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Sulfated galactans from the red seaweed *Gracilaria fisheri* exerts anti-migration effect on cholangiocarcinoma cells

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ABSTRACT

Background: Seaweeds have a long history of use in Asian countries as functional foods, medicinal herbs, and the treatment of cancer. Polysaccharides from various seaweeds have shown anti-tumor activity. Cholangiocarcinoma (CCA), often with metastatic disease, is highly prevalent in Thailand as a consequence of liver fluke infection. Recently, we extracted sulfated galactans (SG) from *Gracilaria fisheri (G. fisheri),* a south east Asian seaweed, and found it exhibited anti-proliferation effect on CCA cells.

Purpose: In the present study, we evaluated the anti-migration activity of SG on CCA cells and its underlined mechanism.

Methods: CCA cells were treated with SG alone or drugs targeting to epidermal growth factor (EGF) receptor (EGFR) or pretreated with SG prior to incubation with EGF. Anti-migration activity was determined using a scratch wound-healing assay and zymography. Immunofluorescence staining and western blotting were used to investigate EGFR signaling mediators.

Results: Under basal condition, SG reduced the migration rate of CCA, which was correlated with a decrease in the active-form of matrix metalloproteinases-9. SG decreased expression of phosphorylated focal adhesion kinase (FAK), but increased expression of E-cadherin to promote cells stasis. Moreover, phosphorylation of EGFR and extracellular signal-regulated kinases (ERK), known to stimulate growth of cancer cells, was blocked in a comparable way to EGFR inhibitors Cetuximab and Erlotinib. Pretreatment cells with SG attenuated EGF induced phosphorylation of EGFR, ERK and FAK.

Conclusion: This study reveals that SG from *G. fisheri* retards migration of CCA cells, and its mechanism of inhibition is mediated, to some extent, by inhibitory effects on MAPK/ERK signal transduction pathway. Our findings suggest that there may be a therapeutic potential of SG in CCA treatment.

Introduction

Cholangiocarcinoma (CCA) arising in the bile duct is associated with infection of the liver fluke, *Opisthorchis viverrini,* and endemic in the Northeastern part of Thailand (Sripa et al., 2011). CCA rates are high throughout Thailand and Laos and southwestern Vietnam – there are 20,000 cases of CCA per year, many with metastatic disease, as it often presents late, despite the recent implementation of screening programs. CCA patients with metastatic disease are resistant to radiation and chemotherapy and five-year survival is very low (< 2%) (Benavides et al., 2015). Previous studies have proven that high malignancy CCA cells are associated with increased levels of matrix metalloproteinases (MMP) and focal adhesion kinase (FAK) (Pongchairerk et al., 2005; Hua et al., 2011). Thus, these molecules are considered as biomarkers of CCA response to treatment as they suppress CCA migration/invasion. In addition, the mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) pathway has been shown to promote CCA cell invasion (Menakongka and Suthiphongchai, 2010). Indeed, MMP especially MMP-2 and −9, have a capacity to degrade collagen, leading to cancer cell migration (Mook et al., 2004) while FAK promotes cancer cell migration, resulting in increasing cancer cell invasion (Pongchairerk et al., 2005). E-

Abbreviations: E-cadherin, epithelial cadherin; ECM, extracellular matrix; FAK, focal adhesion kinase; MMP, matrix metalloproteinases

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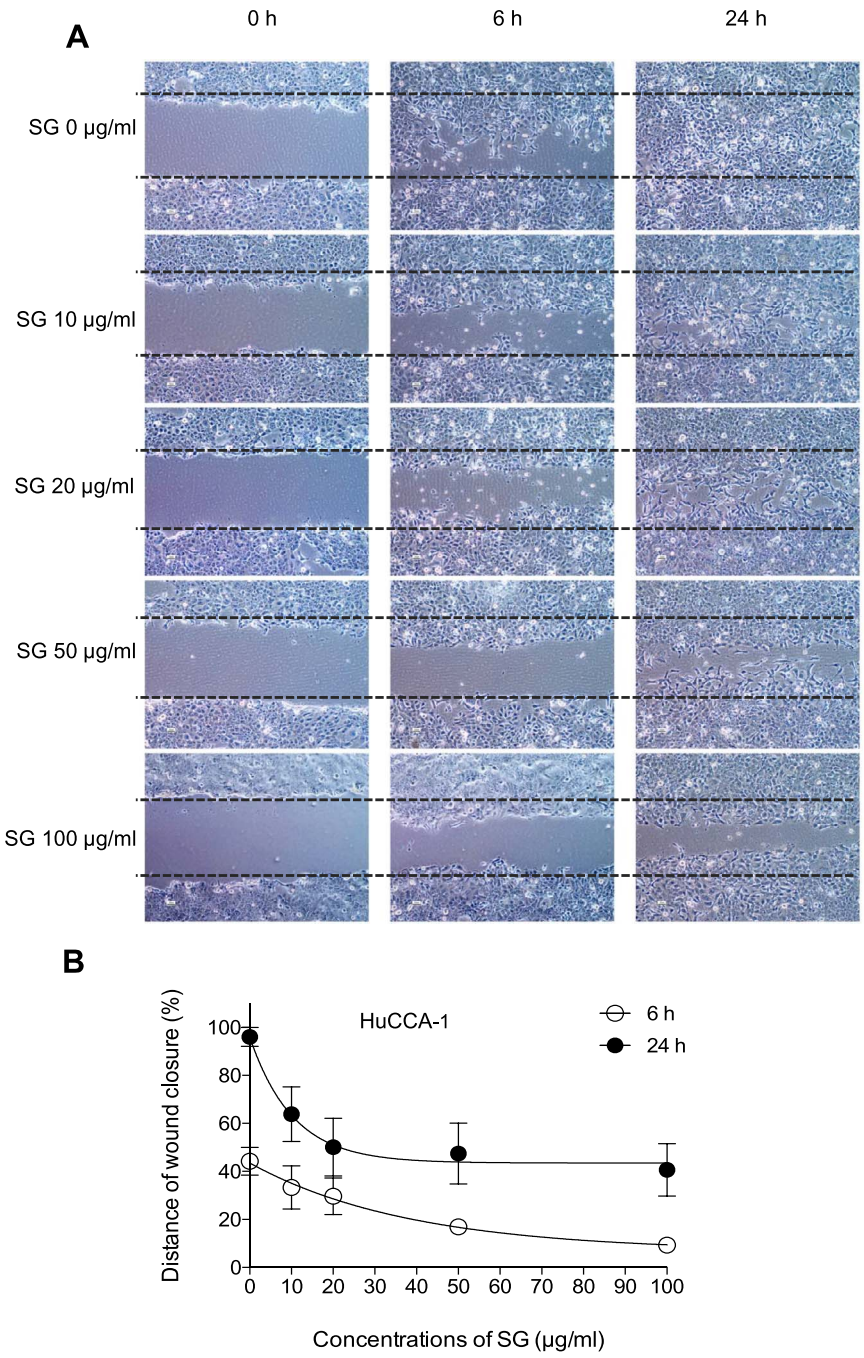

Fig. 1. The effect of SG on HuCCA-1 cell migration by scratch wound-healing assay. Cells were scratch wounded and then treated with SG (10, 20, 50 and 100 µg/ml), and photographs were recorded at 0, 6 and 24 h after scratching. (A) Phase contrast micrographs showing the size of the scratch wound in different treatment groups compared with control. (B) The distance of wound closure in HuCCA-1 cells expressed relatively to the initial gap of control cells in each time point. Results are presented as a mean ± SEM of triplets independent experiments.

cadherin is considered as a key marker of response to cellular adhesion; loss of function of this protein has been associated with higher tumor invasion (Jeanes et al., 2008).

Bioactive polysaccharides from higher plants, seaweeds (Vishchuk et al., 2011) and mushrooms (Zhang et al., 2012) have demonstrated a potential role in cancer therapy in a number of cancer types. For instance, a polysaccharide from mushrooms *Cordyceps sinensis* significantly inhibited breast cancer metastasis and epithelial-mesenchymal transition (Lin et al., 2016); one from *Coriolus versicolor* inhibited migration of mouse mammary carcinoma 4T1 cells by down-regulating MMP-9 (Luo et al., 2014). Sulfated polysaccharides (SPs) from brown seaweed inhibited migration of human lung cancer cells by down-regulating expression of ERK1/2 pathways, leading to decreased MMP and FAK expression and increased E-cadherin (Lee et al., 2012a). Moreover, certain polysaccharides have been subjected to early clinical trials in human (Oba et al., 2007). It is noted that the difference in biological activity of polysaccharides is primarily attributed to their different structure or conformation and composition (Mueller et al., 2000). As such, the search for novel, nontoxic, and effective natural polysaccharide compounds with multiple anti-cancer activities is a significant goal for anticancer therapy.

Recently, we have extracted sulfated galactans (SG) from red seaweed *Gracilaria fisheri*, a south east Asian seaweed; the structure of SG is a polysaccharide of galactose backbone and contains a high percentage of sulfation (Wongprasert et al., 2014). It has been reported that the polysaccharides of galactose with *O*-glycosidic linkage and sulfation provide various potential biological activities such as anti-angiogenic activity (Liu et al., 2012). We have reported biological activities of SG including immune stimulating activity (Rudtanatip et al., 2015), and anti-cancer activity, in particular anti-proliferation (Sae-lao et al., 2017). In the present study, we tested the hypothesis that SG had the potential to inhibit migration of CCA cells.

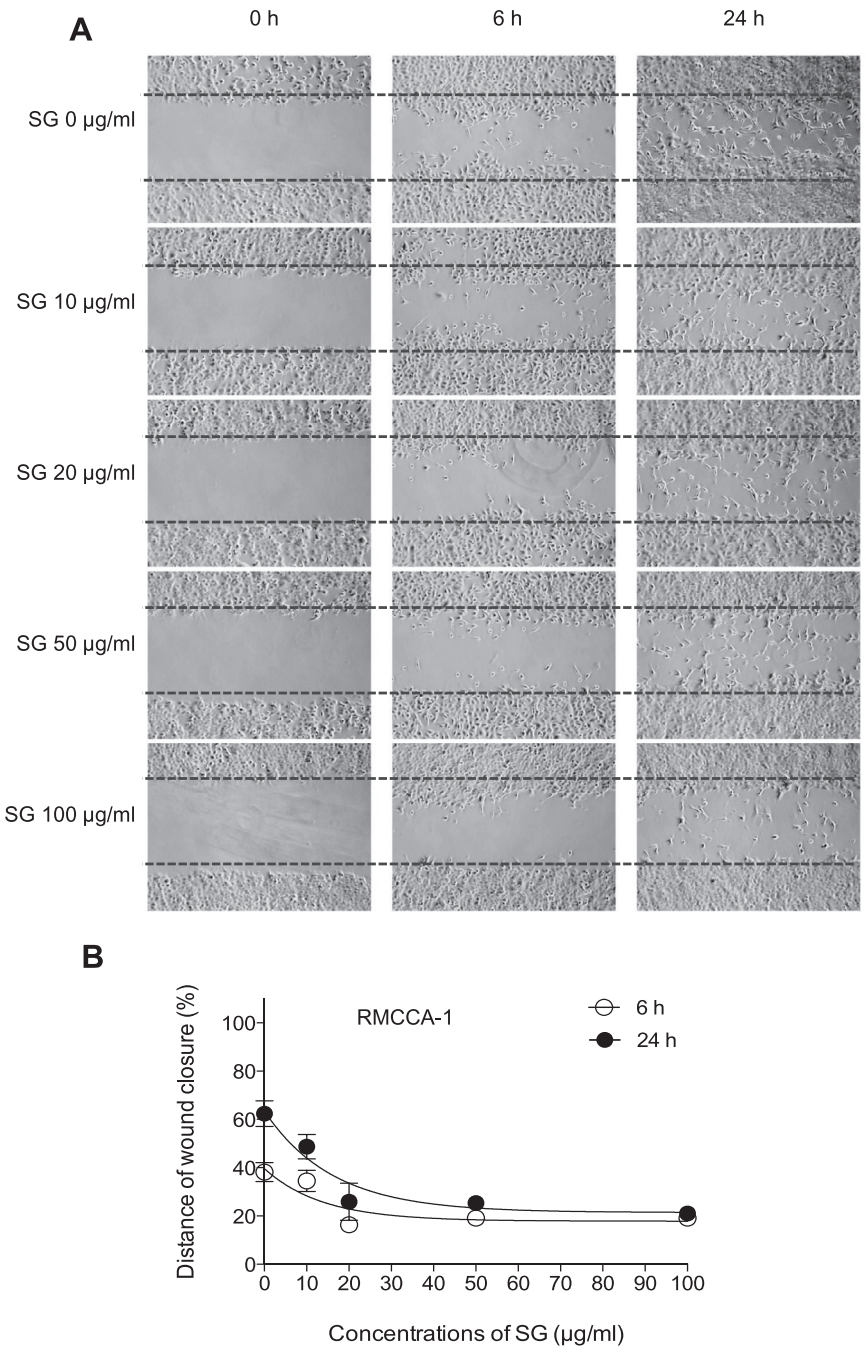

Fig. 2. The effect of SG on RMCCA-1 cell migration by scratch wound-healing assay. Cells were scratch wounded and then treated with SG (10, 20, 50 and 100 µg/ml), and photographs were recorded at 0, 6 and 24 h after scratching. (A) Phase contrast micrographs showing the size of the scratch wound in different treatment groups compared with control. (B) The distance of wound closure expressed as relatively to the initial gap of control cells in each time point. Results are presented as a mean ± SEM of triplets independent experiments.

Materials and methods

Sulfated galactans (SG) from Gracilaria fisheri (G. fisheri)

G. fisheri was collected from Surat Thani Province, Thailand, washed, epiphytes removed, and dried. Dried sample was extracted and purified to obtain SG following previously described protocols (Wongprasert et al., 2014). NMR and FT-IR analysis revealed SG consists of 3-linked-β-D-galactopyranose (G) and 4-linked 3,6-anhydro-α-L-galactose (LA) or α-L-galactose-6-sulfate (L6S) with partial methylation (CH_3) at C-2 of LA and C-6 of G, and sulfation of C-4 and C-6 of D-galactose units (G4S and G6S). HPLC analysis of SG showed about 90% purity.

Cell culture

CCA cells (HuCCA-1 and RMCCA-1) established from CCA tissue fragments of Thai patients were tested for anti-migration effects of SG. HuCCA-1 is derived from a patient with intrahepatic bile duct CCA (Sirisinha et al., 1991). RMCCA-1 is derived from a patient with peripheral CCA (Rattanasinganchan et al., 2006). CCA cells were grown in 6-well plates overnight before treatments. They were cultured at 37 °C, 5% CO_2 in Ham F-12 nutrient mixture (Ham F-12) (Gibco Invitrogen, USA) supplemented with 1 mM L-glutamine, 1.17 g/l sodium bicarbonate ($NaHCO_3$), 10% of FBS (Sigma Aldrich, USA) and penicillin (100 units/ml) plus streptomycin (100 µg/ml) (Wiscent Inc. P.O., Canada).

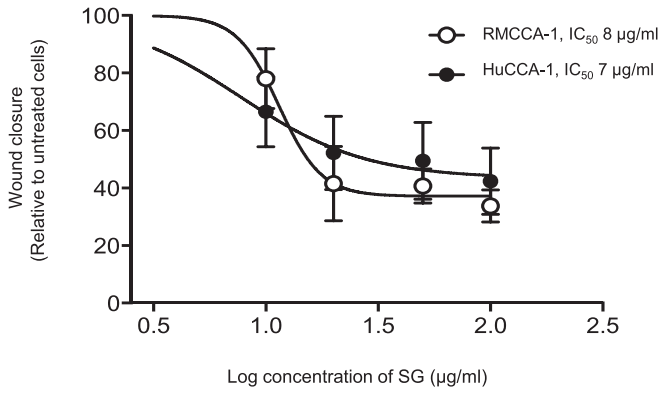

Fig. 3. Dose response curves. HuCCA-1 and RMCCA-1 cells were scratch wounded and treated with SG (10, 20, 50 and 100 µg/ml) for 24 h incubation. Plot depicts percent of wound closure vs. SG concentration. The IC_{50} value for HuCCA-1 was 7 µg/ml, and for RMCCA-1 was 8 µg/ml (log IC_{50} 0.84 ± 0.34 and 0.9 ± 0.07, respectively).

MTT assay

To examine anti-migration activity of SG, MTT assay was performed to determine concentrations of SG that would not cause CCA cell death. HuCCA-1 and RMCCA-1 were grown overnight in a 96-well plate at density 1×10^4 cells/well. Cells were incubated with different concentrations of SG (0, 10, 20, 50 and 100 µg/ml) for 24 h. After incubation cell viability was determined using methyl thiazolium bromide (MTT) assay. Briefly, 100 µl of MTT solution (0.5 mg/ml) (Sigma Aldrich, USA) was added to each well and incubated for 4 h at 37 °C in the dark. After incubation, 100 µl of dimethyl sulfoxide (DMSO) (Merck, Germany) was added to each well, and the absorbance of the sample was measured at OD 490 nm by a Versamax microplate reader using SoftMax® Pro 4.8 analysis software (Molecular Devices, USA).

Scratch wound-healing assay

HuCCA-1 and RMCCA-1 cells were seeded at a density of 1×10^4 cells/well and cultured overnight in 6-well plates. The culture medium was aspirated and then a sterile pipette tip used to create a scratch wound, and 5% FBS fresh medium added before treatment with SG (0, 10, 20, 50 and 100 µg/ml). The cells were photographed immediately and at 6 and 24 h after scratching under a phase-contrast microscope. The percentage of distance wound closure was measured, using the data from time 0 (T_0), the wound area (T_t: 6 and 24 h) by the following formula (Kapoor et al., 2008):

Percentage of distance wound closure

$$= 1 - (\text{wound area at } T_t / \text{wound area at } T_0)] \times 100$$

MMP-2/−9 activities using zymography

After seeding HuCCA-1 cells overnight, cells in FBS-free culture medium were scratched by a sterile pipette tip and treated as described in scratch wound healing assay, and incubated for 12 h. At the designated time, culture supernatant was collected, lyophilized and protein concentration was determined. Protein sample (20 µl) was mixed with a loading dye without dithiothreitol (DTT) (Bio-Rad, USA), loaded into 10% SDS-PAGE gel containing 2% gelatin. The gel was washed twice with renaturing buffer containing 2.5% (w/v) triton X-100, then placed in zymograph-incubation buffer (0.05 M Tris buffer, pH 7.5 containing 200 mM NaCl, 5 mM $CaCl_2$ and 0.05% NaN_3) at 37 °C for 18 h. The gel was stained with 0.5% Coomassie brilliant blue R-250 (Bio-Rad, USA) in 5% methanol and 10% acetic acid for 2 h and destained with 30% methanol and 10% acetic acid for 2 h at room temperature (RT). The MMP activity was quantified as a percentage of MMP expression compared to the untreated cells. Protein standards were run concurrently, and approximate molecular weights were determined relative to the known proteins.

Immunocytochemistry of p-FAK and E-cadherin

HuCCA-1 cells were grown on coverslips in a 24-well plate, and treatment groups were divided as for the scratch wound-healing assay. At the designated time, cells grown on coverslips were washed in PBS,

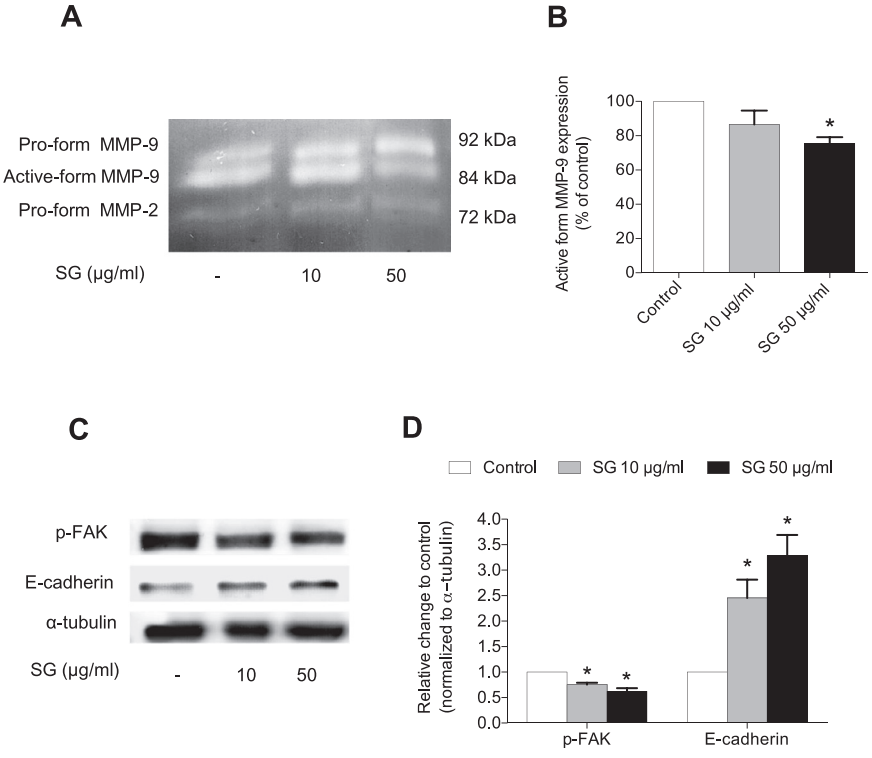

Fig. 4. MMP activity of HuCCA-1 cells estimated using zymography and effect of SG on FAK phosphorylation and E-cadherin expression in CCA cells. (A) Representative gelatinolytic bands of MMP-2 and −9 after scratching and treating cells with SG (10 and 50 µg/ml) for 12 h. (B) Relative intensity of the active-form of MMP-9 was decreased to 86.5 ± 8.11% and 74.5 ± 3.61% of control. Subsequently, FAK phosphorylation and E-cadherin expression were determined. HuCCA-1 cells were treated with SG (10 and 50 µg/ml) for 6 h. (C) The levels of p-FAK and E-cadherin were analyzed by western blot analysis and α-tubulin protein was used as a loading control. (D) For SG at 10 and 50 µg/ml, the relative intensity of *p*-FAK was decreased to 0.75 ± 0.04 and 0.63 ± 0.06 fold of control, respectively whereas E-cadherin was increased to 2.5 ± 0.36 and 3.3 ± 0.41 fold of control. Results are presented as a mean ± SEM of triplets of independent experiments; * ($p < .05$) indicates significantly different from control.

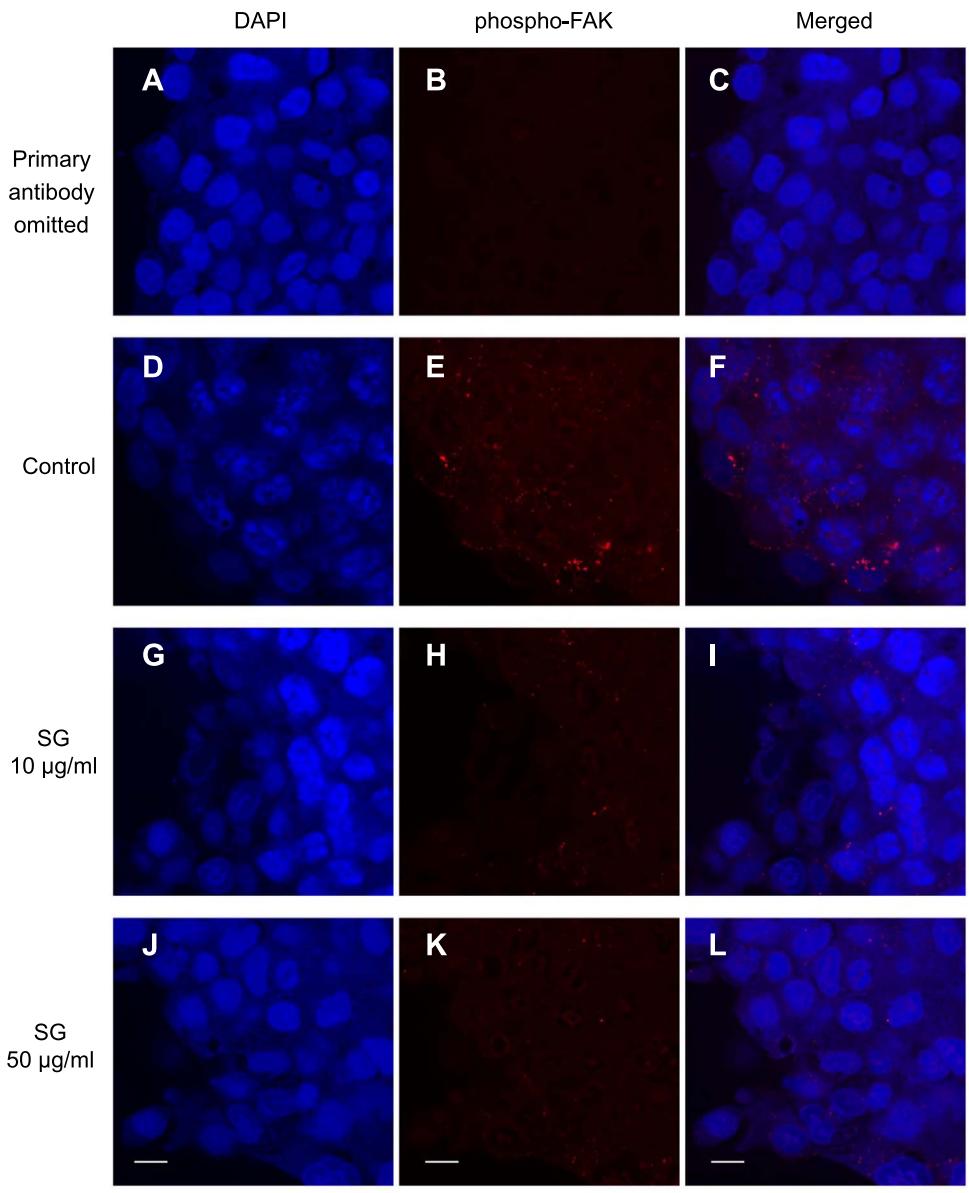

Fig. 5. Immunofluorescent micrographs showing localization of *p*-FAK in HuCCA-1 cells underwent scratched wounded and treated with or without SG for 24 h. (A–C) Cells omitted anti p-FAK primary antibody. (D–F) Control cells cultured with normal media. (G–I) cells treated with 10 µg/ml SG. (J–L) Cells treated with 50 µg/ml SG. The primary antibody was anti p-FAK antibody and the secondary antibody was CFL555-conjugated secondary antibody (red). Nuclei were counterstained with DAPI (blue). Scale bars = 10 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pH 7.4 and fixed with 4% paraformaldehyde in PBS, pH 7.4 for 10 min. HuCCA-1 cell membranes were permeabilized with 0.1% Triton X-100 in PBS (PBS-T) for 3–5 min, blocked with PBS and 3% bovine serum in PBS-T for 2 h, then incubated with anti-p-FAK or anti-E-cadherin antibodies or without primary antibodies (all at 1:500) (Cell Signaling Technology, USA) in blocking solution, at 4 °C overnight in the dark moist chamber. Cells were incubated with goat anti-rabbit IgG-CFL555-conjugated secondary antibody (1:1000) (Cell Signaling Technology, USA) in PBS-T for 1 h, counterstained with 1 µg/ml DAPI (Sigma Aldrich, USA) for 5–10 min, and examined under a confocal laser scanning microscope (FV10i-DOC, Olympus, Japan).

Western blot analysis

HuCCA-1 cells were grown in 6-well plates (1×10^6 cells/well), for 24 h. Cells underwent a scratch wound-healing assay. Cells were treated with SG (10 and 50 µg/ml) or EGFR inhibitors (Cetuximab, 100 µg/ml, Merck, Germany; Erlotinib, 5 µM, Selleckchem, Texas, USA) or EGF (20 ng/ml, cell signaling Technology, USA) or pretreated with SG prior to incubation with EGF. Cells were then collected and cell lysates were prepared in lysis buffer (3 mM $MgCl_2$, 1 mM EGTA, 10 mM sodium pyrophosphate (NaPpi), 10 mM sodium orthovanadate (Na_3VO_4), 50 mM sodium fluoride (NaF) and 100 X protease inhibitor solution) and centrifuged at 8500 × *g* for 15 min at 4 °C. The supernatant was collected to determine protein concentration by BCA assay using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA). Proteins were separated on 10–12.5% gel SDS-PAGE, blotted onto a nitrocellulose membrane (Merck, Germany), and incubated with primary antibodies: -p-FAK, E-cadherin (Cell Signaling Technology, USA), EGFR, phosphorylated-EGFR (p-EGFR), and phosphorylated-ERK (p-ERK) antibodies (Santa Cruz Biotechnology, USA), followed by incubation with horseradish-peroxidase-conjugated (HRP) secondary antibody. Anti-alpha (α)-tubulin antibody (Santa Cruz Biotechnology, USA) was also probed in all blots as an internal control. Proteins were detected using the Enhanced Chemiluminescence (ECL) kit (GE Healthcare, UK) and visualized on Hyperfilm ECL (Piscataway, USA). Expression of protein was quantified by ImageJ analysis program (from NIH website by Scion Corporation, Frederick, MD).

Statistical analysis

All methods were performed in triplicate. Data are presented as

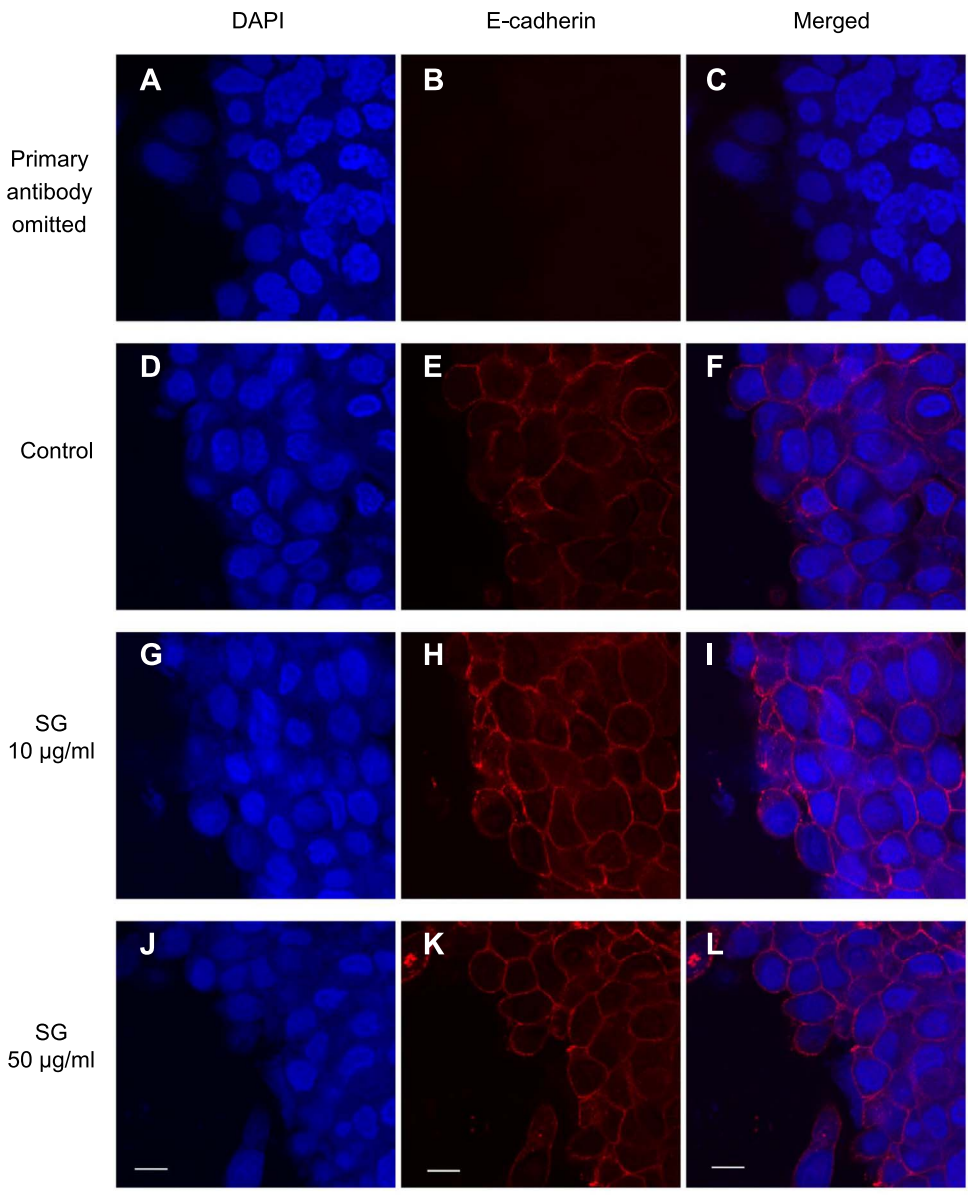

Fig. 6. Immunofluorescent micrographs showing localization of E-cadherin in HuCCA-1 cells underwent scratched wounded and treated with or without SG for 24 h. (A–C) Cells omitted anti-E-cadherin primary antibody. (D–F) Control cells cultured with normal media. (G–I) cells treated with 10 µg/ml SG. (J–L) Cells treated with 50 µg/ml SG. The primary antibody was anti E-cadherin antibody and the secondary antibody was CFL555-conjugated secondary antibody (red). Nuclei were counterstained with DAPI (blue). Scale bars = 10 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

means ± SEM and statistically analyzed by one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison tests and two-way ANOVA in GraphPad Prism program version 6 (GraphPad software, USA). Difference with *p*-values less than 0.05 were considered statistically significant. IC_{50} were calculated from a least squares fit of normalized data to a four-parameter inhibitor non-linear curve fit (Prism 7.0).

Results

SG retarded the migration of CCA cells

The MTT assay showed that SG at the tested concentrations (0, 10, 20, 50 and 100 µg/ml) did not induce CCA cell death. In addition, cells maintained normal morphology after treatment (data not shown). Therefore, these doses of SG were further employed for investigating the effect of SG on CCA cell migration.

The HuCCA-1 and RMCCA-1 cell lines were treated with the scratch wound, followed by treatment with SG (0, 10, 20, 50 and 100 µg/ml). The phase contrast micrographs revealed that at 24 h after scratching SG dose dependently retarded the migration of HuCCA-1 (Fig. 1A) and RMCCA-1 cells (Fig. 2A) compared with control. For HuCCA-1 cells, the distance of wound closure at 6 and 24 h after scratching in the control cells were 44.3 ± 5.84 and 95.9 ± 4.02% of the initial gap, respectively. While cell treatment with SG at concentrations of 10, 20, 50 and 100 µg/ml the distance of wound closure at 6 h were 33.3 ± 9.03, 29.7 ± 7.62, 16.9 ± 1.81, and 9.3 ± 1.33% of the initial gap, respectively, and at 24 h were 63.8 ± 11.36, 50.1 ± 12.06, 47.4 ± 12.68, and 40.6 ± 10.91% of the initial gap, respectively (Fig. 1B).

For RMCCA-1 cells, the distance of wound closure at 6 and 24 h after scratching in the control cells were 38.2 ± 3.99 and 62.4 ± 5.34% of the initial gap, respectively. While cell treatment with SG at concentrations of 10, 20, 50 and 100 µg/ml, the distances of wound closure at 6 h were 34.5 ± 4.41, 16.4 ± 0.82, 19.08 ± 1.16, and 19.07 ± 1.58% of the initial gap, respectively, and at 24 h were 48.7 ± 5.02, 25.9 ± 7.75, 25.4 ± 2.99, and 21.1 ± 3.01% of the initial gap, respectively (Fig. 2B). The IC_{50} for HuCCA-1 was 7 µg/ml and for RMCCA-1 was 8 µg/ml (log IC_{50} 0.844 ± 0.34 and 0.9 ± 0.07, respectively (Fig. 3)).

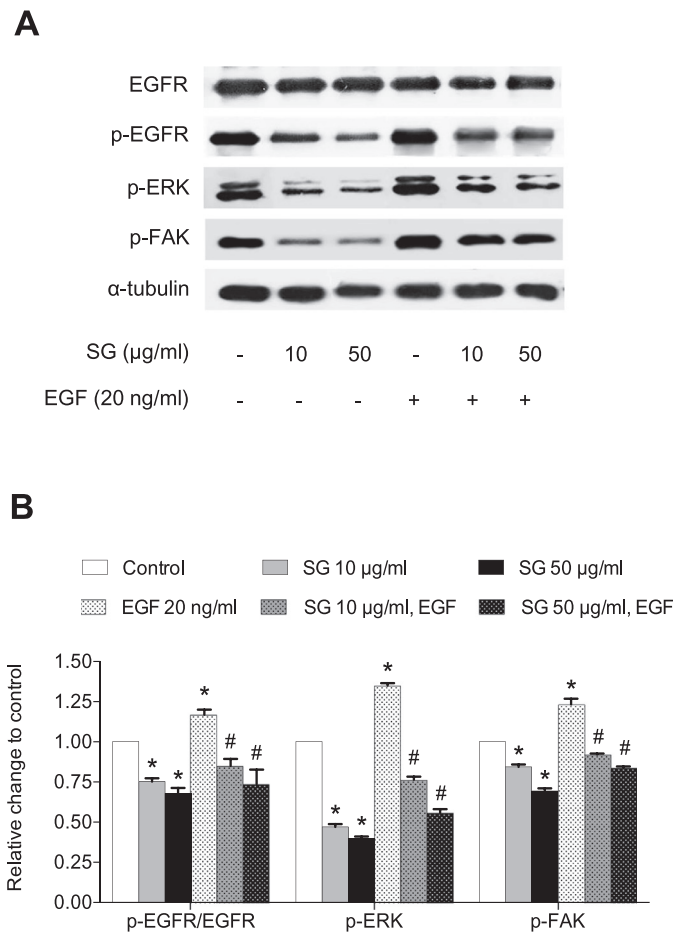

Fig. 7. Effect of SG on the expression of EGFR/MAPK/ERK downstream signaling protein in HuCCA-1 cells. (A, B) The representative immunoblots and relative intensity of signaling proteins in control cells, cells treated with SG for 6 h, cells pretreated with SG for 6 h followed with EGF for 2 h. EGF stimulated increased expression of p-EGFR/EGFR, p-ERK and p-FAK. Cells treated with either SG alone or pretreating with SG prior to EGF showed down-regulation of p-EGFR/EGFR, p-ERK and p-FAK from control. Results are presented as a mean ± SEM of three independent experiments; * ($p < .05$) indicates significantly different from control. # ($p < .05$) indicates significantly different from EGF treated group.

SG decreased the active-form of MMP-9 activity in HuCCA-1 cells

MMP activity of HuCCA-1 cells after SG treatment was analyzed by zymography. The results showed that HuCCA-1 cell treatment with SG at doses of 10 and 50 μg/ml decreased the amount of the active-form of MMP-9 to 86.5 ± 8.11 and 74.5 ± 3.61% of control, respectively (Fig. 4A, B) whereas the MMP-2 activity was not different from control cells.

SG down-regulated the expression of p-FAK and up-regulated the expression of E-cadherin proteins

The effect of SG on p-FAK and E-cadherin, molecules known to regulate the cell migration process, was determined in HuCCA-1 cells after a scratch wound assay. The results showed that treating HuCCA-1 cells with SG (10 and 50 μg/ml) significantly decreased expression of p-FAK to 0.75 ± 0.04 and 0.63 ± 0.06 fold of control, respectively, and increased expression of E-cadherin to 2.5 ± 0.36 and 3.3 ± 0.41 fold of control, respectively (Fig. 4C, D). Levels of p-FAK and E-cadherin were also determined from images from immuno-stained cells. The results demonstrated that HuCCA-1 cell treatment with SG showed substantially less immunoreactivity of p-FAK (Fig. 5) and more immunoreactivity of E-cadherin compared with control cells (Fig. 6).

SG suppressed HuCCA-1 cell migration by down-regulating signaling molecules in EGFR/MAPK/ERK pathway

Our findings showed that SG inhibited HuCCA-1 cells migration by regulating p-FAK, E-cadherin expression, and MMP-9 activity. A number of findings have revealed that the MAPK/ERK signal transduction pathway regulates CCA cell migration and invasion (Menakongka and Suthiphongchai, 2010). The pathway is activated through surface receptors such as EGFR that consequently activate downstream ERK phosphorylation. We thus examined the manipulation of SG on these molecules in this pathway. The results showed that treating HuCCA-1 cells for 6 h with SG alone (10 and 50 μg/ml) did not significantly alter basal EGFR expression (Fig. 7A) but showed marked inhibition of p-EGFR compared to control. The ratio of p-EGFR/EGFR expression decreased to 0.75 ± 0.02, and 0.68 ± 0.03 fold of control, respectively. Expression of the downstream signaling molecules of EGFR, p-ERK and p-FAK were also down-regulated (p-ERK: 0.47 ± 0.01 and 0.39 ± 0.03 fold of control; p-FAK: 0.84 ± 0.02 and 0.69 ± 0.02 fold of control, respectively) (Fig. 7A, B). We next determined whether SG could suppress EGF induced EGFR activation by pretreating the cells with SG for 6 h prior to incubation with EGF for 2 h. The results showed that EGF stimulated overexpression of p-EGFR/EGFR followed by increased activation of downstream signaling p-ERK and p-FAK (1.17 ± 0.01, 1.35 ± 0.01, and 1.23 ± 0.03 fold of control, respectively). However, these events were markedly inhibited by pretreating the cells with SG (10 and 50 μg/ml) in a dose dependent manner (p-EGFR/EGFR: 0.85 ± 0.04 and 0.74 ± 0.09 fold of control; p-ERK; 0.77 ± 0.03 and 0.56 ± 0.04 fold of control; p-FAK: 0.92 ± 0.02 and 0.84 ± 0.02 fold of control, respectively) (Fig. 7A, B). These results suggest that SG inhibited EGFR activation and signal transduction. The effects of the two EGFR inhibitors, the monoclonal antibody against EGFR Cetuximab and the tyrosine kinase inhibitor Erlotinib were also tested on HuCCA-1 cells. Treating the cells with the

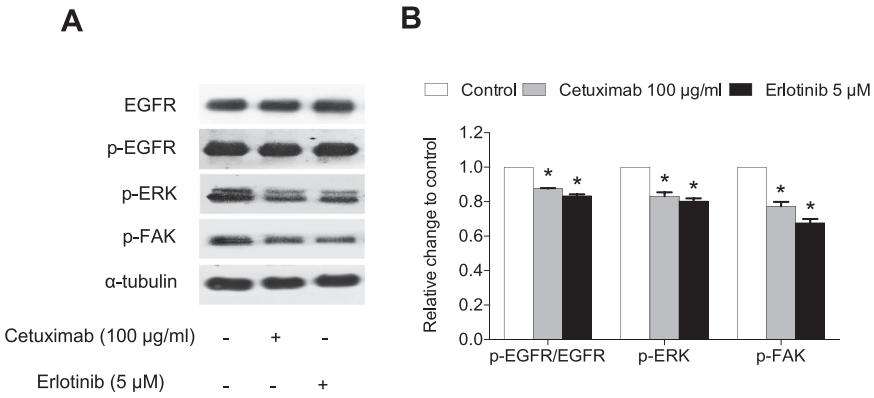

Fig. 8. Effect of drugs targeting EGFR (Cetuximab or Erlotinib) on the expression of EGFR/MAPK/ERK downstream signaling protein in HuCCA-1 cells. (A, B) The representative immunoblots and relative intensity of signaling proteins in control cells, cells treated with Cetuximab or Erlotinib for 6 h. The results revealed that HuCCA-1 cells treated with Cetuximab or Erlotinib showed down-regulation of p-EGFR/EGFR, p-ERK and *p*-FAK from control. Results are presented as a mean ± SEM of three independent experiments; * ($p < .05$) indicates significantly different from control.

Cetuximab or Erlotinib for 6 h caused a similar down regulation of p-EGFR/EGFR, p-ERK and p-FAK (Cetuximab: p-EGFR/EGFR, 0.88 ± 0.02 fold of control; p-ERK, 0.83 ± 0.04 fold of control; p-FAK, 0.77 ± 0.02 fold of control while Erlotinib: p-EGFR/EGFR, 0.83 ± 0.01 fold of control; p-ERK, 0.80 ± 0.03 fold of control; p-FAK, 0.68 ± 0.02 fold of control) (Fig. 8A, B).

Discussion

Cholangiocarcinoma (CCA) is a rare and devastating malignancy, and the incidence of CCA has increased worldwide with a very poor prognosis due to their resistance to radiotherapy and chemotherapy (Zografos et al., 2011). Current cancer treatments are moving towards agents more specifically targeting the tumor drivers and ways to inhibit these drivers. To date, interesting approaches include strategies targeting the EGFR family and the mitogen-activated protein kinases, (MAPK) signaling cascade. The RAS/RAF/MEK/ERK tyrosine kinases pathway is frequently dysregulated in cancers, including CCA, promoting cancer cell growth and survival (O'Neill and Kolch, 2004). Sulfated polysaccharides (SPs), the complex and heterogeneous macromolecules found at high concentrations in seaweed, have shown anticancer activity against various cancer cell types (Zhang et al., 2011). Many natural SPs have structure similar to heparan sulfate proteoglycans (HSPGs) and imitate the function of HSPGs (Zhang et al., 2010). They can bind to growth factor/receptor and co-receptors, resulting in suppression of the activation of receptor downstream signaling pathways (Lee et al., 2008a). Previously, we isolated sulfated galactans (SG) from *G. fisheri* and found its structure similar to HSPGs. SG inhibit proliferation of human cholangiocarcinoma cells, HuCCA-1 by interacting with EGFR and inactivating EGFR signaling. In this study we aimed to determine whether there was an anti-migration effect of SG on human cholangiocarcinoma cells in particular through the RAS/RAF/MEK/ERK signaling system.

Our study revealed that SG was able to retard the migration rates of two different CCA cell lines, HuCCA-1 and RMCCA-1 cells. HuCCA-1 is moderately invasive (Sirisinha et al., 1991) while RMCCA-1 is a low invasive CCA (Rattanasinganchan et al., 2006). We further evaluated the underlined anti-migration effect of SG on HuCCA-1 cells. Movements of cancer cells are mostly regulated by matrix metalloproteinases (MMP), especially MMP-2 and MMP-9 to degrade the collagen components of extracellular matrix. High level of MMP expression is important for CCA migration and indicates a more severe invasiveness of cancer cells (Fava, 2010). Cell treatment with SG attenuated activity of the active form of MMP-9 and restored the pro-form of MMP. These effects of SG on MMP have also been reported in human bladder cancer cells (Cho et al., 2014).

Focal adhesion kinase (FAK) and E-cadherin are important molecules working in opposite ways to promote cancer cell migration. FAK up-regulation enhances cell motility whereas E-cadherin down-regulation occurs during cancer cell migration (Schaller, 2001). It is evidenced that EGFR activation by EGF up-regulates EGFR phosphorylation and downstream signaling molecules such as ERK and PI3K/AKT. Indeed, ERK is a downstream effector of FAK and E-cadherin (Bae et al., 2013). Our results revealed that HuCCA-1 cells constitutively expressed p-EGFR and p-FAK. At basal level, SG could inhibit EGFR and FAK phosphorylation and stimulated expression of E-cadherin, which corresponded to a decrease in HuCCA-1 cells migration rate and correlated with the decrease in expression of MMP-9. Consistent with previous studies, SPs from sea cucumber inhibited migration of gastric adenocarcinoma cell lines (Tian et al., 2005) and pancreatic cell lines (Shimizu et al., 2013) by suppressing phosphorylation of FAK. In addition, SPs from the cuttlefish ink induced E-cadherin expression (Zong et al., 2013).

The MAPK/ERK pathway is among the key mechanisms that transmit signals upon receptor activation from the cell surface to nucleus, eliciting proliferative and migration signals in cancer cells. Indeed, EGF binds to EGFR then stimulates ERK phosphorylation (p-ERK), which then translocates to the nucleus, where p-ERK stimulates several nuclear targets including FAK, MMP, and E-cadherin and promotes migration of tumor cells (Sun et al., 2015). It has been reported that activation of the MAPK/ERK pathway promotes CCA cell invasion (Menakongka and Suthiphongchai, 2010). We, therefore, examined whether the anti-migration activity of SG in HuCCA-1 cells was due to inhibition of ERK phosphorylation, a major driver of cancer cell migration. Our study showed that SG down-regulated basal p-EGFR and p-ERK while the expression of EGFR remained unchanged. These results suggest that SG showed inhibitory effect on EGFR activation not on EGFR expression. In agreement with previous study, fucoidan, SP extracted from brown seaweed suppressed the EGF-induced the expression of p-EGFR and p-ERK1/2 but did not alter the EGFR level in normal mouse epidermal cells (Lee et al., 2008b). Moreover, SG showed a potent inhibitor against exogenous EGF induced EGFR activation and its downstream signaling to p-ERK, as shown that it suppressed expressions of p-FAK and p-ERK which levels were less than control untreated cells.

EGFR is overexpressed in all types of CCA (Yoshikawa et al., 2008). EGFR targeted drugs have been tested in CCA clinical trials including Cetuximab and Erlotinib. Cetuximab is a chimeric monoclonal antibody which blocks binding of EGF and TGFα to the EGFR. Erlotinib is an active tyrosine kinase inhibitor of EGFR. They both inhibit EGFR activation and its downstream signaling pathway proteins (Goldstein et al., 1995; Gordon et al., 2005). Our study showed that HuCCA-1 cells were sensitive to both Cetuximab and Erlotinib, an effect comparable to SG treatment. However, previous phase II clinical trials showed that advanced CCA patients had a low responsiveness to a single Cetuximab (Philip et al., 2006) or Erlotinib treatments (Sprinzl et al., 2006). Satisfactory CCA treatments were mostly accomplished by combined drug treatments. For instance, combination of Cetuximab with GEMOX (Gemcitabine plus Oxaliplatin) (Gruenberger et al., 2010) or Erlotinib with GEMOX provided more effective treatments in advanced or poor prognosis CCA patients (Lee et al., 2012b).

In conclusion, the present study suggests that SG from *G. fisheri* can reduce HuCCA-1 cell migration, possibly by inhibiting EGFR and ERK phosphorylation in EGFR/MAPK/ERK signaling pathway similar to the effect of the EGFR inhibitors Cetuximab and Erlotinib. This inhibiting activity of SG provides a significance application as an adjuvant for enhancing effectiveness in treatment to prevent cancer recurrence or metastasis, in particular those associated with ERK.

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Conflict of interest

The authors declare that there is no conflict of interest.

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