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Quercetin and EGCG Exhibit Chemopreventive Effects in Cholangiocarcinoma Cells via Suppression of JAK/STAT Signaling Pathway

Laddawan Senggunprai,[1]* Veerapol Kukongviriyapan,[1] Auemduan Prawan[1] and Upa Kukongviriyapan[2]

[1]Department of Pharmacology, Faculty of Medicine, and Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, 40002, Thailand
[2]Department of Physiology, Faculty of Medicine, Khon Kaen University, 40002, Thailand

Quercetin and epigallocatechin-3-gallate (EGCG) are dietary phytochemicals with antiinflammatory and antitumor effects. In the present study, we examined the effects of these two compounds on Janus-like kinase (JAK)/signal transduction and transcription (STAT) pathway of cholangiocarcinoma (CCA) cells, because CCA is one of the aggressive cancers with very poor prognosis and JAK/STAT pathway is critically important in inflammation and carcinogenesis. The results showed that the JAK/STAT pathway activation by proinflammatory cytokine interleukin-6 and interferon-γ in CCA cells was suppressed by pretreatment with quercetin and EGCG, evidently by a decrease of the elevated phosphorylated-STAT1 and STAT3 proteins in a dose-dependent manner. The cytokine-mediated up-regulation of inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1) via JAK/STAT cascade was abolished by both quercetin and EGCG pretreatment. Moreover, these flavonoids also could inhibit growth and cytokine-induced migration of CCA cells. Pretreatment with specific JAK inhibitors, AG490 and piceatannol, abolished cytokine-induced iNOS and ICAM-1 expression. These results demonstrate beneficial effects of quercetin and EGCG in the suppression of JAK/STAT cascade of CCA cells. Quercetin and EGCG would be potentially useful as cancer chemopreventive agents against CCA. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: JAK/STAT; quercetin; EGCG; cholangiocarcinoma; phytochemical; chemopreventive.

INTRODUCTION

Epidemiological studies have shown that the amount of consumption of vegetables and fruits rich in flavonoids inversely associated with the risk of human cancers (Riboli and Norat, 2003). The antiinflammatory effect of flavonoids is recognized as an important action for their cancer chemoprevention property (Garcia-Lafuente *et al*., 2009). Quercetin, the most abundant flavonoid in the human diet, and epigallocatechin-3-gallate (EGCG), the biologically most active constituent in green tea, are proposed to have cancer-preventive activities (HemaIswarya and Doble, 2006). The potential chemopreventive effects of these two compounds have been attributed to various mechanisms including anti-oxidative activity as well as capability to modulate cellular signal transduction pathways involving antiinflammatory, anti-proliferative and induction of apoptosis of tumor cells (Shanmugam *et al*., 2011). Quercetin and EGCG can interact with non-receptor protein kinases and receptor tyrosine kinases such as epidermal growth factor receptor and vascular endothelial growth factor receptor (Shanmugam *et al*., 2011). At present, quercetin and EGCG have been used in clinical trials for prevention of several types of cancers such as breast, lung and prostate cancers (http://www.clinicaltrials.gov/).

* Correspondence to: Laddawan Senggunprai, Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
E-mail: laddas@kku.ac.th

Cholangiocarcinoma (CCA) is a malignant epithelial neoplasm of the biliary tree with very poor prognosis. It is a rare type of cancer worldwide; however, populations residing in the Southeast Asian region are at very high risk. The high incidence of CCA in this region is associated with background conditions particularly liver fluke infection (i.e. *Opisthorchis viverrini* and *Chlonorchis sinensis*) that causes long-standing inflammation, cell injury and reparative biliary epithelial cell proliferation (Sripa and Pairojkul, 2008). Persistent inflammation can create a local environment enriched with cytokines and other growth factors that primes for cells to develop autonomous proliferative signaling and enhanced production of mitogenic factors (Wise *et al*., 2008). Excretory/secretory products of liver flukes can stimulate epithelial bile duct cells to secrete proinflammatory cytokine interleukin-6 (IL-6) (Ninlawan *et al*., 2010). In addition, serum interferon-gamma (IFN-γ) and its expression level in the liver of mice were increased by *O. viverrini* infection (Nair *et al*., 2011). These suggest that various inflammatory mediators, particularly proinflammatory cytokines, are common and important contributors for cholangiocarcinogenesis.

Proinflammatory cytokines activate the signal transduction and transcription (STAT) proteins, which are considered to be oncogenic transcription factors (Hodge *et al*., 2005). Stimuli to cell surface cytokine receptors activate the Janus-like kinase (JAK) family of protein kinases, which, in turn, phosphorylates and activates latent cytoplasmic STAT proteins to an active dimer, leading to nuclear translocation and DNA binding and subsequently modulating gene transcription (Darnell,

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1997). Several other kinases including members of the Src and Abl family have also been implicated in the phosphorylation of STATs (Darnell, 1997). Among seven known mammalian STAT proteins, STAT1 and STAT3 are the most widely studied isoforms because of their constitutively activated state in many tumors but not in normal cells (Hodge *et al.*, 2005). STAT1 is relatively specific to IFNs and a pivotal transcription factor in IFN-γ-induced expression of inflammatory genes, whereas STAT3 is mainly activated by IL-6 and other gp130-related cytokines (Yoshimura, 2006).

The crucial roles of STATs, particularly STAT1 and STAT3, in inflammation and tumorigenesis have been demonstrated in several studies such as inflammation-associated gastric tumorigenesis (Ernst *et al.*, 2008) and colitis-associated tumorigenesis (Grivennikov *et al.*, 2009). The activation of JAK/STAT cascade triggered by inflammatory cytokines produced by tumor-infiltrating immune cells can lead to the expression of STAT-regulated genes including proinflammatory enzymes and proteins in which then enhance inflammatory condition (Dalwadi *et al.*, 2005). In addition, the activation of JAK/STAT pathway also results in induction of genes that mediate cell proliferation, suppression of apoptosis and promotion of angiogenesis (Barton *et al.*, 2004).

Presently, there is neither effective treatment for patients with the advanced stage of CCA nor any effective agents for chemoprevention of CCA. Similar to several cancers, the JAK/STAT signaling pathway is involved in the development of CCA (Isomoto *et al.*, 2007). Thus, STAT signaling pathway inhibitors may be useful for prevention and treatment of CCA. Currently, only few studies addressed the role of JAK/STAT signaling pathway in carcinogenesis of CCA.

In the present study, we have investigated the effects of quercetin and EGCG on JAK/STAT pathway by using CCA cells treated with proinflammatory cytokine mixture, IL-6 and IFN-γ, for mimicking inflammatory condition (Kaur *et al.*, 2003). The results show that both quercetin and EGCG could inhibit JAK/STAT signaling cascade. They also could suppress cytokine-induced expression of inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1), the key molecules involving in inflammatory and tumorigenesis processes. Moreover, they inhibited growth and migration of CCA cells. Our findings provide new insights into the intervention strategy for prevention of CCA.

MATERIALS AND METHODS

Reagents. Quercetin, EGCG and piceatannol were purchased from Sigma Chemical (St. Louis, MO, USA). AG490 was obtained from Calbiochem (San Diego, CA, USA). IL-6, IFN-γ and the primary antibodies against STAT1, STAT3, phospho-STAT1 (Tyr^{701}) and phospho-STAT3 (Tyr^{705}) were purchased from Cell Signaling Technology (Danvers, MA, USA). RIPA buffer was from Amresco (Solon, OH, USA). The pGL4 basic and pGL4[*hRluc*] vector, Lipofectamine™ 2000 and Dual-Luciferase® Reporter Assay Kit were obtained from Promega (Madison, WI, USA). Reagents for cell culture were from Gibco BRL Life Technologies (Grand Island, NY, USA).

Cell lines and cell cultures. The human CCA cell lines, KKU100, KKU-M139 and KKU-M213, used in this study were kindly provided by Dr Banchob Sripa of the Department of Pathology, Faculty of Medicine, Khon Kaen University. These cell lines were cultured in complete media consisting of Ham's F12 media, supplemented with 10% fetal calf serum, 12.5 mM HEPES, pH 7.3, 100 U/mL penicillin G and 100 μg/mL streptomycin, and maintained under an atmosphere of 5% CO_2 at 37 °C. The cells were subcultured every 3 days using 0.25% trypsin-EDTA.

Cell treatment. At about 70% confluency, cultures were starved in serum-free medium for 16 h and then treated with cytokine mixture, combination of IL-6 and IFN-γ (Kaur *et al.*, 2003), in serum-free medium for further 1 to 24 h. The concentrations used for both IL-6 and IFN-γ were 100 ng/mL. In experiments involving flavonoids or JAK inhibitors, the cells were pretreated with various concentrations of quercetin (1, 10, 50 and 100 μM) or EGCG (1, 10, 25 and 50 μM) or piceatannol (JAK1 inhibitor) (Barton *et al.*, 2004) (1, 10 and 100 μM) or AG490 (JAK2 inhibitor) (Barton *et al.*, 2004) (1, 10 and 100 μM) in DMSO for 2 h and then further treated with cytokine mixture. An equal amount of DMSO (vehicle) was present in each treatment, including control. DMSO concentration did not exceed 0.1% (v/v) in any treatment.

Protein extraction and western blot analysis. After treatments for designated periods, medium was aspirated, cells were washed twice with cold PBS and whole cell lysates were prepared using RIPA cell lysis buffer (Amresco, OH, USA) according to the manufacturer's instructions. The proteins were resolved by SDS-PAGE using 10% polyacrylamide gel, and the separated proteins on the gel were electrophoretically transferred to a PVDF membrane. The membranes were blocked with 5% (w/v) bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 for 2 h at room temperature, followed by incubation with primary antibody against STAT1 (1:1000), phospho-STAT1 (Tyr^{701}) (1:1000), STAT3 (1:1000), phospho-STAT3 (Tyr^{705}) (1:1000) and β-actin (1:5000) at 4 °C overnight. After washing, the membranes were incubated with the appropriate horseradish peroxidase conjugated secondary antibody to detect bands by Amersham™ ECL™ Prime (Amersham Biosciences Corp, NJ, USA). The densities of the specific protein bands were visualized and captured by ImageQuant™ 400.

Real-time polymerase chain reaction. Total RNA was extracted from cells by using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. One microgram of total RNA was then reverse transcribed to single-stranded cDNA by the ImProm-II™ reverse transcription system (Promega, WI, USA). Polymerase chain reaction (PCR) was carried out using specific primers as follows: iNOS forward 5′-GTTCTCACGGCACAGGTCTC-3′ and iNOS reverse 5′-GCAGGTCACTTATGTCACTTATC-3′ (Wu *et al.*, 2011), ICAM-1 forward

5′-CAAGGCCTCAGTCAGTGTGA-3′ and ICAM-1 reverse 5′-CCTCTGGCTTCGTCAGAATC-3′ (Kim *et al.*, 2010), and GAPDH forward 5′-GTGGTGGACCTGACCTGC-3′ and GAPDH reverse 5′-TGAGCTTGACAAAGTGGTCG-3′ (Kim *et al.*, 2010). The relative expression of iNOS and ICAM-1 was analyzed using quantitative real-time PCR with GAPDH as an internal control. The PCR was performed with SsoFast™ EvaGreen Supermix with low Rox (Bio-Rad, CA, USA) using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) under the following conditions: denaturation at 95 °C for 3 min and amplification by cycling 40 times at 95 °C for 15 s and 60 °C for 31 s.

Cell growth assay. Sulforhodamine B (SRB) was used to measure the effect of quercetin and EGCG on the proliferation of CCA cell lines as described previously (Prawan *et al.*, 2009). Briefly, KKU100 cells were plated in a 96-well plate for 24 h. After exposure of cultured cells to quercetin or EGCG at various concentrations for 48 h, the culture medium was removed, with 10% cold trichloroacetic acid added for 1 h at 4 °C, and subsequently washed five times with deionized H_2O. The plate was air dried, and 0.4% SRB in 1% acetic acid was added for 30 min. Unbound dye was washed out five times with 1% acetic acid. After air drying, SRB dye within cells was solubilized with 200 μL of 10 mM unbuffered Tris base solution. The plate was shaken for 10 min, and the absorbance was measured at 540 nm by using a microplate reader. Cell growth inhibition was expressed in terms of percentage of untreated control absorbance following subtraction of mean background absorbance. The IC_{50} concentration (50% inhibition of cell growth values) was calculated from the dose–response curves.

Wound healing assay. KKU100 cells (1.5×10^5) were seeded into a 24-well plate and incubated overnight in Ham's F12 medium, supplemented with 10% fetal calf serum. A scratch wound was made with a sterile 200 μL pipette tip. After washing with PBS to remove any detached cell, a series of images of the scratched wound were taken from 0 to 24 h. The cells were pretreated with quercetin (10 μM) or EGCG (5 μM) or vehicle for 2 h, then with cytokine mixture afterward. The closing of scratched wound, which represented the migration process, was determined by capture of the denuded area along the scratch using Image-Pro Plus software (Media Cybernetics, LP, USA). The wound distance was calculated by dividing the area by the length of scratch.

STAT3 luciferase reporter assay. To construct the STAT3 luciferase vector, the STAT3 transcriptional regulatory element 5′-GTCGACATTTCCCGTAAATCGTCGA-3′ (Bergad *et al.*, 2000) containing NheI/HindIII restriction sites was subcloned into the multiple cloning site of the pGL4 luciferase reporter plasmid (Promega, WI, USA). The sequences of the constructs were verified by the MegaBACE DNA Analysis System (Amersham Biosciences Corp, NJ, USA). For reporter assay, the cells (1×10^4) were seeded into each well of 96-well plates and cultured for 24 h. The cells were transfected with 0.2 μg of STAT3-pGL4 reporter vector according to the manufacturer's protocols using X-tremeGene HP (Roche) for KKU100 cells or Lipofectamine™ 2000 (Promega, WI, USA) for KKU-M139 and KKU-M213 cells. The cells were also cotransfected with 0.01 μg of pGL4[*hRluc*/TK] vector containing the *Renilla luciferase* gene (Promega) to control transfection efficiency. After 6 h of transfection, cells were pretreated with flavonoids for 2 h and then stimulated with the cytokine mixture for additional 18 h before being washed and lysed in luciferase lysis buffer (Promega). Luciferase activity was measured with a luminometer by using a Dual-Luciferase® Reporter Assay Kit (Promega).

RESULTS

Cytokine mixture induced STAT1 and STAT3 activation in KKU100 cells

To clarify whether JAK/STAT signaling is intact in CCA cells, the effect of cytokine mixture (100 ng/mL IL-6 and

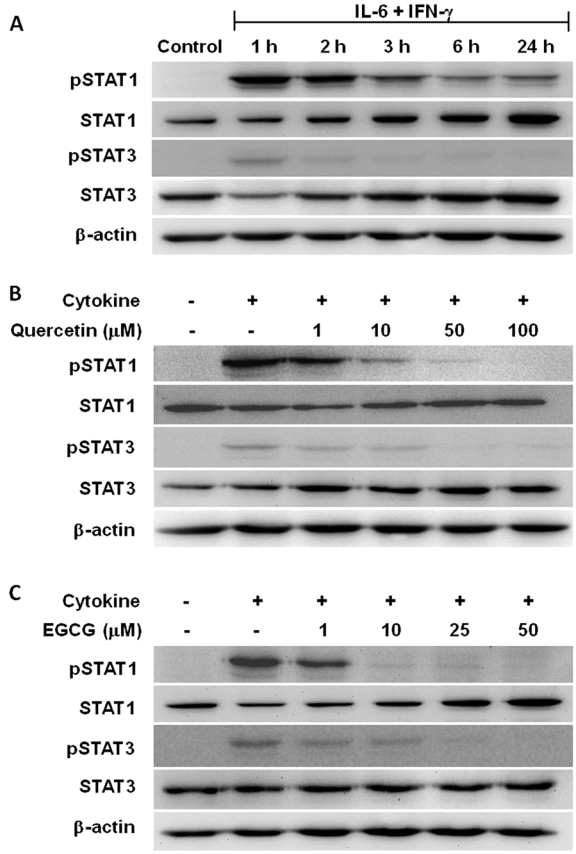

Figure 1. Effects of cytokine mixture on the activation of signal transduction and transcription (STAT) and effects of quercetin and epigallocatechin-3-gallate (EGCG) on cytokine-induced STAT activation. (A) KKU100 cells were incubated with cytokine combination (100 ng/mL interleukin-6 [IL-6] and 100 ng/mL interferon-γ [IFN-γ]) for various time points (1 to 24 h). (B and C) KKU100 cells were pretreated with the indicated concentrations of (B) quercetin or (C) EGCG for 2 h and then treated with cytokine mixture for 1 h. Whole cell extracts were prepared, and 20 μg aliquots of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto PVDF membranes and probed using the indicated antibodies as described under the Materials and Methods section. Anti-β-actin antibody was used as a loading control. Results shown are representative of at least two reproducible independent experiments.

100 ng/mL IFN-γ) on STAT levels in KKU100 cells was determined at various time points (1 to 24 h). In separate experiments, we confirmed that the dose range of cytokine mixture used in this study did not affect the cell viability (data not shown). After treatment with the cytokine mixture, phosphorylation of STAT1 (Tyr^{701}) in KKU100 cells was dramatically increased as early as within the first hour and then gradually decreased afterwards, although it was still detectable at 24 h (Fig. 1A). For STAT3, the cytokine mixture induced transient STAT3 (Tyr^{705}) phosphorylation in KKU100 cells within the first hour, but the phospho-STAT3 was diminished rapidly within 2 h (Fig. 1A). From these results, functional JAK/STAT signaling cascade is present in CCA cells.

Quercetin and EGCG inhibited cytokine-induced STAT1 and STAT3 phosphorylation

To determine whether quercetin and EGCG can suppress the proinflammatory cytokine-induced JAK/STAT phosphorylation, KKU100 cells were pre-incubated with various concentrations of quercetin or EGCG for 2 h and then stimulated with the cytokine mixture for 1 h. Under these conditions, the cell viability was not affected (data not shown). As shown in Fig. 1, when KKU100 cells were pretreated with quercetin (Fig. 1B) or EGCG (Fig. 1C), cytokine-induced STAT1 and STAT3 phosphorylation was markedly suppressed in a dose-dependent manner, although STAT1 and STAT3 protein levels were not affected.

Quercetin and EGCG suppressed cytokine mixture-induced iNOS and ICAM-1 expression

It is known that iNOS and ICAM-1 play a critical role in inflammatory and tumorigenesis processes (Yamada *et al*., 2006; Kostourou *et al*., 2011). Therefore, we first examined the effect of cytokine treatment on the expression of iNOS and ICAM-1 in CCA cells by using real-time PCR. The results showed that cytokine

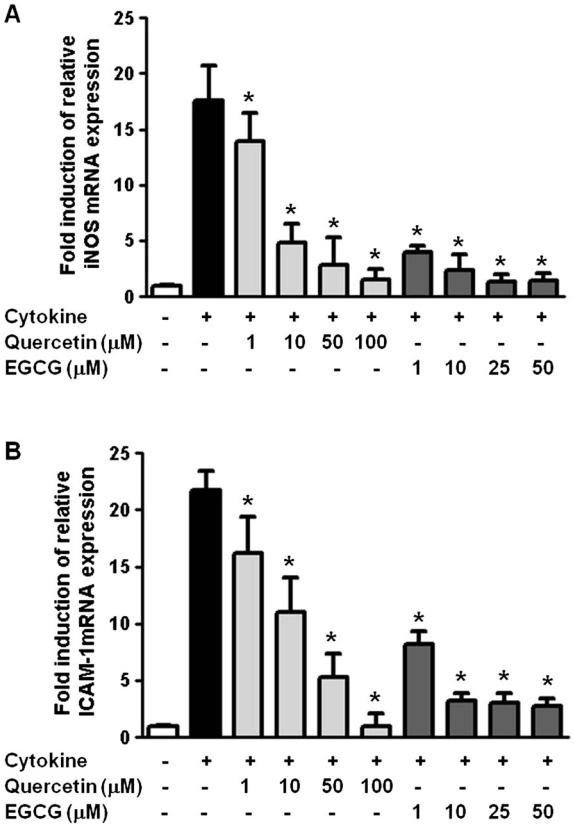

Figure 2. Effects of quercetin and epigallocatechin-3-gallate (EGCG) on cytokine-induced expression of inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1). KKU100 cells were pretreated with various concentrations of quercetin (1–100 μM) or EGCG (1–50 μM) for 2 h and then treated with cytokine combination (100 ng/mL IL-6 and 100 ng/mL IFN-γ) for 3 h. At the end of treatments, total RNA of cells were collected, and real-time polymerase chain reaction was carried out for (A) iNOS and (B) ICAM-1 expression. The bars represent relative expression of indicated genes normalized with GAPDH. The data are expressed as mean ± SD, each from three independent experiments. *, $p < 0.05$ vs cytokine treatment alone.

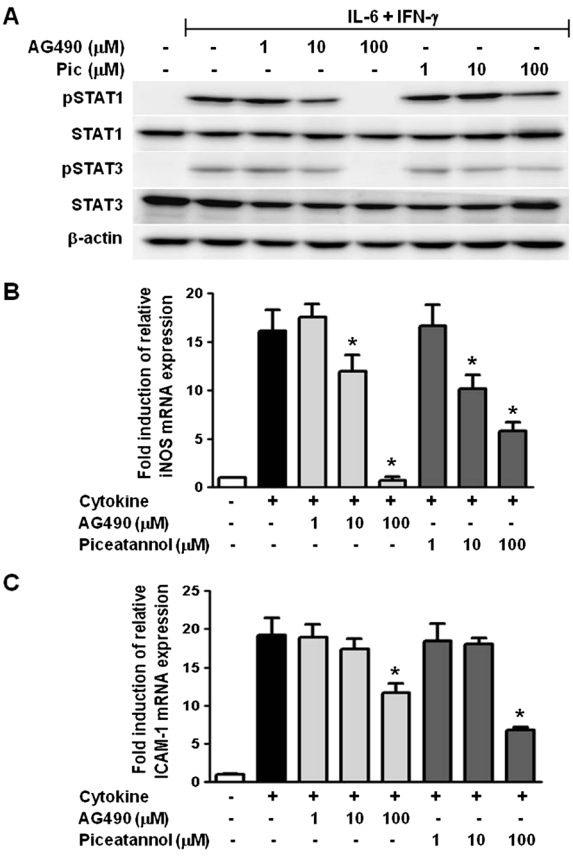

Figure 3. Effects of Janus-like kinase inhibitors on cytokine-mediated phosphorylation of signal transduction and transcription (STAT) and cytokine-induced inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1) expression. (A) KKU100 cells were pretreated with the indicated concentrations of AG490 or piceatannol for 2 h and then treated with cytokine mixture for 1 h. Whole cell extracts were prepared, and 20 μg aliquots of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto PVDF membranes and probed using the indicated antibodies as described under the Materials and Methods section. Anti-β-actin antibody was used as a loading control. Results shown are representative of at least two reproducible independent experiments. (B and C) KKU100 cells were pretreated with indicated concentrations (1–100 μM) of AG490 or piceatannol for 2 h and then treated with cytokine combination (100 ng/mL interleukin-6 [IL-6] and 100 ng/mL interferon-γ [IFN-γ]) for 3 h. At the end of treatments, total RNA of cells was collected and analyzed for (B) iNOS and (C) ICAM-1 expression by real-time polymerase chain reaction. The bars represent relative expression of indicated genes normalized with GAPDH. The data are expressed as mean ± SD, each from three independent experiments. *, $p < 0.05$ vs cytokine treatment alone.

treatment markedly up-regulated the mRNA expression of iNOS and ICAM-1 (Fig. 2). Pretreatment with quercetin and EGCG significantly inhibited cytokine-mediated up-regulation of iNOS and ICAM-1 expression in a dose-dependent manner (Fig. 2A and B, respectively). The inhibitory effect of EGCG was more potent than that of quercetin.

JAK1 and JAK2 inhibition attenuated cytokine-induced expression of iNOS and ICAM-1

To validate whether JAK/STAT signaling contributes to the up-regulation of iNOS and ICAM-1, the effects of piceatannol (JAK1 inhibitor) and AG490 (JAK2 inhibitor) on the cytokine-induced STAT1 and STAT3 phosphorylation in KKU100 cells were examined. As illustrated in Fig. 3A, pretreatment with these inhibitors could abrogate STAT1 and STAT3 phosphorylation in a dose-dependent manner. Subsequently, KKU100 cells were pre-incubated with various concentrations of piceatannol or AG490 for 2 h and then treated with the cytokine mixture for 3 h. After that, the mRNA expressions of iNOS and ICAM-1 were evaluated. The results showed that cytokine-induced iNOS expressions in KKU100 cells were suppressed dose-dependently by piceatannol and AG490 pretreatment (Fig. 3B). Similarly, piceatannol and AG490 could suppress the up-regulation of ICAM-1 expression, although the higher concentration was required for the inhibition compared with the effects on iNOS expression (Fig. 3C). Under these conditions, the cell viability was only slightly reduced (data not shown).

Quercetin and EGCG inhibited growth and migration of CCA cell lines

The anticancer effect of quercetin and EGCG was further evaluated by examining some downstream effects of JAK/STAT signaling, that is, anti-proliferation and anti-migration. The anti-proliferative activity was assayed on KKU100 cells by using SRB assay. Quercetin and EGCG had similar potency to inhibit KKU100 cell growth with the IC_{50} values of 31.1 ± 7.1 and 24.3 ± 3.1 μM, respectively (Fig. 4A).

Because quercetin and EGCG showed strong inhibitory effect on cytokine-induced expression of ICAM-1, an adhesion molecule involved in cell migration and invasion, the effect of these two compounds on CCA cell migration was investigated by wound healing assay using the monolayer culture of KKU100 cells. The results are shown in Fig. 4B and C. Closure of the scratched wound was stimulated by cytokine mixture, determined at 24 h. The cytokine-mediated stimulation of cell migration was suppressed by quercetin and EGCG, as the migration distance of cultured cells in those flavonoid-treated groups was shorter than that of cytokine treatment only.

Quercetin and EGCG inhibited STAT3-dependent luciferase activity in CCA cells

The suppressive effects of quercetin and EGCG on JAK/STAT signaling were confirmed further by STAT3-luciferase reporter assay. Firstly, we demonstrated that quercetin and EGCG suppressed cytokine-induced STAT3

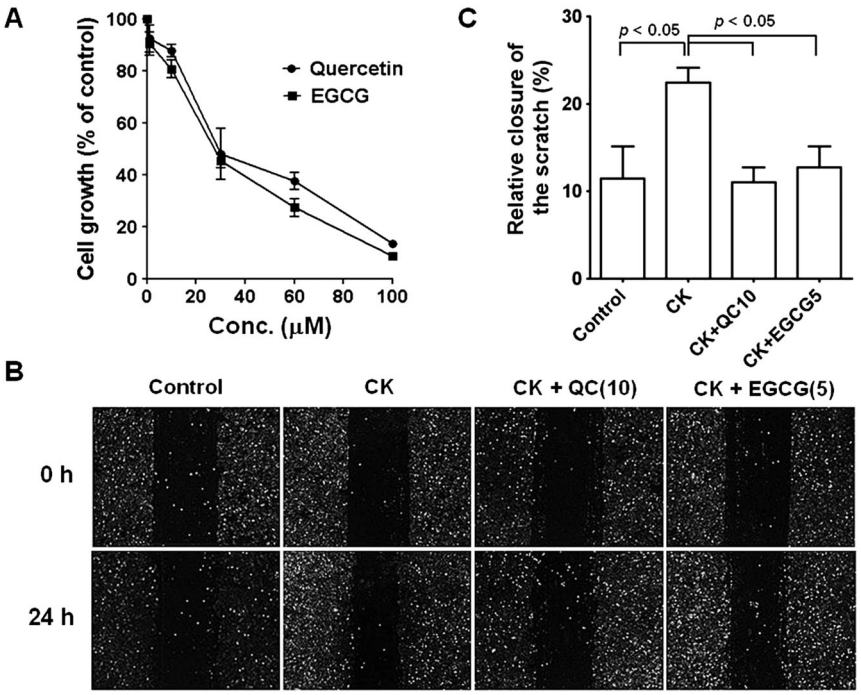

Figure 4. Effects of quercetin and epigallocatechin-3-gallate (EGCG) on the growth and cytokine-induced migration of cholangiocarcinoma cells. (A) KKU100 cells were treated with indicated concentration of quercetin or EGCG for 48 h, and cell numbers were determined by sulforhodamine B assay. The results are presented as percentage of control. The data are the mean ± SD averaged from three independent experiments. (B) Scratched wounds of monolayer KKU100 cells were pretreated with vehicle or 10 μM of quercetin or 5 μM of EGCG for 2 h and then treated with cytokine mixture. Cell migration was monitored under phase-contrast microscopy (×4 magnification). Representative images of wound healing were obtained at the time of the scratch and 24 h later. (C) The graph shows the level of cell migration into the wound scratch quantified as the percentage of wound closure at 24 h. The data are mean ± SD averaged from quadruplets of one experiment. CK, cytokine; QC, quercetin.

luciferase activity in KKU100 cells (Fig. 5A). To explore whether the suppressive effects of flavonoids on STAT3 signaling is common in various CCA cells, in addition to KKU100 cells, KKU-M139 and KKU-M213 cells were employed in this experiment. These three human CCA cell lines were established from primary tumors of liver fluke-associated CCA patients with different histological types, that is, poorly differentiated, adeno-squamous, and mixed papillary and non-papillary adenocarcinoma, respectively. As was expected, cytokine-induced STAT3 activity in KKU-M139 and KKU-M213 cells were strongly suppressed by quercetin and EGCG as shown in Fig. 5B and C, respectively, suggesting that the effects of the flavonoids are not restricted to a special cell type.

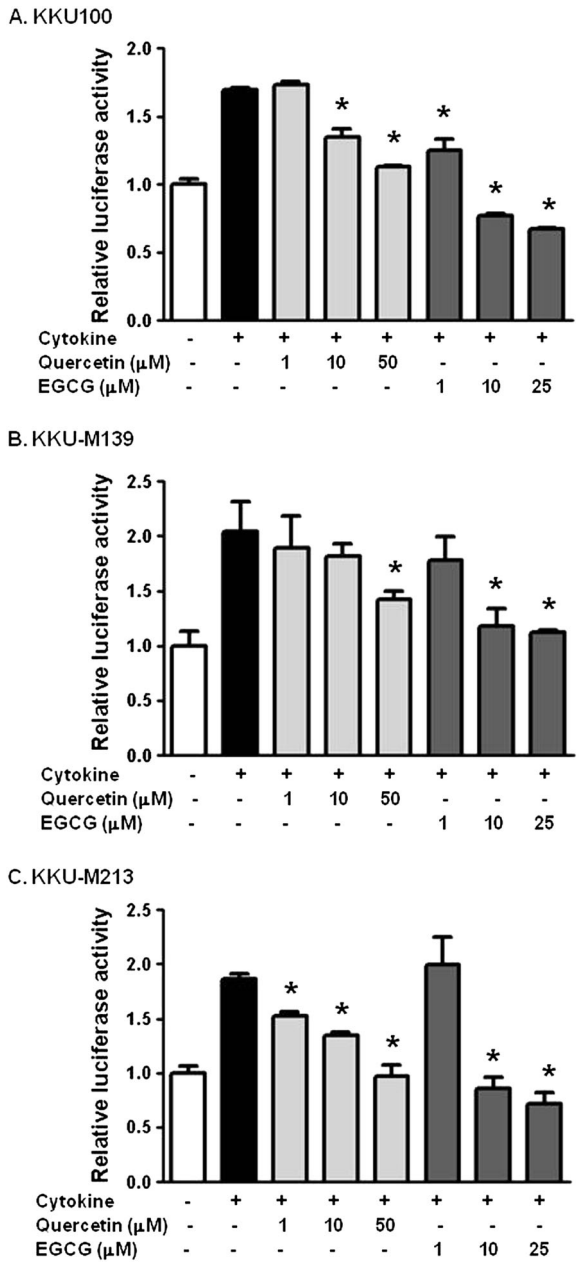

Figure 5. Effects of quercetin and epigallocatechin-3-gallate (EGCG) on cytokine-induced transcriptional activity of STAT3. The cholangiocarcinoma cells (A) KKU100, (B) KKU-M139 and (C) KKU-M213 were transfected with STAT3-pGL4 reporter vector together with *Renilla luciferase* plasmid. After 6 h of transfection period, cells were pretreated with indicated concentrations of quercetin or EGCG for 2 h before exposure to cytokine combination (100 ng/mL interleukin-6 and 100 ng/mL interferon-γ) for additional 18 h. After that, the luciferase activity was measured and presented as fold induction to the control cells without cytokine treatment. Each bar indicates the mean ± SD of five to six determinations. *, $p < 0.05$ vs cytokine treatment alone. STAT, signal transduction and transcription.

DISCUSSION

Chemoprevention and chemotherapy of CCA are so far quite disappointing. Several efforts have been made to identify and develop new agents for both treatment and prevention of this deadly disease. These include targeted therapy on some critical molecules of cancer cells, such as NQO1 and HO-1, where inhibition of the enzymes makes CCA cells sensitive to chemotherapeutic agents (Buranrat *et al.*, 2010; Kongpetch *et al.*, 2012). In the present study, we have demonstrated that JAK/STAT signaling pathway may be an important target for inhibition of CCA. JAK/STAT signaling plays an important role in the expression of iNOS and ICAM-1, both of which are known critical molecules involved in inflammation and carcinogenesis processes. Quercetin and EGCG inhibited JAK/STAT signaling cascade in CCA cells leading to the down-regulation of iNOS and ICAM-1 and suppression of cell migration. In addition, these two phytochemical compounds could also inhibit the growth of CCA cells. The results provide evidence supporting the use of these two flavonoids as chemopreventive agents for CCA.

Quercetin and EGCG are potent inhibitors of tumor growth and inflammation and have traditionally been used to treat many inflammatory disorders (Garcia-Lafuente *et al.*, 2009). Quercetin and EGCG have been shown to exert their effects through inhibiting several signaling pathways such as NF-κB (Priyadarsini and Nagini, 2012), AP-1 (Shanmugam *et al.*, 2011) and JAK/STAT (Tedeschi *et al.*, 2002; Michaud-Levesque *et al.*, 2012). We also found that quercetin and EGCG are potent inhibitors of cytokine-induced STAT1 as well as STAT3 activation in CCA cells.

In the present study, we demonstrated that the activation of JAK/STAT pathway is associated with the increase in expression of iNOS and ICAM-1. The iNOS has critical functions in inflammation-related diseases. Sustained and excess nitric oxide generation, most of which is attributable to iNOS expression, often is pathogenic to cause inflammation, angiogenesis and neoplasia (Kostourou *et al.*, 2011). Quercetin and EGCG are known to exhibit suppression of iNOS expression in various cancer cells (Gerhauser *et al.*, 2003). It has been reported that iNOS gene promoter has binding sites for STAT, AP-1, NF-κB and HIF-1α (Chittezhath *et al.*, 2008). In this study, we demonstrated that cytokine-induced STAT activation is an important determinant of iNOS expression in CCA cells, as JAK inhibitors markedly inhibited the expression. The inhibition of STAT activation by quercetin correlated with the decreases in iNOS mRNA levels. Similar inhibitory patterns were observed after EGCG treatment, and the inhibitory effect of EGCG was stronger than quercetin. These results indicate that the inhibitory effects of quercetin and EGCG on cytokine-induced iNOS expression are mediated partly via suppression of JAK/STAT pathway. Because no other pathways were tested in the present study, further studies are required

to elucidate the action of quercetin and EGCG on the other signaling pathways involved in iNOS expression.

ICAM-1, of which expression is regulated by JAK/STAT pathway, plays a critical role in inflammation-related diseases by promoting trafficking of leukocytes across endothelia and epithelial barriers. It also implicated in carcinogenic processes by contributing in tumor metastasis (Yamada *et al*., 2006). The modulation of ICAM-1 expression is, therefore, an important target for chemoprevention and chemotherapy, as shown by the beneficial effect of ICAM-1 inhibitors on the tumorigenesis and cancer progression (Lee *et al*., 2012). Similar to iNOS, transcription factors important to the activation of ICAM-1 expression include AP-1, NF-κB and STAT (van de Stolpe and van der Saag, 1996).

In this study, we found that although AG490 at 100 μM profoundly suppressed the phosphorylation of STAT1 and STAT3, this treatment caused a significant but small suppression on ICAM-1 expression. These results suggest that ICAM-1 expression may be modulated by multiple signaling pathways. The suppressive effect of piceatannol on ICAM-1 expression was also incomplete. It should be noted that piceatannol may inhibit NF-κB that is also involved in the regulation of ICAM-1 expression (Ashikawa *et al*., 2002). In this study, we found that quercetin and EGCG both dramatically suppress cytokine-induced ICAM-1 expression, where EGCG has higher inhibitory effect than quercetin. Quercetin and EGCG exert their suppressive effects on cytokine-induced ICAM-1 up-regulation, at least in part, through modulating the JAK/STAT cascade in CCA cells.

Enhanced cell migration and invasion are important aspects of cancer phenotypes, where ICAM-1 plays an important role (Yamada *et al*., 2006). In this study, we found that cytokine-induced migration of CCA cells was associated with ICAM-1 expression. Quercetin and EGCG could significantly abolish the cytokine-dependent CCA cell migration. It should be noted that the anti-migratory effect of flavonoids was detected at low concentrations, which minimally inhibited cell growth. In fact, the modulation of cell migration ability is a target for chemopreventive compound. Thus, our findings provide supportive evidence that quercetin and EGCG are potentially useful as cancer chemopreventive agents for CCA.

To ascertain that the inhibition of JAK/STAT signaling by quercetin and EGCG is not confined to only one cell type, we performed the reporter assay with other two CCA cell lines. It is clearly demonstrated that both flavonoids inhibited cytokine-induced STAT3 luciferase activity in all three CCA cell lines examined. These results imply that quercetin and EGCG may efficiently inhibit JAK/STAT signaling pathway in most CCA cells, and this confers a high potential therapeutic opportunity.

In summary, the present study showed that JAK/STAT signaling cascade is functioning in CCA cells, and upon activation, it induces the expression of iNOS and ICAM-1. Quercetin and EGCG can suppress STAT1- and STAT3-phosphorylation and also suppress iNOS and ICAM-1 expression. Moreover, these two compounds can inhibit cytokine-induced CCA cell migration as well. Given that JAK/STAT cascade plays a critical role in the inflammation and carcinogenesis processes, inhibition of JAK/STAT signaling pathway could be a valuable chemopreventive target of natural compounds to protect against inflammation-associated cancer.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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