Sulfated Galactans from Red Seaweed *Gracilaria fisheri* Target Epidermal Growth Factor Receptor (EGFR) and Inhibit Cholangiocarcinoma Cells (CCA) Proliferation

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Abstract

Cholangiocarcinoma (CCA) is increasing in incidence worldwide and is resistant to chemotherapeutic agents, making treatment of CCA a major challenge. Previous studies reported that natural sulfated polysaccharides (SPs) disrupted growth factor receptor activation in cancer cells. The present study, therefore, aimed at investigating the anti-proliferation effect of sulfated galactans (SG) isolated from the red seaweed *Gracilaria fisheri* (*G. fisheri*) on CCA cell lines. Direct binding activity of SG to CCA cells, epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR) were determined. The effect of SG on proliferation of CCA cells was investigated. Cell cycle analyses and expression of signaling molecules associated with proliferation were also determined. The results demonstrated that SG bound directly to EGFR. SG inhibited proliferation of various CCA cell lines by inhibiting EGFR and extracellular signal-regulated kinases (ERK) phosphorylation, and inhibited EGF-induced increased cell proliferation. Cell cycle analyses showed that SG induced cell cycle arrest at the G_0/G_1 phase, down-regulated cell cycle genes and proteins (cyclin-D, cyclin-E, Cdk-4, Cdk-2), and up-regulated the tumor suppressor protein P53 and the cyclin-dependent kinase inhibitor P21. Taken together, these data demonstrate that SG from *G. fisheri* inhibited proliferation of CCA cells, and its mechanism of inhibition is mediated, to some extent, by inhibitory effects on EGFR activation and EGFR/ERK signaling pathway. SG presents a potential EGFR targeted molecule, which may be further clinically developed in a combination therapy for CCA treatment.

Keywords: Cholangiocarcinoma; EGFR; *Gracilaria fisheri*; Sulfated galactans; Anti-proliferation

Introduction

Cholangiocarcinoma (CCA) is a malignant transformation of cholangiocytes, the epithelial cells lining the biliary tree (Khan *et al*, 2005). In the Northeastern part of Thailand, CCA is associated with infection of the liver fluke, *Opisthorchis viverrini* (Sriamporn *et al*., 2004; Sripa *et al*., 2011). The disease has a high fatality rate due to a combination of late diagnosis and lack of treatment options (Tushar, 2002). Combinations of radiation and chemotherapy are used in an attempt to improve survival of patients, but, these therapies have many side-effects and in patients with metastatic or inoperable disease, survival is not significantly improved (Benavides *et al*., 2015). Current standard of care is gemcitabine and cisplatin, but this only increased survival by 2-3 months in trials in the US and Europe, and data on trials in Thailand is still missing.

Heparin-binding EGF-like growth factor (HB-EGF) is a member of epidermal growth factor (EGF) family. Generally, it binds epidermal growth factor receptor (EGFR) using co-receptor heparan sulfate proteoglycans (HSPGs) (Iwamoto *et al*., 2010). CCA from various sources have been shown to express the EGFR and other heparin sulfate binding growth factors (Yoshikawa *et al*., 2007; Hoffmann *et al*., 2013; Clapéron *et al*., 2014) and inhibition of EGFR activation has been shown to prevent growth of CCA cells in vitro. However, a clinical trial of the EGFR antibody panitumumab, carried out on patients in Europe did not find any effect of EGFR inhibition on patient survival. A number of new trials are ongoing in Europe and the US, including a combination of gemcitabine and cisplatin, with vandetanib (Kessler *et al*., 2016), an antagonist to EGFR and vascular endothelial growth factor receptor (VEGFR). Vandetanib has also been shown to decrease the growth of human CCA cell lines (Yoshikawa *et al*., 2009). These results indicate that heparin-binding growth factors such as EGF and vascular endothelial growth factor (VEGF) could play a role in cholangiocarcinoma

progression and could be potential targets for therapy. However, all these trials, and most of the cell biology is undertaken on CCA from European and US patients, which have been shown to be drastically different in their biology from CCA from Thai patients. The molecular etiology of *Opisthorchis* related CCA has recently been shown to be different from elsewhere in the world (Chan-on *et al*., 2013), with much higher levels of p53 mutation in Thai CCA patients than European ones. It is therefore important to determine the effect of heparin binding factor inhibition in CCA from patients with *O. viverrini* associated cancers.

Sulfated polysaccharides (SPs) are compounds found in extracts from various natural sources such as terrestrial (Silva *et al*., 2012), and marine plants (Vishchuk *et al*., 2011), mushrooms (Zhang *et al*., 2012), and animals (Chen *et al*., 2012). SPs have been shown to regulate proliferation, migration, angiogenesis and differentiation in a variety of cells (Costa *et al*., 2010). SPs trigger signaling pathways involving in anti-proliferation and migration (Wu *et al*., 2006; Wu *et al*., 2011). They have been shown to inhibit cancer cell proliferation by induction of cell cycle arrest (Wong *et al*., 2007) and by decreased growth factor secretion in cancer cells (Cao & Lin, 2006).

Previous studies reported that SPs could either be stimulatory or inhibitory. For instance, SPs extracted from an edible herbal plant, enhanced the binding of fibroblast growth factor and its receptor, leading to the proliferation of neural stem/progenitor cells (Zhang *et al*., 2010). In contrast, inhibitory effects of SPs on the binding of basic-fibroblast growth factor and its co-receptor, leading to decreased proliferation of cancer cells (Xiong-Zhi *et al*, 2011). SPs extracted from brown seaweed namely, fucoidan interrupted EGF-induced cell transformation by blockage the EGF and EGFR interaction (Lee *et al*, 2008). This suggests that SPs could act either as activators or competitors in the recognition of growth factors by their co-receptors, depending on cell type and SPs type.

Recently, sulfated galactans (SG) obtained from *Gracilaria fisheri (G. fisheri),* a red seaweed cultivated in South East Asia, have been isolated (Wongprasert *et al*, 2014). Several

biological activities have been reported including anti-viral (Rudtanatip *et al*., 2014), anti-coagulant (Pereira *et al*., 2005), and immune stimulating activities (Rudtanatip *et al*., 2015). Various species of *Gracilaria* have been claimed to exhibit anti-malignant activity against breast cancer and colon cancer (Zandi *et al*., 2010). We previously demonstrated that the structure of SG extracted from *G. fisheri* is a polysaccharide of galactose backbone and contains a high percentage of sulfates (Wongprasert *et al*., 2014), similar in structure to HSPGs. Due to the structural similarity of SG and HSPGs, we hypothesized that SG could imitate a co-receptor on the cell membrane and interact with growth factors or their receptors, effecting an inhibitory effect on cancer cell activity. Therefore, this study aimed to evaluate any anti-proliferation activity of SG in CCA cells lines, and determine any underlying anti-proliferation mechanism of SG, especially through EGF-EGFR interaction

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Materials and Methods

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

G. fisheri was collected from Suratthani Province, Thailand, washed, epiphytes removed, and dried. Dried sample was extracted to obtain SG following the previously described protocol, and the SG yielded was 3% of the seaweed dry weight (Wongprasert *et al*., 2014). The structure of SG analyzed by NMR and FT-IR consists of 3-linked-β-D-galactopyranose (G) and 4-linked 3,6-anhydro-α-L-galactopyranose (LA) or α-L-galactose-6-sulfate (L6S) with partial methylation (CH_3) at C-2 of LA and C-6 of G, and presence of sulfation on C-4 and C-6 of D-galactopyranose units (G4S and G6S) (Fig. 1).

Cell culture

CCA cells (HuCCA-1, RMCCA-1 and KKU-M213) established from CCA tissue fragments of Thai patients were tested for an anti-proliferation effect of SG. HuCCA-1 (Sirisinha *et al*., 1991) and KKU-M213 are derived from a patient with intrahepatic bile duct CCA. RMCCA-

1 is derived from a patient with peripheral CCA (Rattanasinganchan *et al*., 2006). They were cultured at 37°C, 5% CO_2 in Ham F-12 nutrient mixture (Ham F-12) (Gibco Invitrogen, USA) containing 1.17 g/L sodium bicarbonate ($NaHCO_3$), 5% FBS (Sigma Aldrich, USA) and penicillin (100 units/mL) plus streptomycin (100 µg/mL) (Wiscent Inc. P.O., Canada).

Direct binding of FITC-SG to HuCCA-1 cells by confocal laser scanning microscopy (CLSM)

SG conjugated FITC was prepared as previously described (Rudtanatip *et al*., 2015). Briefly, one hundred milligrams of SG was dissolved in 1 mL of DMSO containing 15 µL of pyridine. The SG mixture was mixed with powdered FITC (40 mg) and 2 mg/mL of dibutylin dilaurate, and then heated at 95 °C for 2 h. After precipitation with absolute ethanol overnight at 4 °C, the mixture was centrifuged at 900×*g* for 15 min, and supernatant discarded. The pellet was dissolved in 2-4 mL of PBS pH 7.4, the unbound FITC was removed with an Amicon ®Ultra Centrifugal Filter (Ultracel-30K) (Merck, Germany), centrifuged at 900×*g*, for 5-10 min, and then the solution freeze-dried by Freeze Dry Supermodulyo-230 (Thermo Scientific, USA).

HuCCA-1 cells were grown on poly-L-lysine-coated-coverslip in a 24-well plate overnight at 37 °C. Cells were incubated with FITC without SG as a control or with FITC-SG at 37 °C for 2 h in the dark, then washed thrice with PBS pH 7.4. The coverslips were fixed with 4% paraformaldehyde in PBS for 10 min at RT. After washing in PBS, they were mounted with mounting medium containing TO-PRO-3 (Sigma Aldrich, USA), and examined under a Confocal Laser Scanning Microscope (FV10i-DOC) (Olympus, Japan).

The SG-EGF binding using FAR-Western blot analysis

The binding activity of SG with EGF protein was determined by Far-Western blot analysis as previously described (Rudtanatip *et al*., 2015). The recombinant EGF protein, lectin proteins (positive controls) and 2% BSA (a negative control) were separated on 15% gel-SDS-PAGE,

stained with Coomassie blue, and blotted onto nitrocellulose membrane. The membrane was incubated with 100 µg/mL of SG overnight at 4°C. After washing with 0.05% Tween-20 in PBS (PBS-T), membrane was blocked with 10% non-fat dry milk in PBS-T for 2-4 h at room temperature. The membrane was incubated with primary anti LM_5 monoclonal antibody (Plant Probes, UK), which is specific to (1➔4)-β-D-galactans of SG, overnight at 4°C, followed by incubation with goat anti-rat HRP conjugated secondary antibody for 2 h at room temperature. Complex of SG-proteins was visualized using the ECL kit and visualized on Hyperfilm ECL.

Co-immunoprecipitation for SG-EGFR binding

Cell membrane protein was extracted in cold TE buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, pH 8.0), homogenized with a hypodermic needle gauge size 26, ultracentrifuged at 40,000×*g*, 4 °C for 10 min, the supernatant removed and pellet re-suspended in cold TE buffer containing 2% Triton X-100 and protease inhibitor. After ultracentrifugation at 100,000×*g*, 4 °C for 30 min, the supernatant was collected, and protein concentration determined.

Pull down assay: The membrane protein lysate was incubated with SG (ratio 1:1), shaking overnight at 4 °C. The Co-IP was performed using SureBeads™ Protein G magnetic Beads (Bio-Rad, USA) following the manufacturer's protocol. Briefly, 100 µL of SureBeads was washed with 0.1% Tween-20 in PBS pH 7.4 (PBS-T), magnetized and supernatant discarded three times. The beads were then incubated with 100 µl of anti LM_5 monoclonal antibody or IgG (Santa Cruz Biotechnology, USA) on a rotator for 10 min at room temperature, magnetized, and supernatant discarded. The membrane protein lysate was incubated with SG (ratio 1:1), shaken overnight at 4 °C, and then mixed with the beads, rotated for 60 min at room temperature. After washing with PBS-T three times, 20 µL of glycine (20 mM) pH 2.0 was added to the tube, incubated 5 min at room temperature,

magnetized, and then eluent containing immunocomplex protein collected. The eluent was neutralized in 2 µL of 1 M phosphate buffer (0.05 M dibasic sodium phosphate, 0.05 M monobasic sodium phosphate), pH 7.4. The immunocomplex protein was separated on a 10% polyacrylamide gel by SDS-PAGE, blotted onto nitrocellulose membrane (Merck Germany), incubated with the primary anti EGFR antibody or IgG (Santa Cruz Biotechnology, USA) then followed with HRP-conjugated secondary antibody. Immunoprecipitated proteins were detected using the Enhanced Chemiluminescence (ECL) kit (GE Healthcare, UK) and visualized on Hyperfilm ECL (Piscataway, USA). The protein lysate without co-IP was also immunoblotted with anti-EGFR antibody as a control. To confirm binding, EGFR in the cell lysate was pulled down using Surebeads conjugated with anti-EGFR antibody or IgG, and the eluent containing EGFR was separated on 10% SDS-PAGE gel and blotted onto nitrocellulose membrane. The membrane was incubated with 100 µg/mL of SG for overnight at 4°C, immunoblotted with the anti LM_5 monoclonal antibody or IgG, incubated with the HRP-conjugated secondary antibody, detected using ECL kit, and visualized on Hyperfilm ECL.

MTT assay

CCA cells (HuCCA-1, RMCCA-1 and KKU-M213) 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 48 h. After incubation cell proliferation 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).

To determine an ability of SG to inhibit epidermal growth factor (EGF) induced CCA cell growth, HuCCA-1 cells were treated with SG (10 and 50 µg/mL) or EGF (5 ng/mL) (Cell signaling Technology, USA) or both SG and EGF. After incubation for 48 h, cell proliferation was determined by MTT assay.

Cell cycle analysis using flow cytometry

HuCCA-1 cells were starved with free fetal bovine serum (FBS) overnight to synchronize cells to quiescent stage. Cells were incubated with or without SG (10, 50 µg/mL) for 24 h, then collected, washed twice with phosphate buffer saline (PBS), pH 7.4 and centrifuged. Cells were suspended in 70% cold ethanol in PBS pH 7.4 at -20° C for 30 min, washed twice in 1 mL of PBS, and then 5 µL of 10 mg/mL of RNase (Roche Diagnostics, USA) was added. After incubation at 37° C for 30 min, cells were stained in 100 µL of 0.5 mg/mL propidium iodide (PI) (Sigma Aldrich, USA) at 4°C in the dark for 10 min. DNA content of cells was determined using BD FACSCanto™ flow cytometer (BD Biosciences, USA).

Reverse transcription PCR of cyclin-D, cyclin-E, cdk-4 and cdk-2

The key regulators driving cells from G_0/G_1 phase to S phase include cyclin-D, cyclin-E, cdk-4, and cdk-2. Therefore, we determined the mRNA expression of these genes. After 24 h incubation, cells were collected and washed twice with PBS. Total RNA was extracted using Trizol reagent as manufacturer's instruction (Molecular research Center, Inc, USA). The concentration and purity of RNA were determined using a Nano drop-2000C spectrophotometer (Thermo Scientific, USA). Total RNA (1 µg) was reverse-transcribed to cDNA in a total volume of 20µl system by using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer's protocol. The PCR product was obtained by using Thermo Scientific Phusion High-Fidelity DNA polymerase (Thermo Scientific, USA) following the manufacturer's protocol. The PCR conditions were as follows:

1 cycle of initial denaturation at 98 °C for 30 sec, 27-35 cycles of denaturation at 98 °C for 10 sec, annealing temperature and number of cycles for each particular genes, extension at 72 °C for 30 sec, and final extension 1 cycle at 72 °C for 5 min. The specific PCR primer sequences and amplification conditions of cyclin-D, cyclin-E, cdk-4 and cdk-2 are shown in Table 1. The PCR products were separated on 1.5% W/V agarose gel, tris-borate-ethylenediaminetetraacetic acid (TBE)-buffered, containing 0.5 µg/mL of ethidium bromide. The PCR bands were visualized using UVP EpiChemi III *Darkroom* (UVP Bioimaging Systems, USA). Expression of cyclin-D, cyclin-E, cdk-4 and cdk-2 were quantified by ImageJ analysis program (from NIH website by Scion Corporation, Frederick, MD).

Western blot analysis

The cyclin/cdk complexes are negatively regulated by cdk inhibitor, P21; and transcription of P21 is induced by a tumor suppressor protein P53. EGFR activation by its ligands EGF leads to EGFR phosphorylation, thereby stimulating downstream signaling cascades using the MAPK/ERK pathway involved in cell proliferation. We, therefore, determined the protein expression levels of the key regulators, P21, P53, p-EGFR and p-ERK by Western blotting. HuCCA-1 cells were incubated with or without SG (10, 50 µg/mL) for 24 h. Cells were collected and whole 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 8,500×*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:- cyclin-D, cyclin-E, P53, P21, EGFR, p-EGFR, and 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).

To determine whether SG could decrease EGF induced EGFR activation, cells were treated with SG (10, 50 µg/mL) with or without EGF (5 ng/mL) or with only EGF for 24 h at 37 °C. In addition, to determine whether SG mediated inhibiting effects through EGFR, cells were pretreated with the anti EGFR antibody (Sigma Aldrich, USA) to neutralize EGFR for 2 h prior exposure to SG or EGF for 24 h. After incubation, cells were collected for protein extraction and expression of p-EGFR determined.

Statistical analysis

All methods were performed in three independent experiments. Data are presented as means ± SEM and statistically analyzed by one-way ANOVA followed by Turkey's multiple comparison tests using GraphPad Prism program version 6 (GraphPad software, USA). Difference with *p*-values less than 0.05 were considered statistically significant.

Results

The interaction of SG with HuCCA-1 cells

The binding activity of SG to HuCCA-1 cells was determined by immunofluorescence and Co-IP experiments. The CLSM micrographs revealed that FITC conjugated SG could bind to HuCCA-1 cells whereas FITC by itself showed no binding activity (Fig. 2A). SG interaction with EGFR was examined by Co-IP experiments. SG was allowed to bind with HuCCA-1 cell membrane protein lysate and the SG-protein complex was pulled down using anti LM_5 antibody (a specific antibody against SG). The result demonstrated that SG-protein complex

showed a positive immunoblot with anti-EGFR antibody. In the converse experiment, cell lysate immunoprecipitated with anti EGFR antibody, incubated with SG, and followed with immunoblotting with the anti-LM_5 antibody also showed an immunoreactive band at the same size (Fig. 2B). The cell membrane lysate without co-IP (a positive control) probed with anti-EGFR antibody showed a positive EGFR band, while the cell membrane lysate from co-IP probed with IgG of the same species of the antibodies revealed a negative immunoblot. These results suggested that SG could interact with EGFR. Moreover, an ability of SG to bind with EGF was evaluated by Far-Western blot analysis. The result revealed that SG could interact to controls including commercial lectin, RCA and WGA, but could not interact with the recombinant EGF tested (Fig. 2C).

SG inhibited CCA cell proliferation and EGFR activation

Various CCA cell lines included HuCCA-1, RMCCA-1 and KKU-M213 cells were used to test the anti-proliferation effect of SG. The results revealed that SG at different concentrations (0, 10, 20, 50 and 100 µg/mL) significantly decreased proliferation in all tested CCA cell lines (Fig. 3 A-C). Proliferation, migration/invasion and angiogenesis activities in various cancer cells are mediated through activation of EGFR signaling pathway. We therefore investigated the EGFR-MAPKs/ERK signaling pathway, a major downstream signaling cascade of EGFR activation. The results demonstrated that cells treated with SG showed levels of EGFR expression not different from control but decreased level of p-EGFR (Fig. 4A) and p-ERK (Fig. 4B).

SG suppressed EGF induced EGFR activation

We further determined whether SG could inhibit EGF induced HuCCA-1 cell proliferation. To test this, cells were pretreated with or without SG (10 and 50 µg/mL) before stimulating with EGF (5 ng/mL). The results showed that treatment of cells with SG alone at

concentrations of 10 and 50 µg/mL decreased cell number to 78 ± 2.4 and 75 ± 6.4% of control, respectively. Treatment of cells with EGF alone increased cell number to 128 ± 6.4% of control. Treatment of cells with SG before stimulation with EGF decreased cell number to 94 ± 2.7 and 88 ± 1.2% of control, respectively, significantly less than cells treated with EGF alone (Fig. 5A). Cells treated with SG alone or with SG prior exposure to EGF decreased levels of p-EGFR expression from control. Both showed that phosphorylation of EGFR was less than in cells treated with EGF alone (Fig. 5B). Moreover, when EGFR was neutralized with anti EGFR antibody prior SG treatment, cells restored the level of p-EGFR to that of control (Fig. 6). Collectively, the results suggested that SG might interact with EGFR and mediate inhibition of cell proliferation, in part, by preventing endogenous activation of the EGFR-MAPK/ERK pathway.

SG inhibited CCA cells proliferation by arresting cells at G_0/G_1 phase

To investigate the effect of SG on cell cycle, HuCCA-1 cells were treated with SG (10 and 50 µg/mL) and cell populations determined. Flow cytometry showed that cells treated with SG significantly increased the percentage of cells in G_0/G_1 phase and decreased those in S phase compared with controls (Fig. 7). The results suggested that SG retarded the cells at G_0/G_1 phase.

We further determined expressions of the key regulators in the G_0/G_1 phase transition and tumor suppressor proteins that control cell cycle in HuCCA-1 cells. RT-PCR analysis showed that cells treated with SG induced a dose dependent decrease in mRNA transcripts of cyclin-D, cyclin-E, cdk-4 and cdk-2 (Fig. 8). Western blot analysis showed that cells treated with SG decreased protein levels of cyclin-D, cyclin-E (Fig. 9A, B), and concurrently increased expression levels of the tumor suppressor protein P53 and cyclin-dependent kinase inhibitor P21 compared with control (Fig. 9C, D). These results indicated that SG induced

309 HuCCA-1 cells arrested at G_0/G_1 phase by downregulating cyclin-D, cyclin-E, cdk-4 and
310 cdk-2 and upregulating P53 and P21.
311
312 **Discussion and Conclusion**
313 Cholangiocarcinoma (CCA) is a malignant biliary epithelial cell transformation, which has
314 very poor prognosis due to its resistance to radiotherapy and chemotherapy (Zografos *et al*.,
315 2011). CCA is more common in North East Thailand than anywhere else in the world due to
316 endemic *O. viverrini* infection. Critically, while most trials of anti-cancer drugs have been
317 tried in western cholangiocarcinoma, CCA from Thailand are genetically distinct, and have
318 different activated signal transduction pathways. Therefore it is critical to understand the
319 biology of CCA derived from patients with *O. viverrini* associated carcinoma. It is well
320 established that growth factors and their receptor activation are important signals in
321 regulating cancer cell bioactivities. Many growth factor receptors require HSPGs as a co-
322 receptor to bind and activate them (Afratis *et al*., 2012). EGFR is a member of the ErbB
323 family of receptor tyrosine kinases, and plays a critical role in development and cancer cell
324 progression. Dimerization and phosphorylation of EGFR by EGF activates a series of
325 intracellular signaling cascades to affect transcription of genes regulating cancer cell
326 proliferation, reduced apoptosis, invasion and metastasis and also stimulates tumor-induced
327 angiogenesis (Hynes & MacDonald, 2009). Previous studies have shown that EGFR is
328 overexpressed in CCA human samples (Harder *et al*., 2009). EGFR activation triggers the
329 MAPK-ERK signaling pathway in cholangiocytes (Yoon *et al*., 2004).
330 It has been reported that natural SPs have structures similar to HSPGs and imitate the
331 function of HSPGs. They can act to block the binding of growth factor/receptor and co-
332 receptor, resulting in suppression the activation of receptor downstream signaling pathway in
333 cancer cells (Lee *et al*., 2008; Cheng *et al*., 2012). Recently, we have isolated SPs –
334 specifically SG - from red seaweed *Gracilaria fisheri* (*G. fisheri*) with structure similar to

heparan sulfate (Wongprasert *et al*., 2014). SG might compete for binding with the growth factor/receptor or co-receptor due to its structural similarity to HSPGs. Therefore, we have a hypothesis that SG might interact with EGFR or EGF, thereby preventing EGFR activation, and thus decreasing CCA cells proliferation. In the present study, we show that SG could bind to HuCCA-1 cells. Additionally, far western blotting and Co-IP assay revealed that SG did not interact directly with EGF but interacted with EGFR. SG demonstrated the anti-proliferation effects against three different CCA cell lines derived from Thai patients, (HuCCA-1, RMCCA-1 and KKU-M213). We investigated the effects of SG on EGFR signaling cascades regulating proliferation in HuccA-1 cells. Our results reveal that SG has no effect on EGFR expression but down-regulated phosphorylation of EGFR and ERK in HuCCA-1 cells. Moreover, it inhibited EGF-induced proliferation. Additionally, when EGFR was neutralized, (reducing pEGFR on the cell membrane) and followed by SG treatment, cells restored the levels of p-EGFR to normal. These results suggest that SG could not downregulate EGFR but required interaction with EGFR to reduce EGFR activation. This competitive binding of SG might interfere with receptor dimerization thus decreasing the level of p-EGFR and p-ERK, the signaling molecules in EGFR/MAPK/ERK pathway controlling cell cycle. The specific binding site of SG on EGFR and the underlined scenario by which SG mediated suppress EGFR activation need further investigation.

SG exhibited anti-cancer activity against Thai cholangiocarcinoma by modulating cell cycle regulators and inhibiting ERK expression. This is consistent with findings from other groups on other SPs (Zhang *et al*., 2012; Park *et al*., 2015). SG inhibited HuCCA-1 cells proliferation by arresting cells at G_0/G_1 phase, with no apoptosis (no sub-G_1 peak in cell cycle). This is in contrast with a previous study, which reported that fucoidans, SPs from brown seaweed, induced apoptosis and inhibited cell viability of bladder cancer cells, T24 (Park *et al*., 2014). At G_0/G_1 phase, the main cell-cycle regulators are cyclin/cdk complexes (cyclin-D/cdk-4, cdk-6, cyclin-E/cdk-2) for transition to S-phase. These regulators are

negatively regulated by cdk inhibitors (CDKI), P21 (Dobashi *et al*., 2003) which are important for abnormal or cancer cells being able to evade the G_1 restriction point, and continue to proliferate in S, G_2 and M phases (Fuster & Esko, 2005). P21 binds to and inhibits the kinase activity of CDKs leading to growth arrest at specific stages in the cell (Sherr & Roberts, 1999). Indeed, transcription of p21 can be induced by a tumor suppressor protein P53, and thus it acts as an indirect effector of tumor suppressor pathways for promoting cell cycle arrest (Benson *et al*., 2014). Previous studies in Thai CCA cell lines have shown that CCA cell treatment with chemotherapeutic agents such as doxorubicin and gemcitabine up-regulated P53 and P21 expression (Zeekpudsa *et al*., 2014), and dicoumarol at non-cytotoxic concentrations enhanced the level of P53 protein (Buranrat *et al*., 2010), expression of which was associated with the strong anti-proliferative effect. p53 is the most commonly mutated tumor suppressor gene associated with the development of human cancer and has been implicated in cholangiocarcinoma development by various studies. It is noted that the Thai CCA cell lines and CCA tissues expressed both wild type and mutant p53 (Nutthasirikul *et al*., 2013). Mutant p53 was non-functional, while wild type p53 mediated p53 transcriptional activation. Our results showed that increased expression of P21 was associated with P53 proteins suggesting that SG might induce P21 up-regulation via activation of wild type p53 transcription, and the inhibiting effect of SG on CCA cell proliferation can occur in cells expressing mutant p53. However, this study could not identify specific P53 isoforms due to the limitation of the P53 antibody in Western blot analysis. Collectively, the results suggest that SG reduced proliferation of HuCCA-1 cells by arresting the cells at G_1 phase through the down-regulation of cyclin-D, cyclin-E, cdk-4 and cdk-2, and also by induction of P53 and P21.

The Ras/extracellular-signal-regulated kinase (ERK) mitogen activated protein (MAP) kinase signaling pathway is among the key mechanisms that transmit signals upon receptor activation from the cell surface to the nucleus, eliciting proliferative and survival

signals in cancer cells. In particular, its role in cell cycle progression in G_1 phase and cell proliferation is well established. Growth factors induce phosphorylation and activation of ERK, which subsequently is translocated from the cytoplasm to the nucleus, where p-ERK activates several nuclear ERK targets (Sun *et al*., 2015). Here we show that SG decreased HuCCA-1 cell proliferation by inhibiting cell cycle progression was correlated with decreased expression of p-EGFR and p-ERK. Our results are consistent with a previous study in mouse epidermal JB6Cl41 cells that marine SPs from *Laminaria guryanovae* decreased expression of p-EGFR (Lee *et al*., 2008). The previous reports demonstrated that ERK activation is required for induction of cyclin-D up-regulation and reduction of P53 and P21 for driving cells from G_0/G_1 to S phase (Massagué, 2004). Our study suggests that SG might inhibit ERK activation leading to the reduction of cyclin-D, along with the induction of the cdk inhibitor P21 to stabilize cyclin-D/cdk4 complexes, thus cells fail to enter S-phase.

Taken together, this study demonstrates the anti-proliferation effect of SG from *G. fisheri* against HuCCA-1 cells is by arresting the cell at G_1 phase, and its inhibition mechanism is mediated, to a lesser extent, through EGFR and EGFR/MAPK/ERK signaling. SG presents a potential EGFR targeted molecule, which may be further clinically developed as an adjuvant for enhancing the efficacy of chemotherapeutic agents for CCA treatment. Moreover, the SG demonstrates a potential to overcome drug resistance in CCA with mutated p53 treatment.

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554

555 **Legends**

556 **Fig. 1** The structural feature of sulfated galactans (SG) from *G. fisheri*. SG is a partially
557 pyruvated and methylated agarose structure, which consists of 3-linked-β-d-galactopyranose
558 (G) and 4-linked 3,6-anhydro-α-L-galactopyranose (LA) or α-l-galactose-6-sulfate (L6S)
559 with partial methylaion (CH_3) at C-2 of LA and C-6 of G, and presence of sulfation on C-4
560 and C-6 of d-galactopyranose units (G4S and G6S) (Wongprasert *et al*., 2014).
561

562 **Fig. 2** Representative data of SG interaction with CCA cells. (A) Confocal laser scanning
563 micrographs showed the adherence of FITC-SG to HuCCA-1 cells in X-Y and X-Z axis
564 whereas control cells incubated with FITC only showed no green fluorescence. FITC-SG is
565 represented in green color and nuclei stained with TO-PRO-3 represent in red color. Scale
566 bars = 150 μm. (B) Left: cell membrane lysate was allowed to bind with SG, and proteins
567 bound with SG were pulled down using anti-LM5 antibody. The eluent was then
568 immunoblotted with anti-EGFR antibody. Middle: Cell membrane lysate without co-IP was
569 blotted with anti-EGFR antibody as an EGFR blotted control. Right: in the converse
570 experiment, cell membrane lysate was pulled down with anti-EGFR antibody, and then the
571 eluent was incubated with SG before immunoblotted with anti-LM_5 antibody. The results
572 revealed the interaction of SG with EGFR. The lysate was also probed with IgG of the same
573 species of the antibodies raised, and the results were negative. (C) Right: SDS-PAGE gels
574 showing coomassie-brilliant blue staining of lectins (RCA, WGA) as positive controls,
575 recombinant EGF and BSA (negative control). Left: Far-Western blotting showing SG
576 bound to lectins (RCA and WGA), but not BSA and the recombinant EGF.

577

578 **Fig. 3** The effect of SG on CCA cell proliferation. (A) HuCCA-1, (B) RMCCA-1 and (C)
579 KKU-M213 were treated with different concentrations of SG (0, 10, 20, 50 and 100 μg/mL)
580 for 48 h. Cell viability was measured using MTT assay. Results are presented as a mean ±
581 SEM of triplets independent experiments; *$p < 0.05$ compared to control.

582

583 **Fig. 4** Western blot analysis showing the relative expressions of (A) EGFR and p-EGFR and
584 (B) p-ERK to α -tubulin protein in HuCCA-1 cells after treatment with SG for 24 h. Results
585 are presented as a mean ± SEM of triplets independent experiments; *$p < 0.05$ compared to
586 the respective control.

587

588 **Fig. 5** SG inhibited EGF induced HuCCA-1 cells proliferation and EGFR activation. (A) Cell
589 viability of HuCCA-1 cells measured by MTT assay. HuCCA-1 cells were pretreated with
590 SG (10 and 50 μg/mL) followed with or without EGF (5 ng/mL) for 24 h. (B) Western blot
591 analysis of p-EGFR in HuCCA-1 cells and relative expression of p-EGFR to α -tubulin in
592 different treatment groups. Cells were pre-treated with or without SG and then post-treated
593 with or without EGF for 24 h. Results are presented as a mean ± SEM of three independent
594 experiments; *$p < 0.05$ compared to control, #$p < 0.05$ compared to EGF-treated group.

595

596 **Fig. 6** Western blot analysis showed the amount of p-EGFR relative to α -tubulin in different
597 treatment groups. HuCCA-1 cells were treated with or without anti-EGFR antibody for 2 h
598 prior to exposure to SG for 24 h, and p-EGFR was determined. Results are presented as a
599 mean ± SEM of triplets independent experiments; *$p < 0.05$ compared to control.

600

601 **Fig. 7** Representative experiments of flow cytometry showing the cell cycle distribution in
602 HuCCA-1 cells. Cells treated with SG or without SG for 24 h and mean percentages of cells
603 in the G_0/G_1, S, and G_2/M phases of cell cycle.

604

605 **Fig. 8** (A) RT-PCR bands showing expression at the transcriptional level of cyclin-D, cyclin-
606 E, cdk-4 and cdk-2 in HuCCA-1 cells after treatment with SG for 24 h. (B) Densitometry
607 values of cyclin-D, cyclin-E, cdk-4 and cdk-2 mRNA relative to GAPDH. Results are
608 presented as a mean ± SEM of triplets independent experiments; $*p < 0.05$ compared to the
609 respective control.

610

611 **Fig. 9** (A) Western blot analysis showed the expression of cyclin-D and cyclin-E proteins. (B)
612 The relative expression of cyclin-D and cyclin-E proteins to α -tubulin protein. (C) Western
613 blot analysis showed the expression of P53 and P21 proteins. (D) The relative expression of
614 P53 and P21 proteins to α -tubulin protein. Results are presented as a mean ± SEM of three
615 independent experiments; $*p < 0.05$ compared to the respective control.

616

617 **Table 1.** Specific primers and conditions for determination of the expression at the
618 transcriptional level of cyclin-D, cyclin-E, cdk-4 and cdk-2 in HuCCA-1 treated with SG.