Estrogen activates *Cyclooxygenase-2* and *CD44* expressions and promotes cholangiocarcinoma cell invasion

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ABSTRACT

Cholangiocarcinoma (CCA) is one of the worst prognosis cancers. CCA usually presents in advanced stage according to its high metastatic property. Until present, the factors involving CCA metastasis are still unclear. Estrogen has been revealed as a stimulator of CCA invasiveness. *Cyclooxygenase-2* (*COX-2*), an estrogen targeted gene, associates with cancer invasion and metastasis. This study aims to investigate the role of COX-2 in estrogen-stimulated invasion of CCA cells. KKU-100 and KKU-M213 CCA cell lines were treated with 17β-estradiol (E2), as active form of estrogen, and NS-398, a selective COX-2 inhibitor, and the invasive property of cancer cells was measured as well as the expression levels of some metastatic genes. The results indicated that E2 could stimulate CCA cell invasion and this process was inhibited by NS-398. In addition, in E2-stimulated CCA cells, the levels of COX-2 and CD44 were increased. These results indicate the impact of COX-2-mediated *CD44* expression in E2-driven CCA invasion. The application of using COX-2 inhibitor to attenuate E2-stimulated invasion of CCA is of great interest to be proposed as an effective management of the disease.

Keywords: cholangiocarcinoma; invasion; estrogen; cyclooxygenase-2; NS-398

INTRODUCTION

Cholangiocarcinoma (CCA) is a type of carcinoma that originating from biliary epithelial cell (Kabbach *et al*., 2015). Its prognosis worldwide is one of the worst among other cancers with the median survival rate of 7 months if the patient did not undergo surgery (Esnaola *et al*., 2016). While the epidemiology of CCA is determined as a rare disease, it is an endemic disease in Thailand especially in the northeastern part and generates a major health problem in this area (Miwa *et al*., 2014). This phenomenon could be explained by the endemic infection of liver fluke *"Opisthorchis viverrini* (*Ov*)*"* which presents in the same area of CCA and has been proven as a causative agent of CCA (Miwa *et al*., 2014). There is no effective treatment for the advanced stage CCA (Brandi *et al*., 2016). While surgery is the major therapeutic method for the early stage CCA, most patients came to the hospital at the advanced stage which surgery is not effective (Esnaola *et al*., 2016). In addition, the tumor usually resists to both chemotherapy and radiation (Esnaola *et al*., 2016). Since metastasis is an important process in tumor progression, the control of metastasis may be an alternative method to improve survival rate for CCA patients.

Our previous study determined the increased level of serum estrogen in CCA patients and that it is associated with poor prognosis (Hunsawong *et al*., 2012). Moreover, we have also demonstrated that estrogen could stimulate CCA cell invasion via the expression of Trefoil factor 1 (TFF1) confirmed by si*TFF1*-transfected cells. To explain this phenomenon in CCA, it has been reported that the level of 17β-hydroxysteroid dehydrogenase type 2 is decreased compared to the level in normal liver (Narasaka *et al*., 2000). The function of 17β-hydroxysteroid dehydrogenase type 2 enzyme is to catabolize 17β-estradiol (E2), the active form of estrogen, to the inactive form as estrone or E1. Therefore, the reduction of this enzyme in the liver tissue may associate with E2 accumulation in CCA patients' sera. Estrogen action is usually via the binding to cytoplasmic estrogen receptors and then activates expression of downstream genes including metastatic genes.

Cyclooxygenase-2 (COX-2) is a multifunctional enzyme involving in carcinogenesis in a various types of tumors including CCA (Wu, 2005; Ghosh *et al*., 2010). COX-2 has been revealed to associate with the development and progression of CCA by activation of cell growth in human HuCCT1 CCA cell line (Han *et al*., 2004). In addition, there were many reports showing the association between estrogen and COX-2 expression in cancer (Ho *et al*., 2008; Li et al., 2008; Samir et al., 2010). Moreover, oxysterols were reported to induce COX-2 in cholangiocyte (Yoon *et al*., 2004). There

was a report showing that COX-2 could mediate the activation of kidney and colonic epithelial cell invasion by trefoil peptides (Rodrigues *et al*., 2001). Taken together, it is interesting to investigate the activation effect of E2 on invasiveness of CCA cell lines and expression of the metastatic genes associated with TFF1 and COX-2.

COX-2 inhibitors are commonly utilized in the clinical practice, celecoxib, for example. Therefore, COX-2 and its targeted metastatic genes were selected to study for their roles in CCA invasion in this study. To determine the mechanism of E2 and COX-2 in stimulating the invasion, the expressions of metastatic genes including *TFF1*, *TFF3*, *ERα*, *ERβ*, *COX-2* and *CD44* were measured along with the *36B4* ribosomal protein mRNA as an internal control. *TFF1* and *TFF3* are metastasis-related genes (Thuwajit *et al*., 2007; Pandey *et al*., 2014). *ERα* and *ERβ* are the receptors for estrogen. *CD44* has been reported as a downstream effector of COX-2 (Kinugasa *et al*., 2004). In addition, the association between CD44 and CCA progression has also been reported (Pongcharoen *et al*., 2011). The results of this study may provide an improved method for CCA therapy using COX-2 inhibitor.

In this study, E2 was utilized to stimulate CCA cells *in vitro*. *COX-2* expression was measured by real PCR and inhibited its function using a highly selective COX-2 inhibitor, *N*-(2-(cyclohexyloxy)-4-nitrophenyl) methanesulfonamide (NS-398), and then the invasiveness of the cells was measured using *in vitro* invasion assay compared to cells without E2 treatment. The results suggested the role of COX-2 and CD44 in E2 stimulated CCA cells invasion and the inhibition by NS-398. So, the application of COX-2 inhibitor as the adjuvant therapeutic agent for CCA in clinical practice may be considered.

MATERIALS AND METHODS

Cholangiocarcinoma cell lines

CCA cell lines, KKU-100 and KKU-M213 used in this experiment were established as previously described (Sripa *et al*., 2005) and were kindly provided by Professor Dr. Banchob Sripa, Division of Experimental Pathology, Department of Pathology, Faculty of Medicine, Khon Kaen University. They derived from different originating pathological tissues: primary tumors of poorly differentiated adenocarcinoma for KKU-100 and mixed-type differentiated adenocarcinoma for KKU-M213. The cell lines were developed from male patients admitted at Srinagarind Hospital and the samples were collected with approval from the Human Research Ethics Committee, Khon Kaen University (protocol number HE450526). Cells were cultured using regular Dulbecco's Modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) (GIBCO, Invitrogen, Carlsbad, CA, USA) and changed to phenol red-free DMEM with 10% FBS (GIBCO) for at least 48 hours when performed the experiment to avoid the estrogenic effect of the phenol red (Berthois *et al*., 1986).

E2 and NS-398 treatments

The concentration of NS-398 (SigmaAldrich, St. Louis, MO, USA) used to treat the CCA cells in this study was determined by toxicity test. The maximal concentration of NS-398 with non-significant effect on cell count compared to the untreated sample would be classified as minimal toxic to cells and used. Both CCA cell liness were treated with 0.1, 1, 10 µM of NS-398 for 72 hours then stained with trypan blue and counted for viable cell by hemacytometer under light microscope. The concentration of E2 (β-Estradiol-Water Soluble; SigmaAldrich) used in this experiment for both cell lines was 1 nM according to the previous study (Hunsawong *et al*., 2012). Cells were treated with E2 for 48 hours and then of NS-398 was added for 24 hours. Cells were trypsinized and collected for the *in vitro* invasion assay and metastatic gene expression measurement.

In vitro invasion assay

The assay was performed in BD Biocoat Matrigel Invasion Chamber® (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were prepared as suspension approximately $4x10^4$ cells/500 µl/well in phenol red-free DMEM with E2 and NS-398 and were applied in the upper chamber while the lower chambers were filled with the same media as the upper chamber. Then KKU-M213 and KKU-100the cells were incubated in humidified tissue culture incubator at 37°C in 5% CO_2 for 28 and 45 hours, respectively. Invading cells use proteinase enzyme to digest the matrix and went down through the lower part of the apparatus. After incubation, the non-invading cells would be removed from the upper chamber by mechanical scrubbing and cells in the lower surface of the membrane were fixed with 25% methanol for 30 minutes and stained with 1% crystal violet. The invading cells were count using inverted light microscope and then the percent invasion was determined as following formula:

Folding of invasion = Mean of invading cell number$^{(Treated)}$/Mean of invading cell number$^{(Untreated\ control)}$

Semi-quantitative real time reverse transcriptase (RT)-polymerase chain reaction (PCR)

Semi-quantitative real time RT-PCR was used to determine the expression levels of metastasis genes in treated cells and the control cells. First, total RNA was extracted from treated cells by TRIZOL® reagent (Invitrogen) following the manufacturer's instructions. Then, total RNA was treated with DNase (Promega, Madison, WI, USA) to destroy the contaminated genomic DNA. After that, RNA was converted to cDNA using AMV reverse transcription system (Promega). Semi-quantitative real time PCR with SYBR green® technique was performed using Applied Biosystems 7300/7500 Real time PCR machine (Applied Biosystems, Foster, CA, USA) to measure the expression levels of *TFF3, ERα, ERβ, COX-2*, *CD44*, and *36B4*. Ribosomal protein 36B4 mRNA, which has minimal effect from estrogen (Laborda, 1991), was used as the internal control in this study. Taqman® probe (Applied Biosystems) (Catalog No. Hs00170216_m1) was used to determine the expression level of *TFF1*. This product contained a pair of primers and a Taqman® probe for *TFF1* cDNA (NCBI reference sequence number NM_003225.2 from position 123 to 227, amplicon size 105 nucleotides). Threshold cycle (Ct) of each reaction was recorded and calculated as relative quantification to determine the level of gene expression. The relative quantification was calculated as $2^{-\Delta\Delta Ct}$ (ΔCt = average Ct of interesting gene - average Ct of internal control and $\Delta\Delta$Ct = average ΔCt of sample - average ΔCt of control). Primers used in this study (excluded *TFF1*) are listed in Table 1.

Statistical analysis

The statistical analysis was performed by SigmaStat® software version 3.11 (Systat Software, Richmond, CA, USA). Student *t*-test was used to determine the significance of NS-398 toxicity. One-Way ANOVA was used for comparison among conditions in the *in vitro* invasion assay. Differences were considered as statistically significant at p-value < 0.05.

RESULTS

Effects of E2 and NS-398 on invasiveness of KKU-100 and KKU-M213 CCA cell lines

The results showed that the percentages of viable cells of KKU-100 cell line after treated with 0.1, 1.0 and 10.0 µM of NS-398 were 97.3%, 91.4% and 91.1%, respectively, compared to the untreated control. The percentages of viable cells of KKU-M213 cell line were 97.6%, 96.1% and 92.7%, respectively (Figure 1). No statistically NS-398 with no toxicity to CCA cells in this experiment significant difference of each concentration compared to control was observed. The results suggested that there was no significant toxicity of NS-398 in this experiment. The maximum concentration of was 10 µM.

Results of the *in vitro* invasion assay of both the KKU-100 and KKU-M213 CCA cells treated with 1 nM E2 and 10 µM NS-398 were revealed as the folding of invasive cells compared to that of the control without treatment (Figure 2). The results showed that E2 could stimulate invasiveness in both CCA cell lines as 4.67 and 1.38-fold for KKU-100 and KKU-M213 cells, respectively. However, only the KKU-100 showed statistical significance of the E2-induced invasion. NS-398 alone did not make significant change of invasion in both CCA cell lines. However, NS-398 reduced the E2-induced invasive effect of both cell lines and had statistical significance reduction in the KKU-100 CCA cells.

Table 1 List of primer sequences for semi-quantitative real time RT-PCR.

Gene/Forward or Reverse	Sequences
TFF3/Forward	5′-AACCGGGGCTGCTGCTTTG-3′
TFF3/Reverse	5′-GAGGTGCCTCAGAAGGTGC-3′
ERα/Forward	5′-GAATCTGCCAAGGAGACTCGC-3′
ERα/Reverse	5′-ACTGGTTGGTGGCTGGACAC-3′
ERβ/Forward	5′-TGTCTGCAGCGATTACGCA-3′
ERβ/Reverse	5′-GCGCCGGTTTTTATCGATT-3′
COX-2/Forward	5′-CTATGGCTACAAAAGCTGGGAA-3′
COX-2/Reverse	5′-GTGCTGGGCAAAGAATGCAA-3′
CD44/Forward	5′-AAGGTGGAGCAAACACAACC-3′
CD44/Reverse	5′-AGCTTTTTCTTCTGCCCACA-3′
36B4/Forward	5′-CTTCCCACTTGCTGAAAAG-3′
36B4/Reverse	5′-CCAAATCCCATATCCTCGT-3′

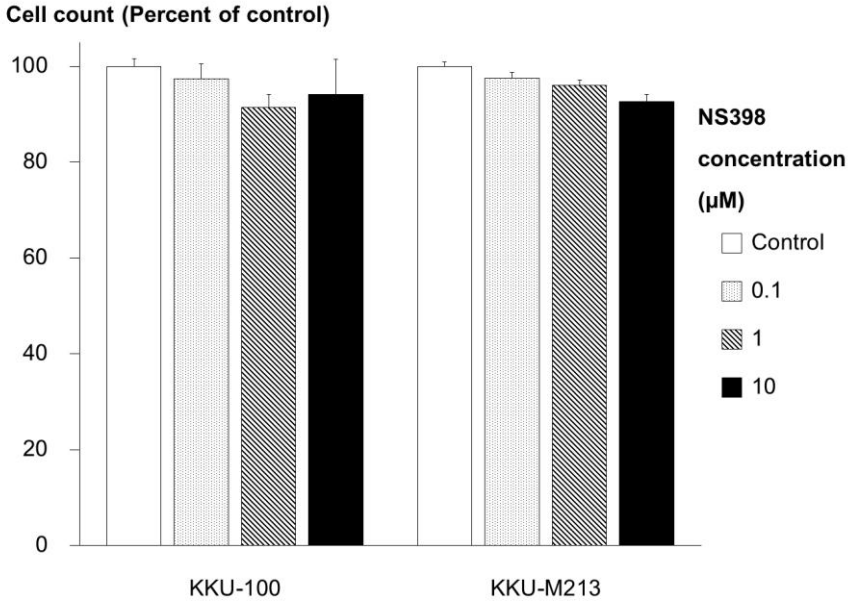

Figure 1 Toxicity analyses of NS-398 on the KKU-100 and KKU-M213 CCA cell lines. CCA cells were treated with various concentrations of NS-398 (0.1-10 μM) for 72 hours for cytotoxicity assay. Cell count was measured by trypan blue staining with hemacytometer under light microscope. Bars represent mean ± SEM of duplicated independent experiments. No statistical significance was observed when compared cells treated with any concentration to control.

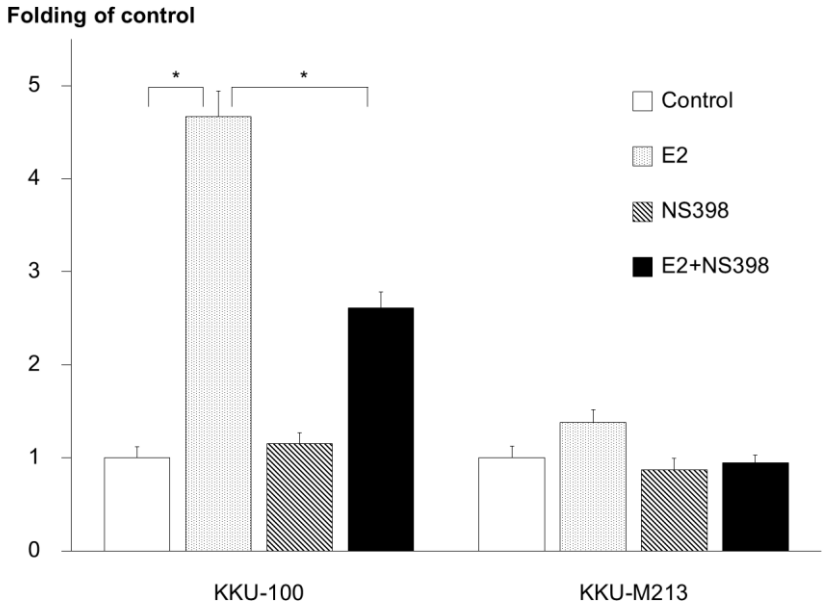

Figure 2 Effects of E2 and NS-398 on the invasiveness of the KKU-100 and KKU-M213 CCA cell lines. Cells were treated with 1 nM E2 for 48 hours, then 10 μM NS-398 was added for another 24 hours. The invasion of cells in different treatment conditions was compared to the control condition and was represented as the folds of control in y-axis. Bars represent mean ± SEM of duplicated independent experiments. **P*-value of less than 0.05.

Effect of E2 and NS-398 on metastatic gene expressions in the KKU-100 and KKU-M213 CCA cell lines

The expression levels of metastatic genes including *TFF1, TFF3, ERα, ERβ, COX-2* and *CD44* were measured in the KKU-100 and KKU-M213 CCA cells treated with various conditions of E2 and NS-398. Relative quantification of each gene was calculated and shown as folding of expression compared to the control without treatment. The results exhibited that E2 increased the expressions of all the mentioned genes in both CCA cell lines (Figure 3). NS-398, by itself alone, had no marked effect on the expression of these genes. However, NS-398 altered the expressions of these genes in both CCA cell lines pre-treated with E2. The E2-induced *TFF1* expression level was increased by NS-398 treatment in both cell lines. In contrast, the E2-induced *TFF3* expression level was reduced by NS-398 treatment in the KKU-M213 CCA cells, but not in the KKU-100 CCA cells. For the *ERα* and *ERβ* genes, as expected, their levels were dramatically increased in the E2-treated CCA cells and NS-398 attenuated this effect. In the similar pattern, NS-398 reduced the *CD44* expression level in both cell lines either with or without pre-treatment with E2. Surprisingly, NS-398 increased *COX-2* expression in the KKU-100 cells treated with either NS-398 alone or NS-398+E2 pre-treated cells. Notably, this effect was not observed in the KKU-M213 CCA cells. In consistence of the findings in both CCA cell types used herein, NS-398 markedly attenuated the effect of E2-induced expressions of the *ERα* and *CD44* genes.

DISCUSSION

CCA is one of carcinomas with the worse prognosis. One of the reasons is that it is usually diagnosed in the late stage. There has been no effective therapeutic method for CCA (Brandi *et al*., 2016). While *Ov* is endemic, CCA is remained as an important health problem in Thailand (Miwa *et al*., 2014). Metastasis is the mortality process of cancer progression that leads to the advanced stage. Therefore, the control of metastasis may be an alternative method to treat cancer patients. From our previous study, estrogen accumulation has been observed in CCA patients and could promote cancer metastasis *in vitro* (Hunsawong *et al*., 2012). Hence, understanding the mechanism of estrogen-stimulated metastasis may introduce the way to control metastasis in CCA. Our previous study has also shown that TFF1 was an estrogen mediator that stimulated the invasion in CCA cell lines (Hunsawong *et al*., 2012). On the other hand, COX-2 has been also reported to be associated with estrogen and CCA progression (Yoon *et al*., 2004). Since COX-2 has specific inhibitors used as drugs in clinical practice, thus it is interesting to study COX-2 as a mediator of estrogen-associated CCA invasion and the method to control the process.

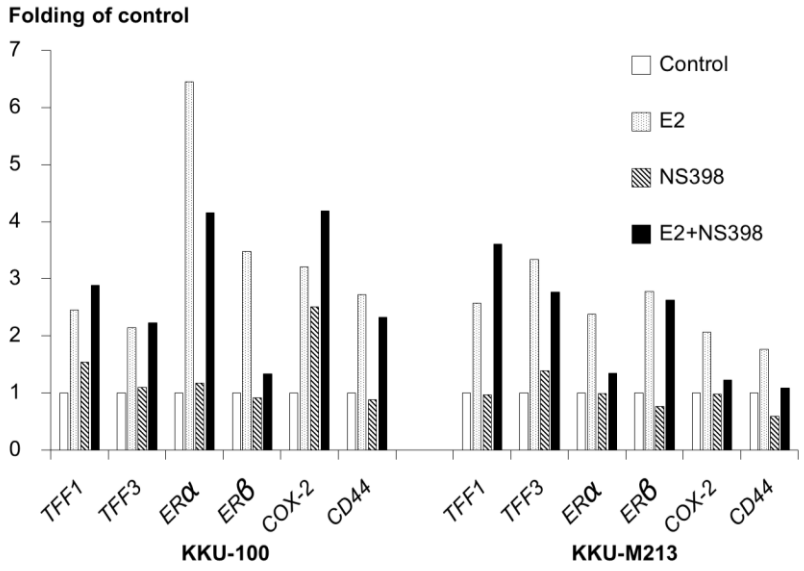

Figure 3 Metastatic genes expression determination in the E2-treated and NS-398-treated KKU-100 and KKU-M213 CCA cell lines. *TFF1*, *TFF3*, *ERα*, *ERβ*, *COX-2* and *CD44* expressions were measured using semi-quantitative real time RT-PCR method and normalized by the expression of *36B4* ribosomal protein mRNA. E2 could induce the expression of all of the tested genes. Only *ERα* and *CD44* showed the response to both E2 and NS-398 in the manner of cell invasiveness in both CCA cell lines. Bars represent folds of the gene expression level by the setting the expression of the control untreated cells to be one.

COX-2 is an enzyme that catalyzes the reaction of prostaglandins syntheses and plays major roles in inflammation (Patrono, 2016). Selective COX-2 inhibitors have been used in clinical practice to relief pain and inflammation, and are classified as non-steroidal anti-inflammatory drugs (NSAIDs). COX-2 is usually found in the inflammatory sites and its inhibitors are suggested to generate less gastrointestinal adverse effects such as peptic ulcer. Examples of COX-2 inhibitor are celecoxib, etoricoxib and lumiracoxib. However, there were reports of atherothrombotic event in cardiovascular diseases and hypertension developed from COX-2 inhibitors usage and some of them were withdrawn from the US market. The mechanism could be explained by the imbalance of tissue prostaglandin I2, one of COX-2 products which has restraining effect on platelet activation. Only celecoxib is remaining in the US drug market under close observation for the adverse effect.

Since many types of cancers have presented with chronic inflammation, COX-2 is interesting to study the involvement in cancer progression. COX-2 showed the roles in cancer progression in various types of cancers, including breast cancer (Esbona *et al*., 2016), prostate cancer (Pruthi and Wallen, 2005), colorectal cancer (Tuncer and Banerjee, 2015), lung cancer (Huang and Chen, 2011), hepatocellular carcinoma (Cervello and Montalto, 2006) and CCA (Itatsu *et al*., 2009). Roles of COX-2 in promoting cancer progression cover growth, metastasis and angiogenesis (Sahib *et al*., 2009). The usefulness of COX-2 inhibitors in cancer treatment and prevention has been reported (Harris, 2009). Celecoxib is approved by the US-FDA for chemoprevention of colorectal carcinoma for high-risk patients with familial adenomatous polyposis (Half and Arber, 2009). There have been many clinical trials of COX-2 inhibitors used in treatment and prevention of cancer, including colorectal cancer, lung cancer, prostate cancer, breast cancer (Harris, 2009) and CCA (Watkins *et al*., 2009). COX-2 inhibitors were also reported to improve the efficacy of radiotherapy in cancer (Salehifar and Hosseinimehr, 2016). The basic study of using COX-2 inhibitors to suppress cancer progression should support the idea of how to use COX-2 inhibitors as adjuvant therapy in CCA.

In this study, E2 was used to activate the invasiveness of the KKU-100 and KKU-M213 CCA cell lines and a selective COX-2 inhibitor, NS-398, was used to inhibit the process. NS-398 is the prototype of selective COX-2 inhibitor commonly used in experimental laboratory but not in clinical use, while celecoxib is the first selective COX-2 inhibitor drug in clinical use but should be cautious in patients with heart disease. The mechanism of NS-398 inhibition was determined by measuring the expression levels of a certain metastasis-related genes including *TFF1*, *TFF3*, *ERα*, *ERβ*, *COX-2* and *CD44*, using semi-quantitative real time RT-PCR method. The results confirmed the previous findings that E2 could stimulate the invasiveness of the KKU-100 and KKU-M213 CCA cell lines (Hunsawong *et al*., 2012) and NS-398 could significantly suppress this phenotype. Since only NS-398 alone did not significantly change the invasiveness of both cell lines compared to the untreated control, thus it indicates that the suppression of cell invasion from NS-398 depends on E2 stimulation.

Results from this study showed that E2 stimulated all metastasis-related genes in both CCA cell types. However, only *ERα* and *CD44* expression levels were correspond to the interpretation that their up-regulated expression was controlled by E2 and this up-regulation was attenuated when COX-2 activation was blocked by adding NS-398. Hence, these suggest the role of COX-2 in E2-stimulated CCA cell invasion through CD44 (Figure 4). ERα was reported to have increased expression in CCA tissues (Hunsawong *et al*., 2012). ERα is a well-known estrogen receptor and was reported to have the activation response from estrogen (Tübel *et al*., 2016).

CD44, a stem cell marker, is a surface molecule that interacts with extracellular matrix, in particular hyaluronic acids, and exhibits cellular response (Thapa and Wilson, 2016). *CD44* expression could be responding to COX-2 action and considered as a downstream effector of COX-2 activity (Kinugasa *et al*., 2004). In CCA, CD44 overexpression and the association with poor prognosis have been reported (Kunlabut *et al*., 2012). In addition, suppression of CD44 could reduce cellular migration and invasion of CCA cells in an *in vitro* experiment (Pongcharoen *et al*., 2011). Our findings that CD44 expression increased in the E2-treated cells implies that CD44 may partly involve in the E2-induced invasion of CCA cells (Figure 4). In addition, after applying NS-398 to inhibit COX-2 action, *CD44* expression reduced. These results confirm the theory that CD44 is a downstream molecule of COX-2 and CD44 may then be involved in cancer invasion and metastasis. Taken together, the results suggest that E2 can stimulate CCA cell invasiveness via COX-2-related mechanism partly through CD44.

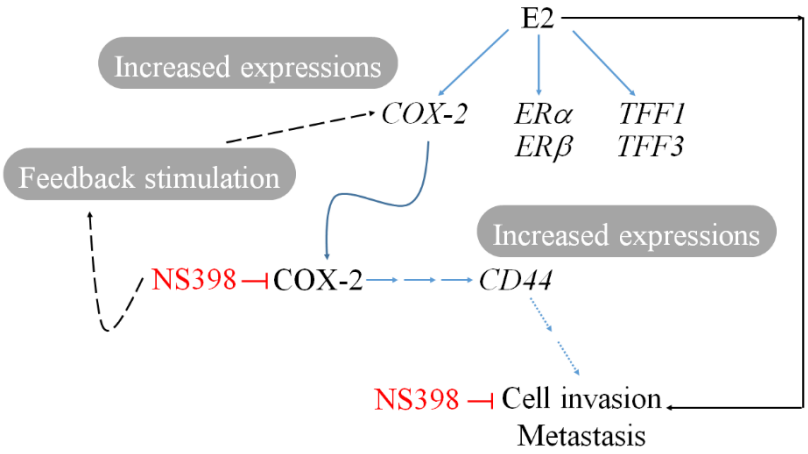

Figure 4 Proposed diagram of estrogen (E2)-stimulated cancer cell invasion and metastasis. NS-398, a COX-2 inhibitor, inhibits COX-2 functions including the function to regulate *CD44* gene expression, but increased *COX-2* expression by positive feedback mechanism.

In conclusion, estrogen accumulation has been reported in CCA patients. Estrogen could stimulate CCA cell invasiveness in an *in vitro* experiment. The obtained findings from the herein study indicate that estrogen-stimulated CCA cell invasion is COX-2 and CD44-dependent pathway and this process can be controlled by a selective COX-2 inhibitor. Therefore, the usage of COX-2 antagonist drugs may help attenuate cancer invasion and metastasis in CCA patients.

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