was scraped with a pipette tip. The debris was removed by PBS washing, then media containing 1% FBS with the given concentrations of DDTC was added, followed by incubation for 4, 8, and 12 h. Images were acquired using an inverted microscope (Keyence, Osaka, Japan). Migration rates were measured by quantifying the migration distance (D) in the same field of the image using Image-Pro Plus software ver. 2.0 (Media Cybernetics, Silver Spring, MD, USA) and calculated using the following equation;

Migration (%)=[D$_{control}$-D$_{treated}$/D$_{control}$]×100

Transwell assay

Single cell migration was measured using transwell chambers as previously described with modification (Hu et al., 2010). Briefly, cells were seeded into the upper chambers in serum-free media containing DDTC. After that the upper chambers were placed into the lower chamber containing DMEM containing 10% FBS. The transwells were then incubated for 16 h. Non-migrated cells were removed from the upper chambers by scraping. Migrated cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Images were acquired using the inverted microscope. The migration rates were measured by quantifying the number of migrated cells with ImageJ software (National Institutes of Health, Bethesda, MD, USA). Values were normalized to the untreated (control) samples.

Adhesion assay

The adhesion assay was modified from Chen et al. (Chen et al., 2004). Briefly, cells were incubated in a 96-well plate in the presence of DDTC for 12 h. Unbound cells were removed and washed with PBS. Adhering cells were fixed and stained with 0.1% crystal violet. Crystal violet-stained cells were lysed with 10% acetic acid. OD_{595} were determined with the ELISA plate reader. Values were normalized to the untreated (control) samples.

Luciferase assay

The transcriptional activity of NF-κB was determined using the dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's manual. Briefly, KKU-M213 cells were transfected with pNF-κB-Luc (Takara Bio., Ohtsu, Japan). Concomitantly, cells were co-transfection with phRG-TK (Promega). pGL3 basic (Promega) was transfected to negative control cells instead of NF-κB promoter construct. Luciferase activity was analyzed using a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany) and values were normalized to the untreated (control) samples.

Electrophoretic mobility shift assay (EMSA)

NF-κB DNA binding ability was determined by EMSA as described previously (Matsuno et al., 2012). Cells were treated with DDTC and incubated for 4, 8, and 12 h. Nuclear extracts were prepared as described by Andrews et al. (Andrews and Faller, 1991). EMSA was performed using a second-generation DIG Gel Shift Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Double-stranded 22-mer oligonucleotide probes containing NF-κB binding site; 5'-AGT TGA GGG GAC TTT CCC AGG C-3', were purchased from Promega. 3'end was labeled with a digoxigenin (DIG)-11-ddUTP. The nuclear extract was incubated with poly d(A-T), poly d(I-C), poly L-lysine, and DIG-labeled double-stranded oligonucleotide. The obtained samples were separated on 6% acrylamide non-denaturing gel in TBE buffer, and then blotted onto a nylon membrane (Roche Diagnostics), cross linking, detected using anti-DIG-AP and chemiluminescent substrate. Signal was detected by ImageQuant LAS 4000 and analyzed by ImageQuant TL 7.0 software (GE Healthcare, Tokyo, Japan).

Western blot analysis

Whole cell extractions of DDTC treated CCA cells

were performed as previously described (Matsuno et al., 2012). Briefly, cells were lysed by NP-40 lysis buffer. Cellular proteins were separated by 10% SDS-PAGE and blotted onto a PVDF membrane (GE Healthcare). Primary antibodies were used as follows: anti-active p65 (MAB3026; EMD Millipore Corporation, Billerica, MA, USA) and anti-actin (sc-8432; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Corresponded secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) were used. Detection was performed using Chemi-Lumi One (NacalaiTesque, Tokyo, Japan).

Quantitative real-time PCR (quantitative RT-PCR)

The mRNA expressions were determined in DDTC-treated KKU-M213 cells compared to the untreated control by quantitative RT-PCR. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and cDNA was prepared using PrimeScript RT-PCR kit (Takara Bio Inc.) according to the manufacturer's recommendation. Quantitative RT-PCR analysis was performed using the Applied Biosystems Step One real-time PCR system (Life Technologies, Foster City, CA, USA). The Ct of each gene was normalized to Ct of *GAPDH* (internal control). The normalized expressions were expressed as relative gene expressions (fold induction compared with control). The oligonucleotide primers of *E-cadherin*, *ICAM-1* and *GADPH* were previously reported (Uthaisar et al., 2012).

Statistical analysis

For data analysis, statistically significant differences were determined using Student's t-test. P values<0.05 were defined as statistically significant. Data were analyzed with SPSS ver. 17.0 software (IBM Corporation, Somers, NY, USA).

## Results

Inhibitory effects of DDTC on CCA cell viability

To determine the effects of DDTC on cell proliferation, three human CCA cell lines were treated with DDTC for 24 h and cell viability was assessed by MTT assay. The suppression of cell viability was observed in a dose-dependent manner (Figure 1). Effective doses varied for each cell line. KKU-M213 and KKU-M214 cell lines responded to DDTC similarly at IC_{50} 5-8 μM. DDTC showed the highest cytotoxicity on KKU-OCA17 cells (IC_{50}=0.6 μM).

*DDTC has inhibitory effects on KKU-M213 cell migration and adhesion*

To examine the effects of DDTC on CCA cell metastasis, a migration assay was performed. KKU-M213 cell line was selected due to moderate effect of DDTC on cell viability, comparable to KKU-M214 cell line. Highly sensitivity of KKU-OCA17 to DDTC would be difficult to verify anti-migration activity without encountering of cell death induction. Monolayer cell migration was examined by wound healing assay. As shown in Figure 2A, the inhibition of wound healing was observed in KKU-M213 cells treated with DDTC. This effect could be observed in both dose- and time-dependent manners (Figure 2B,

2C). To confirm the effect of anti-migration was not due to the insufficient serum component (1% FBS) used in this assay, MTT assay was used to evaluate cell viability under the same condition. We found that DDTC did not affect cell viability at the concentrations 0-10 μM when incubated for 12 h or 16 h (Figure 2D). Inhibitory effects on CCA cell migration without induction of cell death were observed at 10 μM DDTC treatment.

We verified the inhibitory effects of DDTC on KKU-M213 cell migration using the single-cell migration system, transwell assay. As shown in Figure. 3A, migrated cells were indicated by crystal violet stained spots. Inhibition of cell migration could be detected at 0.3 μM.
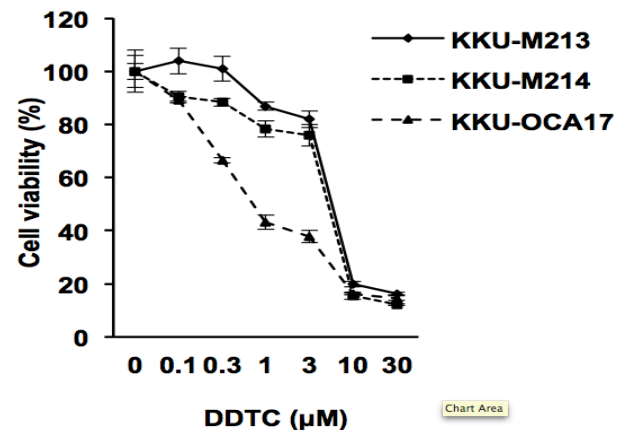
Figure 1. Suppression of Viability of Cholangiocarcinoma (CCA) Cell Lines by DDTC. Three human CCA cell lines, KKU-M213, KKU-M214, and KKU-OCA17, were treated with DDTC for 24 h. Cell viability was analyzed by MTT assay. Values are normalized to the untreated (control) samples. Data are presented as the mean±SD
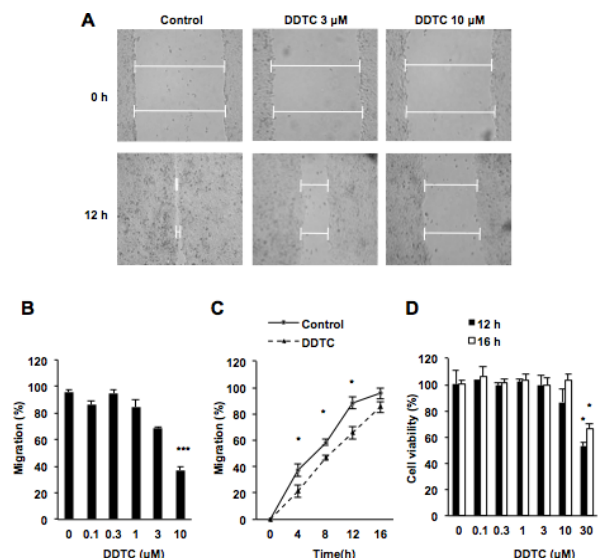
Figure 2. Effects of DDTC on Monolayer Cell Migration of CCA Cells. Confluent monolayer KKU-M213 cells were scratched and treated with DDTC. **A)** Cells were photographed using a Keyence microscope. **B)** Cells were treated with different concentrations of DDTC for 12 h. **C)** Cells were treated with 10 μM DDTC and migrated distances were assessed at 4, 8, 12, and 16 h. Migration rates were calculated and compared to untreated control. **D)** Cell viability of treated cells in D-MEM containing 1% FCS for 12 and 16 h was analyzed by MTT assay. Values are normalized to the untreated (control) samples. Data are presented as the mean±SD with*p<0.05 and ***p<0.001
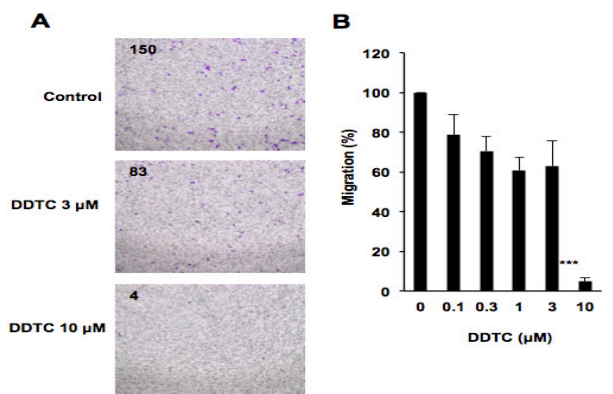
Figure 3. Effects of DDTC on Single-Cell Migration of CCA Cells. Cell migration was determined in KKU-M213 cells by transwell assay after 16 h. **A)** Crystal violet-stained spots represent migrated cells on transwell membrane. **B)** Migration rates were calculated and compared to untreated (control) samples. Data are presented as the mean±SD of five independent experiments with *p<0.05 and ***p<0.001
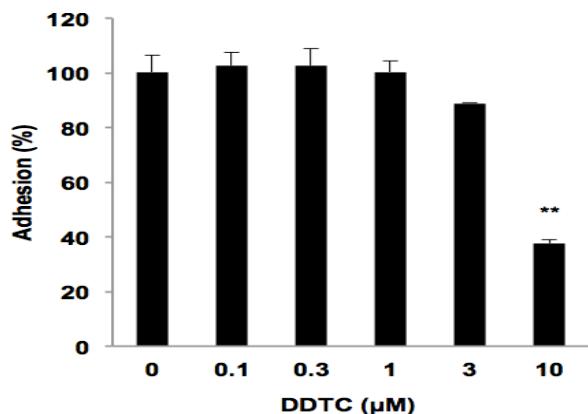

Figure 4. DDTC Inhibits Cell Adhesion of CCA Cells. KKU-M213 cells were treated with DDTC for 12 h. After incubation, adhesion cells were stained with crystal violet and analyzed by spectrometry. Values are normalized to the untreated (control) samples. Data are presented as the mean±SD with **p<0.01

DDTC treatment and more than 90% suppression could be detected at 10 μM DDTC treatment as shown in Figure. 3B.

To investigate anti-metastatic activity in different processes, adhesion assay was determined in KKU-M213 cells. DDTC treatment alleviated KKU-M213 adhesion in a dose-dependent manner (Figure 4). The effective concentration to inhibit KKU-M213 cell adhesion was 10 μM.

DDTC suppresses the NF-κB pathway in KKU-M213 cells

To investigate the inhibitory effects of DDTC on NF-κB function, NF-κB transcriptional activity was investigated by promoter assay. Suppression of NF-κB transcriptional activity was observed in DDTC treated KKU-M213 cells in dose- and time-dependent manners as shown in Figure 5A and 5B. To further clarify the NF-κB inhibitory effects of DDTC, NF-κB-DNA binding capability was determined by EMSA. Inhibition of NF-κB-DNA binding could be observed in a time-dependent manner (Figure 5C). Western blot analysis of active p65
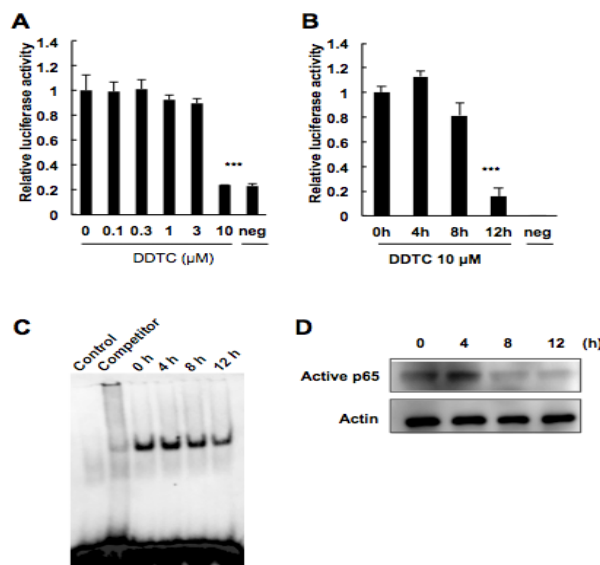
Figure 5. Inhibition of NF-κB Pathway of CCA Cells by DDTC. KKU-M213 cells were treated with DDTC and transcriptional activity, binding affinity, and protein levels were determined in the NF-κB pathway. **A)** Cells were transfected with pNF-κB-Luc. Transfected cells were treated with DDTC for 12 h and analyzed with an assay kit. **B)** Transfected cells were treated with 10 μM DDTC for 4, 8, and 12 h. Relative luciferase activities are normalized to the untreated (control) samples. Data are presented as the mean±SD with **p<0.001. **C)** DNA-binding affinity was determined in treated cells by EMSA using an assay kit. **D)** Cellular proteins were extracted and underwent Western blot analysis. Actin was used as the protein loading control
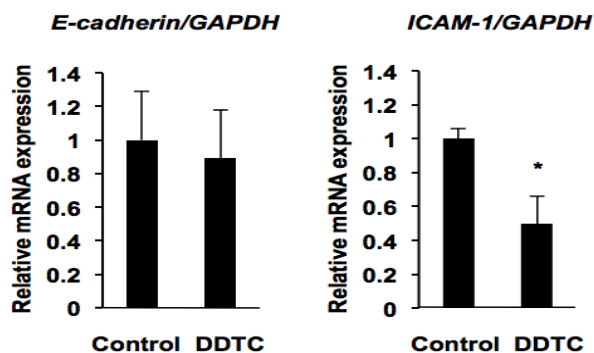
Figure 6. Suppression of *E-cadherin* and *ICAM* Expressions in CCA Cells by DDTC Treatment. KKU-M213 cells were treated with 10 μM DDTC for 12 h and mRNA expressions were measured by quantitative RT-PCR. The gene expression levels were normalized to the levels of *GAPDH* as an internal control. Data are the mean±SE with *p<0.05

suggested the reduction of NF-κB DNA binding and transcriptional activity were caused by reduced active p65 protein (Figure 5D).

DDTC suppressed adhesion gene expression in KKU-M213 CCA cells

E-cadherin and *ICAM-1* are NF-κB target genes which have been reported to play important roles in cell metastasis (Maruo et al., 2002; Margineanu et al., 2008). We measured the mRNA expression of these genes by quantitative RT-PCR in KKU-M213 cells cultured with 10 μM DDTC for 12 h compared to the untreated control. Fifty percent suppression of *ICAM-1* mRNA expression could be detected in DDTC treated KKU-M213 as shown

in Figure 6. No significant difference of *E-cadherin* was observed in DDTC treated cells compared to untreated control. These results indicated the potential anti-metastatic effect of DDTC on the NF-κB pathway.

Discussion

Diethyldithiocarbamate (DDTC) has been well-documented as a potent NF-κB inhibitor. This study is the first report in which DDTC exhibited potent anti-migration and anti-adhesion effects in CCA cell line. These anti-metastasis potential was due to suppression of the NF-κB pathway. Anti-proliferative effect of DDTC was observed in all CCA cell lines (KKU-M213, KKU-M214, and KKU-OCA17) with different potency. Anti-migration and anti-adhesion effects of DDTC were demonstrated. These were occurred prior the anti-proliferative effect (12-16 h vs. 24 h).The suppressions of active p65 protein, NF-κB-DNA binding and NF-κB activity were clearly observed in DDTC-treated KKU-M213 cells. The inhibition of NF-κB-related gene, ICAM-1, expression suggested DDTC inhibit CCA migration and adhesion through NF-κB suppression.

The inhibitory effects of disulfiram and disulfiram derivative, PDTC, on cancer adhesion, migration, invasion and angiogenesis were previously reported (Shian et al., 2003; Cho et al., 2007; Murai et al., 2010; Zhang et al., 2011). Similar inhibitory effects of DDTC on CCA cell migration and adhesion were observed. These effects were demonstrated at DDTC concentration that did not affect cell proliferation and at shorter treatment duration than duration that exhibited anti-proliferative activity. Disulfiram and disulfiram derivatives; DDTC and PDTC, are well-known as NF-κB inhibitors (Lovborg et al., 2006; Cvek and Dvorak, 2007; Matsuno et al., 2012). The NF-κB inhibitory effects have been evident by the suppression of NF-κB translocation, IκB phosphorylation, and proteasome degradation via their metal complex (Lovborg et al., 2006; Cvek and Dvorak, 2007; Pang et al., 2007; Wang et al., 2011; Matsuno et al., 2012; Skrott and Cvek, 2012). Our results showed the inhibition of NF-κB transcriptional activity by DDTC; however, we could not detect any effect of DDTC on proteasome activity of CCA cell lines (data not shown). This mechanism was supported by the reductions of NF-κB-DNA binding and levels of active p65 proteins in CCA cells after treatment with DDTC.

Since the suppression of NF-κB transcriptional activity could be clearly observed in DDTC-treated KKU-M213 cells, we checked the mRNA expression of NF-κB target genes that play roles in cancer metastasis. *ICAM-1* has been reported to be associated with increased metastasis potential in several cancers (Maruo et al., 2002; Rosette et al., 2005). The over-expression of *ICAM-1* in CCA cell lines and high *ICAM-1* protein serum levels were reported (Janan et al., 2012). In this study, we identified the suppression of *ICAM-1* mRNA expression in DDTC-treated KKU-M213 cells, which supported the inhibitory effect of DDTC on cell adhesion. Inhibition of the migration and adhesion of KKU-M213 cells by DDTC suggested that DDTC demonstrated potential anti-metastasis effects on CCA.

Taken together, the anti-metastasis of DDTC on CCA was suggested herein. These effects were partly explained by suppression of NF-κB transcriptional activity and suppression of NF-κB target gene; ICAM-1, expression. DDTC could offer a potential therapeutic strategy to control CCA progression in advance cancer.

Acknowledgements

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