รายงานวิจัยฉบับสมบูรณ์

ภาวะ oxidative stress กระตุ้นให้เกิด cancer stem-like cells ผ่านการสร้าง polyploid giant cells: เป้าหมายใหม่ของการป้องกันและรักษาโรคมะเร็งท่อน้ำดี

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มีนาคม 2562

สัญญาเลขที่ RSA5980031

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บทคัดย่อ

ภาวะเครียดออกซิเดชันเป็นสาเหตุหลักของโรคที่เกี่ยวข้องกับกระบวนการอักเสบ เช่น โรคมะเร็ง โรคมะเร็งท่อน้ำดีเป็นมะเร็งตับชนิดหนึ่งที่เกิดจากความผิดปกติของเซลล์เยื่อบุท่อทางเดินน้ำดี คณะผู้วิจัยได้ทำการศึกษาก่อนหน้านี้ทั้งในสัตว์ทดลองและในมนุษย์ที่แสดงให้เห็นว่าภาวะเครียดออกซิเดชันนั้นเป็นสาเหตุสำคัญของโรคมะเร็งชนิดนี้ ไฮโดเจนเปอร์ออกไซด์เป็นสารที่กระตุ้นให้เกิดอนุมูลอิสระและภาวะเครียดออกซิเดชันที่จะทำให้เกิดการทำลายของสารชีวโมเลกุลหลายตัว ได้แก่ ลิปิด โปรตีน และกรดนิวคลีอิก ซึ่งก่อให้เกิดการตายของเซลล์ หากแต่เซลล์บางเซลล์สามารถปรับตัวให้มีชีวิตอยู่ในภาวะเครียดออกซิเดชันได้ ซึ่งจะพัฒนาไปเป็นเซลล์มะเร็งได้ต่อไป คณะผู้วิจัยได้ทำการกระตุ้นเซลล์ท่อน้ำดีด้วยไฮโดเจนเปอร์ออกไซด์เป็นเวลานาน และทำให้เซลล์ท่อน้ำดีมีการปรับตัวและเพิ่มคุณสมบัติของการเกิดมะเร็งขึ้นมา เช่น มีการแบ่งเซลล์และการเคลื่อนที่ที่เร็วขึ้น ในการศึกษาครั้งนี้ คณะผู้วิจัยได้ทำการพิสูจน์ว่าการปรับตัวให้อยู่ในภาวะเครียดออกซิเดชั่นของเซลล์ท่อน้ำดี จะมีการปรับตัวให้เซลล์มีขนาดใหญ่ขึ้น มีการแสดงออกของยีน ZNF423 มากขึ้น และมีการแสดงออกของยีน EBF1 ลดลง ซึ่งจะส่งผลให้เซลล์มีคุณสมบัติของเซลล์ต้นกำเนิดและดื้อต่อภาวะเครียดออกซิเดชัน นอกจากนี้การลดลงของ EBF1 ยังส่งผลให้เซลล์มีการตอบสนองต่อฮอร์โมนเอสโตรเจน การแบ่งเซลล์ และการเคลื่อนที่ เพิ่มมากขึ้น คณะผู้วิจัยจึงได้มีการศึกษาเอนไซม์ CYP19A1 ที่ทำหน้าที่สังเคราะห์ฮอร์โมนเอสโตรเจนร่วมด้วย จากการศึกษาพบว่า CYP19A1 ทำให้เซลล์มะเร็งท่อน้ำดีมีการสร้างเอสโตรเจนและการตอบสนองต่อฮอร์โมนดังกล่าวเพิ่มขึ้น ส่งผลให้เซลล์มะเร็งท่อน้ำดีมีการแบ่งตัวและการเคลื่อนที่ที่เร็วขึ้นด้วย กล่าวโดยสรุปจากการศึกษาในครั้งนี้ทำให้ทราบว่า การเกิดเซลล์ตัวใหญ่ การแสดงออกของ ZNF423 และการลดลงของ EBF1 เป็นกระบวนการสำคัญในการเพิ่มคุณสมบัติของเซลล์ต้นกำเนิดและการดื้อต่อภาวะเครียดออกซิเดชันของเซลล์ ทั้งนี้การลดลงของ EBF1 นั้นยังส่งเสริมให้เกิดการตอบสนองที่ดีกับฮอร์โมนเอสโตรเจนและทำให้เซลล์มะเร็งท่อน้ำดีพัฒนามากขึ้น จะเห็นว่าการลดลงของ EBF1 นั้นเป็นปัจจัยสำคัญที่เกิดจากภาวะเครียดออกซิเดชั่นที่ส่งผลต่อพัฒนาการของเซลล์มะเร็ง ดังนั้นในการศึกษาต่อไปคณะผู้วิจัยจะมุ่งเน้นศึกษา EBF1 และ EBF1-related genes ในมะเร็งท่อน้ำดี เพื่อพัฒนาโมเลกุลดังกล่าวสำหรับเป็นเป้าหมายใหม่ในการรักษาโรคมะเร็งท่อน้ำดีต่อไป

คำสำคัญ: ภาวะเครียดออกซิเดชัน, EBF1, ZNF423, CYP19A1, เอสโตรเจน, เซลล์ตัวใหญ่

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Project Title: Oxidative stress induces cancer stem-like cells through the formation of polyploid giant cells: novel targets for cholangiocarcinoma prevention and treatment

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Abstract

Oxidative stress is a cause of inflammation-related diseases, including cancers. Cholangiocarcinoma (CCA) is a liver cancer with bile duct epithelial cell phenotypes. Our previous studies in animal and human models indicated that oxidative stress is a major cause of CCA development. Hydrogen peroxide (H_2O_2) can generate hydroxyl radicals, which damage lipids, proteins, and nucleic acids, leading to cell death. However, some cells can survive by adapting to oxidative stress conditions, and selective clonal expansion of these resistant cells would be involved in oxidative stress-related carcinogenesis. We established H_2O_2-resistant cell line from an immortal cholangiocyte cell line (MMNK1) that showed increasing of the oxidative stress-resistant properties and the expressions of the anti-oxidant genes. In this study, we confirmed that the giant cell formation, increase of ZNF423 expression and loss of EBF1 expression involved in oxidative stress-resistant and stem-like cell properties. Notably, down-regulation of EBF1 also increased estrogen response leading to CCA progression. Then, CYP19A1, an estrogen producing enzyme, was also included in this study. CYP19A1 increase estrogen generation and estrogen receptor activation which involved in CCA cell proliferation and migration. These findings suggest that oxidative stress-related CCA genesis through the alterations of EBF1 and ZNF423 expressions and the giant cell formation. Additionally, oxidative stress had a synergistic roles with estrogen-mediated CCA progression. Thus, EBF1 is an oxidative stress responsive gene that exerts a tumor suppressive function against CCA genesis. In next study, we will further identify EBF1-targeted genes in CCA which may be used as new therapeutic targets for CCA chemotherapy and chemoprevention.

Keywords: Oxidative stress, EBF1, ZNF423, CYP19A1, estrogens, giant cells

Executive Summary

Oxidative stress is an imbalance of oxidants and anti-oxidant systems that cause over-production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which play roles in oxidative stress-related diseases such as cancer [1]. Under cellular bombard by ROS and RNS most of cells die, whereas some can adapt to survive. Under this circumstance is called “oxidative stress-resistant cells”. Our proteomic approach and double-fluorescent staining identified high expression and co-localization of albumin and cytokeratin-19 in liver fluke-associated cholangiocarcinoma (CCA) tissues, compared with normal liver, suggesting that CCA has mixed phenotypes of hepatocytes and cholangiocytes as well as bi-potential liver stem-like cells [2]. Recently through TRG5680039 project, early B cell factor 1 (EBF1) and insulin receptor substrate 1 (IRS1) was identified as the epigenetic target of oxidative stress. DNA hypermethylation of EBF1 promoter region causing EBF1 down-regulation was demonstrated to be induced by oxidative stress. Literature reviews suggest that EBF1 plays role in the induction of stem cell differentiations into mature cells including B cells, neuron cells, osteocytes, adipocytes, retinal cells and kidney [3-7], therefore down-regulation of EBF1 may involve in the increasing of stem-like cell properties of CCA tumor initiating cells. Our accumulated findings suggest that CCA is a disease of oxidative stress and stem-like cells. Thus, we hypothesize that the oxidative stress-resistant cells should have stem-like cell property leading to the accumulation of cellular damage by oxidative stress, including DNA mutation, epigenetic changes, genetic instability leading to cancer development. According to our TRF research (TRG5680039), we tried to induce and characterize the oxidative stress-resistant cell lines from immortal cholangiocyte (MMNK1) and CCA (M213 and M214) cell lines. Ox-MMNK1-L cell line is an oxidative stress resistant cell line that was generated from MMNK1 cells [8]. Our pilot studies indicated that polyploid giant CCA cells were increased in ox-MMNK1-L cell lines compared with the parental cell lines. Polyploid giant cancer cells refer to the presence of large atypical cancer cells with multiple copies of DNAs [9]. Recently, cancer stem-like cells were characterized as a polyploid giant cancer cells isolated from human ovarian cancer cell lines and primary ovarian cancer using various concentrations of $CoCl_2$ via hypoxia induction [10].

Therefore, we hypothesize that EBF1 and its related genes as well as the polyploid giant CCA cells involved in oxidative stress-resistant and CCA development. This study aims to prove that down-regulation of EBF1 and the polyploid giant cells formation can be induced oxidative stress. Our results showed that oxidative stress induces giant cell formation, EBF1 suppression and ZNF423 expression lead to increasing of oxidative stress-resistant, stem-like and tumorigenic properties of the cholangiocyte and cholangiocarcinoma cells. Moreover, chronic inflammation also induces CYP19A1 expression which consequently to over production of estrogen resulting in the induction of estrogen response. Taken together our results indicated that oxidative stress and estrogen are the synergistic factors that induces CCA progression with aggressive clinical outcomes.

Moreover, EBF1 is vulnerable to oxidative stress, and that oxidative stress significantly suppresses EBF1 which can induce stem cell and migration properties of the cholangiocyte cells leading to CCA promotion and progression resulting in CCA development with aggressive clinical outcomes such as short survival time. Thus, EBF1 is an oxidative stress responsive gene that exerts a tumor suppressive function against CCA genesis. In next study, we will further identify EBF1-targeted genes in relation with tumorigenic, stem cell and oxidative stress-resistance properties. The outcome of next study is to determine the important linkage between oxidative stress and cancer stem-like cells in liver fluke-induced cholangiocarcinogenesis which could be applied to other model of oxidative-related carcinogenesis. Therefore, EBF1 and EBF1-targeted genes may be used as new therapeutic targets for CCA chemotherapy and chemoprevention. This is the important initiation study which will be generated series of experiments to understand the relationship among key events in carcinogenesis: inflammation, oxidative stress, cancer stem-like cells, and mutant stem cells. Therefore, the light of this study will reveal the basic knowledge for the improvement of novel CCA prevention and therapy.

Full Research Report:

Research title: Oxidative stress induces cancer stem-like cells through the formation of polyploid giant cells: novel targets for cholangiocarcinoma prevention and treatment

1. Introduction

Giant cancer cells (GCCs) refer to the large atypical cancer cells. The giant cells have often been thought to originate from repeated mitosis/cytokinesis failure or as intermediate products of genomic instability. So, the giant cells have not drawn much attention in the cancer research community and their roles in tumorigenesis have been largely untested. Recently, cancer stem-like cells were characterized as polyploid giant cancer cells isolated from human ovarian cancer cell lines and primary ovarian cancer using various concentrations of $CoCl_2$ via hypoxia induction. These indicated GCCs had stem cell properties that may associate with cancer stem-like cells.

Infection and inflammation play important roles in cancer development. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are key players in inflammation-related cancers. Oxidative stress is an imbalance of oxidants and anti-oxidant systems that cause over-production of ROS and RNS. Cholangiocarcinoma (CCA) is a cancer that has bile duct epithelial cell phenotypes. One of the established risk factors for CCA is chronic inflammation of cholangiocytes triggered by infection by the liver fluke, Opisthorchis viverrini, that is commonly found in northeast Thailand [11]. Chronic inflammation induced by O. viverrini infection clearly increased oxidative stress through the highly formation of DNA damage lesions in the bile duct epithelium cells [12, 13]. Oxidative stress causes oxidative damage to biomolecules, tissue remodeling and alteration of gene expressions which are involved in all stages of CCA development [14]. Interestingly, it can result not only in damage to numerous biomolecules that leads to DNA mutation, but it can also induce epigenetic changes and stem cells activation for tissue remodeling [1, 15]. Under cellular bombardment by ROS and RNS most cells die, whereas some can adapt to survive, defined as “oxidative stress-resistant cells” [8]. The induced oxidative stress-resistant cholangiocyte cells gain the properties of tumor genesis such as high proliferation rate [8]. Therefore, many studies strongly support that oxidative stress is the major cause of CCA development which is induced by chronic inflammation [14, 16, 17]. However, the oxidative stress underlining mechanisms and targeted molecules have been under-estimated to date.

Cholangiocarcinoma (CCA) is a liver cancer originating from bile duct epithelium, and is the second most common primary hepatic malignancy worldwide [18]. Thailand has still the highest incidence and mortality rates of CCA in the world, especially in the Northeast Thailand, where the incidence of CCA reaches up to

87.7 per 100,000 in males and 36.3 per 100,000 in females [19]. The high incidence of CCA in this region is associated with chronic inflammation of the biliary tract caused by liver fluke (*Opisthorchis viverrini*) infection, which is also highly prevalent in this area [20]. CCA is known as a “silent killer” because of its relatively silent clinical progression, which renders it difficult to diagnose at its early stages [11]. Therefore, the study on the molecular mechanisms of CCA development and progression is needed for identifying potential prognostic markers and therapeutic targets in CCA.

Early B cell factor 1 (EBF1) is a novel transcriptional factor which recognizes the mb-1 promoter region and is strongly expressed in the early stage of B cell development [21, 22]. EBF1 possesses a number of biological functions in several developmental pathways, for example, EBF1 has been mainly involved in the B cell differentiation [23], bone development [24], adipogenesis [5], retinal cell differentiation [6] and kidney development [3]. Additionally, EBF1 plays an important role in the differentiation of several stem cells to mature cells. Therefore, we proposed that EBF1 may associate with stem cell activation in the process of tissue injury through increased stem cell differentiation leading to mature cells for used in the tissue repaired process whereas down-regulation of EBF1 may inhibit stem cell differentiation leading to increased stem cell properties which may be involved in tumor cell transformation.

Recently, down-regulation of EBF1 has been found in many tumors, and EBF1 is believed to play suppressive roles in cancer promotion and progression. Down-regulation of EBF1 by ZNF423 expression (EBF1 inhibitor) has been shown to induce B cell maturation arrest leading to promotion and progression of various types of leukemia such as acute lymphoblastic leukemia (ALL) [25]. Moreover, mono-allelic deletions of EBF1 may contribute to block differentiation of mature B cells which lead to leukaemogenesis via increasing of immature B cells that are hallmarks of ALL {Mullighan, 2007 #143}. EBF1 was also found to be suppressed in solid cancers of which EBF1 suppression could be achieved in different ways, such as the genomic loss of 5q32 which encodes for EBF1 in breast cancer [26]. In addition, somatic missense mutation that causes the amino acid substitution of arginine for glutamine at position 242 located on DNA binding domain of EBF1 contributes to the EBF1 suppression in pancreatic ductal adenocarcinoma [27]. Interestingly, EBF1 had been proposed to be the negative regulator of estrogen receptors (ERs) [28], and ERs were reported to promote carcinogenesis including CCA [29, 30]. These findings lead us to hypothesize that the down-regulation of EBF1 may play a crucial role in tumor promotion and progression via the induction of estrogen response.

Zinc finger protein 423 (ZNF423) is a transcription factor which belongs to the family of Krüppel-like C2H2 zinc finger proteins. This protein contains 30 domains that are known as binding of DNA and proteins [31] and is implicated in numerous developmental pathways, notably neurogenesis and adipogenesis [32]. The ZNF423 causes neurogenesis by activating early B-cell factor 1 (EBF1) and Notch pathways [33, 34]. ZNF423 promotes adipogenesis via peroxisome proliferator-activated receptor γ (PPARγ) pathway [35, 36]. Besides,

this protein acts as an important transcriptional modulator in several types of cancer, including leukemia [25, 37], nasopharyngeal carcinoma (NPC) [38], neuroblastoma [39] and breast cancer [40, 41]. ZNF423 plays different roles in various cell types depend on its downstream interaction. In leukemia and NPC, ZNF423 plays role as oncogene to drive progression of these cancer types [25, 37, 38]. On the other hand, ZNF423 act as tumor suppressor in neuroblastoma and breast cancer [39, 41].

Estrogen is a group of sex steroid hormones and plays crucial roles in both reproductive and non-reproductive systems. The action of estrogen is mediated through estrogen receptors (ERs) signaling pathways [42]. Increased estrogen levels and the effect of estrogen on tumor progression have been reported in breast, lung, endometrium, liver, bladder and bile duct cancers [29, 30, 43-49]. In CCA, estrogen stimulates cell proliferation, cell invasion and angiogenesis through ERs by inducing the expressions of pAKT, pERK1/2, trefoil factor 1 (TFF1), EGFR/MAPK and vascular endothelial growth factor (VEGF) [30, 48-51]. Male CCA patients have higher serum estrogen levels than age- and sex-matched controls and high serum estrogen level was significantly correlated with poor survival of the patients [30]. These findings strongly suggest that estrogen induces CCA progression and is associated with aggressive clinical outcomes. Thus, estrogen and estrogen-related molecules may be potential molecular targets for diagnosis and treatment of CCA.

In order to test whether the oxidative stress may suppress the expression of EBF1 expression and induce EBF1 inhibitor (ZNF423) and estrogen producing enzymes, contribution to induce CCA promotion and progression via inductions of stem cell properties, tumorigenic properties and estrogen response, the expressions and functions of EBF1, ZNF423 and CYP19A1 were analyzed in CCA tissues and cell lines. We investigated the correlation of the expression patterns and 8-oxodG formation in CCA tissues by immunohistochemical analysis. The functional analysis related to stem cell properties including CD133 and Oct3/4 expressions, cell surviving under oxidative stress, tumorigenic properties including cell proliferation, wound healing, cell migration and estrogen response of EBF1 down-regulation was studied by siRNA technique using highly EBF1 expressing cell line (MMNK1).

2. Materials and Methods

-Human CCA tissues

Cholangiocarcinoma tissues were collected from CCA patients admitted at the surgical wards of Srinagarind Hospital, Khon Kaen University. The study was approved by the Ethics Committee for Human Research, Khon Kaen University (HE571283 and HE611577). The paraffin-embedded CCA tissues were used for immunohistochemistry (n=75). All samples were obtained from the specimen bank of the Cholangiocarcinoma Research Institute, Khon Kaen University.

- Hematoxylin and Eosin staining

Hematoxylin and Eosin staining was performed to evaluate numbers of the giant cells. The 4-μm-thick sections of paraffin-embedded human CCA tissues were deparaffinized by 60 $^{\circ}$C for 30 min then in xylene and rehydrated with descending series of ethanol. The sections were rinsed in running tap water and then incubated hematoxylin for 5 min. After washing using tap water, the sections were bluing in 1.0% lithium carbonate solution for 30 seconds. After washing using tap water, the slides were rinse in 70% alcohol, then counterstain in eosin Y solution for 5 min. Then, the cells were washed with 70% ethanol, 100% ethanol, and Xylene respectively. Finally, the cells were mounted with xylene based mounting medium.

- Giant cancer cells counting

Giant cancer cells are defined as cancer cells with a nucleus that is at least three times larger than that of a diploid cancer cell or multiple nuclei. The counting number of the giant cells was done on the selected hot spot areas. For each case, ten microscopic hot fields were counted at × 400 magnification. The average number was calculated on the top five numbers. The number of giant cell count was classified into low ($\leq$4.7) and high (4.7>) groups to correlation with other parameters.

- Immunohistochemistry

Immunohistochemical staining was performed to determine the expressions and localizations of all targeted proteins (*i.e.*, EBF1, ZNF423, CYP19A1, ERα, ERβ, GPR30 and TFF1) in human CCA tissues. The sections (5 μm thick) of paraffin-embedded human CCA tissues were deparaffinized in xylene and rehydrated with descending series of ethanol. For the heat induced antigen retrieval, the sections were immersed in 10 mM sodium citrate buffer (pH 6) and heat-treated in a pressure cooker for 5 min. Endogenous peroxidase activity was blocked by 0.3% (v/v) hydrogen peroxide in phosphate buffered saline (PBS) at room temperature for 30 min. Then, the sections were incubated for 30 min with 10% skim milk in PBS to block non-specific binding, and were incubated with a primary antibody for 1 h at room temperature and then 4 °C overnight. After washing, the sections were incubated with peroxidase-conjugated Envision™ secondary antibody (Dako, Denmark) for 1 h at room temperature. Peroxidase activity was detected using a DAB (3, 3´-diaminobenzidine tetrahydrochloride) substrate kit (Vector Laboratories, Inc., USA) for 5 min, followed by nuclear counterstaining with hematoxylin. Tissue sections were dehydrated with the ascending ethanol series, cleared in xylene and mounted with mounting solution.

In this study, the following primary antibodies were used: 2.5 µg/ml of rabbit anti-EBF1 polyclonal antibody (Sigma-Aldrich Corp, MO, USA), or 0.1 µg/ml mouse anti-8-oxodG monoclonal antibody (Japan Institute for the Control of Aging, Shizuoka, Japan), Rabbit anti-human ZNF423 (1:100; Abcam, MA, USA), 10 µg/mL rabbit polyclonal anti-CYP19A1 antibody (Sigma-Aldrich, USA), 26.49 µg/mL rabbit monoclonal anti-ERα antibody (Abcam, UK), 2 µg/mL rabbit polyclonal anti-ERβ antibody (Abcam, UK), 1.33 µg/mL rabbit polyclonal anti-GPR30 antibody (Abcam, UK), anti-CD44v6 antibody (1:300), and 1 µg/mL mouse monoclonal anti-TFF1 antibody (Sigma-Aldrich, USA).

The stained sections were reviewed under a light microscope. The DAB-positive staining was seen as brown to dark-brown color. Cell nuclei were appeared as blue-purple colors. The immunohistochemistry score (IHC score) was calculated by the multiplying of the intensities of DAB staining results (scored as: 0 = negative, 1 = weak, 2 = modulate and 3 = strong) and the frequency of the staining results (scored as: 0 = none, +1 = 1 – 25%, +2 = 26 – 50% and +3 = 51 – 75% and +4 = >76%) [52]. Thus, the IHC score ranged from 0 to 12. In this study, the IHC score was used to discriminate high (IHC score > 4) and low (IHC score ≤ 4) expression patterns.

- Cell lines

Two distinct human intrahepatic CCA cell lines, KKU-100 and KKU-213, were established from primary tumors of CCA patients in the Northeast of Thailand and obtained from the Cholangiocarcinoma Research Institute, Khon Kaen University, Thailand. Immortalized human cholangiocyte cell line (MMNK1) was originally obtained from Okayama University, Japan [53] and maintained also in the Cholangiocarcinoma Research Institute, Khon Kaen University. All cell lines were cultured in complete medium which is Ham's F-12 (Gibco®, Life technologies™, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin (Life technologies™, USA). Cell lines were incubated in a humidified incubator with 5% CO2 and 95% relative humidity at 37 °C.

- Small interfering RNA (siRNA) treatments

Specific siRNA for EBF1 (siEBF1), ZNF423 (siZNF423) and CYP19A1 (siCYP19A1) was designed by GE Healthcare Dharmacon (ON-TARGETplus SMARTpool, GE Healthcare Dharmacon Inc., USA). The cells were transfected with siRNA using Lipofectamine RNAiMAX® (Thermo Fisher Scientific, USA) in a 6-well plate with 10^5 cells for 24-72 h transfection. The cells treated with scramble siRNA (ON-TARGETplus Non-targeting Control siRNA, GE Healthcare Dharmacon Inc., USA) were used as controls (scramble). Cells were

harvested using trypsinization for RNA extraction, immunocytochemical analysis, cell migration assay and cell proliferation assay.

- RNA extraction and real time PCR

Total RNA was isolated from cell pellets with Trizol® reagent (Invitrogen, USA) according to the manufacturer's protocol. The quality of RNA was assessed with a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, USA). Then, 2 μg of total RNA was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) as described in the manufacturer's protocol. The expression levels of EBF1, ZNF423, MMP-9, CYP19A1, TFF1, BIRC5 and β-actin mRNA were measured using Taqman gene expression assay with Taqman. The PCR was performed in an ABI-7500 real time PCR system (Applied Biosystems, USA). Relative mRNA expression was analyzed with a cycle threshold (Ct) in the linear range of amplification using β-actin as an internal control.

- Immunocytochemistry

Each cell lines (5.0×10^4 cells/well) were placed in a 48-well plate and incubated for 24 h. The attached cells were fixed with 10% paraformaldehyde in PBS for 30 min at room temperature. After washing, cells were incubated with 0.2% (v/v) Triton-X100 solution for 2 min. The cells were incubated with 0.3% (v/v) hydrogen peroxide in PBS for 30 min to block endogenous hydrogen peroxide activity and subsequently incubated with 3% (w/v) BSA in PBS for 30 min to block non-specific binding. The cells were incubated with each primary antibody at 4 °C overnight. After extensive washing, the cells were incubated with peroxidase-conjugated secondary antibody. The peroxidase activity was detected as brown reaction product using a DAB substrate kit. After dehydration, the stained cells were reviewed under an inverted microscope.

- Cell migration assay

The cell migration assay was performed using a Boyden transwell chamber consisting of a membrane filter insert with 8-μm pore size in a 24-well plate (Corning, USA). In brief, after knockdown treatment with siRNA or scramble (control), 4×10^4 cells were placed into the insert upper chamber with serum free medium. In the lower chamber, complete medium was added. Then, the chambers were incubated for 12 h. Non-migrating cells in the upper chamber were removed. Migrated cells that attached at the undersurface of the filter were fixed with absolute methanol for 1 h and stained with hematoxylin overnight. The membrane was allowed to dry and the number of migrated cells were quantified and analyzed under a light microscope.

- CYP19A1 inhibitors treatment

Exemestane (Pfizer Inc., USA) and letrozole (Novartis, Switzerland) were dissolved in dimethyl sulfoxide (DMSO) and pre-diluted in the complete medium before use. The final concentration of DMSO was adjusted to 0.5% in all conditions. The cell lines were treated with various concentrations of exemestane or letrozole for 48 h. After treatment, the cells were subjected to Sulforhodamine B (SRB) assay.

- Sulforhodamine B (SRB) assay for cell density determination

Cell proliferation capacity was determined using SRB assay. In brief, cells in 96-well plate were fixed with 10% cold trichloroacetic acid at 4 °C for 1 h. Subsequently, the cells were stained with 0.4% (w/v) SRB in 1% (v/v) acetic acid for 45 min and washed with 1% (v/v) acetic acid to remove the unbound SRB. The stained cells were dried in a hot air oven (60 $^{\circ}$C) for 30 min. The SRB-bound proteins were solubilized with 10 mM Tris-base (pH 10.5) at room temperature for 1 h. The absorbance was measured at 540 nm using a microplate reader (Tecan Sunrise, Switzerland).

- Statistical analysis

Statistical analysis was performed using SPSS Statistics software version 17.0 (IBM Cooperation, USA). The Kaplan-Meier estimate with log-rank test was used for survival analysis. The correlations between protein expression patterns in CCA tissues and clinico-pathological data were analyzed using Pearson Chi-square test. The correlations among protein expressions were analyzed by Pearson correlation. The results of mRNA expression, cell migration assay and cell proliferation assay were analyzed using Student's t-test. *P*-value < 0.05 is considered as statistically significant.

3. Results and discussion

Part I: Oxidative stress induces giant cancer cell formation with stem-like cell property

Giant cancer cells in CCA tissues related with oxidative stress and stem cell marker expression

Figure 1 showed the representatives of GCCs (green arrows), CD44v6 staining patterns and 8-oxodG staining patterns, respectively. GCCs were found in all CCA tissues as shown in Figure. 1A. GCCs were high-frequency found at the inflammatory, fibrosis, tumor necrosis and hypoxic areas. CD44v6 was localized in cell membrane and/or cytoplasm of the cancer cells whereas it was not expressed in normal bile ducts cells. Additionally, CD44v6 was positive staining in GCCs (green arrow, Figure 1B). Immunoreactivity of CD44v6 expression in cancer cells was classified as negative, low, and high patterns as shown in Figure. 1B. 8-oxodG was strongly detected in nuclei of CCA cells whereas it was also detected in normal bile duct and hepatocyte cells at the tumor adjacent area. Immunoreactivity of 8-oxodG formation in cancer cells was classified as low and high patterns as shown in Figure. 1C.

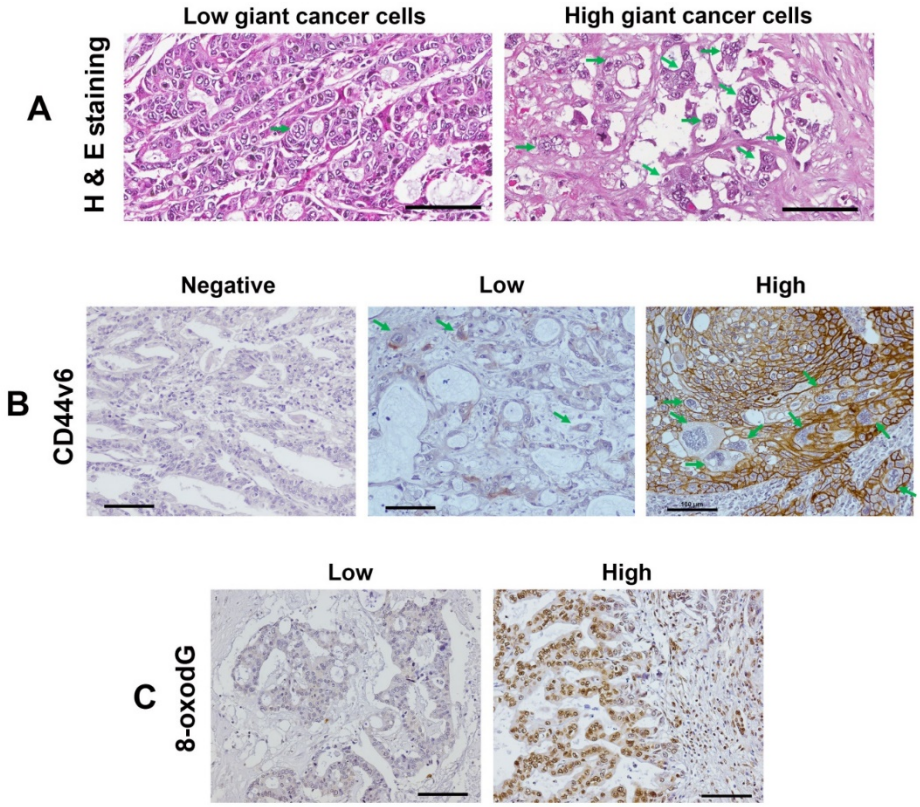

Figure 1 H & E (A) and immunohistochemical staining patterns of CD44v6, a stem cell marker (B) and 8-oxodG, an oxidative stress marker (C) in CCA tissues. Green arrows indicate giant cancer cells. Scale bars are equal to 100 μm.

Table 1 showed the correlation between numbers of GCCs with clinical data, CD44v6 expression and 8-oxodG formation. The numbers of GCCs were significantly increased in CCA tissues with poorly- and moderately-differentiated tubular types and high CD44v6 expression. They had tendency to correlate with 8-oxodG formation. Taken together, numbers of GCCs were significantly correlated with CCA tissues with high CD44v6 expression and 8-oxodG formation. The numbers of GCCs in CCA tissues were not correlated with survival rates, metastasis status, age and sex of CCA patients (data not shown).

Table 1 Correlations of giant cancer cells formation with tumor histology, stem cell marker expression and oxidative stress biomarker

	CCA giant cells		Pearson Chi-Square
	Low (n=43)	High (n=30)	
CD44v6 expression patterns			
Low	32	9	
High	11	21	*P*<0.001*
8-oxodG formation patterns			
Low	22	9	
High	21	21	*P*=0.072

Oxidative stress induces giant cancer cell formation

To verify that oxidative stress induces GCCs formation, CCA cell line was subjected to hydrogen peroxide treatment for mimic oxidative stress condition. Effect of oxidative stress to GCCs formation was showed in Figure. 2. Numbers of GCCs were significantly increased in high concentrations of hydrogen peroxide treatment (100-500 μM). Moreover, the hydrogen peroxide-induced GCCs were positively stained with CD44v6 as shown in Figure 3.

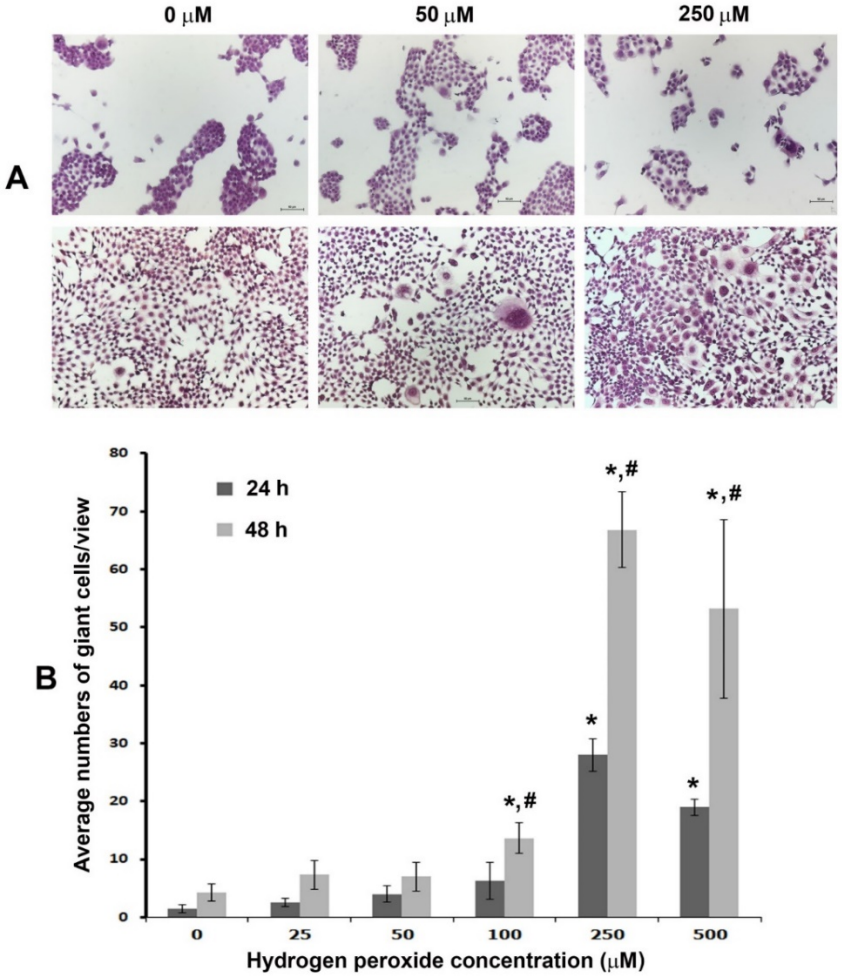

Figure 2 Hydrogen peroxide H_2O_2 treatment of CCA cell line (KKU-213) for 24 h and 48 h. (A) Cell morphology analysis by H&E staining. Scale bars equal to 50 μm. (B) Graphical represents average numbers of giant cancer cells (mean±SD) in 100x pictures of each condition. $^{*}P$-value < 0.05 compared with untreated condition (0 μM) of each time. $^{\#}P$-value<0.05 compared with same condition at 24 h.

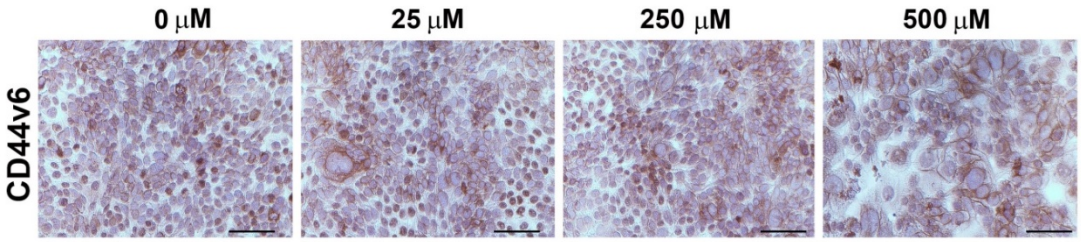

Figure 3 Immunocytochemical analysis of CD44v6 in H_2O_2 (0, 25, 250 and 500 μM) treated CCA cell line (KKU-213) for 48 h. Scale bars are equal to 50 μm.

The GCCs are highly resistant to hydrogen peroxide and could form through endo-reduplication or cell fusion, generating regular-sized cancer cells quickly through budding or bursting similar to simple organisms like fungi detected by In-cell analysis.

Part I: Discussion

GCCs were detected in CCA tissues especially at inflammatory, fibrosis, tumor necrosis and hypoxic areas. Chronic inflammation had been reported to induce tissue injury, necrosis and fibrosis via oxidative stress (ref). HIF-1α, a hypoxia-induced transcription factor, induced NFKB and iNOS expressions that also consequently induced oxidative stress (refs). The numbers of GCCs were trended to correlated with oxidative stress marker. Moreover, GCCs were increased in an oxidative stress-treated CCA cell line, suggesting that oxidative stress induces GCCs formation. In addition, the numbers of GCCs were strongly positive correlated with a stem cell marker (CD44v6) expression in CCA tissues. GCCs had been previously reported that they gain many stem-like cell properties (ref). Therefore, the present results indicated that chronic inflammation and oxidative stress can activate GCCs formation that are potential stem-like cell population in CCA.

Under cellular bombard by oxidative stress most of cells die, whereas some can adapt to survive. We previously induced oxidative stress-resistant cells (ox-MMNK1-L) from the immortal cholangiocyte cell line (MMNK1) and the oxidative stress-resistant cell line had higher cell proliferation rate and increasing the expressions of the anti-oxidant genes and an epigenetics-related gene when compared to the parental cells (ref). In the present results, GCCs were increased in oxidative stress-treated CCA cell lines. At high concentration (500 μM) for 48 h, most of the survival cells are GCCs. Additionally, GCCs were also reported to relate with hypoxia condition, stem cell properties as well as resistant to chemotherapy and radiotherapy (refs). Thus, we hypothesize that the formations of GCCs are one of adaptation process of cells to survive under oxidative stress condition, when several carcinogenic events such as mutation, epigenetic change and genomic instability occur in the GCCs, the cells would gain a property of cancer stem-like cells.

Part II: Prolonged oxidative stress down-regulates Early B cell factor 1 with inhibition of its tumor suppressive function against cholangiocarcinoma genesis

-Immunohistochemical analysis of EBF1 expression and 8-oxodG formation

Figure 4 shows the immunoreactivities of EBF1 expression and 8-oxodG formation obtained. The expression of EBF1 was predominantly detected in cytoplasm and nucleus of hepatocytes and normal bile ducts in adjacent non-tumor areas, while it was weakly observed in hyperplasia and tumor areas. Low EBF1 expression was found in 69% (52/75) of CCA tissues. Additionally, the immunoreactivity of EBF1 staining in CCA cancer cells was significantly reduced compared to the adjacent normal bile ducts ($P < 0.001$, graph not shown). 8-oxodG was highly detected in the nucleus of hyperplasia bile ducts and the cancer cells compared with the individual normal bile ducts. High formation of 8-oxodG was detected in 53% (40/75) of CCA tissues.

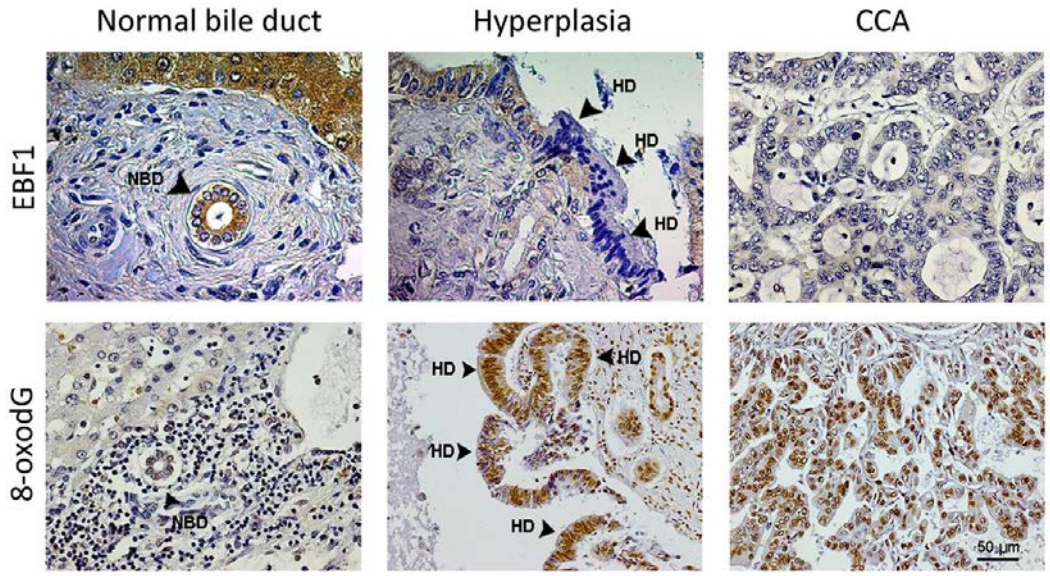

Figure 4 Immunohistochemical analysis of EBF1 expression and 8-oxodG formation in normal bile duct of a tumor adjacent area, hyperplasia and CCA tissues. NBD= normal bile duct, HD= hyperplasia bile duct. An original magnification is 200× for all figures.

-EBF1 expression and 8-oxodG formation in relation to clinico-pathological parameters in human CCA tissues

Among liver sections obtained from 75 patients with intrahepatic CCA examined, 52 (69%) cases were male and 23 (31%) cases were female. The median age of patients was 57.8 years. In our study, the CCA histological types were classified as 47% (35/75) papillary type and 53% (40/75) tubular type. CCA

metastatic stages were classified into two different groups; 56% (42/75) metastasis and 44% (33/75) non-metastasis. The criterion of the classification of CCA histology types and metastatic stages has been described in previous studies [54-57]. No significant correlation of EBF1expression and the 8-oxodG formation with age, gender, histological types and metastatic stages was found.

-Expressions of EBF1 and level of 8-oxodG with survival rate of CCA patients

The Kaplan-Meier method with log rank test showed that CCA patients with low expression of EBF1 or high level of 8-oxodG formation were significantly correlated with poor prognosis (P=0.033 and P=0.007, respectively) as shown in Figures 5A and 5B. Interestingly, CCA patients who had both reduced expression of EBF1 and elevated formation of 8-oxodG showed even greater significant correlation with poor prognosis (P=0.003) when compared among various correlations (Figure 5C).

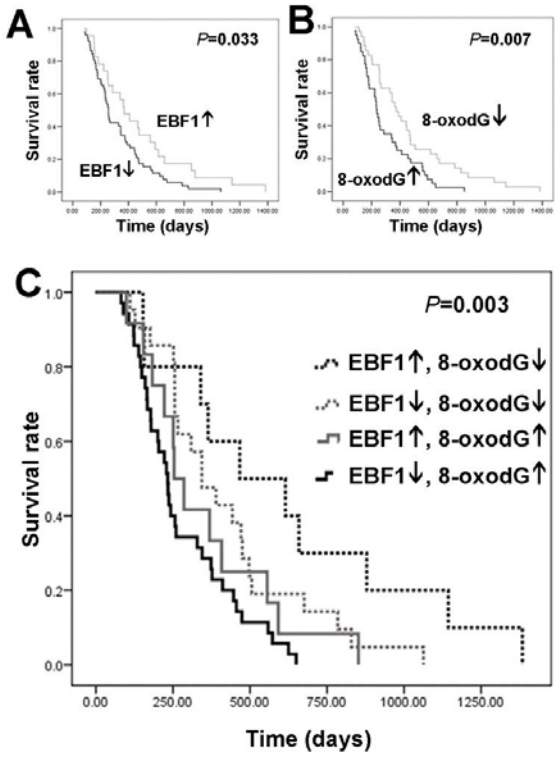

Figure 5 Kaplan-Meier analysis of EBF1 (A), 8-oxodG formation (B), and combined EBF1 expression and 8-oxodG formation (C) with survival rate in CCA. ***P*-value was analyzed by log rank test.** EBF1↓= low EBF1 expression, EBF1↑= high EBF1 expression, 8-oxodG↓= low 8-oxodG formation, and 8-oxodG↑= high 8-oxodG formation in CCA tissues.

-Expressions of EBF1 in cholangiocyte, oxidative stress-resistant and CCA cell lines

Recently, we have established and characterized the oxidative stress-resistant cell line (ox-MMNK1-L cell) by exposing MMNK1 cells to daily hydrogen peroxide treatment, thus ox-MMNK1-L cells can be used as the prolonged oxidative stress response model [8]. The analyses of EBF1 expression was further performed in MMNK1, ox-MMNK1-L, KKU-213, KKU-214 and KKU-156 cells using real time PCR and immunocytochemical technique (Figure 6A and 6B). EBF1 was highly stained in nucleus and cytoplasm of the cholangiocyte cell (MMNK1) compared with all of CCA cell lines (KKU-213, KKU-214 and KKU-156) used in this study, suggesting that down-regulation of EBF1 could be involved in CCA development. Moreover, EBF1 was slightly expressed in ox-MMNK1-L cells compared with MMNK1 cells, suggesting that the prolonged oxidative stress suppress the expression of EBF1.

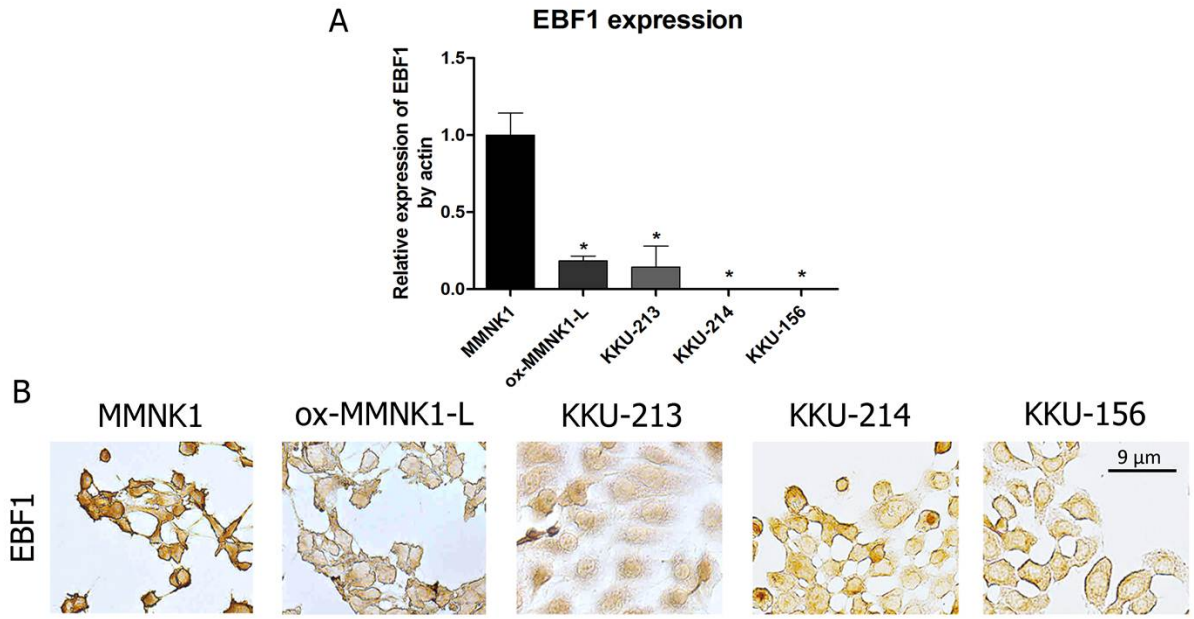

Figure 6 (A) Relative mRNA expression levels of EBF1 was measured by real-time PCR and adjusted by β-actin mRNA expression in MMNK1, ox-MMNK1-L and CCA cell lines. The symbol asterisk (*) indicates statistical significance at $P<0.05$. (B) Immunocytochemical analysis of EBF1 expression.

-EBF1-knockdown effects on stem cell marker expressions of cholangiocyte cells

Figure 7 shows the effect of EBF1 knockdown on stem cell markers expression. The MMNK1 was transfected with 50 nM of EBF1 siRNA compared with lipofectamine and media alone. Following 48 h of

transfection, the EBF1 mRNA was significantly decreased, whereas CD133 and Oct3/4 mRNA levels were significantly increased when compared with the control sets as shown in Figure 7A, 7B and 7C, respectively. Moreover, the protein expressions of EBF1 and CD133 were confirmed in EBF1 knockdown-cholangiocyte cells using the immunocytochemical technique as shown in Figure 7D and 7E. EBF1 protein was slightly expressed, whereas CD133 was highly expressed in EBF1 knockdown-MMNK1 cells when compared to the control, suggesting that suppression of EBF1 associates with stem cell properties.

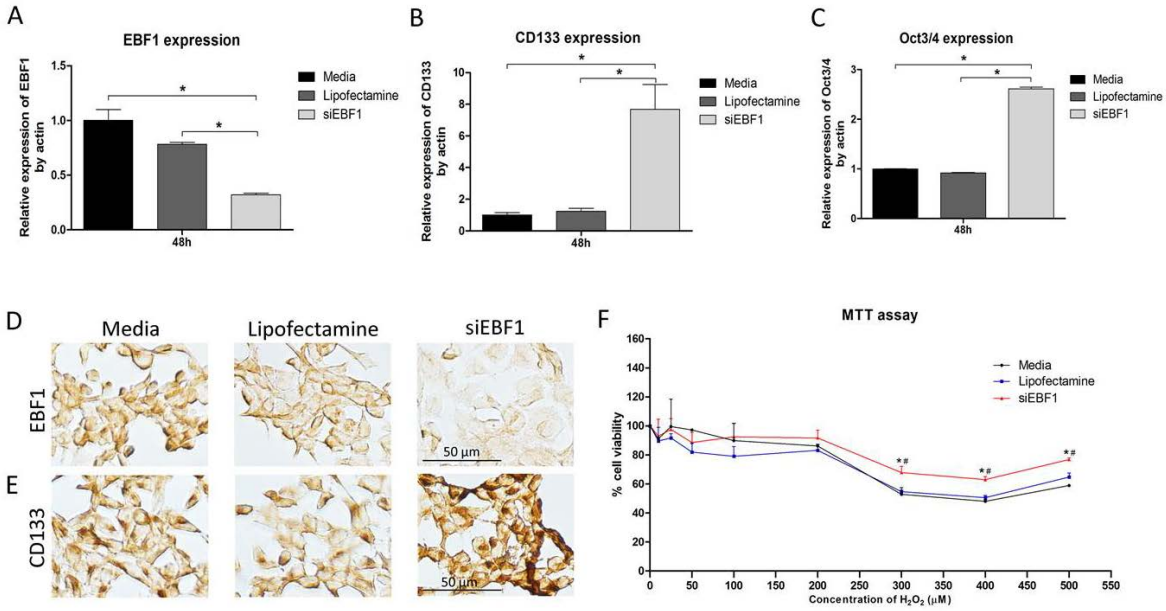

Figure 7 Relative mRNA expression levels of EBF1 (A), CD133 (B) and Oct3/4 (C) were measured by real-time PCR and adjusted by β-actin mRNA expression. The asterisk (*) indicates statistical significance at $P<0.05$. Protein expression levels of EBF1 (D) and CD133 (E) were detected by immunocytochemical staining. An original magnification is 200× for all figures. (F) Viability of EBF1-knockdown cholangiocyte cell line using MTT assay. The asterisk (*) indicates statistical significance at $P<0.05$ (compared with lipofectamine); (#) for $P<0.05$ (compared with media).

-EBF1-knockdown induces cholangiocyte cell line resistant to oxidative stress

To elucidate the relationship between oxidative stress and EBF1 down-regulation, EBF1 gene of MMNK1 cells were silenced and the cells were exposed to various concentrations of H_2O_2. The results show that the number of viable cells was significantly higher in EBF1-knockdown group were than that of control after exposure to 200 μM or higher concentrations of H2O2 for 48 h (Figure 7F). This result suggests that down-regulation of EBF1 strengthens the oxidative stress-resistant property.

-EBF1-knockdown effects on wound healing and migration of cholangiocyte cells

We further investigated the function of EBF1 in MMNK1 measured by wound healing and migration assays. The results showed that siEBF1-transfected MMNK1 cells had a significantly increased in cell migration compared with lipofectamine transfection at 31 h as determined by wound healing assay (Figure 8A) and the graphical data represented the percentage of migration area as shown in Figure 8B. Additionally, we also confirmed the ability of EBF1 knockdown-MMNK1 cell in cell migration using a Boyden chamber transwell consisting of a membrane filter insert in 24-well plate with 8-μm pore size. The result showed that EBF1 knockdown in MMNK1 cell was significantly increased in cell migration numbers when compared with the controls ($P < 0.001$) (Figure 8C and 8D).

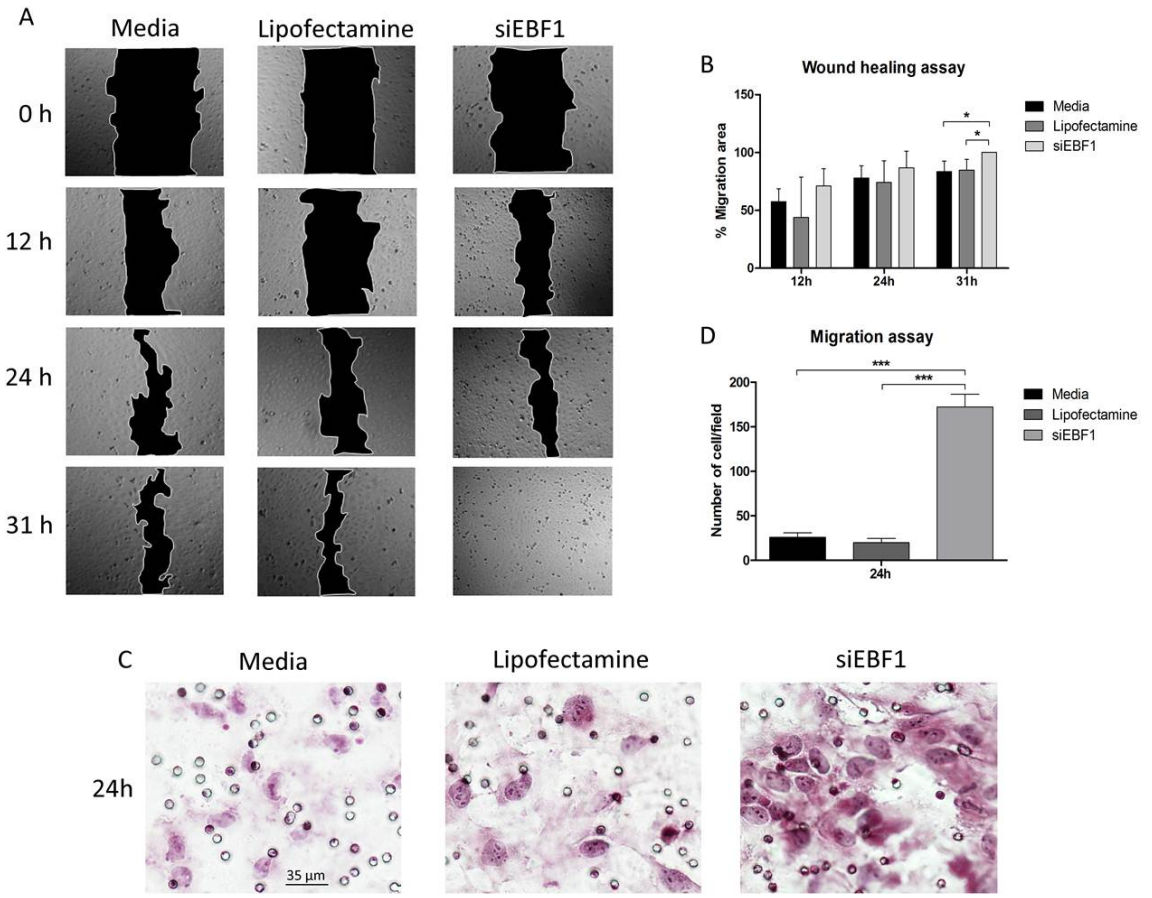

Figure 8 (A) Wound healing assay under microscope (10×). (B) The graphical data represented the percentage of migration area determined by wound healing assay. (C) Hematoxylin-staining migrated cholangiocyte cells after 24 h treated with lipofectamine and siEBF1 using a Boyden chamber transwell consisting of a membrane filter. (D) The graphical data represent the migrated cells detected by the migration assay at 24 h. The asterisk (*) indicates statistical significance at $P<0.05$ and asterisks (***) indicates statistical significance at $P<0.001$.

-Effect of EBF1 knockdown to estrogen response in cholangiocyte cells

Trefoil factor 1 (TFF1) is one of estrogen responsive genes that play critical roles in cell migration and invasion in estrogen-related cancers including CCA [30, 50, 58]. In this study, TFF1 expression was significantly increased in the EBF1-knockdown cholangiocyte cell line as shown in Figure 9A. We also explored the migration of cholangiocyte cells after EBF1 knockdown and treated with 17β-estradiol. Our results showed that a significant increase in the number of migrated cells was observed in EBF1 knockdown-MMNK1 cells after being treated with estradiol for 12 h when compared to the EBF1 knockdown-MMNK1cells alone ($P < 0.001$) as shown in Figure 9B and 9C.

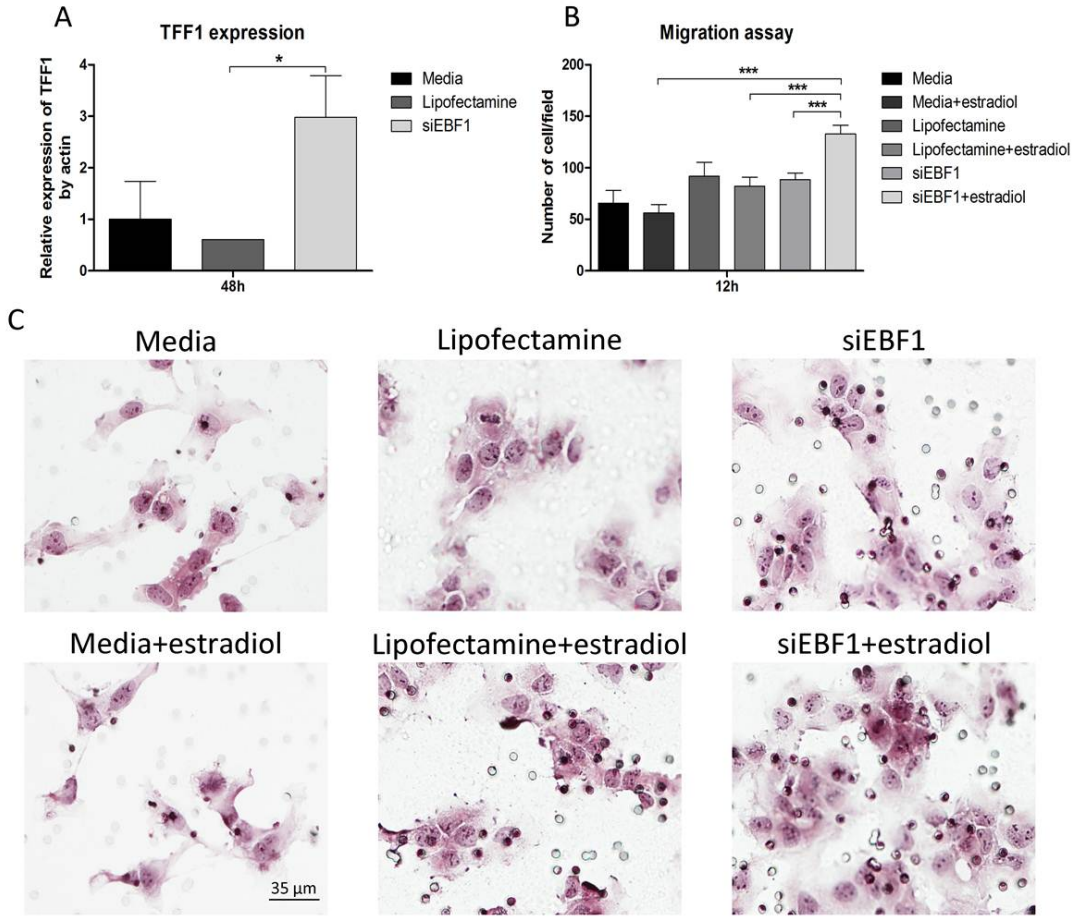

Figure 9 (A) Relative mRNA expression levels of TFF1 was measured by real-time PCR and adjusted by β-actin mRNA expression. (B) The graphical data represent the migrated cells detected by the migration assay. The Y axis represents the number of migrating cells per field and the X axis the experimental group. The asterisks (***) indicates statistical significance at *P*<0.001. (C) Hematoxylin-staining migrated cells of cholangiocyte after 12 h treated with 17β-estradiol using a Boyden chamber transwell consisting of a membrane filter.

Part II: Discussion

In our study, CCA patients with low EBF1 expression and high oxidative stress were significantly correlated with poor survival. Thus, the down-regulation of EBF1 may be caused by oxidative stress and play the important role in CCA development. There are several studies suggesting that EBF1 may function as the potent tumor suppressor [7, 26, 27]. Chronic inflammation mediated by infection is a major risk factor causing carcinogenesis including O. viverrini-driven CCA [14]. In addition, oxidative stress was also reported to induce the alteration of gene expressions via the inductions of mutation, genetic instability and epigenetic changes [17, 59, 60]. Recently, we successfully established the oxidative stress-resistant cell line (ox-MMNK1-L), which originated from the MMNK1 cell line with the long-term daily-exposure to 25 μM H_2O_2 [8]. The oxidative stress-resistant cells could increase not only antioxidant properties but also DNMT1 expression level, suggesting that the epigenetic changes may be triggered as the oxidative stress response condition [8]. Additionally, EBF1 expression in ox-MMNK1-L was decreased compared to the parental cell line of which EBF1 mRNA expression level was increased after acute exposure to H_2O_2 (Figure S1). Related to this, bio-informatics analysis showed that EBF1 was down-regulated in human obese adipose tissues which were exposed to long-term oxidative stress [61]. Our results suggest that prolonged oxidative stress inhibits EBF1 expression in the cholangiocyte cell line as the adaptive response for cell survival under persistent stress situation.

The immunohistochemical analysis in 75 cases of CCA tissues and immunocytochemical analysis of MMNK1, ox-MMNK1-L and 3 CCA cell lines raises the hypothesis that EBF1 down-regulation may be involved in CCA promotion and progression resulting in CCA development with aggressive clinical outcomes. Therefore, EBF1 knockdown by siRNA was performed in MMNK1 cells. EBF1 knockdown-MMNK1 cells had no effect to cell growth (Figure S2) whereas they significantly increased wound healing activity and cell migration numbers. These confirmed that EBF1 down-regulation could induce cancer properties of the cholangiocyte cells through the induction of cell migration activities.

Stem cells are cells that possess the ability to unlimited self-renewal and to generate mature cells of a particular tissue by differentiation [62]. EBF1 play roles in hematopoietic stem cells differentiate into mature B-cells [63], and is involved in mesenchymal stem cell (MSC) differentiation which induces MSC differentiation into adipocytes, whereas it suppresses differentiation into osteocytes [64]. In the process of liver development, bipotential liver stem cells could differentiate either into cholangiocytes (bile ducts) and hepatocytes [65]. Recently, we proposed the mechanism that CCA might differentiate from bipotential liver stem cells lining at canal of Hering, biliary ductules, bile duct or progenitor cells from bone marrow-derived circulating cells during tissues repairing process under oxidative stress induced by O. viverrini-chronic inflammation [2]. Moreover, CD133 and Oct3/4 were potentially used as a bipotential liver stem cell marker [2]. Our results showed that

EBF1 was highly detected in the nucleus of normal bile duct and hepatocyte cells, suggesting that EBF1 may play roles in bipotential liver stem cell differentiation into cholangiocytes and hepatocytes. Therefore, we hypothesized that down-regulation of EBF1 during CCA carcinogenesis may play significant role in CCA development via the inhibition of bipotential liver stem cells differentiation into mature cholangiocytes and hepatocytes or induction of stem cell properties in the mature cells leading to increase stem cell property of the tumor initiating cells. This hypothesis was supported by the increasing of CD133 and Oct3/4 expressions after EBF1 suppression on MMNK1 cell line by specific siRNA.

Estrogen is the sex hormone that plays roles in secondary female characteristics. It was reported to promote carcinogenesis especially in breast cancers [29]. ERs are mediated by estrogen. Activated ERs bind to the estrogen response element and promote the expressions of estrogen responsive genes such as TFF1. Serum estrogen levels were significantly increased in male CCA patients and it was reported to play roles in the tumor progression via induction of cell proliferation and migration through ERs and TFF1 expressions [30]. Recently, EBF1 had been proposed to be the negative regulator of ERs [28]. The present results showed that increasing of TFF1 expression was found in EBF1-knockdown cholangiocyte cells, suggesting that down-regulation of EBF1 induced an estrogen response through the induction of ERs activity. Cell proliferation analyzed by the sulforhodamine B (SRB) assay significantly increased in estrogen-treated lipofectamine and estrogen-treated siEBF1 (Figure S3), suggesting that lipofectamine may induce estrogen uptake via increasing of cell membrane surface area and estrogen induced cell proliferation does not associate with EBF1 down-regulation. On the other hand, the numbers of migrated cells significantly increased in the EBF1 knockdown-MMNK1 cells after being treated with 17β-estradiol. This suggests that down-regulation of EBF1 increases the effect of estrogen response via induction of cell migration activities through TFF1 expression resulting in CCA development with aggressive clinical outcomes.

In conclusion, the present results show that EBF1 expression is affected by oxidative stress. Chronic exposure to oxidative stress induces significant suppression of EBF1 expression. Suppression of EBF1 can induce stem cell and migration properties of the cholangiocyte cells leading to CCA promotion and progression resulting in CCA development with aggressive clinical outcomes such as short survival time. Therefore, EBF1 and its related molecules may be used as new therapeutic targets for CCA chemotherapy and chemoprevention.

Part III; Oxidative stress induces Zinc finger protein 423 expression lead to cholangiocarcinoma cell proliferation

-The expression patterns of ZNF423 and the formation of 8-oxodG in CCA tissues

The protein expression levels of ZNF423 in CCA tissues (n=75) were determined using IHC. The results showed that ZNF423 was over-expressed in CCA tissues compared to normal bile ducts ($p<0.001$; graph not shown). Using medium value for the cutoff point, 41% (31/75) of CCA tissues showed high ZNF423 expression pattern as shown in Figure 10A. Moreover, 47% (35/75) of CCA tissues showed high 8-oxodG detection pattern as shown in Figure 10B. The ZNF423 expression patterns in CCA tissues were significantly positive correlated with the 8-oxodG detection patterns as shown in Table 2. Taken together, CCA patients who had high ZNF423 expression and high 8-oxodG detection were significantly correlated with poor prognosis ($p = 0.047$ analyzed by Kaplan-Meier estimate with Log-rank test) as shown in Figure 1C. These data revealed that the over-expression of ZNF423 in CCA may induced by oxidative stress consequently to CCA progression with poor prognosis.

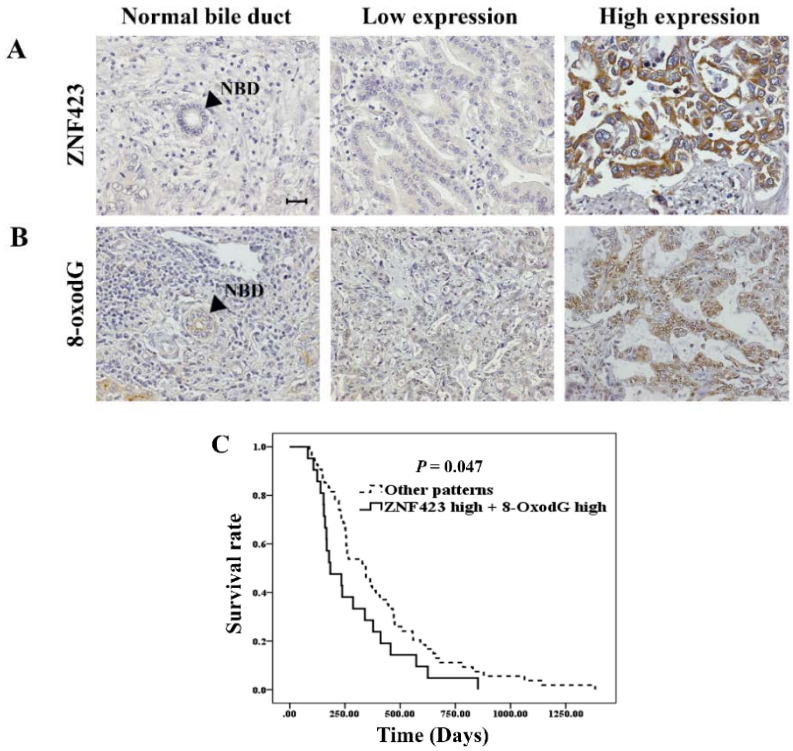

Figure 10 Immunohistochemical analysis ZNF423 (A) and 8-oxodG (B) in normal bile duct (NBD) of tumor adjacent area and CCA. NBD was indicated by the arrows. All figures are 200x original magnification. Scale bar is equal to 50 μm. (C) The expression of ZNF423 and the formation of 8-oxodG with survival rate in CCA analyzed by Kaplan-Meier with log-rank test analyses.

Table 2 The correlation of ZNF423 expression in CCA tissues with clicino-pathological data of CCA patients and 8-oxodG formation.

Variables	ZNF423		*P*-value
	Low	High	
Survival day (median(min-max))	297.5 (89-1384)	260 (82-852)	0.101
Metastasis			
- Non-metastasis	20	13	
- Metastasis	24	18	0.474
Histology			
- Tubular	24	16	
- Papillary	20	15	0.493
Gender			
- male	29	23	
- female	15	8	0.306
Age			
- <57.8	23	13	
- >57.8	21	18	0.259
Age (mean±SD)	56.97±7.72	59.03±6.81	0.228
8-oxodG			
- Low	26	9	
- High	18	22	0.009**

-The expressions of ZNF423 and its correlation with clinico-parameter in tissues of CCA patients

Paraffin-embedded human CCA tissues were obtained from 75 intrahepatic CCA patients. These CCA tissues contained male 69% (52/75) and female 31% (23/75). The median of age was 57.8 years. Moreover, CCA metastasis stages were divided into metastasis stage 56% (42/75) and non-metastasis stage 44% (33/75). In this study, histological types of CCA patients were classified into 53% (40/75) tubular type and 47% (35/75) papillary type. Nevertheless, ZNF423 expression had no correlation with metastasis stages, histology types, gender and age (Table 2).

-Oxidative stress induced up-regulation of ZNF423 in cholangiocyte cell line

To assess the effect of oxidative stress to expression of ZNF423, MMNK1 (low ZNF423 expressed cell line) was treated with H_2O_2 in various concentrations including 0, 25, 50 and 100 μM for 24, 48 and 72 h. The result indicated that mRNA expression levels of ZNF423 were significantly increased with dose and time dependents especially at 48 and 72 h after treatments (Figure 11). These finding strongly demonstrated that over-expression of ZNF423 can be induced by reactive oxygen species (ROS).

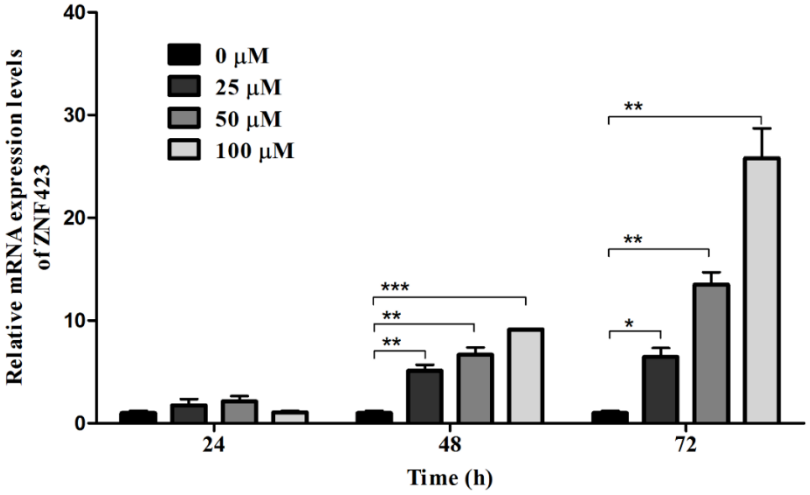

Figure 11 Relative mRNA expression levels of ZNF423 after treatment with H_2O_2 in various concentrations (0, 25, 50 and 100 μM) for 24, 48 and 72 h. Significance was calculated by Student's *t* test compared with untreated condition (* $p<0.05$, ** $p<0.01$, *** $p < 0.001$). mRNA expression levels of ZNF423 were normalized by β-actin.

-ZNF423 induced proliferation and colony forming abilities of CCA cell line

To evaluate the role of ZNF423 in CCA cell line, the ZNF423 high expressed CCA cell line (KKU-100) was selected for ZNF423 knockdown using specific siRNA. The siRNA effectively decreased mRNA expression level of ZNF423 to approximately 70% (Figure 12A) at 72 h after knockdown. After knocked down, proliferation and colony forming ability of the cell lines were measured using cell proliferation assay and clonogenic assay. The result showed that ZNF423-knockdown-KKU100 cells were significantly decreased cells proliferation ($p<0.001$) (Figure 12B) and colony formation abilities compared to the control cells ($p<0.01$) (Figure 12C-D). Our finding suggested that ZNF423 had oncogenic properties to drive proliferation and colony formation of CCA cell line.

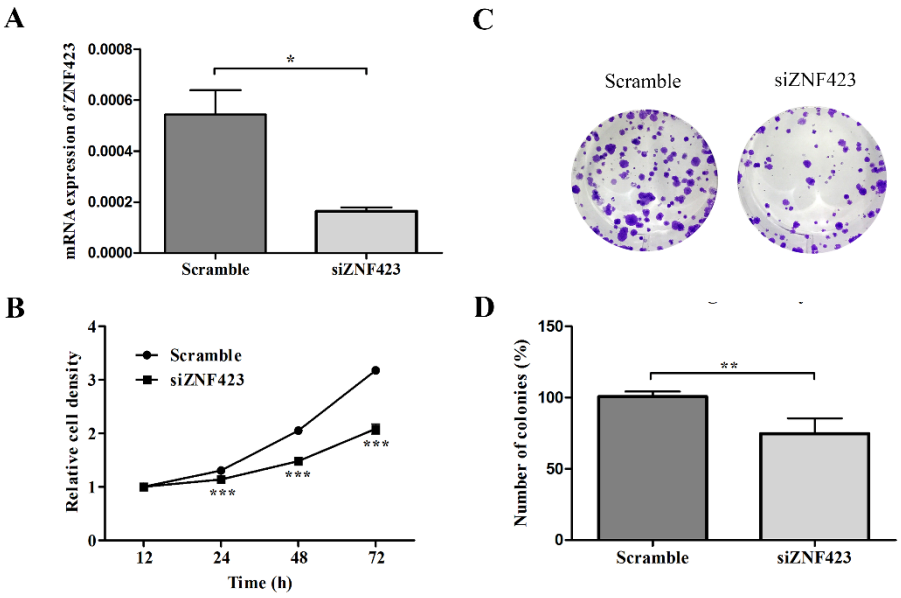

Figure 12 Functional analysis of ZNF423 using specific siRNA in CCA cell line. (A) mRNA expression level of ZNF423 after knockdown experiments in KKU-100 were determined using qPCR. mRNA levels of ZNF423 were normalized by β-actin. (B) Relative cell densities analyzed by SRB assay. (C) Clonogenic assay was stained with crystal violet after cell reseeding for 14 days. (D) Graphical represented the relative number of colonies (%). *P*-values were calculated by Student's *t* test compared with scramble condition (*p<0.05, **p<0.01, and ***p<0.001).

Part III: Discussion

The previously, an oxidative stress induces DNA damage marker (8-oxodG) formation was detected in CCA tissues and the highly formation of 8-oxodG was correlated with poor prognosis of the CCA patients [1, 66]. In this study, ZNF423 was highlighted to be one of an oxidative stress targeted genes which play significant roles in CCA promotion and progression. Because ZNF423 expression could be induced by H2O2 in the cholangiocyte cell line. H2O2 was reported to be an oxidative stress inducing factor via increasing of superoxide anion (O2•—) generation through NADPH oxidase activity [67-69]. Moreover, the expression patterns of ZNF423 in CCA tissues were also correlated with an oxidative stress formation marker (8-oxodG). In addition, ZNF423 act as a CCA progression factor via the induction of cell proliferation and colony formation activities. Notably, this is novel synergistic between oxidative stress and ZNF423 in cancers.

Oxidative stress induced proliferation in several cancer types such as colorectal cancer [70, 71]. This condition triggered cell proliferation through Akt pathway via up-regulated β-catenin and cyclin D1 [70]. Moreover, oxidative stress also induced cell proliferation rate of the cholangiocyte cell line (MMNK1), which could be involved in CCA promotion [8]. Then, we further investigated the functions of ZNF423 in CCA progression. The results revealed that ZNF423 induced proliferation and colonies formation of CCA cells. Furthermore, the function of ZNF423 was studied in other cancers especially in leukemia such as chronic myelogenous leukemia (CML), ZNF423 induced proliferation of CML cell line and developed CML into late severe stage of this cancer type [37]. Thus, we assumed that ZNF423 play important roles in CCA promotion and progression via the increasing of cell proliferation rate of cholangiocyte and CCA cells induced by oxidative stress.

ZNF423 can interacts with all retinoic acid receptor (RAR) types especially retinoid X receptor (RXR) including α, β and γ [72]. Recently, the retinoid X receptor α (RXRα) was reported to play crucial role in CCA. This gene acts as oncogene to enhance growth of CCA cell lines [73]. It activated CCA cell cycle progression through Wnt/β-catenin pathway via up-regulated cyclin D1. Moreover, RXRα play role to drive proliferation of CCA cell lines through NF-ΚB signaling pathway by increase proliferating cell nuclear antigen (PCNA) and p21 [73]. In addition, RXRγ was overexpressed in CCA and act as oncogene to promote proliferation of CCA cell via the Akt/NF-ΚB and Wnt/β-catenin pathways [74]. Therefore, ZNF423 may interacts with RXRs leading to CCA proliferation through Wnt/β-catenin and Akt/NF-ΚB signaling pathway [73]. In conclusion, ZNF423 was an oxidative stress related gene and played role as oncogene to drive CCA genesis. Essentially, the ZNF423 and its related genes might be a novel target therapy in CCA towards to benefit of the patients with this disease.

Part IV: The importance of CYP19A1 in estrogen receptor-positive cholangiocarcinoma

-Expression of CYP19A1 in CCA tissues

Immunohistochemical staining of CCA tissues demonstrated that CYP19A1 was strongly expressed in the cytoplasm of cancer cells but it was slightly expressed in the nucleus (Figure. 13). CYP19A1 was also slightly expressed in the cytoplasm of hepatocytes. The degrees of CYP19A1 expression determined by immunohistochemistry were significantly higher in the CCA cells compared with the normal bile duct epithelial cells located at the adjacent area ($P < 0.001$; graph not shown). More than half of the CCA tissues examined (45/74, 61%) showed high expression of CYP19A1.

- Expression of estrogen receptors in CCA tissues

The expression patterns of three estrogen receptors, ERα, ERβ and GPR30, in CCA tissues were shown in Figure. 13. While ERα and ERβ were seen as the cytoplasmic and nuclear distribution patterns in cancer cells, GPR30 was located at the cytoplasm and the cell membrane of cancer cells. All CCA tissues showed positive staining of ERα, ERβ and/or GPR30 (100%, 74/74). Significantly positive correlation was observed between the expression patterns of ERα and ERβ ($r = 0.263$ and $P = 0.024$; Pearson Correlation analysis). Also, the expression levels of all three estrogen receptors in immunohistochemistry were significantly higher in CCA cells than in the normal bile duct epithelial cells in the tumor adjacent tissues ($P < 0.001$; graph not shown). High expression levels of ERα, ERβ and GPR30 in IHC score were observed in 78% (58/74), 50% (37/74) and 38% (28/74), respectively, of the CCA patients. All estrogen receptors were also highly expressed in the cytoplasm of hepatocytes. In spite of the high expression patterns of ERα, ERβ and GPR30 in CCA tissues, their expression levels were not correlated with clinico-pathological data such as age, sex, metastasis status and CCA histology types.

-Expression of TFF1, as a representative of estrogen-responsive proteins, in CCA tissues

As a representative of estrogen-responsive proteins [50], the expression of TFF1 in both CCA cells and adjacent normal bile duct cells was examined using immunohistochemical staining. As shown in Figure. 13. TFF1 protein was highly expressed in the cytoplasm of CCA cells, whereas it was negative in normal bile duct epithelial cells. Among 74 CCA cases, 39 cases (53%) have high TFF1 expression pattern, although it was not correlated with clinico-pathological data including age, sex and CCA histology types. Moreover, TFF1 expression was significantly correlated with ERβ expression ($r = 0.244$ and $P = 0.036$; Pearson correlation).

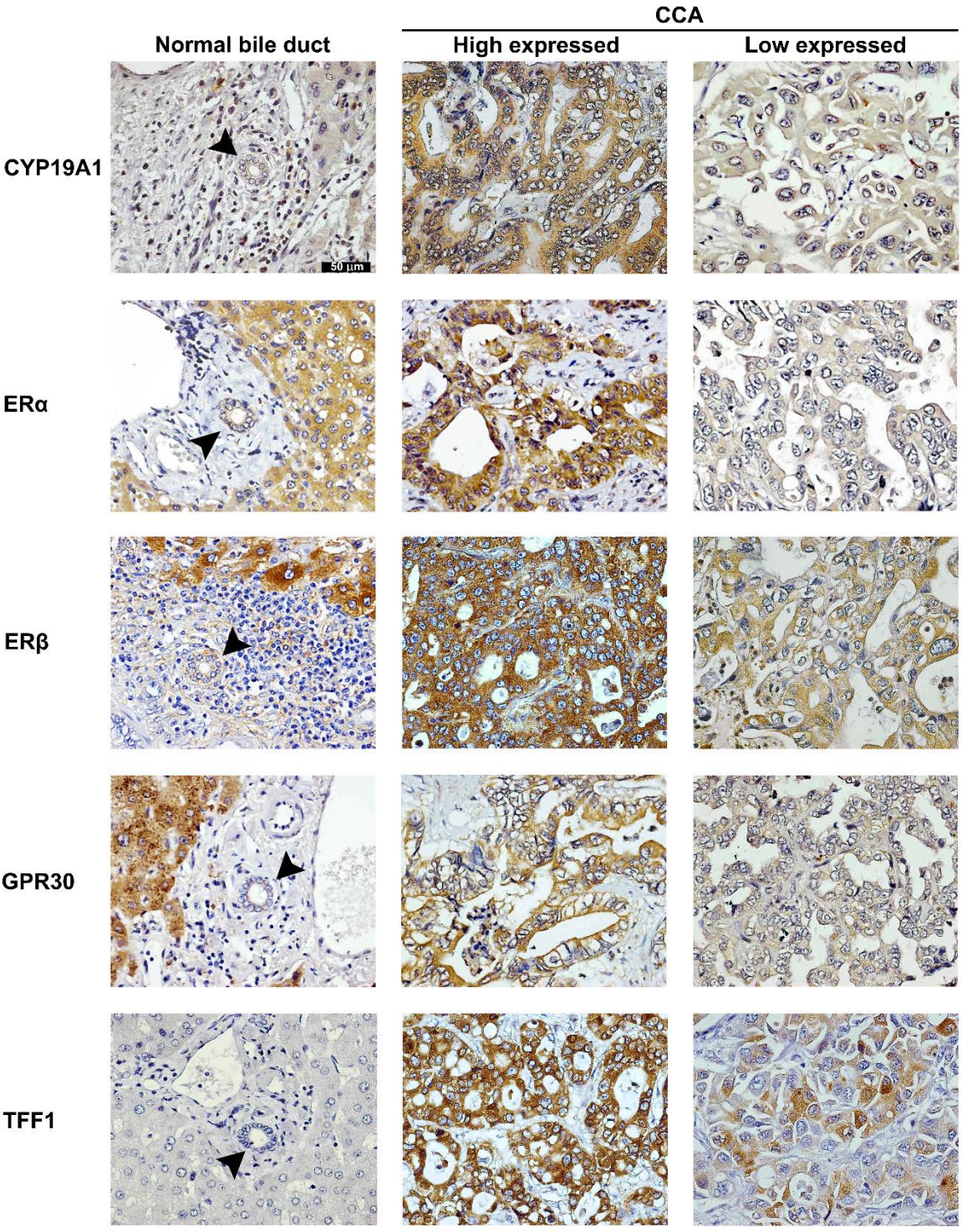

Figure 13 The expressions of CYP19A1, ERα, ERβ, GPR30 and TFF1 in normal bile duct of the tumor adjacent area and CCA tissues (n = 74; original magnification 200×). Arrow heads indicate normal bile ducts. Positive staining-immunoreactivities were represented with brown color. Blue color represented nucleus.

-Correlations of CYP19A1 with clinico-pathological data and estrogen-related proteins

Table 3 shows the correlations between CYP19A1 expression patterns and clinico-pathological data as well as estrogen-related protein expression patterns. CYP19A1 expression patterns were not correlated with the sex and age of CCA patients. The expression of CYP19A1 in CCA tissues was significantly correlated with metastatic status (r = 0.449 and P < 0.001; Pearson correlation) and GPR30 expression (r = 0.284 and P = 0.014; Pearson correlation).

Table 3 Correlations of CYP19A1 expression with estrogen- related protein expressions and clinico-pathological features

Value	CYP19A1 in CCA		
	Low (n=29)	High (n=45)	#*P*-value
Age			
< 57	15	21	0.671
≥ 57	14	24	
Sex			
Male	20	31	0.994
Female	9	14	
Metastasis status			
Non-metastasis	21	12	**<0.001**
Metastasis	8	33	
Histological type			
Tubular	12	27	**0.117**
Papillary	17	18	
Estrogen related protein			
ERα			
Low	7	9	0.673
High	22	36	
ERβ			
Low	18	19	**0.096**
High	11	26	
GPR30			
Low	23	23	**0.015**
High	6	22	
TFF1			
Low	17	18	**0.117**
High	12	27	

#*P*-value was analysis by Pearson Chi-square.

-Correlations between CYP19A1 expression levels and the survival rate of patients

CCA patients with high CYP19A1 expression in their tumor tissues showed no correlation with poor prognosis (P = 0.058; log-rank test) as shown in Figure. 14a. When CCA patients were divided into males and females, male CCA patients showed high expression of CYP19A1 in tumor tissues, which was significantly correlated with poor prognosis (P = 0.040; log-rank test) as shown in Figure. 14b. The estimated median survival time of male CCA patients with high expression of CYP19A1 (233 days) in the cancer tissues was shorter than that of CCA patients with low CYP19A1 expression (447 days). In contrast, no correlation between CYP19A1 expression patterns in tumor tissues and the survival rates was observed in female CCA patients (Figure. 14c) which can be explained by higher circulating concentrations of estrogens in women than in men, although the sample size of females in this study are not being enough to draw any solid conclusion. These results suggested that CYP19A1 plays essential roles in CCA progression and its expression can be used as a CCA prognostic marker, especially for male CCA patients.

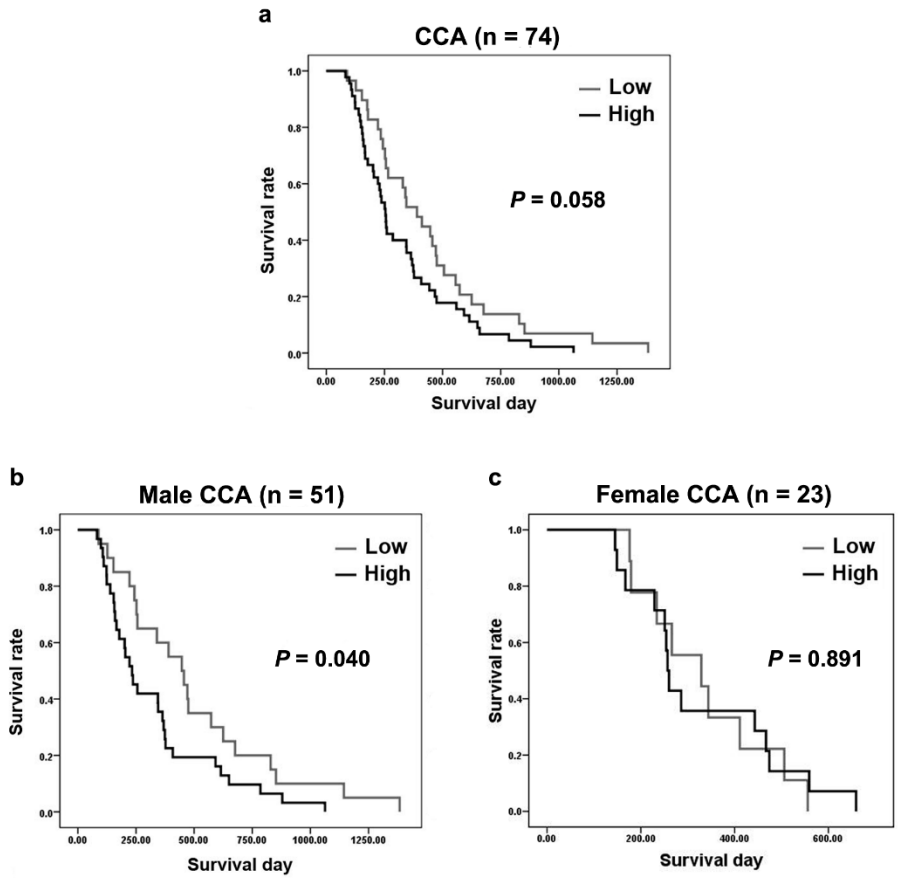

Figure 14 Kaplan-Meier analysis of CYP19A1 in (a) all human CCA tissues (n = 74), (b) male CCA tissues (n = 51) and (c) female CCA tissues (n = 23).

-Expressions of CYP19A1 and estrogen receptors in immortal cholangiocyte and CCA cell lines

The mRNA and protein expression levels of CYP19A1 in two CCA cell lines and in an immortalized cholangiocyte cell line, MMNK1, were examined using real time PCR and immunocytochemistry. CYP19A1 mRNA and protein levels were highly expressed in CCA cell lines (KKU-213 > KKU-100) compared to those in MMNK1 cell line (Figure. 15a and 15b). Likewise, ERα, ERβ and GPR30 were also highly expressed in all CCA cells compared to MMNK1 cells, according to the immunocytochemistry results (Figure. 15b).

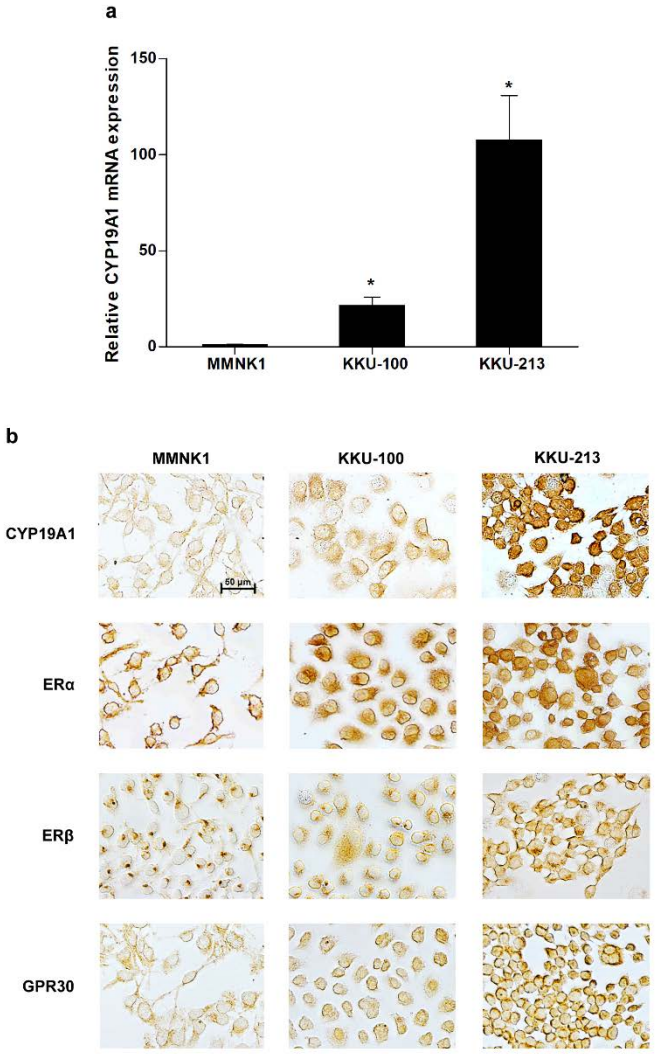

Figure 15 The expressions of CYP19A1, ERα, ERβ and GPR30 in cholangiocyte (MMNK1) and CCA (KKU-100 and KKU-213) cell lines. (a) Relative CYP19A1 mRNA expression levels detected by real time PCR. **P*-value < 0.05 compared to MMNK1. (b) CYP19A1, ERα, ERβ and GPR30 protein expressions detected by immunocytochemistry. Positive staining results were represented in brown color.

-Effect of CYP19A1 gene silencing by siRNA on cell proliferation and migration

Effect of CYP19A1 suppression by siRNA was examined in KKU-213, a high-CYP19A1-expressing CCA cell line. After silencing by specific siRNA (siCYP19A1), both CYP19A1 protein and mRNA levels were decreased at 72 h after treatment when compared with those treated with the scramble (Figure. 16a and 16b). Moreover, after siCYP19A1 transfection, the expression of BIRC5, an anti-apoptotic gene, was found to be decreased (Figure. 16c). Low expression levels of BIRC could be related to a decrease in CCA cell proliferation rates at 24, 48 and 72 h after re-seeding (Figure. 16d). However, adding exogenous estradiol to the culture media could restore the reduction of proliferative activity of CYP19A1-knockdown-KKU213 cells (Figure. 16e). On the other hand, siCYP19A1 treatment significantly could reduce the migration activity of cancer cells at 12 h after re-seeding (Figure. 16f and 16g). Thus, our results confirm that CYP19A1 increases estrogen generation, which leads to the up-regulation of the estrogen response and results in CCA progression with aggressive clinical outcomes.

-Effects of CYP19A1 inhibitors on cell viability of CCA cell lines

The effects of exemestane and letrozole on cell viability of two different CCA cell lines, i.e., CYP19A1-low-KKU-100 and CYP19A1-high-KKU-213, were investigated. The selected cell lines were treated with various concentrations of exemestane or letrozole for 48 h. The results showed that the calculated IC50 (mean ± SD) of CYP19A1 inhibitors for KKU-213 were 5.36 ± 2.68 μM of exemestane and 42.00 ± 16.35 μM of letrozole which are more sensitive than those of KKU-100 with IC50 76.42 ± 12.11 μM of exemestane and 125.59 ± 31.64 μM of letrozole (Figure. 17). These results suggest that both CYP19A1 inhibitors are more effective in suppressing cell viabilities of CYP19A1-high KKU-213 cells than those of CYP19A1-low KKU-100 cells.

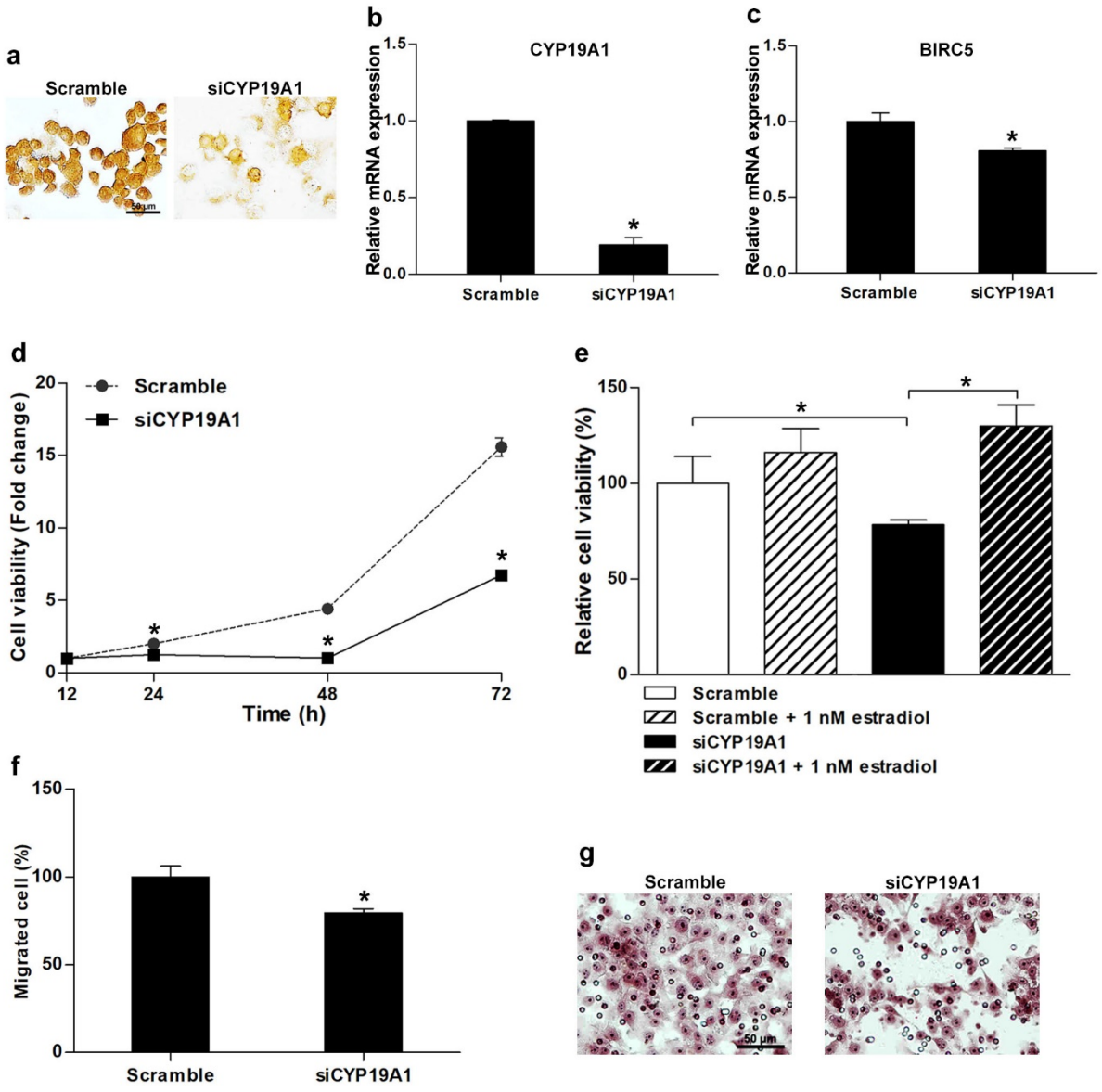

Figure 16 Effect of CYP19A1 silencing on KKU-213 CCA cell proliferation and migration between cells transfected with siCYP19A1 or scramble. (a) Expression levels of CYP19A1 protein (Brown staining) in cells treated with siCYP19A1 or scramble (b) Relative CYP19A1 mRNA expression levels (mean ± SD) detected by real time PCR. (c) Relative BIRC5 mRNA expression levels (mean ± SD) analyzed by real time PCR. (d) Cell viability assays (represented as fold change ± SD relative to scrambled control) in cells treated with siCYP19A1 or scramble. (e) Effect of 17β-estradiol on cell viability, represented as relative percentage ± SD, analyzed using MTT assay at 48 h post treatment. (f) Effect of siCYP19A1 on the cell migration expressed as a percentage of migrated cells (mean ± SD) at 12 h post migration and (g) hematoxylin staining of migrated cells (red-purple color) at 12 h post migration. * *P*-value < 0.05 compared to a scramble control (b, c, d, f). Scale bars are equal to 50 μm.

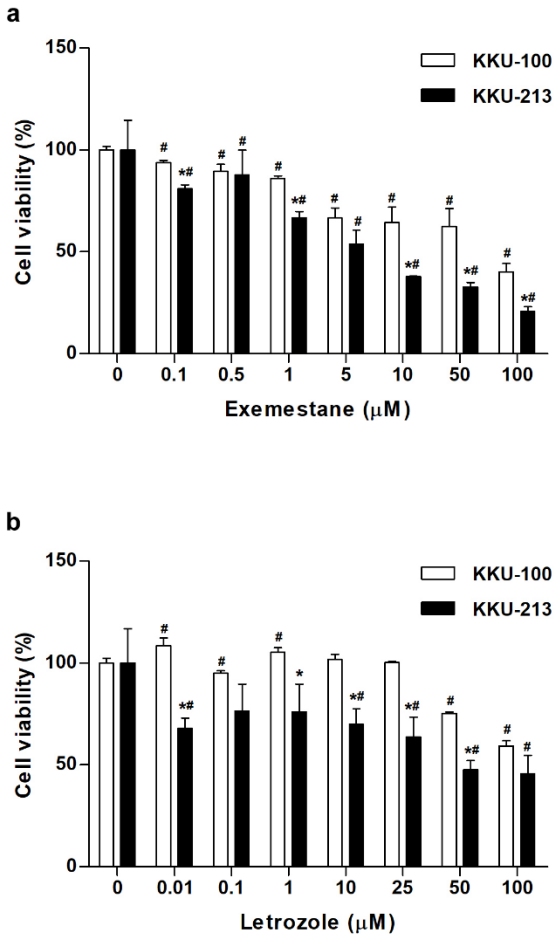

Figure 17 Effect of exemestane and letrozole on cell viability of CCA cell lines. The percentage of relative cell viabilities (mean ± SD) were investigated by MTT assay in KKU-100 and KKU-213 treated with various concentrations of exemestane or letrozole for 48 h. **P*-value < 0.05 compared to KKU-100 at the same concentrations. $^{\#}$*P*-value < 0.05 compared to 0 μM of drug in each cell type.

Part IV: Discussion

Estrogen receptors and estrogen responsive proteins play important roles in CCA progression via induction of cell proliferation, cell invasion and angiogenesis [30, 48, 49]. Moreover, serum estrogen levels of male CCA patients were significantly higher than in healthy male subjects [30]. The present results demonstrated that CYP19A1 was overexpressed in CCA cells compared with normal bile duct cells in the adjacent normal tissues. The majority of CCA patients have high expression levels of CYP19A1 and estrogen receptors (ERα, ERβ and/or GPR30). A positive correlation between the expression levels of CYP19A1 and estrogen receptors was observed. These results suggest that overexpressed CYP19A1 may up-regulate the

estrogen production by converting androgen to estrogen resulting in high concentrations of estrogen in CCA cells. These processes promote the induction of estrogen responses of CCA cells and could be, at least in part, a possible cause of elevated serum estrogen levels of CCA patients.

CYP19A1 over-expression is involved in the progression of various types of estrogen-related cancers such as breast, lung and bladder cancers [75-78]. In the present study, over-expression of CYP19A1 was significantly correlated with metastatic status of CCA patients. CYP19A1 may promote CCA progression through induction of cell invasion and migration. This finding was further confirmed by knockdown of CYP19A1 in CCA cell lines using specific siRNA. CYP19A1-silencing significantly reduced the cell migration activity of CCA cell lines. The reduction of cell migration activity was rescued by adding external estradiol to the cell culture media. Related to this, the expression of CYP19A1 in CCA tissues was not significantly correlated with poor prognosis in general. However, statistically significant correlation was observed in male CCA patients. These findings suggested that CYP19A1 is involved in CCA progression and is associated with aggressive clinical outcomes, especially in male CCA patients. Furthermore, CYP19A1 expression was positively correlated with GPR30 expression. GPR30 modulates both genomic transcriptional action and rapid non-genomic action of estrogen [79] and is involved in progression of estrogen-related cancers such as breast, endometrium and ovarian cancers through the activations of EGFR/MAPK and PI3K/Akt signaling pathways [80-82]. Since the EGFR/MAPK and PI3K/Akt signaling pathways are implicated in the progression of CCA [51, 83, 84], the activation of GPR30 by estrogen is associated with CCA progression via modulation of EGFR/MAPK and PI3K/Akt signaling pathways.

Since over-expression of CYP19A1 has been reported in estrogen-related cancers and CYP19A1 inhibitors including anastrazole, letrozole and exemestane, are currently in clinical use for breast cancer treatment [85]. Exemestane is known to reduce cell viability of breast cancer cells via activation of mitochondrial-mediated apoptosis and induction of autophagy [86]. This compound also inhibited tumorigenic properties and caused morphological changes of non-small cell lung cancer cell lines [78, 87]. On the other hand, letrozole could reduce tumor size and induce apoptosis of ER-positive breast cancer cells both in vitro and in vivo [77]. Additionally, plasma estrogen levels of postmenopausal women with breast cancer were down-regulated by CYP19A1 inhibitors [88, 89]. In the present study, two CYP19A1 inhibitors, exemestane and letrozole, could suppress cell proliferation of both high- and low-CYP19A1-expressing CCA cell lines. Drug sensitivities of the cell lines were related to the basal level of CYP19A1 expression. Similarly, silencing of CYP19A1 using specific siRNA reduced cell proliferation of CCA cells via down-regulation of BIRC5 which is an anti-apoptotic gene. This result was consistent with BIRC5 being known as an estrogen-inducible gene in human ovarian and breast cancer cells [90, 91]. Moreover, BIRC5 expression was associated with development and poor prognosis of CCA {Javle, 2004 #2119;Chang, 2004 #2336}, suggesting its anti-apoptotic role in CCA. Interestingly, the reduction of cell proliferation by siCYP19A1 was rescued by the addition of exogenous

estradiol. Therefore, in the case of CCA, it could be suggested that CYP19A1 promoted CCA cell proliferation and inhibited cell apoptosis via estrogen-related pathways.

Based on the aforementioned findings, we propose possible mechanisms by which CYP19A1 and downstream estrogen-related proteins can cause CCA progression. Briefly, the over-expression of CYP19A1 permits elevated conversion of androgen to estrogen, resulting in accumulation of estrogen in cancer cells and high serum estrogen levels. High estrogen levels can cause aberrant estrogen-ER interactions, which in turn can trigger the expressions of estrogen responsive genes via activating nuclear estrogen receptors (ERs) and/or membrane receptor (GPR30) signaling cascade and promoting ERs/GPR30-dependent transcription factor changes in response to estrogen, leading to CCA progression with aggressive clinical outcomes. Therefore, CYP19A1 inhibitors could be applied for the targeted therapy of CCA.

4. Summary

In summary, our results show that oxidative stress induces giant cell formation, EBF1 suppression and ZNF423 expression lead to increasing of oxidative stress-resistant, stem-like and tumorigenic properties of the cholangiocyte and cholangiocarcinoma cells. Moreover, chronic inflammation also induces CYP19A1 expression which consequently to over production of estrogen resulting in the induction of estrogen response. Taken together our results indicated that oxidative stress and estrogen are the synergistic factors that induces CCA progression with aggressive clinical outcomes (Figure 18). Moreover, EBF1 is vulnerable to oxidative stress, and that oxidative stress significantly suppresses EBF1 which can induce stem cell and migration properties of the cholangiocyte cells leading to CCA promotion and progression resulting in CCA development with aggressive clinical outcomes such as short survival time. Thus, EBF1 is an oxidative stress responsive gene that exerts a tumor suppressive function against CCA genesis. In next study, we will further identify EBF1-targeted genes in relation with tumorigenic, stem cell and oxidative stress-resistance properties. The outcome of next study is to determine the important linkage between oxidative stress and cancer stem-like cells in liver fluke-induced cholangiocarcinogenesis which could be applied to other model of oxidative-related carcinogenesis. Therefore, EBF1 and EBF1-targeted genes may be used as new therapeutic targets for CCA chemotherapy and chemoprevention.

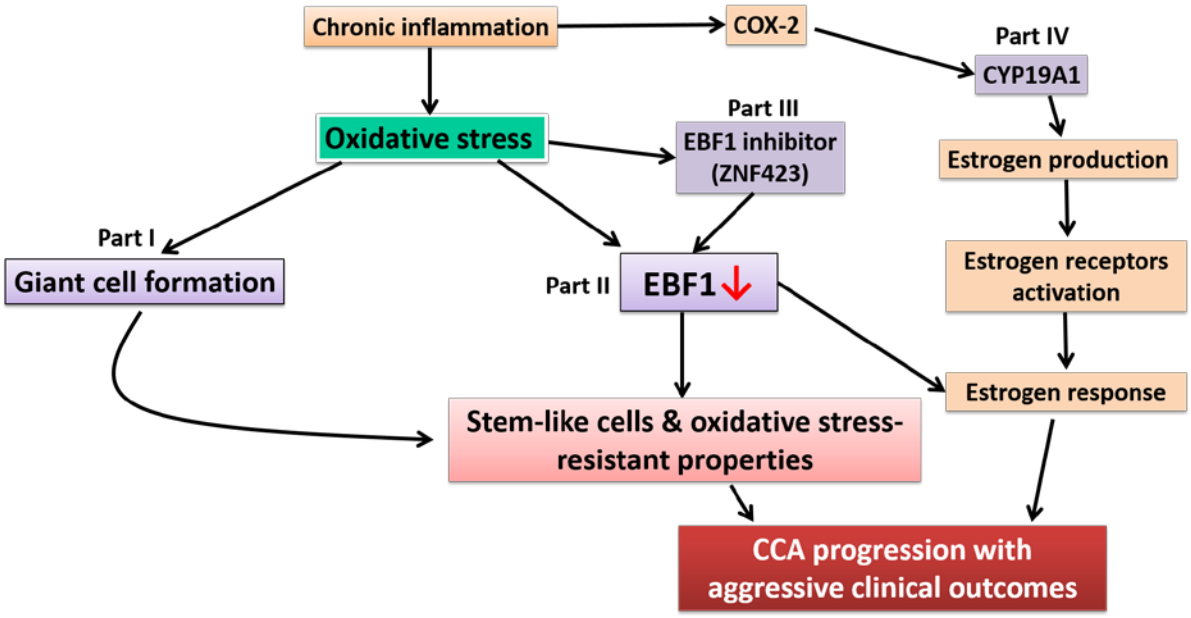

Figure 18 Summary of our finding in this project

5. Reference

[1] R. Thanan, S. Oikawa, Y. Hiraku, S. Ohnishi, N. Ma, S. Pinlaor, P. Yongvanit, S. Kawanishi, M. Murata, Oxidative stress and its significant roles in neurodegenerative diseases and cancer, International journal of molecular sciences, 16 (2015) 193-217.

[2] R. Thanan, C. Pairojkul, S. Pinlaor, N. Khuntikeo, C. Wongkham, B. Sripa, N. Ma, K. Vaeteewoottacharn, A. Furukawa, H. Kobayashi, Y. Hiraku, S. Oikawa, S. Kawanishi, P. Yongvanit, M. Murata, Inflammation-related DNA damage and expression of CD133 and Oct3/4 in cholangiocarcinoma patients with poor prognosis, Free radical biology & medicine, 65 (2013) 1464-1472.

[3] J.A. Fretz, T. Nelson, H. Velazquez, Y. Xi, G.W. Moeckel, M.C. Horowitz, Early B-cell factor 1 is an essential transcription factor for postnatal glomerular maturation, Kidney international, 85 (2014) 1091-1102.

[4] S. Garel, F. Marin, R. Grosschedl, P. Charnay, Ebf1 controls early cell differentiation in the embryonic striatum, Development, 126 (1999) 5285-5294.

[5] D.G. Hesslein, J.A. Fretz, Y. Xi, T. Nelson, S. Zhou, J.A. Lorenzo, D.G. Schatz, M.C. Horowitz, Ebf1-dependent control of the osteoblast and adipocyte lineages, Bone, 44 (2009) 537-546.

[6] K. Jin, M. Xiang, Ebf1 deficiency causes increase of Muller cells in the retina and abnormal topographic projection at the optic chiasm, Biochemical and biophysical research communications, 414 (2011) 539-544.

[7] D. Liao, Emerging roles of the EBF family of transcription factors in tumor suppression, Molecular cancer research : MCR, 7 (2009) 1893-1901.

[8] R. Thanan, A. Techasen, B. Hou, W. Jamnongkan, N. Armartmuntree, P. Yongvanit, M. Murata, Development and characterization of a hydrogen peroxide-resistant cholangiocyte cell line: A novel model of oxidative stress-related cholangiocarcinoma genesis, Biochemical and biophysical research communications, 464 (2015) 182-188.

[9] N.J. Ganem, Z. Storchova, D. Pellman, Tetraploidy, aneuploidy and cancer, Current opinion in genetics & development, 17 (2007) 157-162.

[10] S. Zhang, I. Mercado-Uribe, Z. Xing, B. Sun, J. Kuang, J. Liu, Generation of cancer stem-like cells through the formation of polyploid giant cancer cells, Oncogene, 33 (2014) 116-128.

[11] P. Sithithaworn, P. Yongvanit, K. Duenngai, N. Kiatsopit, C. Pairojkul, Roles of liver fluke infection as risk factor for cholangiocarcinoma, Journal of hepato-biliary-pancreatic sciences, 21 (2014) 301-308.

[12] S. Pinlaor, N. Ma, Y. Hiraku, P. Yongvanit, R. Semba, S. Oikawa, M. Murata, B. Sripa, P. Sithithaworn, S. Kawanishi, Repeated infection with Opisthorchis viverrini induces accumulation of 8-nitroguanine and 8-oxo-7,8-dihydro-2'-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase, Carcinogenesis, 25 (2004) 1535-1542.

[13] S. Pinlaor, P. Yongvanit, Y. Hiraku, N. Ma, R. Semba, S. Oikawa, M. Murata, B. Sripa, P. Sithithaworn, S. Kawanishi, 8-nitroguanine formation in the liver of hamsters infected with Opisthorchis viverrini, Biochemical and biophysical research communications, 309 (2003) 567-571.

[14] P. Yongvanit, S. Pinlaor, H. Bartsch, Oxidative and nitrative DNA damage: key events in opisthorchiasis-induced carcinogenesis, Parasitology international, 61 (2012) 130-135.

[15] S. Ohnishi, N. Ma, R. Thanan, S. Pinlaor, O. Hammam, M. Murata, S. Kawanishi, DNA damage in inflammation-related carcinogenesis and cancer stem cells, Oxidative medicine and cellular longevity, 2013 (2013) 387014.

[16] S. Pinlaor, Y. Hiraku, N. Ma, P. Yongvanit, R. Semba, S. Oikawa, M. Murata, B. Sripa, P. Sithithaworn, S. Kawanishi, Mechanism of NO-mediated oxidative and nitrative DNA damage in hamsters infected with Opisthorchis viverrini: a model of inflammation-mediated carcinogenesis, Nitric oxide : biology and chemistry, 11 (2004) 175-183.

[17] M. Murata, R. Thanan, N. Ma, S. Kawanishi, Role of nitrative and oxidative DNA damage in inflammation-related carcinogenesis, Journal of biomedicine & biotechnology, 2012 (2012) 623019.

[18] T. Ito, M. Sakurai-Yageta, A. Goto, C. Pairojkul, P. Yongvanit, Y. Murakami, Genomic and transcriptional alterations of cholangiocarcinoma, Journal of hepato-biliary-pancreatic sciences, 21 (2014) 380-387.

[19] N. Khuntikeo, N. Chamadol, P. Yongvanit, W. Loilome, N. Namwat, P. Sithithaworn, R.H. Andrews, T.N. Petney, S. Promthet, K. Thinkhamrop, C. Tawarungruang, B. Thinkhamrop, C. investigators, Cohort profile: cholangiocarcinoma screening and care program (CASCAP), BMC Cancer, 15 (2015) 459.

[20] B. Sripa, C. Pairojkul, Cholangiocarcinoma: lessons from Thailand, Current opinion in gastroenterology, 24 (2008) 349-356.

[21] J. Hagman, M.J. Gutch, H. Lin, R. Grosschedl, EBF contains a novel zinc coordination motif and multiple dimerization and transcriptional activation domains, The EMBO journal, 14 (1995) 2907-2916.

[22] J. Hagman, C. Belanger, A. Travis, C.W. Turck, R. Grosschedl, Cloning and functional characterization of early B-cell factor, a regulator of lymphocyte-specific gene expression, Genes & development, 7 (1993) 760-773.

[23] J. Hagman, K. Lukin, Early B-cell factor 'pioneers' the way for B-cell development, Trends in immunology, 26 (2005) 455-461.

[24] M. Kieslinger, S. Folberth, G. Dobreva, T. Dorn, L. Croci, R. Erben, G.G. Consalez, R. Grosschedl, EBF2 regulates osteoblast-dependent differentiation of osteoclasts, Developmental cell, 9 (2005) 757-767.

[25] L. Harder, G. Eschenburg, A. Zech, N. Kriebitzsch, B. Otto, T. Streichert, A.S. Behlich, K. Dierck, B. Klingler, A. Hansen, M. Stanulla, M. Zimmermann, E. Kremmer, C. Stocking, M.A. Horstmann, Aberrant ZNF423 impedes B cell differentiation and is linked to adverse outcome of ETV6-RUNX1 negative B precursor acute lymphoblastic leukemia, The Journal of experimental medicine, 210 (2013) 2289-2304.

[26] R.M. Neve, K. Chin, J. Fridlyand, J. Yeh, F.L. Baehner, T. Fevr, L. Clark, N. Bayani, J.P. Coppe, F. Tong, T. Speed, P.T. Spellman, S. DeVries, A. Lapuk, N.J. Wang, W.L. Kuo, J.L. Stilwell, D. Pinkel, D.G. Albertson, F.M. Waldman, F. McCormick, R.B. Dickson, M.D. Johnson, M. Lippman, S. Ethier, A. Gazdar, J.W. Gray, A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes, Cancer cell, 10 (2006) 515-527.

[27] S. Jones, X. Zhang, D.W. Parsons, J.C. Lin, R.J. Leary, P. Angenendt, P. Mankoo, H. Carter, H. Kamiyama, A. Jimeno, S.M. Hong, B. Fu, M.T. Lin, E.S. Calhoun, M. Kamiyama, K. Walter, T. Nikolskaya, Y. Nikolsky, J. Hartigan, D.R. Smith, M. Hidalgo, S.D. Leach, A.P. Klein, E.M. Jaffee, M. Goggins, A. Maitra, C. Iacobuzio-Donahue, J.R. Eshleman, S.E. Kern, R.H. Hruban, R. Karchin, N. Papadopoulos, G. Parmigiani, B. Vogelstein, V.E. Velculescu, K.W. Kinzler, Core signaling pathways in human pancreatic cancers revealed by global genomic analyses, Science, 321 (2008) 1801-1806.

[28] T.P. Le, M. Sun, X. Luo, W.L. Kraus, G.L. Greene, Mapping ERbeta genomic binding sites reveals unique genomic features and identifies EBF1 as an ERbeta interactor, PloS one, 8 (2013) e71355.

[29] J. Russo, I.H. Russo, The role of estrogen in the initiation of breast cancer, The Journal of steroid biochemistry and molecular biology, 102 (2006) 89-96.

[30] T. Hunsawong, E. Singsuksawat, N. In-chon, W. Chawengrattanachot, C. Thuwajit, B. Sripa, A. Paupairoj, S. Chau-in, P. Thuwajit, Estrogen is increased in male cholangiocarcinoma patients' serum and stimulates invasion in cholangiocarcinoma cell lines in vitro, Journal of cancer research and clinical oncology, 138 (2012) 1311-1320.

[31] J. Turner, M. Crossley, Mammalian Kruppel-like transcription factors: more than just a pretty finger, Trends in biochemical sciences, 24 (1999) 236-240.

[32] L. Harder, A.C. Puller, M.A. Horstmann, ZNF423: Transcriptional modulation in development and cancer, Molecular & cellular oncology, 1 (2014) e969655.

[33] A. Hata, J. Seoane, G. Lagna, E. Montalvo, A. Hemmati-Brivanlou, J. Massague, OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways, Cell, 100 (2000) 229-240.

[34] G. Masserdotti, A. Badaloni, Y.S. Green, L. Croci, V. Barili, G. Bergamini, M.L. Vetter, G.G. Consalez, ZFP423 coordinates Notch and bone morphogenetic protein signaling, selectively up-regulating Hes5 gene expression, The Journal of biological chemistry, 285 (2010) 30814-30824.

[35] O.A. MacDougald, M.D. Lane, Transcriptional regulation of gene expression during adipocyte differentiation, Annual review of biochemistry, 64 (1995) 345-373.

[36] Q.Y. Yang, J.F. Liang, C.J. Rogers, J.X. Zhao, M.J. Zhu, M. Du, Maternal obesity induces epigenetic modifications to facilitate Zfp423 expression and enhance adipogenic differentiation in fetal mice, Diabetes, 62 (2013) 3727-3735.

[37] K. Miyazaki, N. Yamasaki, H. Oda, T. Kuwata, Y. Kanno, M. Miyazaki, Y. Komeno, J. Kitaura, Z. Honda, S. Warming, N.A. Jenkins, N.G. Copeland, T. Kitamura, T. Nakamura, H. Honda, Enhanced expression of p210BCR/ABL and aberrant expression of Zfp423/ZNF423 induce blast crisis of chronic myelogenous leukemia, Blood, 113 (2009) 4702-4710.

[38] G.T. Chung, R.W. Lung, A.B. Hui, K.Y. Yip, J.K. Woo, C. Chow, C.Y. Tong, S.D. Lee, J.W. Yuen, S.W. Lun, K.K. Tso, N. Wong, S.W. Tsao, T.T. Yip, P. Busson, H. Kim, J.S. Seo, B. O'Sullivan, F.F. Liu, K.F. To, K.W. Lo, Identification of a recurrent transforming UBR5-ZNF423 fusion gene in EBV-associated nasopharyngeal carcinoma, The Journal of pathology, 231 (2013) 158-167.

[39] S. Huang, J. Laoukili, M.T. Epping, J. Koster, M. Holzel, B.A. Westerman, W. Nijkamp, A. Hata, S. Asgharzadeh, R.C. Seeger, R. Versteeg, R.L. Beijersbergen, R. Bernards, ZNF423 is critically required for retinoic acid-induced differentiation and is a marker of neuroblastoma outcome, Cancer cell, 15 (2009) 328-340.

[40] H.M. Bond, S. Scicchitano, E. Chiarella, N. Amodio, V. Lucchino, A. Aloisio, Y. Montalcini, M. Mesuraca, G. Morrone, ZNF423: A New Player in Estrogen Receptor-Positive Breast Cancer, Frontiers in endocrinology, 9 (2018) 255.

[41] J.N. Ingle, M. Liu, D.L. Wickerham, D.J. Schaid, L. Wang, T. Mushiroda, M. Kubo, J.P. Costantino, V.G. Vogel, S. Paik, M.P. Goetz, M.M. Ames, G.D. Jenkins, A. Batzler, E.E. Carlson, D.A. Flockhart, N. Wolmark, Y. Nakamura, R.M. Weinshilboum, Selective estrogen receptor modulators and pharmacogenomic variation in ZNF423 regulation of BRCA1 expression: individualized breast cancer prevention, Cancer discovery, 3 (2013) 812-825.

[42] J. Cui, Y. Shen, R. Li, Estrogen synthesis and signaling pathways during aging: from periphery to brain, Trends Mol Med, 19 (2013) 197-209.

[43] B. Chen, Y. Wang, S.E. Kane, S. Chen, Improvement of sensitivity to tamoxifen in estrogen receptor-positive and Herceptin-resistant breast cancer cells, J Mol Endocrinol, 41 (2008) 367-377.

[44] H. Niikawa, T. Suzuki, Y. Miki, S. Suzuki, S. Nagasaki, J. Akahira, S. Honma, D.B. Evans, S. Hayashi, T. Kondo, H. Sasano, Intratumoral estrogens and estrogen receptors in human non-small cell lung carcinoma, Clin Cancer Res, 14 (2008) 4417-4426.

[45] L.P. Stabile, A.L. Davis, C.T. Gubish, T.M. Hopkins, J.D. Luketich, N. Christie, S. Finkelstein, J.M. Siegfried, Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor alpha and beta and show biological responses to estrogen, Cancer Res, 62 (2002) 2141-2150.

[46] Z. Zhang, D. Zhou, Y. Lai, Y. Liu, X. Tao, Q. Wang, G. Zhao, H. Gu, H. Liao, Y. Zhu, X. Xi, Y. Feng, Estrogen induces endometrial cancer cell proliferation and invasion by regulating the fat mass and obesity-associated gene via PI3K/AKT and MAPK signaling pathways, Cancer letters, 319 (2012) 89-97.

[47] S.S. Shen, C.L. Smith, J.T. Hsieh, J. Yu, I.Y. Kim, W. Jian, G. Sonpavde, G.E. Ayala, M. Younes, S.P. Lerner, Expression of estrogen receptors-alpha and -beta in bladder cancer cell lines and human bladder tumor tissue, Cancer, 106 (2006) 2610-2616.

[48] A. Mancino, M.G. Mancino, S.S. Glaser, G. Alpini, A. Bolognese, L. Izzo, H. Francis, P. Onori, A. Franchitto, S. Ginanni-Corradini, E. Gaudio, D. Alvaro, Estrogens stimulate the proliferation of human cholangiocarcinoma by inducing the expression and secretion of vascular endothelial growth factor, Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver, 41 (2009) 156-163.

[49] D. Alvaro, B. Barbaro, A. Franchitto, P. Onori, S.S. Glaser, G. Alpini, H. Francis, L. Marucci, P. Sterpetti, S. Ginanni-Corradini, A. Onetti Muda, D.E. Dostal, A. De Santis, A.F. Attili, A. Benedetti, E. Gaudio, Estrogens and insulin-like growth factor 1 modulate neoplastic cell growth in human cholangiocarcinoma, The American journal of pathology, 169 (2006) 877-888.

[50] P. Thuwajit, W. Chawengrattanachot, C. Thuwajit, B. Sripa, F.E. May, B.R. Westley, N.N. Tepsiri, A. Paupairoj, S. Chau-In, Increased TFF1 trefoil protein expression in Opisthorchis viverrini-associated cholangiocarcinoma is important for invasive promotion, Hepatol Res, 37 (2007) 295-304.

[51] K. Kosriwong, T.R. Menheniott, A.S. Giraud, P. Jearanaikoon, B. Sripa, T. Limpaiboon, Trefoil factors: tumor progression markers and mitogens via EGFR/MAPK activation in cholangiocarcinoma, World journal of gastroenterology, 17 (2011) 1631-1641.

[52] R. Thanan, M. Murata, N. Ma, O. Hammam, M. Wishahi, T. El Leithy, Y. Hiraku, S. Oikawa, S. Kawanishi, Nuclear localization of COX-2 in relation to the expression of stemness markers in urinary bladder cancer, Mediators of inflammation, 2012 (2012) 165879.

[53] M. Maruyama, N. Kobayashi, K.A. Westerman, M. Sakaguchi, J.E. Allain, T. Totsugawa, T. Okitsu, T. Fukazawa, A. Weber, D.B. Stolz, P. Leboulch, N. Tanaka, Establishment of a highly differentiated immortalized human cholangiocyte cell line with SV40T and hTERT, Transplantation, 77 (2004) 446-451.

[54] Y. Nakanuma, Y. Sato, K. Harada, M. Sasaki, J. Xu, H. Ikeda, Pathological classification of intrahepatic cholangiocarcinoma based on a new concept, World journal of hepatology, 2 (2010) 419-427.

[55] B.R. Blechacz, G.J. Gores, Cholangiocarcinoma, Clinics in liver disease, 12 (2008) 131-150, ix.

[56] S.A. Khan, B.R. Davidson, R. Goldin, S.P. Pereira, W.M. Rosenberg, S.D. Taylor-Robinson, A.V. Thillainayagam, H.C. Thomas, M.R. Thursz, H. Wasan, G. British Society of, Guidelines for the diagnosis and treatment of cholangiocarcinoma: consensus document, Gut, 51 Suppl 6 (2002) VI1-9.

[57] B. Blechacz, M. Komuta, T. Roskams, G.J. Gores, Clinical diagnosis and staging of cholangiocarcinoma, Nature reviews. Gastroenterology & hepatology, 8 (2011) 512-522.

[58] K.J. Stabile, H.J. Pfaeffle, M.M. Tomaino, The Essex-Lopresti fracture-dislocation factors in early management and salvage alternatives, Hand Clin, 18 (2002) 195-204.

[59] K.V. Donkena, C.Y. Young, D.J. Tindall, Oxidative stress and DNA methylation in prostate cancer, Obstetrics and gynecology international, 2010 (2010) 302051.

[60] W. Wongpaiboonwattana, P. Tosukhowong, T. Dissayabutra, A. Mutirangura, C. Boonla, Oxidative stress induces hypomethylation of LINE-1 and hypermethylation of the RUNX3 promoter in a bladder cancer cell line, Asian Pacific journal of cancer prevention : APJCP, 14 (2013) 3773-3778.

[61] L. Li, S. Borges, R.D. Jason, C. Shen, Z. Desta, D. Flockhart, A penalized mixture model approach in genotype/phenotype association analysis for quantitative phenotypes, Cancer Inform, 9 (2010) 93-103.

[62] T. Reya, S.J. Morrison, M.F. Clarke, I.L. Weissman, Stem cells, cancer, and cancer stem cells, Nature, 414 (2001) 105-111.

[63] H. Kikuchi, M. Nakayama, Y. Takami, F. Kuribayashi, T. Nakayama, EBF1 acts as a powerful repressor of Blimp-1 gene expression in immature B cells, Biochemical and biophysical research communications, 422 (2012) 780-785.

[64] J.M. Stephens, The fat controller: adipocyte development, PLoS biology, 10 (2012) e1001436.

[65] T. Roskams, Liver stem cells and their implication in hepatocellular and cholangiocarcinoma, Oncogene, 25 (2006) 3818-3822.

[66] N. Armartmuntree, M. Murata, A. Techasen, P. Yongvanit, W. Loilome, N. Namwat, C. Pairojkul, C. Sakonsinsiri, S. Pinlaor, R. Thanan, Prolonged oxidative stress down-regulates Early B cell factor 1 with inhibition of its tumor suppressive function against cholangiocarcinoma genesis, Redox biology, 14 (2018) 637-644.

[67] J.S. Beckman, T.W. Beckman, J. Chen, P.A. Marshall, B.A. Freeman, Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide, Proceedings of the National Academy of Sciences of the United States of America, 87 (1990) 1620-1624.

[68] C.H. Coyle, K.N. Kader, Mechanisms of H2O2-induced oxidative stress in endothelial cells exposed to physiologic shear stress, ASAIO journal, 53 (2007) 17-22.

[69] W.A. Pryor, G.L. Squadrito, The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide, The American journal of physiology, 268 (1995) L699-722.

[70] K.A. Kang, K.C. Kim, S.C. Bae, J.W. Hyun, Oxidative stress induces proliferation of colorectal cancer cells by inhibiting RUNX3 and activating the Akt signaling pathway, International journal of oncology, 43 (2013) 1511-1516.

[71] S. Reuter, S.C. Gupta, M.M. Chaturvedi, B.B. Aggarwal, Oxidative stress, inflammation, and cancer: how are they linked?, Free radical biology & medicine, 49 (2010) 1603-1616.

[72] H. Gronemeyer, A. Zelent, Fingering modulators of retinoic acid signaling identifies new prognostic marker for neuroblastoma, Cancer cell, 15 (2009) 249-251.

[73] G.L. Huang, W. Zhang, H.Y. Ren, X.Y. Shen, Q.X. Chen, D.Y. Shen, Retinoid X receptor alpha enhances human cholangiocarcinoma growth through simultaneous activation of Wnt/beta-catenin and nuclear factor-kappaB pathways, Cancer science, 106 (2015) 1515-1523.

[74] G.L. Huang, Q. Luo, G. Rui, W. Zhang, Q.Y. Zhang, Q.X. Chen, D.Y. Shen, Oncogenic activity of retinoic acid receptor gamma is exhibited through activation of the Akt/NF-kappaB and Wnt/beta-catenin pathways in cholangiocarcinoma, Molecular and cellular biology, 33 (2013) 3416-3425.

[75] S.E. Bulun, D. Chen, M. Lu, H. Zhao, Y. Cheng, M. Demura, B. Yilmaz, R. Martin, H. Utsunomiya, S. Thung, E. Su, E. Marsh, A. Hakim, P. Yin, H. Ishikawa, S. Amin, G. Imir, B. Gurates, E. Attar, S. Reierstad, J. Innes, Z. Lin, Aromatase excess in cancers of breast, endometrium and ovary, The Journal of steroid biochemistry and molecular biology, 106 (2007) 81-96.

[76] D.P. Nguyen, P. O'Malley, B. Al Hussein Al Awamlh, M.A. Furrer, N.P. Mongan, B.D. Robinson, G.J. Wang, D.S. Scherr, Association of Aromatase With Bladder Cancer Stage and Long-Term Survival: New Insights Into the Hormonal Paradigm in Bladder Cancer, Clin Genitourin Cancer, (2016).

[77] K.D. Mukhopadhyay, Z. Liu, A. Bandyopadhyay, N.B. Kirma, R.R. Tekmal, S. Wang, L.Z. Sun, Aromatase expression increases the survival and malignancy of estrogen receptor positive breast cancer cells, PloS one, 10 (2015) e0121136.

[78] E. Giannopoulou, K.E. Siatis, D. Metsiou, I. Kritikou, D.J. Papachristou, M. Kalofonou, A. Koutras, G. Athanassiou, H.P. Kalofonos, The inhibition of aromatase alters the mechanical and rheological properties of non-small-cell lung cancer cell lines affecting cell migration, Biochim Biophys Acta, 1853 (2015) 328-337.

[79] D. Wang, L. Hu, G. Zhang, L. Zhang, C. Chen, G protein-coupled receptor 30 in tumor development, Endocrine, 38 (2010) 29-37.

[80] E.J. Filardo, J.A. Quinn, A.R. Frackelton, Jr., K.I. Bland, Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis, Mol Endocrinol, 16 (2002) 70-84.

[81] S. Fujiwara, Y. Terai, H. Kawaguchi, M. Takai, S. Yoo, Y. Tanaka, T. Tanaka, S. Tsunetoh, H. Sasaki, M. Kanemura, A. Tanabe, Y. Yamashita, M. Ohmichi, GPR30 regulates the EGFR-Akt cascade and predicts lower survival in patients with ovarian cancer, J Ovarian Res, 5 (2012) 35.

[82] X. Ge, R. Guo, Y. Qiao, Y. Zhang, J. Lei, X. Wang, L. Li, D. Hu, The G protein-coupled receptor GPR30 mediates the nontranscriptional effect of estrogen on the activation of PI3K/Akt pathway in endometrial cancer cells, Int J Gynecol Cancer, 23 (2013) 52-59.

[83] S. Padthaisong, M. Thanee, A. Techasen, N. Namwat, P. Yongvanit, A. Liwatthakun, K. Hankla, S. Sangkhamanon, W. Loilome, Nimotuzumab Inhibits Cholangiocarcinoma Cell Metastasis via Suppression of the Epithelial-Mesenchymal Transition Process, Anticancer Res, 37 (2017) 3591-3597.

[84] S. Yothaisong, H. Dokduang, A. Techasen, N. Namwat, P. Yongvanit, V. Bhudhisawasdi, A. Puapairoj, G.J. Riggins, W. Loilome, Increased activation of PI3K/AKT signaling pathway is associated with cholangiocarcinoma metastasis and PI3K/mTOR inhibition presents a possible therapeutic strategy, Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine, 34 (2013) 3637-3648.

[85] S. Chumsri, Clinical utilities of aromatase inhibitors in breast cancer, Int J Womens Health, 7 (2015) 493-499.

[86] C. Amaral, M. Borges, S. Melo, E.T. da Silva, G. Correia-da-Silva, N. Teixeira, Apoptosis and autophagy in breast cancer cells following exemestane treatment, PloS one, 7 (2012) e42398.

[87] A. Koutras, E. Giannopoulou, I. Kritikou, A. Antonacopoulou, T.R. Evans, A.G. Papavassiliou, H. Kalofonos, Antiproliferative effect of exemestane in lung cancer cells, Mol Cancer, 8 (2009) 109.

[88] J. Geisler, N. King, G. Anker, G. Ornati, E. Di Salle, P.E. Lonning, M. Dowsett, In vivo inhibition of aromatization by exemestane, a novel irreversible aromatase inhibitor, in postmenopausal breast cancer patients, Clin Cancer Res, 4 (1998) 2089-2093.

[89] J. Geisler, B. Haynes, G. Anker, M. Dowsett, P.E. Lonning, Influence of letrozole and anastrozole on total body aromatization and plasma estrogen levels in postmenopausal breast cancer patients evaluated in a randomized, cross-over study, J Clin Oncol, 20 (2002) 751-757.

[90] J. Zhu, X. Lu, K.Q. Hua, H. Sun, Y.H. Yu, Y.J. Feng, Oestrogen receptor alpha mediates 17beta-estradiol enhancement of ovarian cancer cell motility through up-regulation of survivin expression, Arch Gynecol Obstet, 286 (2012) 729-737.

[91] X. Yu, X. Zhang, I.B. Dhakal, M. Beggs, S. Kadlubar, D. Luo, Induction of cell proliferation and survival genes by estradiol-repressed microRNAs in breast cancer cells, BMC Cancer, 12 (2012) 29.

6. Research outcomes

1. N. Armartmuntree, M. Murata, A. Techasen, P. Yongvanit, W. Loilome, N. Namwat, C. Pairojkul, C. Sakonsinsiri, S. Pinlaor, R. Thanan*, Prolonged oxidative stress down-regulates Early B cell factor 1 with inhibition of its tumor suppressive function against cholangiocarcinoma genesis, Redox Biol 14 (2018) 637-644. (Impact factor 2017= 7.126, Q1 Biochemistry and Molecular Biology)

2. W. Kaewlert, C. Sakonsinsiri, N. Namwat, K. Sawanyawisuth, P. Ungarreevittaya, N. Khuntikeo, N. Armartmuntree, R. Thanan*, The Importance of CYP19A1 in Estrogen Receptor-Positive Cholangiocarcinoma, Horm Cancer 9 (2018) 408-419. (Impact factor 2017= 2.5, Q2 Oncology)

7. Appendix

7.1 Reprints of accepted manuscripts

7.2 In preparation manuscripts

7.3 List of awards

7.4 List of oral and poster presentation

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Contents lists available at ScienceDirect

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Research paper

Prolonged oxidative stress down-regulates Early B cell factor 1 with inhibition of its tumor suppressive function against cholangiocarcinoma genesis

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ABSTRACT

Early B cell factor 1 (EBF1) is a transcription factor involved in the differentiation of several stem cell lineages and it is a negative regulator of estrogen receptors. EBF1 is down-regulated in many tumors, and is believed to play suppressive roles in cancer promotion and progression. However, the functional roles of EBF1 in carcinogenesis are unclear. Liver fluke-infection-associated cholangiocarcinoma (CCA) is an oxidative stress-driven cancer of bile duct epithelium. In this study, we investigated EBF1 expression in tissues from CCA patients, CCA cell lines (KKU-213, KKU-214 and KKU-156), cholangiocyte (MMNK1) and its oxidative stress-resistant (ox-MMNK1-L) cell lines. The formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) was used as an oxidative stress marker. Our results revealed that EBF1 expression was suppressed in cancer cells compared with the individual normal bile duct cells at tumor adjacent areas of CCA tissues. CCA patients with low EBF1 expression and high formation of 8-oxodG were shown to correlate with poor survival. Moreover, EBF1 was suppressed in the oxidative stress-resistant cell line and all of CCA cell lines compared to the cholangiocyte cell line. This suggests that prolonged oxidative stress suppressed EBF1 expression and the reduced EBF1 level may facilitate CCA genesis. To elucidate the significance of EBF1 suppression in CCA genesis, EBF1 expression of the MMNK1 cell line was down-regulated by siRNA technique, and its effects on stem cell properties (CD133 and Oct3/4 expressions), tumorigenic properties (cell proliferation, wound healing and cell migration), estrogen responsive gene (TFF1), estrogen-stimulated wound healing, and cell migration were examined. The results showed that CD133, Oct3/4 and TFF1 expression levels, wound healing, and cell migration of EBF1 knockdown-MMNK1 cells were significantly increased. Also, cell migration of EBF1-knockdown cells was significantly enhanced after 17β-estradiol treatment. Our findings suggest that EBF1 down-regulation via oxidative stress induces stem cell properties, tumorigenic properties and estrogen responses of cholangiocytes leading to CCA genesis with aggressive clinical outcomes.

1. Introduction

Infection and inflammation play important roles in cancer development. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are key players in inflammation-related cancers. Oxidative stress is an imbalance of oxidants and anti-oxidant systems that cause overproduction of ROS and RNS. Cholangiocarcinoma (CCA) is a cancer that has bile duct epithelial cell phenotypes. One of the established risk factors for CCA is chronic inflammation of cholangiocytes triggered by infection by the liver fluke, *Opisthorchis viverrini*, that is commonly found in northeast Thailand [1]. Chronic inflammation induced by *O. viverrini* infection clearly increased oxidative stress through the highly formation of DNA damage lesions in the bile duct epithelium cells [2,3]. Oxidative stress causes oxidative damage to biomolecules, tissue

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remodeling and alteration of gene expressions which are involved in all stages of CCA development [4]. Interestingly, it can result not only in damage to numerous biomolecules that leads to DNA mutation, but it can also induce epigenetic changes and stem cells activation for tissue remodeling [5,6]. Under cellular bombardment by ROS and RNS, most cells die, whereas some can adapt to survive, defined as “oxidative stress-resistant cells” [7]. The induced oxidative stress-resistant cholangiocyte cells gain the properties of tumor genesis such as high proliferation rate [7]. Therefore, many studies strongly support that oxidative stress is the major cause of CCA development which is induced by chronic inflammation [2,4,8]. However, the oxidative stress underlining mechanisms and targeted molecules have been under-estimated to date.

Early B cell factor 1 (EBF1) is a novel transcriptional factor which recognizes the mb-1 promoter region and is strongly expressed in the early stage of B cell development [9,10]. EBF1 possesses a number of biological functions in several developmental pathways, for example, EBF1 has been mainly involved in the B cell differentiation [11], bone development [12], adipogenesis [13], retinal cell differentiation [14] and kidney development [15]. Additionally, EBF1 plays an important role in the differentiation of several stem cells to mature cells. Therefore, we proposed that EBF1 may associate with stem cell activation in the process of tissue injury through increased stem cell differentiation, leading to mature cells for used in the tissue repaired process; whereas down-regulation of EBF1 may inhibit stem cell differentiation, leading to increased stem cell properties which may be involved in tumor cell transformation.

Recently, down-regulation of EBF1 has been found in many tumors, and EBF1 is believed to play suppressive roles in cancer promotion and progression. Down-regulation of EBF1 by ZNF423 expression (EBF1 inhibitor) has been shown to induce B cell maturation arrest, leading to promotion and progression of various types of leukemia such as acute lymphoblastic leukemia (ALL) [16]. Moreover, mono-allelic deletions of EBF1 may contribute to block differentiation of mature B cells which lead to leukaemogenesis via increasing of immature B cells that are hallmarks of ALL [17]. EBF1 was also found to be suppressed in solid cancers of which EBF1 suppression could be achieved in different ways, such as the genomic loss of 5q32 which encodes for EBF1 in breast cancer [18]. In addition, somatic missense mutation that causes the amino acid substitution of arginine for glutamine at position 242 located on DNA binding domain of EBF1 contributes to the EBF1 suppression in pancreatic ductal adenocarcinoma [19]. Interestingly, EBF1 had been proposed to be the negative regulator of estrogen receptors (ERs) [20], and ERs were reported to promote carcinogenesis including CCA [21,22]. These findings lead us to hypothesize that the down-regulation of EBF1 may play a crucial role in tumor promotion and progression via the induction of estrogen response.

In order to test whether the oxidative stress may suppress the expression of EBF1, contribution to induce CCA promotion and progression via inductions of stem cell properties, tumorigenic properties and estrogen response, the expression and function of EBF1 were analyzed in CCA tissues and cell lines. We investigated the correlation of EBF1 expression and 8-oxodG formation in CCA tissues by immunohistochemical analysis. The functional analysis related to stem cell properties including CD133 and Oct3/4 expressions, cell surviving under oxidative stress, tumorigenic properties including cell proliferation, wound healing, cell migration and estrogen response of EBF1 down-regulation was studied by siRNA technique using highly EBF1 expressing cell line (MMNK1).

2. Materials and methods

2.1. Human cholangiocarcinoma tissues

Cholangiocarcinoma tissues were collected from CCA patients admitted at the surgical wards of Srinagarind Hospital, Khon Kaen University. The study was approved by the Ethics Committee for Human Research, Khon Kaen University (HE571283). The paraffin-embedded CCA tissues were used for immunohistochemistry (n = 75). All samples were obtained from the specimen bank of the Cholangiocarcinoma Research Institute, Khon Kaen University.

2.2. Immunohistochemistry

Immunohistochemical analysis was performed to determine the expression pattern of EBF1 and the formation of 8-oxodG. The paraffin-embedded human liver CCA tissues were de-paraffinized and rehydrated with stepwise-decreasing concentration of ethanol. Antigen retrieval was performed using a microwave (Sharp Microwave Oven, R-129, Thailand) treatment in 10 mM sodium citrate buffer with 0.5% Tween pH 6.0 at low power setting for 10 min, then sections were immersed for 30 min in 0.3% (v/v) H_2O_2-containing phosphate-buffered saline (PBS) for endogenous hydrogen peroxide activity blocking. Non-specific binding was blocked using 10% skim milk in PBS for 30 min. Sections were incubated with the primary antibodies, [2.5 μg/ml of rabbit anti-EBF1 polyclonal antibody (Sigma-Aldrich Corp, MO, USA), or 0.1 μg/ml mouse anti-8-oxodG monoclonal antibody (Japan Institute for the Control of Aging, Shizuoka, Japan)] at room temperature for overnight. The sections were washed in PBS with 0.1% Tween (three times) and incubated with peroxidase-conjugated Envision™ secondary antibody (DAKO, Glostrup, Denmark) at room temperature for 1 h. After washing in PBS with 0.1% Tween (three times), the color was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Vector Laboratories, Inc., CA, USA) for 6 min, then counter stained with Mayer's haematoxylin. The sections were dehydrated with stepwise-increasing concentrations of ethanol and mounted with permounting solution. The stained sections were examined under a light microscope.

The immune-reactivity was evaluated by calculating the total immunostaining index (IHC score) as the product of frequency and intensity score. The frequency score described the estimated fraction of positive stained tumor cells (0 = none; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; 4 = > 75%). The intensity score represented the estimated staining intensity (0 = negative staining; 1 = weak; 2 = moderate; 3 = strong). These scores were calculated by multiplying the frequency score and intensity score. The IHC score ranged from 0 to 12. The mean of the IHC score was defined as the cut-off value of low and high expression [23]. In the present study, 8-oxodG levels were measured semi-quantitatively using IHC method. The main reason is the limited availability of sufficient amounts of sample specimens to extract DNA and measure 8-oxodG using HPLC coupled with electrochemical detector (HPLC-ECD) simultaneously. We already confirmed in our previous studies that the formation of 8-oxodG in the livers of liver fluke-infected hamsters [3], and the increase of 8-oxodG in human cholangiocarcinoma tissues [24] could be detected by both IHC and HPLC-ECD with the comparable results.

2.3. Cell lines and cell culture

CCA cell lines, KKU-213, KKU-214 and KKU-156 were established in-house from the tumor of CCA patients of Srinagarind Hospital, Khon Kaen University. The immortalized cholangiocyte cell line, MMNK1 was established and characterized at Okayama University [25]. Ox-MMNK1-L cells were established and characterized by our previous study [7]. All cell lines were cultured in Ham F′12 (Invitrogen, CA, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 g/ml streptomycin (complete medium) at 37 °C in a humidified incubator maintained with an atmosphere of 5% CO_2. A subculture was conducted when the cells reached the confluent stage and the media were changed once every two days.

2.4. Immumocytochemistry of cell lines

MMNK1 (60,000 cells/well), ox-MMNK1-L (60,000 cells/well), CCA cell lines (30,000 cells/well) were placed on 48-well plates for overnight. The cells were fixed with 4% paraformaldehyde-containing PBS for 30 min at room temperature. After washing, 0.2% (v/v) Triton-X100 solution was added. The cells were washed with PBS and incubated for 30 min with PBS containing 0.3% (v/v) hydrogen peroxide for endogenous hydrogen peroxide activity blocking and non-specific binding was blocked by 3% (w/v) BSA in PBS for 30 min. Cells were incubated with 10 μg/ml of rabbit anti-EBF1 polyclonal antibody (Abcam, MA, USA) or 9 μg/ml of rabbit anti-CD133 (Abcam, MA, USA) at room temperature for overnight followed by peroxidase-conjugated Envision™ secondary antibody (DAKO, Glostrup, Denmark). The color was developed with DAB substrate kit (Vector Laboratories, Inc., CA, USA) and washed with distilled water. The stained cells were dehydrated with stepwise (5 min/step) increasing concentrations of ethanol (70%→80%→90%→100%) and air dried overnight. The stained cells were examined under an inverted microscope.

2.5. EBF1 knockdown by siRNA

MMNK1 cell line was maintained in the culture medium without penicillin and streptomycin at 37 °C in a humidified incubator maintained at an atmosphere of 5% CO_2. The cells were transfected with siRNA (ON-TARGETplus Human EBF1 siRNA, Dharmacon, CO, USA) using Lipofectamine RNAiMAX® (Thermo Fisher Scientific, MA, USA) in 6-well plates with 10^5 cells for 48-h transfection. Cells were then harvested by trypsinization for RNA extraction, immunocytochemistry technique, wound healing and migration assay. Untreated cells were used as the negative control. Lipofectamine-treated cells were used as vehicle control.

2.6. Detection of mRNA levels by real time PCR

Total RNA was isolated from cell pellets with Trizol® reagent (Invitrogen, CA, USA) following the manufacturer's protocol. The quality of RNA was assessed with NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, DE, USA). Then, 2 μg total RNA was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) following the manufacturer's protocol. EBF1, CD133, Oct3/4, TFF1 and β-actin mRNA expression levels were analyzed with Taqman gene expression assay using Taqman probes (EBF1, Hs00395513_m1, CD133, Hs01009257_m1, Oct3/4, Hs04260367_gH, TFF1, Hs00907239_m1 and β-actin, Hs99999903_m1) on an ABI-7500 real time PCR system (Applied Biosystems, CA, USA). Relative mRNA expression (fold changes) was analyzed with a cycle threshold (Ct) in the linear range of amplification and using β-actin as an internal control.

2.7. Wound healing assay

Cells were cultured in complete medium at 37 °C in a humidified incubator maintained at an atmosphere of 5% CO_2 in 24 well plates until cells were confluent or nearly (> 90%) confluent. Cell monolayers were scratched by using a 200-μl pipette tip, and then rinsed three times with PBS to remove cell debris. Cell migration in the wound area was observed by phase contrast microscopy at 0–31 h and digitally photographed. Wound healing was measured on the images and the migration area was calculated by the area of original wound minus the area of wound during healing divided by the area of original wound.

2.8. Cell migration assay and estrogen treatment

The cell migration assay was performed using a Boyden transwell chamber consisting of a membrane filter insert in 24-well plate with 8-μm pore size (Corning, NY, USA). For the functional experiment, 4×10^4 cells at 48 h after knockdown experiments (media, lipofectamine and siEBF1) were plated into the insert upper chamber with serum free medium. At the lower chamber, complete medium was added and the cells were incubated for 24 h, whereupon, non-migrating cells in the upper chamber were removed. Migrating cells that attached at the underside of the filter were fixed with absolute methanol for 1 h and stained with haematoxylin for overnight. The transwell membrane was allowed to dry and the quantification of migrating cells was analyzed by counting under a light microscope.

For estrogen response experiment, the MMNK1 cells of 48 h after knockdown experiments (media, lipofectamine and siEBF1) were pretreated with or without 1 nM 17β-estradiol (Sigma Aldrich, MO, USA) for 30 min before seeding into the insert upper chamber with serum free medium with or without 1 nM 17β-estradiol and the lower chamber with Ham F′12 supplemented with 5% fetal calf serum, 100 U/ml penicillin, and 100 g/ml streptomycin for 12 h. Then, non-migrating cells in the upper chamber were removed. Migrating cells that attached at the underside of the filter were fixed with absolute methanol for 1 h and stained with haematoxylin for overnight. The transwell membrane was allowed to dry and the quantification of migrating cells was analyzed by counting under a light microscope.

2.9. Hydrogen peroxide treatment

To confirm whether EBF1 down-regulation is involved in oxidative stress-resistant property; 1×10^4 cells at 48 h after EBF1 knockdown treatment (media, lipofectamine and siEBF1) were plated into 96 well plates in triplicate. The cells were maintained in the complete media at 37 °C in a humidified incubator maintained at an atmosphere of 5% CO_2. After 12 h, the cells were treated with complete media containing various concentrations of H_2O_2 (0, 10, 25, 50, 100, 200, 300, 400, and 500 μM) for 48 h. Then, the number of viable cells was measured using a standard MTT method. In brief, 100 μl MTT (Sigma-Aldrich Corp, MO, USA) was added to cells with a final concentration of 0.5 mg/ml, and incubated at 37 °C for 4 h. After that the MTT solution was removed and replaced with 100 μl of DMSO to dissolve the dark blue crystals and measure the optical density (OD) at 540 nm using a microplate reader.

2.10. Statistical analysis

Statistical analysis was performed using SPSS software version 17.0 (IBM Corporation, USA). The associations of protein expressions and 8-oxodG formation in CCA tissues with patients' clinico-pathological factors were assessed by Fisher's exact test. The survival analysis was performed using Kaplan-Meier estimate with Log-rank test. Levels of mRNA and protein expressions were compared by Student's *t*-test. A *P*-value < 0.05 was considered as statistical significance.

3. Results

3.1. Immunohistochemical analysis of EBF1 expression and 8-oxodG formation

Fig. 1 shows the immunoreactivities of EBF1 expression and 8-oxodG formation obtained. The expression of EBF1 was predominantly detected in cytoplasm and nucleus of hepatocytes and normal bile ducts in non-tumor areas, while it was weakly observed in hyperplasia and tumor areas. Low EBF1 expression was found in 69% (52/75) of CCA tissues. Additionally, the immunoreactivity of EBF1 staining in CCA cancer cells was significantly reduced compared to the adjacent normal bile ducts ($P < 0.001$, graph not shown). 8-oxodG was highly detected in the nucleus of hyperplasia bile ducts and the cancer cells compared with the individual normal bile ducts. High formation of 8-oxodG was detected in 53% (40/75) of CCA tissues.

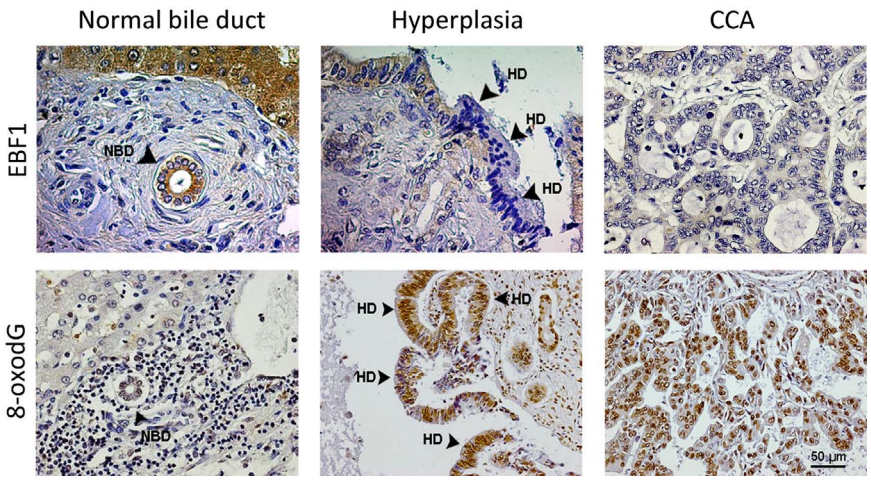

Fig. 1. Immunohistochemical analysis of EBF1 expression and 8-oxodG formation in normal bile duct of a tumor adjacent area, hyperplasia and CCA tissues. NBD = normal bile duct, HD = hyperplasia bile duct. An original magnification is 200× for all figures.

3.2. EBF1 expression and 8-oxodG formation in relation to clinico-pathological parameters in human CCA tissues

Among liver sections obtained from 75 patients with intrahepatic CCA examined, 52 (69%) cases were male and 23 (31%) cases were female. The median age of patients was 57.8 years. In our study, the CCA histological types were classified as 47% (35/75) papillary type and 53% (40/75) tubular type. CCA metastatic stages were classified into two different groups; 56% (42/75) metastasis and 44% (33/75) non-metastasis. The criterion of the classification of CCA histology types and metastatic stages has been described in previous studies [26–29]. No significant correlation of EBF1expression and the 8-oxodG formation with age, gender, histological types and metastatic stages was found (Table S1).

3.3. Expressions of EBF1 and level of 8-oxodG with survival rate of CCA patients

The Kaplan-Meier method with Log-rank test showed that CCA patients with low expression of EBF1 or high level of 8-oxodG formation were significantly correlated with poor prognosis ($P = 0.033$ and $P = 0.007$, respectively) as shown in Fig. 2A and B. Interestingly, CCA patients who had both reduced expression of EBF1 and elevated formation of 8-oxodG showed even greater significant correlation with poor prognosis ($P = 0.003$) when compared among various correlations (Fig. 2C). Multivariate analyses of all clinical data with survival rate are shown in Table S2.

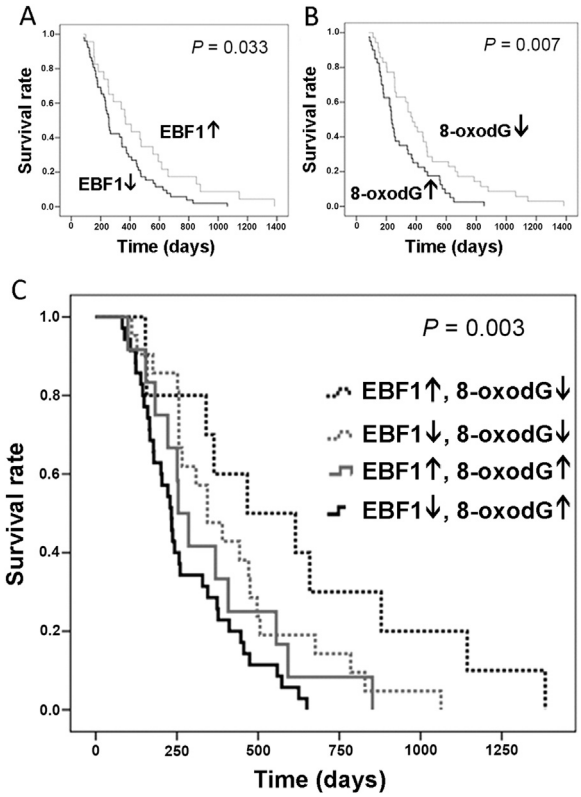

Fig. 2. Kaplan-Meier analysis of EBF1 (A), 8-oxodG formation (B), and combined EBF1 expression and 8-oxodG formation (C) with survival rate in CCA. *P*-value was analyzed by Log-rank test. EBF1↓= low EBF1 expression, EBF1↑= high EBF1 expression, 8-oxodG↓= low 8-oxodG formation, and 8-oxodG↑= high 8-oxodG formation in CCA tissues.

3.4. Expressions of EBF1 in cholangiocyte, oxidative stress-resistant and CCA cell lines

Recently, we have established and characterized the oxidative stress-resistant cell line (ox-MMNK1-L cell) by exposing MMNK1 cells to daily hydrogen peroxide treatment, thus ox-MMNK1-L cells can be used as the prolonged oxidative stress response model [7]. The analyses of EBF1 expression was further performed in MMNK1, ox-MMNK1-L, KKU-213, KKU-214 and KKU-156 cells using real time PCR and immunocytochemical technique (Fig. 3A and B). EBF1 was highly stained in nucleus and cytoplasm of the cholangiocyte cell (MMNK1) compared with all of CCA cell lines (KKU-213, KKU-214 and KKU-156) used in this study, suggesting that down-regulation of EBF1 could be involved in CCA development. Moreover, EBF1 was slightly expressed in ox-MMNK1-L cells compared with MMNK1 cells, suggesting that the prolonged oxidative stress suppress the expression of EBF1.

3.5. EBF1-knockdown effects on stem cell marker expressions of cholangiocyte cells

Fig. 4 shows the effect of EBF1 knockdown on stem cell markers expression. The MMNK1 was transfected with 50 nM of EBF1 siRNA compared with lipofectamine and media alone. Following 48 h of transfection, the EBF1 mRNA was significantly decreased, whereas CD133 and Oct3/4 mRNA levels were significantly increased when compared with the control sets as shown in Figs. 4A, 4B and 4C, respectively. Moreover, the protein expressions of EBF1 and CD133 were confirmed in EBF1 knockdown-cholangiocyte cells using the immunocytochemical technique as shown in Figs. 4D and 4E. EBF1 protein was slightly expressed, whereas CD133 was highly expressed in

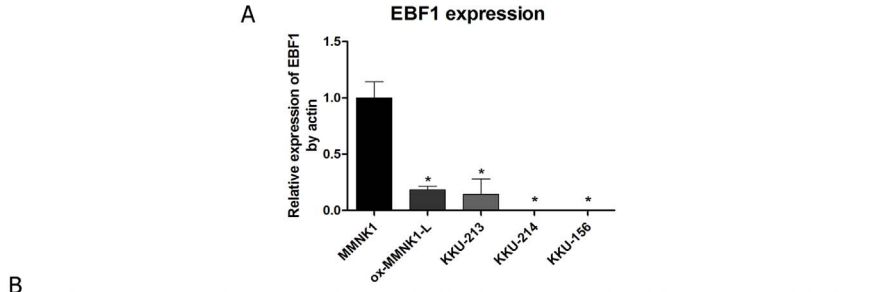

Fig. 3. (A) Relative mRNA expression levels of EBF1 was measured by real-time PCR and adjusted by β-actin mRNA expression in MMNK1, ox-MMNK1-L and CCA cell lines. The symbol asterisk (*) indicates statistical significance at $P < 0.05$. (B) Immunocytochemical analysis of EBF1 expression.

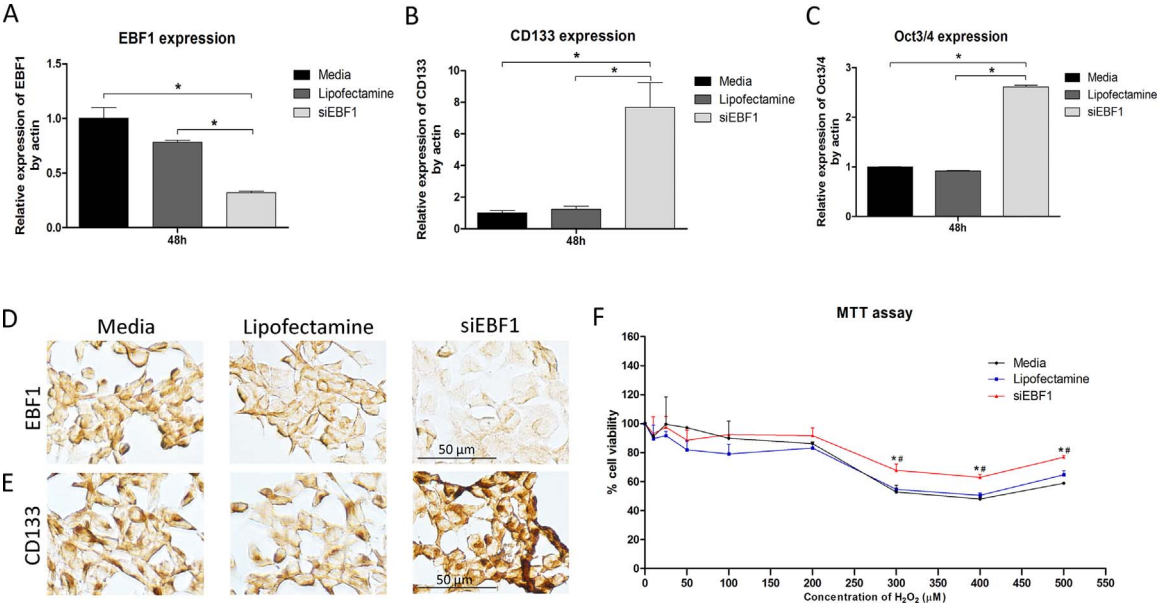

Fig. 4. Relative mRNA expression levels of EBF1 (A), CD133 (B) and Oct3/4 (C) were measured by real-time PCR and adjusted by β-actin mRNA expression. The asterisk (*) indicates statistical significance at $P < 0.05$. Protein expression levels of EBF1 (D) and CD133 (E) were detected by immunocytochemical staining. An original magnification is 200× for all figures. (F) Viability of EBF1-knockdown cholangiocyte cell line using MTT assay. The asterisk (*) indicates statistical significance at $P < 0.05$ (compared with lipofectamine); (#) for $P < 0.05$ (compared with media).

EBF1 knockdown-MMNK1 cells when compared to the control, suggesting that suppression of EBF1 associates with stem cell properties.

3.6. EBF1-knockdown induces cholangiocyte cell line resistant to oxidative stress

To elucidate the relationship between oxidative stress and EBF1 down-regulation, EBF1 gene of MMNK1 cells were silenced and the cells were exposed to various concentrations of H_2O_2. The results show that the number of viable cells was significantly higher in EBF1-knockdown group than that of control after exposure to 200 µM or higher concentrations of H_2O_2 for 48 h (Fig. 4F). This result suggests that down-regulation of EBF1 strengthens the oxidative stress-resistant property.

3.7. EBF1-knockdown effects on wound healing and migration of cholangiocyte cells

We further investigated the function of EBF1 in MMNK1 measured by wound healing and migration assays. The results showed that siEBF1-transfected MMNK1 cells had a significantly increased in cell migration compared with lipofectamine transfection at 31 h as determined by wound healing assay (Fig. 5A) and the graphical data represented the percentage of migration area as shown in Fig. 5B. Additionally, we also confirmed the ability of EBF1 knockdown-MMNK1 cell in cell migration using a Boyden chamber transwell consisting of a membrane filter insert in 24-well plate with 8-µm pore size. The result showed that EBF1 knockdown in MMNK1 cell was significantly increased in cell migration numbers when compared with the controls ($P < 0.001$) (Figs. 5C and 5D).

3.8. Effect of EBF1 knockdown to estrogen response in cholangiocyte cells

Trefoil factor 1 (TFF1) is one of estrogen responsive genes that play critical roles in cell migration and invasion in estrogen-related cancers including CCA [22,30,31]. In this study, TFF1 expression was significantly increased in the EBF1-knockdown cholangiocyte cell line as shown in Fig. 6A. We also explored the migration of cholangiocyte cells after EBF1 knockdown and treated with 17β-estradiol. Our results showed that a significant increase in the number of migrated cells was observed in EBF1 knockdown-MMNK1 cells after being treated with estradiol for 12 h when compared to the EBF1 knockdown-MMNK1cells

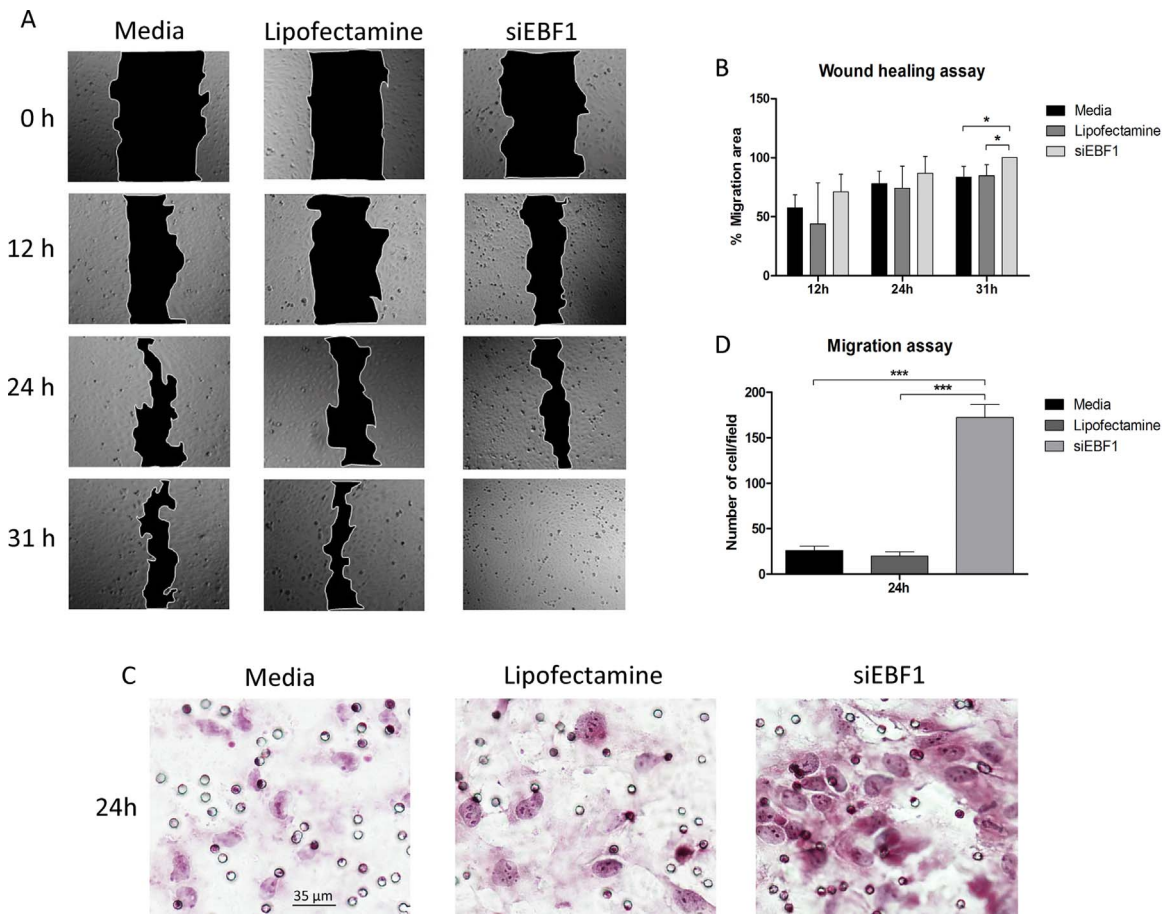

Fig. 5. (A) Wound healing assay under microscope (10×). (B) The graphical data represented the percentage of migration area determined by wound healing assay. (C) Hematoxylin-staining migrated cholangiocyte cells after 24 h treated with lipofectamine and siEBF1 using a Boyden chamber transwell consisting of a membrane filter. (D) The graphical data represent the migrated cells detected by the migration assay at 24 h. The asterisk (*) indicates statistical significance at $P < 0.05$ and asterisks (***) indicates statistical significance at $P < 0.001$.

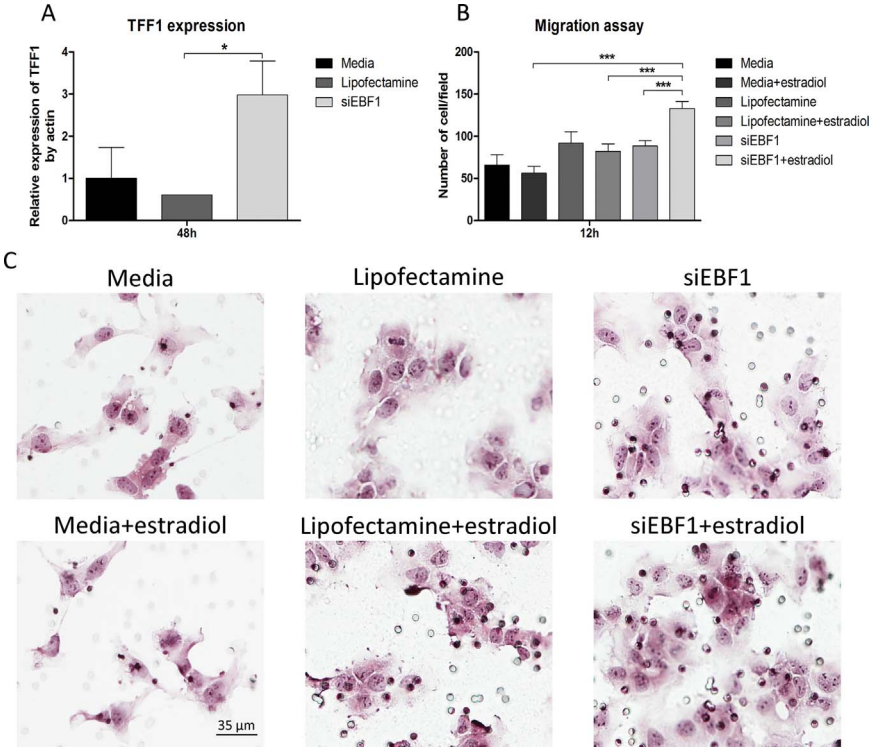

Fig. 6. (A) Relative mRNA expression levels of TFF1 was measured by real-time PCR and adjusted by β-actin mRNA expression. (B) The graphical data represent the migrated cells detected by the migration assay. The Y axis represents the number of migrating cells per field and the X axis the experimental group. The asterisk (*) indicates statistical significance at $P < 0.05$ and asterisks (***) indicates statistical significance at $P < 0.001$. (C) Hematoxylin-staining migrated cells of cholangiocytes after 12 h treated with 17β-estradiol using a Boyden chamber transwell consisting of a membrane filter.

alone ($P < 0.001$) as shown in Figs. 6B and 6C.

4. Discussion

In our study, CCA patients with low EBF1 expression and high oxidative stress were significantly correlated with poor survival. Thus, the down-regulation of EBF1 may be caused by oxidative stress and play the important role in CCA development. There are several studies suggesting that EBF1 may function as the potent tumor suppressor [18,19,32]. Chronic inflammation mediated by infection is a major risk factor causing carcinogenesis including *O. viverrini*-driven CCA [4]. In addition, oxidative stress was also reported to induce the alteration of gene expressions via the inductions of mutation, genetic instability and epigenetic changes [8,33,34]. Recently, we successfully established the oxidative stress-resistant cell line (ox-MMNK1-L), which originated from the MMNK1 cell line with the long-term daily-exposure to 25 μM H_2O_2 [7]. The oxidative stress-resistant cells could increase not only antioxidant properties but also DNMT1 expression level, suggesting that the epigenetic changes may be triggered as the oxidative stress response condition [7]. Additionally, EBF1 expression in ox-MMNK1-L was decreased compared to the parental cell line of which EBF1 mRNA expression level was increased after acute exposure to H_2O_2 (Fig. S1). Related to this, bio-informatics analysis showed that EBF1 was down-regulated in human obese adipose tissues which were exposed to long-term oxidative stress [35]. Our results suggest that prolonged oxidative stress inhibits EBF1 expression in the cholangiocyte cell line as the adaptive response for cell survival under persistent stress situation.

The immunohistochemical analysis in 75 cases of CCA tissues and immunocytochemical analysis of MMNK1, ox-MMNK1-L and 3 CCA cell lines raise the hypothesis that EBF1 down-regulation may be involved in CCA promotion and progression resulting in CCA development with aggressive clinical outcomes. Therefore, EBF1 knockdown by siRNA was performed in MMNK1 cells. EBF1 knockdown-MMNK1 cells had no effect to cell growth (Fig. S2) whereas they significantly increased wound healing activity and cell migration numbers. These confirmed that EBF1 down-regulation could induce cancer properties of the cholangiocyte cells through the induction of cell migration activities.

Stem cells are cells that possess the ability to unlimited self-renewal and to generate mature cells of a particular tissue by differentiation [36]. EBF1 play roles in hematopoietic stem cells differentiate into mature B cells [37], and is involved in mesenchymal stem cell (MSC) differentiation which induces MSC differentiation into adipocytes, whereas it suppresses differentiation into osteocytes [38]. In the process of liver development, bipotential liver stem cells could differentiate either into cholangiocytes (bile ducts) and hepatocytes [39]. Recently, we proposed the mechanism that CCA might differentiate from bipotential liver stem cells lining at canal of Hering, biliary ductules, bile duct or progenitor cells from bone marrow-derived circulating cells during tissues repairing process under oxidative stress induced by *O. viverrini*-chronic inflammation [24]. Moreover, CD133 and Oct3/4 were potentially used as bipotential liver stem cell markers [24]. Our results showed that EBF1 was highly detected in the nucleus of normal bile duct and hepatocyte cells, suggesting that EBF1 may play roles in bipotential liver stem cell differentiation into cholangiocytes and hepatocytes. Therefore, we hypothesized that down-regulation of EBF1 during CCA genesis may play a significant role in CCA development via the inhibition of bipotential liver stem cells differentiation into mature cholangiocytes and hepatocytes or induction of stem cell properties in the mature cells leading to increased stem cell property of the tumor initiating cells. This hypothesis was supported by the increasing of CD133 and Oct3/4 expressions after EBF1 suppression on MMNK1 cell line by specific siRNA.

Estrogen is the sex hormone that plays roles in secondary female characteristics. It was reported to promote carcinogenesis especially in breast cancer [21]. ERs are mediated by estrogen. Activated ERs bind to the estrogen response element and promote the expressions of estrogen responsive genes such as TFF1. Serum estrogen levels were significantly increased in male CCA patients and it was reported to play roles in the tumor progression via induction of cell proliferation and migration through ERs and TFF1 expressions [22]. Recently, EBF1 had been proposed to be the negative regulator of ERs [20]. The present results showed that increasing of TFF1 expression was found in EBF1-knockdown cholangiocyte cells, suggesting that down-regulation of EBF1 induced an estrogen response through the induction of ERs activity. Cell proliferation analyzed by the sulforhodamine B (SRB) assay significantly increased in estrogen-treated lipofectamine and estrogen-treated siEBF1 (Fig. S3), suggesting that lipofectamine may induce estrogen uptake via increasing of cell membrane surface area and estrogen-induced cell proliferation does not associate with EBF1 down-regulation. On the other hand, the number of migrated cells significantly increased in the EBF1 knockdown-MMNK1 cells after being treated with 17β-estradiol. This suggests that down-regulation of EBF1 increases the effect of estrogen response via induction of cell migration activities through TFF1 expression resulting in CCA development with aggressive clinical outcomes.

In conclusion, the present results show that EBF1 expression is affected by oxidative stress. Chronic exposure to oxidative stress induces significant suppression of EBF1 expression. Suppression of EBF1 can induce stem cell and migration properties of the cholangiocytes leading to CCA promotion and progression resulting in CCA development with aggressive clinical outcomes such as short survival time. Therefore, EBF1 and its related molecules may be used as new therapeutic targets for CCA chemotherapy and chemoprevention.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.11.011.

References

[1] P. Sithithaworn, P. Yongvanit, K. Duenngai, N. Kiatsopit, C. Pairojkul, Roles of liver fluke infection as risk factor for cholangiocarcinoma, J. Hepatobiliary Pancreat. Sci. 21 (2014) 301–308.

[2] S. Pinlaor, N. Ma, Y. Hiraku, P. Yongvanit, R. Semba, S. Oikawa, M. Murata, B. Sripa, P. Sithithaworn, S. Kawanishi, Repeated infection with *Opisthorchis viverrini* induces accumulation of 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase, Carcinogenesis 25 (2004) 1535–1542.

[3] S. Pinlaor, P. Yongvanit, Y. Hiraku, N. Ma, R. Semba, S. Oikawa, M. Murata, B. Sripa, P. Sithithaworn, S. Kawanishi, 8-nitroguanine formation in the liver of hamsters infected with *Opisthorchis viverrini*, Biochem. Biophys. Res. Commun. 309 (2003) 567–571.

[4] P. Yongvanit, S. Pinlaor, H. Bartsch, Oxidative and nitrative DNA damage: key events in opisthorchiasis-induced carcinogenesis, Parasitol. Int. 61 (2012) 130–135.

[5] S. Ohnishi, N. Ma, R. Thanan, S. Pinlaor, O. Hammam, M. Murata, S. Kawanishi, DNA damage in inflammation-related carcinogenesis and cancer stem cells, Oxid. Med. Cell Longev. 2013 (2013) 387014.

[6] R. Thanan, S. Oikawa, Y. Hiraku, S. Ohnishi, N. Ma, S. Pinlaor, P. Yongvanit, S. Kawanishi, M. Murata, Oxidative stress and its significant roles in

neurodegenerative diseases and cancer, Int. J. Mol. Sci. 16 (2015) 193–217.
[7] R. Thanan, A. Techasen, B. Hou, W. Jamnongkan, N. Armartmuntree, P. Yongvanit, M. Murata, Development and characterization of a hydrogen peroxide-resistant cholangiocyte cell line: a novel model of oxidative stress-related cholangiocarcinoma genesis, Biochem. Biophys. Res. Commun. 464 (2015) 182–188.
[8] M. Murata, R. Thanan, N. Ma, S. Kawanishi, Role of nitrative and oxidative DNA damage in inflammation-related carcinogenesis, J. Biomed. Biotechnol. 2012 (2012) 623019.
[9] J. Hagman, M.J. Gutch, H. Lin, R. Grosschedl, EBF contains a novel zinc coordination motif and multiple dimerization and transcriptional activation domains, EMBO J. 14 (1995) 2907–2916.
[10] J. Hagman, C. Belanger, A. Travis, C.W. Turck, R. Grosschedl, Cloning and functional characterization of early B-cell factor, a regulator of lymphocyte-specific gene expression, Genes Dev. 7 (1993) 760–773.
[11] J. Hagman, K. Lukin, Early B-cell factor 'pioneers' the way for B-cell development, Trends Immunol. 26 (2005) 455–461.
[12] M. Kieslinger, S. Folberth, G. Dobreva, T. Dorn, L. Croci, R. Erben, G.G. Consalez, R. Grosschedl, EBF2 regulates osteoblast-dependent differentiation of osteoclasts, Dev. Cell. 9 (2005) 757–767.
[13] D.G. Hesslein, J.A. Fretz, Y. Xi, T. Nelson, S. Zhou, J.A. Lorenzo, D.G. Schatz, M.C. Horowitz, Ebf1-dependent control of the osteoblast and adipocyte lineages, Bone 44 (2009) 537–546.
[14] K. Jin, M. Xiang, Ebf1 deficiency causes increase of Muller cells in the retina and abnormal topographic projection at the optic chiasm, Biochem. Biophys. Res. Commun. 414 (2011) 539–544.
[15] J.A. Fretz, T. Nelson, H. Velazquez, Y. Xi, G.W. Moeckel, M.C. Horowitz, Early B-cell factor 1 is an essential transcription factor for postnatal glomerular maturation, Kidney Int. 85 (2014) 1091–1102.
[16] L. Harder, G. Eschenburg, A. Zech, N. Kriebitzsch, B. Otto, T. Streichert, A.S. Behlich, K. Dierck, B. Klingler, A. Hansen, M. Stanulla, M. Zimmermann, E. Kremmer, C. Stocking, M.A. Horstmann, Aberrant ZNF423 impedes B cell differentiation and is linked to adverse outcome of ETV6-RUNX1 negative B precursor acute lymphoblastic leukemia, J. Exp. Med. 210 (2013) 2289–2304.
[17] C.G. Mullighan, S. Goorha, I. Radtke, C.B. Miller, E. Coustan-Smith, J.D. Dalton, K. Girtman, S. Mathew, J. Ma, S.B. Pounds, X. Su, C.H. Pui, M.V. Relling, W.E. Evans, S.A. Shurtleff, J.R. Downing, Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia, Nature 446 (2007) 758–764.
[18] R.M. Neve, K. Chin, J. Fridlyand, J. Yeh, F.L. Baehner, T. Fevr, L. Clark, N. Bayani, J.P. Coppe, F. Tong, T. Speed, P.T. Spellman, S. DeVries, A. Lapuk, N.J. Wang, W.L. Kuo, J.L. Stilwell, D. Pinkel, D.G. Albertson, F.M. Waldman, F. McCormick, R.B. Dickson, M.D. Johnson, M. Lippman, S. Ethier, A. Gazdar, J.W. Gray, A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes, Cancer Cell. 10 (2006) 515–527.
[19] S. Jones, X. Zhang, D.W. Parsons, J.C. Lin, R.J. Leary, P. Angenendt, P. Mankoo, H. Carter, H. Kamiyama, A. Jimeno, S.M. Hong, B. Fu, M.T. Lin, E.S. Calhoun, M. Kamiyama, K. Walter, T. Nikolskaya, Y. Nikolsky, J. Hartigan, D.R. Smith, M. Hidalgo, S.D. Leach, A.P. Klein, E.M. Jaffee, M. Goggins, A. Maitra, C. Iacobuzio-Donahue, J.R. Eshleman, S.E. Kern, R.H. Hruban, R. Karchin, N. Papadopoulos, G. Parmigiani, B. Vogelstein, V.E. Velculescu, K.W. Kinzler, Core signaling pathways in human pancreatic cancers revealed by global genomic analyses, Science 321 (2008) 1801–1806.
[20] T.P. Le, M. Sun, X. Luo, W.L. Kraus, G.L. Greene, Mapping ERbeta genomic binding sites reveals unique genomic features and identifies EBF1 as an ERbeta interactor, PLoS One 8 (2013) e71355.
[21] J. Russo, I.H. Russo, The role of estrogen in the initiation of breast cancer, J. Steroid Biochem. Mol. Biol. 102 (2006) 89–96.
[22] T. Hunsawong, E. Singsuksawat, N. In-chon, W. Chawengrattanachot, C. Thuwajit, B. Sripa, A. Paupairoj, S. Chau-in, P. Thuwajit, Estrogen is increased in male cholangiocarcinoma patients' serum and stimulates invasion in cholangiocarcinoma cell lines in vitro, J. Cancer Res. Clin. Oncol. 138 (2012) 1311–1320.
[23] R. Thanan, M. Murata, N. Ma, O. Hammam, M. Wishahi, T. El Leithy, Y. Hiraku, S. Oikawa, S. Kawanishi, Nuclear localization of COX-2 in relation to the expression of stemness markers in urinary bladder cancer, Mediat. Inflamm. 2012 (2012) 165879.
[24] R. Thanan, C. Pairojkul, S. Pinlaor, N. Khuntikeo, C. Wongkham, B. Sripa, N. Ma, K. Vaeteewoottacharn, A. Furukawa, H. Kobayashi, Y. Hiraku, S. Oikawa, S. Kawanishi, P. Yongvanit, M. Murata, Inflammation-related DNA damage and expression of CD133 and Oct3/4 in cholangiocarcinoma patients with poor prognosis, Free Radic. Biol. Med. 65 (2013) 1464–1472.
[25] M. Maruyama, N. Kobayashi, K.A. Westerman, M. Sakaguchi, J.E. Allain, T. Totsugawa, T. Okitsu, T. Fukazawa, A. Weber, D.B. Stolz, P. Leboulch, N. Tanaka, Establishment of a highly differentiated immortalized human cholangiocyte cell line with SV40T and hTERT, Transplantation 77 (2004) 446–451.
[26] Y. Nakanuma, Y. Sato, K. Harada, M. Sasaki, J. Xu, H. Ikeda, Pathological classification of intrahepatic cholangiocarcinoma based on a new concept, World J. Hepatol. 2 (2010) 419–427.
[27] B.R. Blechacz, G.J. Gores, Cholangiocarcinoma, Clin. Liver Dis. 12 (2008) 131–150.
[28] S.A. Khan, B.R. Davidson, R. Goldin, S.P. Pereira, W.M. Rosenberg, S.D. Taylor-Robinson, A.V. Thillainayagam, H.C. Thomas, M.R. Thursz, H. Wasan, G. British, Society of, Guidelines for the diagnosis and treatment of cholangiocarcinoma: consensus document, Gut 51 (Suppl 6) (2002) (VI1-9).
[29] B. Blechacz, M. Komuta, T. Roskams, G.J. Gores, Clinical diagnosis and staging of cholangiocarcinoma, Nat. Rev. Gastroenterol. Hepatol. 8 (2011) 512–522.
[30] P. Thuwajit, W. Chawengrattanachot, C. Thuwajit, B. Sripa, F.E. May, B.R. Westley, N.N. Tepsiri, A. Paupairoj, S. Chau-In, Increased TFF1 trefoil protein expression in Opisthorchis viverrini-associated cholangiocarcinoma is important for invasive promotion, Hepatol. Res. 37 (2007) 295–304.
[31] S.J. Prest, F.E. May, B.R. Westley, The estrogen-regulated protein, TFF1, stimulates migration of human breast cancer cells, FASEB J. 16 (2002) 592–594.
[32] D. Liao, Emerging roles of the EBF family of transcription factors in tumor suppression, Mol. Cancer Res. 7 (2009) 1893–1901.
[33] K.V. Donkena, C.Y. Young, D.J. Tindall, Oxidative stress and DNA methylation in prostate cancer, Obstet. Gynecol. Int. 2010 (2010) 302051.
[34] W. Wongpaiboonwattana, P. Tosukhowong, T. Dissayabutra, A. Mutirangura, C. Boonla, Oxidative stress induces hypomethylation of LINE-1 and hypermethylation of the RUNX3 promoter in a bladder cancer cell line, Asian Pac. J. Cancer Prev. 14 (2013) 3773–3778.
[35] X. Li, P.A. Thomason, D.J. Withers, J. Scott, Bio-informatics analysis of a gene co-expression module in adipose tissue containing the diet-responsive gene Nnat, BMC Syst. Biol. 4 (2010) 175.
[36] T. Reya, S.J. Morrison, M.F. Clarke, I.L. Weissman, Stem cells, cancer, and cancer stem cells, Nature 414 (2001) 105–111.
[37] H. Kikuchi, M. Nakayama, Y. Takami, F. Kuribayashi, T. Nakayama, EBF1 acts as a powerful repressor of Blimp-1 gene expression in immature B cells, Biochem. Biophys. Res. Commun. 422 (2012) 780–785.
[38] J.M. Stephens, The fat controller: adipocyte development, PLoS Biol. 10 (2012) e1001436.
[39] T. Roskams, Liver stem cells and their implication in hepatocellular and cholangiocarcinoma, Oncogene 25 (2006) 3818–3822.

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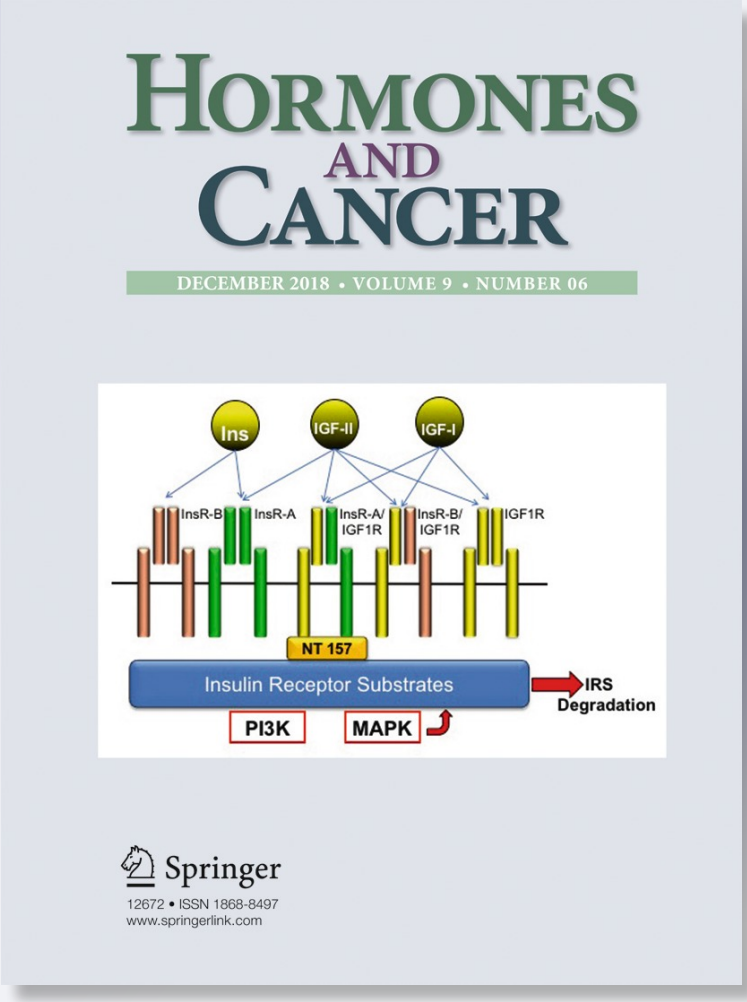

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ORIGINAL PAPER

The Importance of CYP19A1 in Estrogen Receptor-Positive Cholangiocarcinoma

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Abstract

CYP19A1, also called aromatase, is a key enzyme for converting androgens to estrogens of estrogen synthesis. Elevated serum estrogen and high expression levels of estrogen-related proteins are found in cholangiocarcinoma (CCA; bile duct cancer). However, the expression of CYP19A1 in relation to estrogen-related proteins, including estrogen receptors (ERα, ERβ, and GPR30) and an estrogen response protein (TFF1), has never been explored in CCA. In this study, we investigated the expressions of CYP19A1 and estrogen-related proteins in CCA tissues ($n = 74$; 51 males and 23 females) using immunohistochemistry. The results showed that CYP19A1 was overexpressed in CCA cells compared with that in normal bile duct cells in the adjacent tissues. High expression of CYP19A1 was correlated with the metastatic status of the patients. High CYP19A1 expression was also positively correlated with GPR30 expression. Correlation between high CYP19A1 expression in the tumor tissues and shorter survival time was more prominent in male than in female CCA patients. To elucidate further, the effect of CYP19A1 knockdown on a CCA cell line was examined using a specific siRNA. When CYP19A1 gene expression was suppressed, migration and proliferation activities of CCA cells were significantly reduced. Moreover, the cell proliferation of high CYP19A1-expressing KKU-213 cells was more profoundly suppressed by CYP19A1 inhibitors (exemestane and letrozole) than low CYP19A1-expressing KKU-100 cells. Thus, CYP19A1 promotes CCA progression with aggressive clinical outcomes via increased migration and proliferation activities of cancer cells. CYP19A1 can be a potential chemotherapeutic target for CCA, especially in male patients.

Keywords Cholangiocarcinoma · CYP19A1 · Aromatase · Estrogen · Estrogen receptors · Tumor progression

Introduction

Cholangiocarcinoma (CCA) is a liver cancer originating from bile duct epithelium and is the second most common primary hepatic malignancy worldwide [1]. Thailand has still the highest incidence and mortality rates of CCA in the world, especially in the Northeast of Thailand, where the incidence of CCA reaches up to 87.7 per 100,000 in males and 36.3 per 100,000 in females [2]. The high incidence of CCA in this region is associated with chronic inflammation of the biliary tract caused by liver fluke (*Opisthorchis viverrini*) infection, which is also highly prevalent in this area [3]. CCA is known as a "silent killer" because of its relatively silent clinical progression, which renders it difficult to diagnose at its early stages [4]. Therefore, the study on the molecular mechanisms of CCA development and progression is needed for identifying potential prognostic markers and therapeutic targets in CCA.

Estrogen is a group of sex steroid hormones and plays crucial roles in both reproductive and non-reproductive systems. The action of estrogen is mediated through estrogen receptor (ER) signaling pathways [5]. Increased estrogen levels and the effect of estrogen

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on tumor progression have been reported in breast, lung, endometrium, liver, bladder, and bile duct cancers [6–14]. In CCA, estrogen stimulates cell proliferation, cell invasion, and angiogenesis through ERs by inducing the expressions of pAKT, pERK1/2, trefoil factor 1 (TFF1), EGFR/MAPK, vascular endothelial growth factor (VEGF), and E26 transformation-specific variant 4 (ETV4) [12–17]. Male CCA patients have higher serum estrogen levels than age- and sex-matched controls, and high serum estrogen level was significantly correlated with poor survival of the patients [12]. These findings strongly suggest that estrogen induces CCA progression and is associated with aggressive clinical outcomes. Thus, estrogen and estrogen-related molecules may be potential molecular targets for diagnosis and treatment of CCA.

Cytochrome P450 family 19 subfamily A member 1 (CYP19A1; also called aromatase), a member of cytochrome P450 superfamily, is a key enzyme in estrogen biosynthesis, catalyzing the aromatization of aromatizable androgens (androstenedione and testosterone) to estrogens [18]. The overexpression of CYP19A1 is involved in progression of estrogen-related cancers such as breast, lung, and bladder cancers [8, 19, 20]. For example, in non-small cell lung cancer, high expression of CYP19A1 in the tumor tissues was correlated with higher estrogen levels in tumor tissues, higher cell proliferation rates, and poor prognosis of the patients [8, 21]. CYP19A1 expression has been shown to play an important role in the development and progression of ER-positive breast cancer [20]. Conversely, suppression of CYP19A1 reduced cell proliferation, migration, and invasion and increased apoptosis of non-small cell lung cancer cells [22, 23]. Thus, upregulation of CYP19A1 induces tumor progression of estrogen-related cancers.

Biliary obstruction and reduced estrogen turnover rates have been reported to be associated with the elevation of serum estrogen levels in CCA patients [12, 24, 25]. We further hypothesized that an estrogen-producing enzyme (CYP19A1) is upregulated in ER-positive CCA cells and could be a possible cause of elevated serum estrogen levels of CCA patients. To confirm this hypothesis, the expression patterns of CYP19A1 and estrogen-related proteins including estrogen receptors (ERα, ERβ, and GPR30) and an estrogen response protein (TFF1) in CCA tissues were determined using immunohistochemistry. In addition, the correlations between the protein expressions and the clinicopathological features (i.e., age, sex, histological types, metastatic status, and the survival rates of CCA patients) were analyzed. To further validate the importance of CYP19A1 in proliferation and metastatic activities of CCA cell lines, silencing CYP19A1 gene was performed by treatment of CCA cells with either a specific siRNA or CYP19A1 inhibitors (exemestane and letrozole). The overall results show that overexpression of CYP19A1 plays important roles in proliferation and migration of ER-positive CCA cells and can be used to determine the prognosis of CCA patients.

Materials and Methods

Human CCA Tissues

Paraffin-embedded human CCA tissues ($n = 74$; 51 males and 23 females) were obtained from the Cholangiocarcinoma Research Institute, Khon Kaen University, Thailand.

Immunohistochemistry

Immunohistochemical staining was performed to determine the expressions and localizations of all targeted proteins (i.e., CYP19A1, ERα, ERβ, GPR30, and TFF1) in human CCA tissues. The sections (5 μm thick) of paraffin-embedded human CCA tissues were deparaffinized in xylene and rehydrated with descending series of ethanol. For the heat-induced antigen retrieval, the sections were immersed in 10 mM sodium citrate buffer (pH 6) and heat-treated in a pressure cooker for 5 min. Endogenous peroxidase activity was blocked by 0.3% (*v*/*v*) hydrogen peroxide in phosphate-buffered saline (PBS) at room temperature for 30 min. Then, the sections were incubated for 30 min with 10% skim milk in PBS to block non-specific binding and were incubated with a primary antibody for 1 h at room temperature and then 4 °C overnight. After washing, the sections were incubated with peroxidase-conjugated Envision™ secondary antibody (Dako, Denmark) for 1 h at room temperature. Peroxidase activity was detected using a DAB (3, 3′-diaminobenzidine tetrahydrochloride) substrate kit (Vector Laboratories, Inc., USA) for 5 min, followed by nuclear counterstaining with hematoxylin. Tissue sections were dehydrated with the ascending ethanol series, cleared in xylene, and mounted with mounting solution.

In this study, the following primary antibodies were used: 10 μg/mL rabbit polyclonal anti-CYP19A1 antibody (Sigma-Aldrich, USA), 24.15 μg/mL rabbit monoclonal anti-ERα antibody (Abcam, UK), 2 μg/mL rabbit polyclonal anti-ERβ antibody (Abcam, UK), 1.33 μg/mL rabbit polyclonal anti-GPR30 antibody (Abcam, UK), and 1 μg/mL mouse monoclonal anti-TFF1 antibody (Sigma-Aldrich, USA).

The stained sections were reviewed under a light microscope. The DAB-positive staining was seen as brown to dark-brown color. Cell nuclei were appeared as blue-purple colors. The immunohistochemistry score (IHC score) was calculated by the multiplying of the intensities of DAB staining

results (scored as 0 = negative, 1 = weak, 2 = modulate, and 3 = strong) and the frequency of the staining results (scored as 0 = none, + 1 = 1–25%, + 2 = 26–50%, + 3 = 51–75%, and + 4 = > 75%) [26]. Thus, the IHC score ranged from 0 to 12. In this study, the IHC score was used to discriminate high (IHC score > 4) and low (IHC score ≤ 4) expression patterns.

Cell Lines

Two distinct human intrahepatic CCA cell lines, KKU-100 and KKU-213, were established from primary tumors of CCA patients in the Northeast of Thailand and obtained from the Cholangiocarcinoma Research Institute, Khon Kaen University, Thailand. Immortalized human cholangiocyte cell line (MMNK1) was originally obtained from Okayama University, Japan [27], and maintained also in the Cholangiocarcinoma Research Institute, Khon Kaen University. All cell lines were cultured in complete medium which is Ham's F-12 (Gibco®, Life technologies™, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (Life technologies™, USA). Cell lines were incubated in a humidified incubator with 5% CO_2 and 95% relative humidity at 37 °C.

Small Interfering RNA Against CYP19A1

Specific siRNA for CYP19A1 (siCYP19A1) was designed by GE Healthcare Dharmacon (ON-TARGETplus SMARTpool Human CYP19A1 siRNA, GE Healthcare Dharmacon Inc., USA). The cells were transfected with siCYP19A1 using Lipofectamine RNAiMAX® (Thermo Fisher Scientific, USA) in a 6-well plate with 10^5 cells for 72-h transfection. The cells treated with scramble siRNA (ON-TARGETplus Non-targeting Control siRNA, GE Healthcare Dharmacon Inc., USA) were used as controls (scramble). Cells were harvested using trypsinization for RNA extraction, immunocytochemical analysis, cell migration assay, and cell viability assay.

RNA Extraction and Real-time PCR

Total RNA was isolated from cell pellets with Trizol® reagent (Invitrogen, USA) according to the manufacturer's protocol. The quality of RNA was assessed with a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, USA). Then, 2 μg of total RNA was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) as described in the manufacturer's protocol. The expression levels of CYP19A1, BIRC5, and β-actin mRNA were measured using TaqMan gene expression assay with TaqMan probes (Hs00903411_m1 CYP19A1, Hs04194392_s1 BIRC5, and Hs99999903_m1 β-actin). The PCR was performed in an ABI-7500 real-time PCR system (Applied Biosystems, USA). Relative mRNA expression was analyzed with a cycle threshold (Ct) in the linear range of amplification using β-actin as an internal control.

Immunocytochemistry

Each cell lines (5.0×10^4 cells/well) were placed in a 48-well plate and incubated for 24 h. The attached cells were fixed with 10% paraformaldehyde in PBS for 30 min at room temperature. After washing, cells were incubated with 0.2% (*v*/*v*) Triton-X100 solution for 2 min. The cells were incubated with 0.3% (*v*/*v*) hydrogen peroxide in PBS for 30 min to block endogenous hydrogen peroxide activity and subsequently incubated with 3% (*w*/*v*) BSA in PBS for 30 min to block non-specific binding. The cells were incubated with each primary antibody: 10 μg/mL rabbit polyclonal anti-CYP19A1 antibody (Sigma-Aldrich, USA), 24.15 μg/mL rabbit monoclonal anti-ERα antibody (Abcam, UK), 2 μg/mL rabbit polyclonal anti-ERβ antibody (Abcam, UK), or 1.33 μg/mL rabbit polyclonal anti-GPR30 antibody (Abcam, UK) at 4 °C overnight. After extensive washing, the cells were incubated with peroxidase-conjugated secondary antibody. The peroxidase activity was detected as brown reaction product using a DAB substrate kit. After dehydration, the stained cells were reviewed under an inverted microscope.

Cell Migration Assay

The cell migration assay was performed using a Boyden transwell chamber consisting of a membrane filter insert with 8-μm pore size in a 24-well plate (Corning, USA). In brief, 72 h after knockdown treatment with siCYP19A1 or scramble (control), 4×10^4 cells were placed into the insert upper chamber with serum-free medium. In the lower chamber, complete medium was added. Then, the chambers were incubated for 12 or 18 h. Non-migrating cells in the upper chamber were removed. Migrated cells that attached at the undersurface of the filter were fixed with absolute methanol for 1 h and stained with hematoxylin overnight. The membrane was allowed to dry and the number of migrated cells were quantified and analyzed under a light microscope.

Cell Viability Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to investigate CCA cell proliferation.

Cells in each well of a 96-well flat-bottom microtiter plate (in triplicate) were incubated with 0.5 mg/mL MTT solution in a complete medium at 37 °C for 4 h in the dark. After washing, formazan crystal was solubilized by adding 100 μL DMSO and the plate was shaken using an orbital shaker for 10 min in the dark. The absorbance was measured at 540 nm using a microplate reader (Tecan Sunrise, Switzerland).

Estrogen Treatment

After 72 h of siCYP19A1 transfection, KKU-213 cells were reseeded and treated with or without 1 nM of 17β-estradiol (Sigma-Aldrich, USA) in the cell culture conditions. Cell migration was assessed by Boyden transwell chamber assay and cell viability was analyzed by MTT assay.

CYP19A1 Inhibitor Treatment

Exemestane (Pfizer Inc., USA) and letrozole (Novartis, Switzerland) were dissolved in dimethyl sulfoxide (DMSO) and pre-diluted in the complete medium before use. The final concentration of DMSO was adjusted to 0.5% in all conditions. The cell lines were treated with various concentrations of exemestane or letrozole for 48 h. After treatment, the cells were subjected to MTT assay.

Statistical Analysis

Statistical analysis was performed using SPSS Statistics software version 19.0 (IBM Cooperation, USA). The Kaplan-Meier estimate with log-rank test was used for survival analysis. The correlations between protein expression patterns in CCA tissues and clinicopathological data were analyzed using

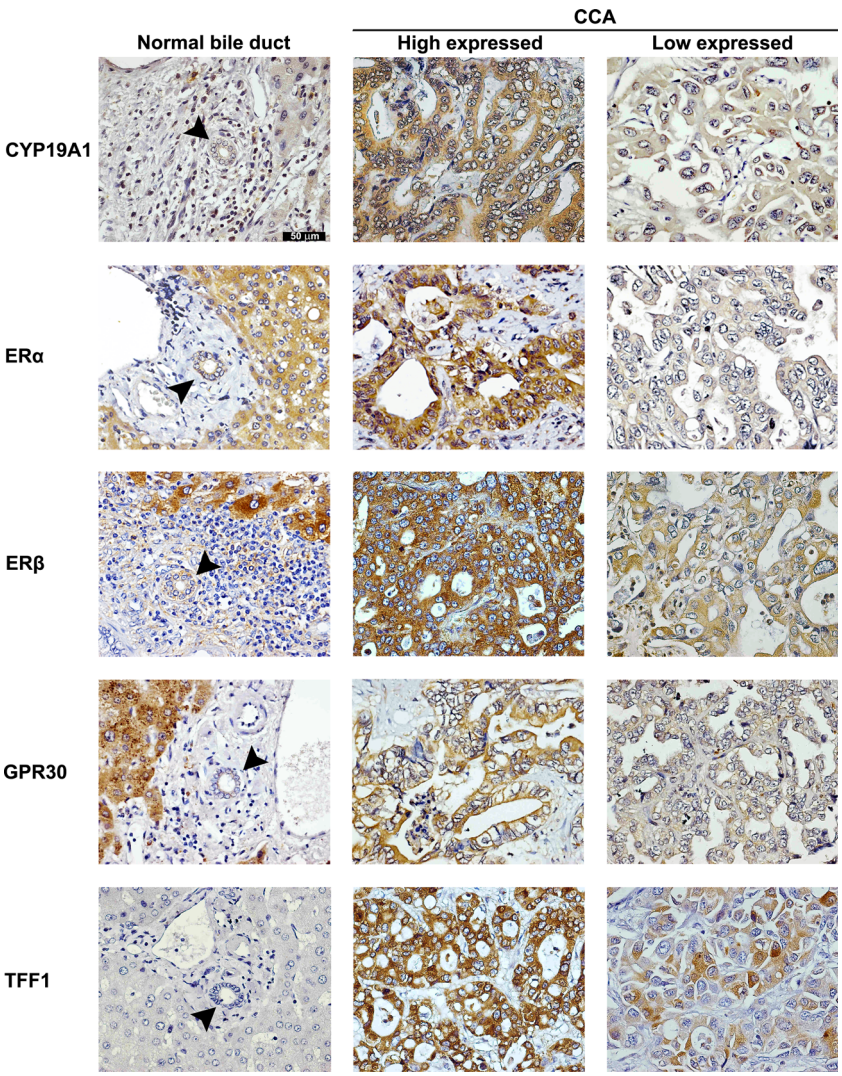

Fig. 1 The expressions of CYP19A1, ERα, ERβ, GPR30, and TFF1 in normal bile duct of the tumor adjacent area and CCA tissues ($n = 74$; original magnification × 200). Arrow heads indicate normal bile ducts. Positive staining immunoreactivities were represented with brown color. Blue color represented the nucleus

Pearson's chi-square test. The correlations among protein expressions were analyzed by Pearson correlation. The results of mRNA expression, cell migration assay, and cell proliferation assay were analyzed using Student's *t* test. *P* value < 0.05 is considered statistically significant.

Results

Expression of CYP19A1 in CCA Tissues

Immunohistochemical staining of CCA tissues demonstrated that CYP19A1 was strongly expressed in the cytoplasm of cancer cells but it was slightly expressed in the nucleus (Fig. 1). CYP19A1 was also slightly expressed in the cytoplasm of hepatocytes. The degrees of CYP19A1 expression determined by immunohistochemistry were significantly higher in the CCA cells compared with those in the normal bile duct epithelial cells located at the adjacent area (P < 0.001; graph not shown). More than half of the CCA tissues examined (45/74, 61%) showed high expression of CYP19A1.

Expression of Estrogen Receptors in CCA Tissues

The expression patterns of three estrogen receptors, ERα, ERβ, and GPR30, in CCA tissues were shown in Fig. 1. While ERα and ERβ were seen as the cytoplasmic and nuclear distribution patterns in cancer cells, GPR30 was located at the cytoplasm and the cell membrane of cancer cells. All CCA tissues showed positive staining of ERα, ERβ, and/or GPR30 (100%, 74/74). Significantly positive correlation was observed between the expression patterns of ERα and ERβ ($r = 0.263$ and $P = 0.024$; Pearson correlation analysis). Also, the expression levels of all three estrogen receptors in immunohistochemistry were significantly higher in CCA cells than in the normal bile duct epithelial cells in the tumor adjacent tissues (P < 0.001; graph not shown). High expression levels of ERα, ERβ, and GPR30 in IHC score were observed in 78% (58/74), 50% (37/74), and 38% (28/74), respectively, of the CCA patients. All estrogen receptors were also highly expressed in the cytoplasm of hepatocytes. In spite of the high expression patterns of ERα, ERβ, and GPR30 in CCA tissues, their expression levels were not correlated with clinicopathological data such as age, sex, metastasis status, and CCA histology types.

Expression of TFF1, as a Representative of Estrogen-Responsive Proteins, in CCA Tissues

As a representative of estrogen-responsive proteins [15], the expression of TFF1 in both CCA cells and adjacent normal bile duct cells was examined using immunohistochemical staining. As shown in Fig. 1. TFF1 protein was highly expressed in the cytoplasm of CCA cells, whereas it was negative in normal bile duct epithelial cells. Among 74 CCA cases, 39 cases (53%) have high TFF1 expression pattern, although it was not correlated with clinicopathological data including age, sex, and CCA histology types. Moreover, TFF1 expression was significantly correlated with ERβ expression ($r = 0.244$ and $P = 0.036$; Pearson correlation).

Correlations of CYP19A1 with Clinicopathological Data and Estrogen-Related Proteins

Table 1 shows the correlations between CYP19A1 expression patterns and clinicopathological data as well as estrogen-related protein expression patterns. CYP19A1 expression patterns were not correlated with the sex and age of CCA patients. The expression of CYP19A1 in CCA tissues was significantly correlated with metastatic status ($r = 0.449$ and $P < 0.001$; Pearson correlation) and GPR30 expression ($r = 0.284$ and $P = 0.014$; Pearson correlation).

Table 1 Correlations of CYP19A1 expression with estrogen-related protein expressions and clinicopathological features

Value	CYP19A1 in CCA		
	Low (n = 29)	High (n = 45)	#P value
Age			
< 57	15	21	0.671
≥ 57	14	24	
Sex			
Male	20	31	0.994
Female	9	14	
Metastasis status			
Non-metastasis	21	12	< 0.001
Metastasis	8	33	
Histological type			
Tubular	12	27	0.117
Papillary	17	18	
Estrogen-related protein			
ERα			
Low	7	9	0.673
High	22	36	
ERβ			
Low	18	19	0.096
High	11	26	
GPR30			
Low	23	23	0.015
High	6	22	
TFF1			
Low	17	18	0.117
High	12	27	

P value was analyzed by Pearson's chi-square test

Correlations Between CYP19A1 Expression Levels and the Survival Rate of Patients

CCA patients with high CYP19A1 expression in their tumor tissues showed no correlation with poor prognosis ($P = 0.058$; log-rank test) as shown in Fig. 2a. When CCA patients were divided into males and females, male CCA patients showed high expression of CYP19A1 in tumor tissues, which was significantly correlated with poor prognosis ($P = 0.040$; log-rank test) as shown in Fig. 2b. The estimated median survival time of male CCA patients with high expression of CYP19A1 (233 days) in the cancer tissues was shorter than that of CCA patients with low CYP19A1 expression (447 days). In contrast, no correlation between CYP19A1 expression patterns in tumor tissues and the survival rates was observed in female CCA patients (Fig. 2c) which can be explained by higher circulating concentrations of estrogens in women than in men, although the sample size of females in this study is not being enough to draw any solid conclusion. These results suggested that CYP19A1 plays essential roles in CCA progression and its expression can be used as a CCA prognostic marker, especially for male CCA patients.

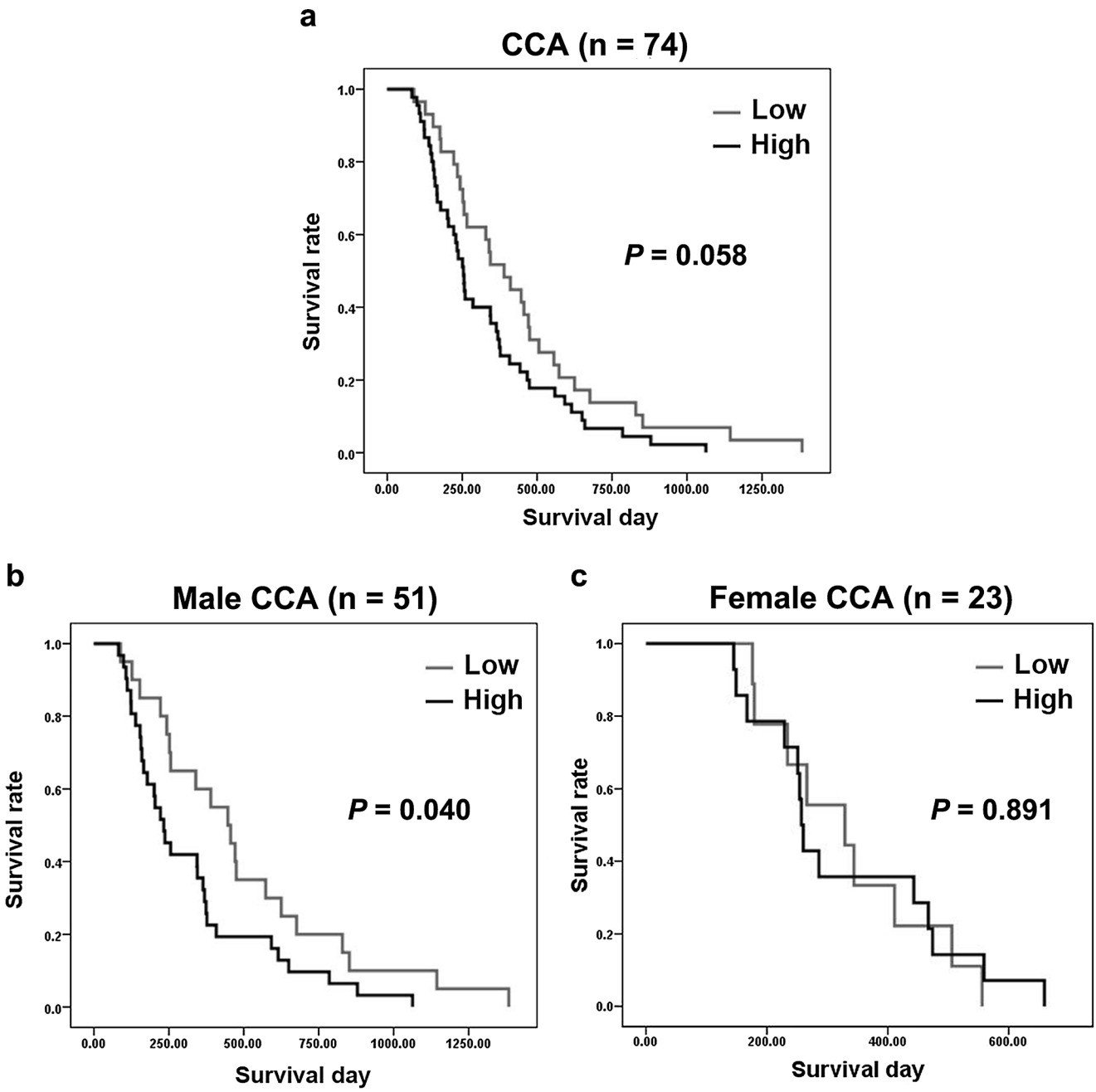

Fig. 2 Kaplan-Meier analysis of CYP19A1 in **a** all human CCA tissues ($n = 74$), **b** male CCA tissues ($n = 51$), and **c** female CCA tissues ($n = 23$)

Expressions of CYP19A1 and Estrogen Receptors in Immortal Cholangiocyte and CCA Cell Lines

The mRNA and protein expression levels of CYP19A1 in two CCA cell lines and in an immortalized cholangiocyte cell line, MMNK1, were examined using real-time PCR and immunocytochemistry. CYP19A1 mRNA and protein levels were highly expressed in CCA cell lines (KKU-213 > KKU-100) compared to those in MMNK1 cell line (Fig. 3a, b). Likewise, ERα, ERβ, and GPR30 were also highly expressed in all CCA cells compared to those in MMNK1 cells, according to the immunocytochemistry results (Fig. 3b).

Effect of CYP19A1 Gene Silencing by siRNA on Cell Proliferation and Migration

Effect of CYP19A1 suppression by siRNA was examined in KKU-213, a high-CYP19A1-expressing CCA cell line. After silencing by specific siRNA (siCYP19A1), both CYP19A1 protein and mRNA levels were decreased at 72 h after treatment when compared with those treated with the scramble (Fig. 4a, b). Moreover, after siCYP19A1 transfection, the expression of BIRC5, an anti-apoptotic gene, was found to be decreased (Fig. 4c). Low expression levels of BIRC could be related to a decrease in CCA cell proliferation rates at 24, 48, and 72 h after

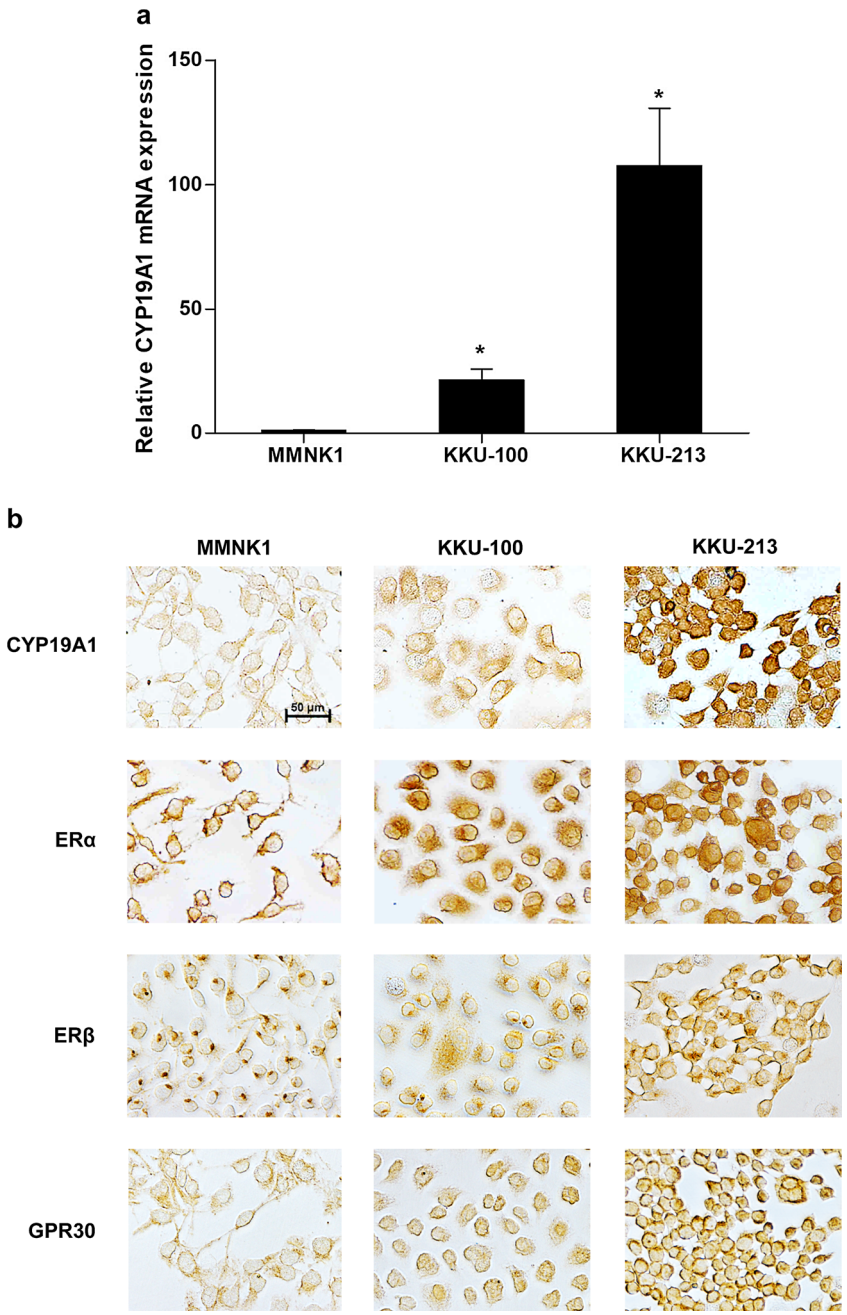

Fig. 3 The expressions of CYP19A1, ERα, ERβ, and GPR30 in cholangiocyte (MMNK1) and CCA (KKU-100 and KKU-213) cell lines. **a** Relative CYP19A1 mRNA expression levels detected by real-time PCR. *P value < 0.05 compared to MMNK1. **b** CYP19A1, ERα, ERβ, and GPR30 protein expressions detected by immunocytochemistry. Positive staining results were represented in brown color

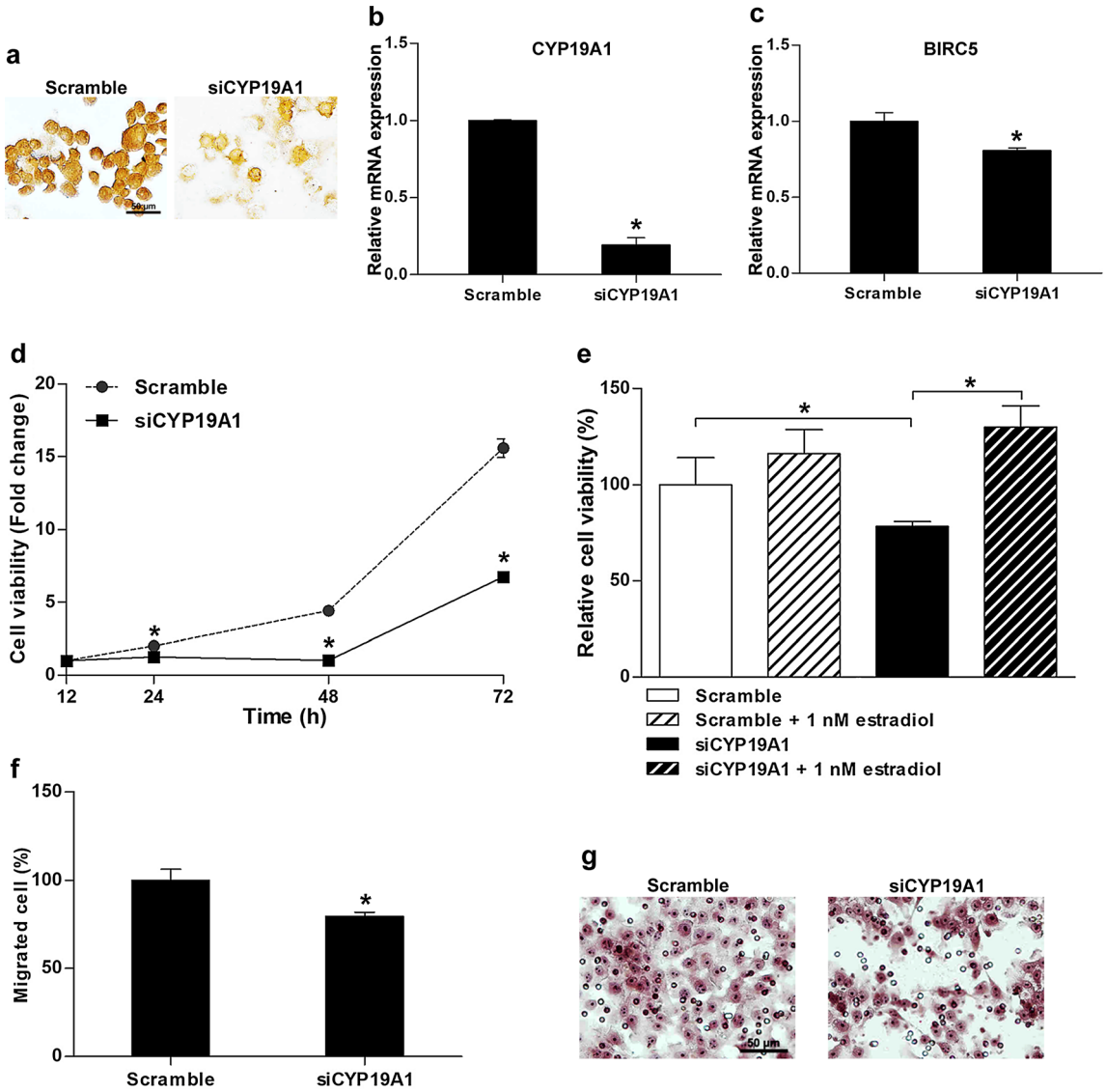

Fig. 4 Effect of CYP19A1 silencing on KKU-213 CCA cell proliferation and migration between cells transfected with siCYP19A1 or scramble. **a** Expression levels of CYP19A1 protein (brown staining) in cells treated with siCYP19A1 or scramble. **b** Relative CYP19A1 mRNA expression levels (mean ± SD) detected by real-time PCR. **c** Relative BIRC5 mRNA expression levels (mean ± SD) analyzed by real-time PCR. **d** Cell viability assays (represented as fold change ± SD relative to scrambled control) in cells treated with siCYP19A1 or scramble. **e** Effect of 17β-estradiol on cell viability, represented as relative percentage ± SD, analyzed using MTT assay at 48 h post treatment. **f** Effect of siCYP19A1 on the cell migration expressed as a percentage of migrated cells (mean ± SD) at 12 h post migration and **g** hematoxylin staining of migrated cells (red-purple color) at 12 h post migration. *P value < 0.05 compared to a scramble control (**b**–**d**, **f**). Scale bars are equal to 50 μm

re-seeding (Fig. 4d). However, adding exogenous estradiol to the culture media could restore the reduction of proliferative activity of CYP19A1-knockdown-KKU213 cells (Fig. 4e). On the other hand, siCYP19A1 treatment significantly could reduce the migration activity of cancer cells at 12 h after re-seeding (Fig. 4f, g). However, the reduced migration activity was rescued by adding external estradiol, as evidence by an increase in the number of migrated cells in scramble- or siCYP19A1-treated cells as shown in Fig. S1 (see Supporting Information). Thus, our results confirm that CYP19A1 increases estrogen generation, which leads to the upregulation of the estrogen response and results in CCA progression with aggressive clinical outcomes.

Effects of CYP19A1 Inhibitors on Cell Viability of CCA Cell Lines

The effects of exemestane and letrozole on cell viability of two different CCA cell lines, i.e., CYP19A1-low-KKU-100 and CYP19A1-high-KKU-213, were investigated. The selected cell lines were treated with various concentrations of exemestane or letrozole for 48 h. The results showed that the calculated IC_{50} (mean ± SD) of CYP19A1 inhibitors for KKU-213 were 5.36 ± 2.68 μM of exemestane and 42.00 ± 16.35 μM of letrozole which are more sensitive than those of KKU-100 with IC_{50}

76.42 ± 12.11 μM of exemestane and 125.59 ± 31.64 μM of letrozole (Fig. 5). These results suggest that both CYP19A1 inhibitors are more effective in suppressing cell viabilities of CYP19A1-high-KKU-213 cells than those of CYP19A1-low-KKU-100 cells.

Discussion

Estrogen receptors and estrogen responsive proteins play important roles in CCA progression via induction of cell proliferation, cell invasion, and angiogenesis [12–14]. Moreover, serum estrogen levels of male CCA patients were significantly higher than in healthy male subjects [12]. The present results demonstrated that CYP19A1 was overexpressed in CCA cells compared with that in normal bile duct cells in the adjacent normal tissues. The majority of CCA patients have high expression levels of CYP19A1 and estrogen receptors (ERα, ERβ, and/or GPR30). A positive correlation between the expression levels of CYP19A1 and estrogen receptors was observed. These results suggest that overexpressed CYP19A1 may upregulate the estrogen production by converting androgen to estrogen resulting in high concentrations of estrogen in CCA cells. These processes promote the induction of estrogen responses of CCA cells and could be, at least in part, a possible cause of elevated serum estrogen levels of CCA patients.

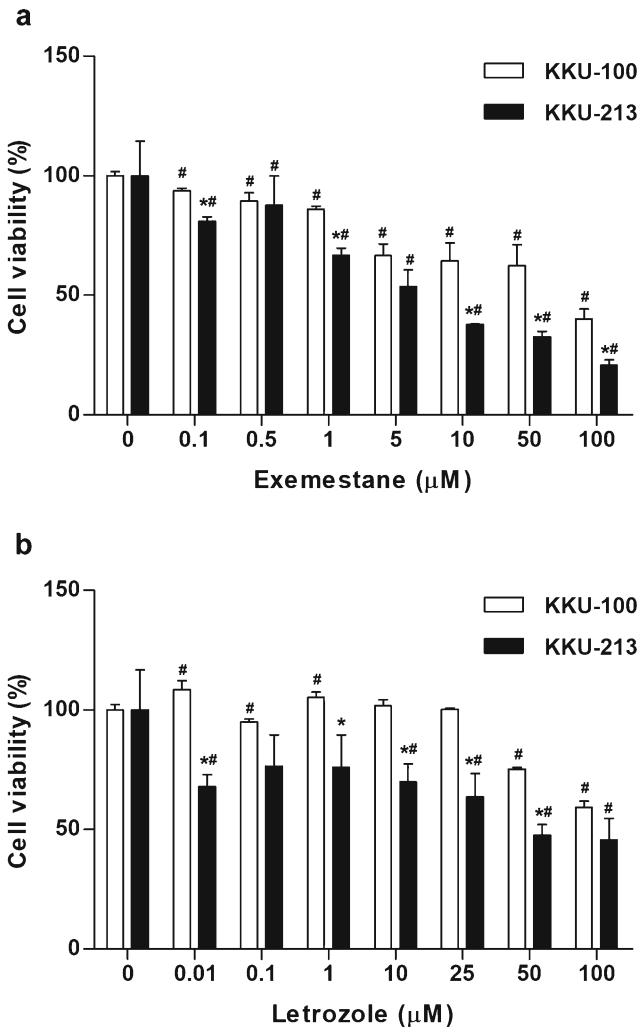

Fig. 5 Effect of exemestane (a) and letrozole (b) on cell viability of CCA cell lines. The percentage of relative cell viabilities (mean ± SD) was investigated by MTT assay in KKU-100 and KKU-213 treated with various concentrations of exemestane or letrozole for 48 h. **P* value < 0.05 compared to KKU-100 at the same concentrations. #*P* value < 0.05 compared to 0 μM of drug in each cell type

CYP19A1 overexpression is involved in the progression of various types of estrogen-related cancers such as breast, lung, and bladder cancers [18–20, 22]. In the present study, overexpression of CYP19A1 was significantly correlated with metastatic status of CCA patients. CYP19A1 may promote CCA progression through induction of cell invasion and migration. This finding was further confirmed by the knockdown of CYP19A1 in CCA cell lines using specific siRNA. CYP19A1 silencing significantly reduced the cell migration activity of CCA cell lines. The reduction of cell migration activity was rescued by adding external estradiol to the cell culture media. Related to this, the expression of CYP19A1 in CCA tissues was not significantly correlated with poor prognosis in general. However, statistically significant correlation was observed in male CCA patients. These findings suggested that CYP19A1 is involved in CCA progression and is associated with aggressive clinical outcomes, especially in male CCA patients. Furthermore, CYP19A1 expression was positively correlated with GPR30 expression. GPR30 modulates both genomic transcriptional action and rapid non-genomic action of estrogen [28] and is involved in progression of estrogen-related cancers such as breast, endometrium, and ovarian cancers through the activations of EGFR/MAPK and PI3K/Akt signaling pathways [29–31]. Since the EGFR/MAPK and PI3K/Akt signaling pathways are implicated in the progression of CCA [16, 32, 33], the activation of GPR30 by estrogen is associated with CCA progression via modulation of EGFR/MAPK and PI3K/Akt signaling pathways.

Since overexpression of CYP19A1 has been reported in estrogen-related cancers and CYP19A1 inhibitors including anastrozole, letrozole, and exemestane are currently in clinical use for breast cancer treatment [34], exemestane is known to reduce cell viability of breast cancer cells via activation of mitochondrial-mediated apoptosis and induction of autophagy [35]. This compound also inhibited tumorigenic properties and caused morphological changes of non-small cell lung cancer cell lines [22, 23]. On the other hand, letrozole could reduce tumor size and induce apoptosis of ER-positive breast cancer cells both in vitro and in vivo [20]. Additionally,

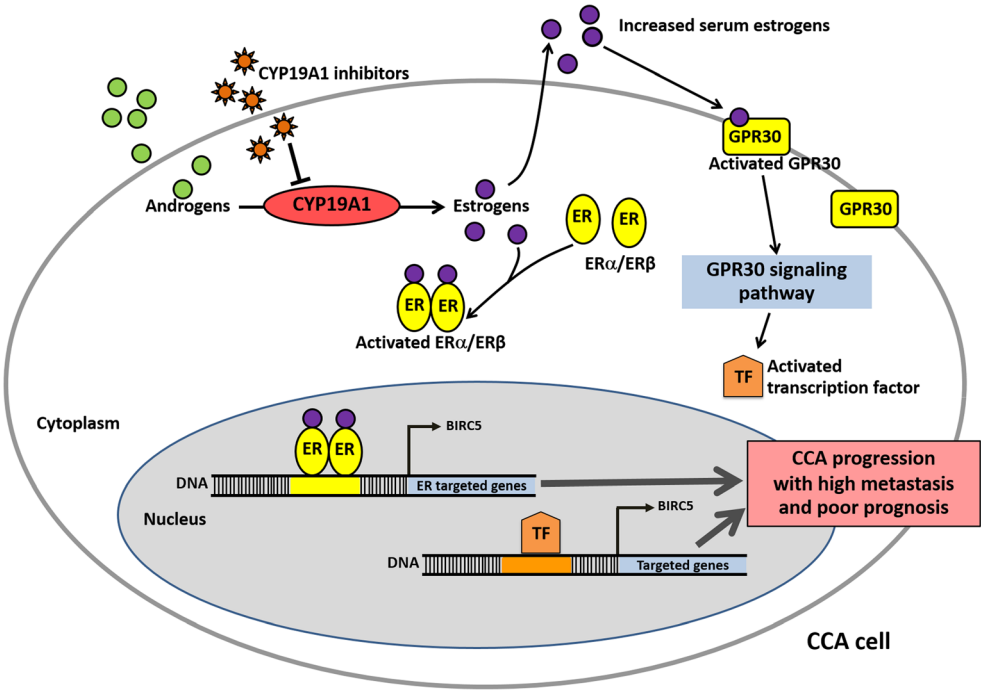

Fig. 6 Proposed mechanisms of CYP19A1 and estrogen-related proteins in CCA progression (ER estrogen receptor)

plasma estrogen levels of postmenopausal women with breast cancer were downregulated by CYP19A1 inhibitors [36, 37]. In the present study, two CYP19A1 inhibitors, exemestane and letrozole, could suppress cell proliferation of both high- and low-CYP19A1-expressing CCA cell lines. Drug sensitivities of the cell lines were related to the basal level of CYP19A1 expression. Similarly, silencing of CYP19A1 using specific siRNA reduced cell proliferation of CCA cells via downregulation of BIRC5 which is an anti-apoptotic gene. This result was consistent with BIRC5 being known as an estrogen-inducible gene in human ovarian and breast cancer cells [38, 39]. Moreover, BIRC5 expression was associated with development and poor prognosis of CCA [40, 41], suggesting its anti-apoptotic role in CCA. Interestingly, the reduction of cell proliferation by siCYP19A1 was rescued by the addition of exogenous estradiol. Therefore, in the case of CCA, it could be suggested that CYP19A1 promoted CCA cell proliferation and inhibited cell apoptosis via estrogen-related pathways.

Based on the aforementioned findings, we propose possible mechanisms by which CYP19A1 and downstream estrogen-related proteins can cause CCA progression (Fig. 6). Briefly, the overexpression of CYP19A1 permits elevated conversion of androgen to estrogen, resulting in accumulation of estrogen in cancer cells and high serum estrogen levels. High estrogen levels can cause aberrant estrogen-ER interactions, which in turn can trigger the expressions of estrogen-responsive genes via activating nuclear estrogen receptor (ER) and/or membrane receptor (GPR30) signaling cascade and promoting ER/GPR30-dependent transcription factor changes in response to estrogen, leading to CCA progression with aggressive clinical outcomes. Therefore, CYP19A1 inhibitors could be applied for the targeted therapy of CCA.

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Author Contributions Designed research topic, drafted research grants, and analyzed data: RT. Drafted and wrote the manuscript: RT and WK. Performed experiments and analyzed data: WK, CS, PU, and NA. Analyzed clinical data of CCA patients: PU and NK. Critically reviewed the manuscript and provided input: NN and KS. All authors are involved in drafting the article critically for important intellectual content and have read and approved the final version of the manuscript.

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Compliance with Ethical Standards

The protocols of sample collection and study plan were approved by the Ethics Committee for Human Research, Khon Kaen University (HE571283). All CCA subjects were provided with the approved informed consent.

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Ito T, Sakurai-Yageta M, Goto A, Pairojkul C, Yongvanit P, Murakami Y (2014) Genomic and transcriptional alterations of cholangiocarcinoma. J Hepatobiliary Pancreat Sci 21:380–387
2. Khuntikeo N, Chamadol N, Yongvanit P, Loilome W, Namwat N, Sithithaworn P, Andrews RH, Petney TN, Promthet S, Thinkhamrop K, Tawarungruang C, Thinkhamrop B, investigators C (2015) Cohort profile: cholangiocarcinoma screening and care program (CASCAP). BMC Cancer 15:459. https://doi.org/10.1186/s12885-015-1475-7
3. Sripa B, Pairojkul C (2008) Cholangiocarcinoma: lessons from Thailand. Curr Opin Gastroenterol 24:349–356
4. Sithithaworn P, Yongvanit P, Duenngai K, Kiatsopit N, Pairojkul C (2014) Roles of liver fluke infection as risk factor for cholangiocarcinoma. J Hepatobiliary Pancreat Sci 21:301–308
5. Cui J, Shen Y, Li R (2013) Estrogen synthesis and signaling pathways during aging: from periphery to brain. Trends Mol Med 19:197–209
6. Russo J, Russo IH (2006) The role of estrogen in the initiation of breast cancer. J Steroid Biochem Mol Biol 102:89–96
7. Chen GG, Zeng Q, Tse GM (2008) Estrogen and its receptors in cancer. Med Res Rev 28:954–974
8. Niikawa H, Suzuki T, Miki Y, Suzuki S, Nagasaki S, Akahira J, Honma S, Evans DB, Hayashi S, Kondo T, Sasano H (2008) Intratumoral estrogens and estrogen receptors in human non-small cell lung carcinoma. Clin Cancer Res 14:4417–4426
9. Stabile LP, Davis AL, Gubish CT, Hopkins TM, Luketich JD, Christie N, Finkelstein S, Siegfried JM (2002) Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor alpha and beta and show biological responses to estrogen. Cancer Res 62:2141–2150
10. Zhang Z, Zhou D, Lai Y, Liu Y, Tao X, Wang Q, Zhao G, Gu H, Liao H, Zhu Y, Xi X, Feng Y (2012) Estrogen induces endometrial cancer cell proliferation and invasion by regulating the fat mass and obesity-associated gene via PI3K/AKT and MAPK signaling pathways. Cancer Lett 319:89–97
11. Shen SS, Smith CL, Hsieh JT, Yu J, Kim IY, Jian W, Sonpavde G, Ayala GE, Younes M, Lerner SP (2006) Expression of estrogen receptors-alpha and -beta in bladder cancer cell lines and human bladder tumor tissue. Cancer 106:2610–2616
12. Hunsawong T, Singsuksawat E, In-chon N, Chawengrattanachot W, Thuwajit C, Sripa B, Paupairoj A, Chau-in S, Thuwajit P (2012) Estrogen is increased in male cholangiocarcinoma patients' serum and stimulates invasion in cholangiocarcinoma cell lines in vitro. J Cancer Res Clin Oncol 138:1311–1320
13. Mancino A, Mancino MG, Glaser SS, Alpini G, Bolognese A, Izzo L, Francis H, Onori P, Franchitto A, Ginanni-Corradini S, Gaudio E, Alvaro D (2009) Estrogens stimulate the proliferation of human cholangiocarcinoma by inducing the expression and secretion of vascular endothelial growth factor. Dig Liver Dis 41:156–163
14. Alvaro D, Barbaro B, Franchitto A, Onori P, Glaser SS, Alpini G, Francis H, Marucci L, Sterpetti P, Ginanni-Corradini S, Onetti Muda A, Dostal DE, De Santis A, Attili AF, Benedetti A, Gaudio E (2006) Estrogens and insulin-like growth factor 1 modulate neoplastic cell growth in human cholangiocarcinoma. Am J Pathol 169:877–888
15. Thuwajit P, Chawengrattanachot W, Thuwajit C, Sripa B, May FE, Westley BR, Tepsiri NN, Paupairoj A, Chau-In S (2007) Increased TFF1 trefoil protein expression in Opisthorchis viverrini-associated cholangiocarcinoma is important for invasive promotion. Hepatol Res 37:295–304
16. Kosriwong K, Menheniott TR, Giraud AS, Jearanaikoon P, Sripa B, Limpaiboon T (2011) Trefoil factors: tumor progression markers and mitogens via EGFR/MAPK activation in cholangiocarcinoma. World J Gastroenterol 17:1631–1641
17. Singsuksawat E, Thuwajit C, Charngkaew K, Thuwajit P (2018) Increased ETV4 expression correlates with estrogen-enhanced proliferation and invasiveness of cholangiocarcinoma cells. Cancer Cell Int 18:25. https://doi.org/10.1186/s12935-018-0525-z
18. Bulun SE, Chen D, Lu M, Zhao H, Cheng Y, Demura M, Yilmaz B, Martin R, Utsunomiya H, Thung S, Su E, Marsh E, Hakim A, Yin P, Ishikawa H, Amin S, Imir G, Gurates B, Attar E, Reierstad S, Innes J, Lin Z (2007) Aromatase excess in cancers of breast, endometrium and ovary. J Steroid Biochem Mol Biol 106:81–96
19. Nguyen DP, O'Malley P, Al Hussein Al Awamlh B, Furrer MA, Mongan NP, Robinson BD, Wang GJ, Scherr DS (2016) Association of aromatase with bladder cancer stage and long-term survival: new insights into the hormonal paradigm in bladder cancer. Clin Genitourin Cancer 15:256–262.e1. https://doi.org/10.1016/j.clgc.2016.05.017
20. Mukhopadhyay KD, Liu Z, Bandyopadhyay A, Kirma NB, Tekmal RR, Wang S, Sun LZ (2015) Aromatase expression increases the survival and malignancy of estrogen receptor positive breast cancer cells. PLoS One 10:e0121136. https://doi.org/10.1371/journal.pone.0121136
21. Skjefstad K, Grindstad T, Khanehkenari MR, Richardsen E, Donnem T, Kilvaer T, Andersen S, Bremnes RM, Busund LT, Al-Saad S (2016) Prognostic relevance of estrogen receptor alpha, beta and aromatase expression in non-small cell lung cancer. Steroids 113:5–13
22. Giannopoulou E, Siatis KE, Metsiou D, Kritikou I, Papachristou DJ, Kalofonou M, Koutras A, Athanassiou G, Kalofonos HP (2015) The inhibition of aromatase alters the mechanical and rheological properties of non-small-cell lung cancer cell lines affecting cell migration. Biochim Biophys Acta 1853:328–337
23. Koutras A, Giannopoulou E, Kritikou I, Antonacopoulou A, Evans TR, Papavassiliou AG, Kalofonos H (2009) Antiproliferative effect of exemestane in lung cancer cells. Mol Cancer 8:109. https://doi.org/10.1186/1476-4598-8-109
24. Narasaka T, Moriya T, Endoh M, Suzuki T, Shizawa S, Mizokami Y, Matsuoka T, Sasano H (2000) 17Beta-hydroxysteroid dehydrogenase type 2 and dehydroepiandrosterone sulfotransferase in the human liver. Endocr J 47:697–705
25. Liossi AK, Aroni KG, Kyrkou KA, Kittas C, Markaki SP (1988) Immunohistochemical study of sex steroid hormones in primary liver cancer. Cancer Detect Prev 13:195–201
26. Thanan R, Murata M, Ma N, Hammam O, Wishahi M, El Leithy T, Hiraku Y, Oikawa S, Kawanishi S (2012) Nuclear localization of COX-2 in relation to the expression of stemness markers in urinary bladder cancer. Mediat Inflamm 2012:165879. https://doi.org/10.1155/2012/165879
27. Maruyama M, Kobayashi N, Westerman KA, Sakaguchi M, Allain JE, Totsugawa T, Okitsu T, Fukazawa T, Weber A, Stolz DB, Leboulch P, Tanaka N (2004) Establishment of a highly differentiated immortalized human cholangiocyte cell line with SV40T and hTERT. Transplantation 77:446–451
28. Wang D, Hu L, Zhang G, Zhang L, Chen C (2010) G protein-coupled receptor 30 in tumor development. Endocrine 38:29–37
29. Filardo EJ (2002) Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. J Steroid Biochem Mol Biol 80:231–238
30. Fujiwara S, Terai Y, Kawaguchi H, Takai M, Yoo S, Tanaka Y, Tanaka T, Tsunetoh S, Sasaki H, Kanemura M, Tanabe A, Yamashita Y, Ohmichi M (2012) GPR30 regulates the EGFR-Akt cascade and predicts lower survival in patients with ovarian cancer. J Ovarian Res 5:35

31. Ge X, Guo R, Qiao Y, Zhang Y, Lei J, Wang X, Li L, Hu D (2013) The G protein-coupled receptor GPR30 mediates the nontranscriptional effect of estrogen on the activation of PI3K/Akt pathway in endometrial cancer cells. Int J Gynecol Cancer 23:52–59
32. Padthaisong S, Thanee M, Techasen A, Namwat N, Yongvanit P, Liwatthakun A, Hankla K, Sangkhamanon S, Loilome W (2017) Nimotuzumab inhibits cholangiocarcinoma cell metastasis via suppression of the epithelial-mesenchymal transition process. Anticancer Res 37:3591–3597
33. Yothaisong S, Dokduang H, Techasen A, Namwat N, Yongvanit P, Bhudhisawasdi V, Puapairoj A, Riggins GJ, Loilome W (2013) Increased activation of PI3K/AKT signaling pathway is associated with cholangiocarcinoma metastasis and PI3K/mTOR inhibition presents a possible therapeutic strategy. Tumour Biol 34:3637–3648
34. Chumsri S (2015) Clinical utilities of aromatase inhibitors in breast cancer. Int J Womens Health 7:493–499
35. Amaral C, Borges M, Melo S, da Silva ET, Correia-da-Silva G, Teixeira N (2012) Apoptosis and autophagy in breast cancer cells following exemestane treatment. PLoS One 7:e42398. https://doi.org/10.1371/journal.pone.0042398
36. Geisler J, King N, Anker G, Ornati G, Di Salle E, Lonning PE, Dowsett M (1998) In vivo inhibition of aromatization by exemestane, a novel irreversible aromatase inhibitor, in postmenopausal breast cancer patients. Clin Cancer Res 4:2089–2093
37. Geisler J, Haynes B, Anker G, Dowsett M, Lonning PE (2002) Influence of letrozole and anastrozole on total body aromatization and plasma estrogen levels in postmenopausal breast cancer patients evaluated in a randomized, cross-over study. J Clin Oncol 20:751–757
38. Zhu J, Lu X, Hua KQ, Sun H, Yu YH, Feng YJ (2012) Oestrogen receptor alpha mediates 17beta-estradiol enhancement of ovarian cancer cell motility through up-regulation of survivin expression. Arch Gynecol Obstet 286:729–737
39. Yu X, Zhang X, Dhakal IB, Beggs M, Kadlubar S, Luo D (2012) Induction of cell proliferation and survival genes by estradiol-repressed microRNAs in breast cancer cells. BMC Cancer 12:29. https://doi.org/10.1186/1471-2407-12-29
40. Javle MM, Tan D, Yu J, LeVea CM, Li F, Kuvshinoff BW, Gibbs JF (2004) Nuclear survivin expression predicts poor outcome in cholangiocarcinoma. Hepatogastroenterology 51:1653–1657
41. Chang Q, Liu ZR, Wang DY, Kumar M, Chen YB, Qin RY (2004) Survivin expression induced by doxorubicin in cholangiocarcinoma. World J Gastroenterol 10:415–418

Manuscript for Biochemical and Biophysical Research Communications (full paper ~4,300 words; limited 4,600 words, 4 tables +figures)

Oxidative stress induces Zinc finger protein 423 expression lead to cholangiocarcinoma cell proliferation

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Abstract (241 words, limited 250 words)

Oxidative stress is an imbalance of oxidant/antioxidant systems that play roles in several diseases including cancer. Cholangiocarcinoma (CCA) is a primary liver cancer which has cholangiocyte cell phenotypes and it was investigated as an oxidative stress-driven carcinogenesis. Up-regulations of oncogenes and down-regulations of tumor suppressor genes are often found in tumorigenesis. ZNF423 is a transcriptional factor that act as oncogene in leukemia and nasopharyngeal carcinoma. In this study, we hypothesized that ZNF423 is an oxidative stress-targeted gene which involve in CCA genesis. ZNF423 expression patterns and 8-oxodG (an oxidative stress marker) formations were investigated using immunohistochemical analysis (IHC). The IHC staining results showed that ZNF423 was overexpressed in CCA cells compared to normal bile duct cells located at tumor adjacent areas. The expression of ZNF423 was also positive correlated with 8-oxodG staining results. CCA patients who had high ZNF423 expression and high 8-oxodG staining in the tumor tissues were significantly correlated with poor prognosis. Moreover, ZNF423 expression was increased in hydrogen peroxide-treated immortal cholangiocyte cell line (MMNK1), suggesting that oxidative stress induces ZNF423 expression. The important roles of ZNF423 in CCA progression were further investigated using specific siRNA for ZNF423 knockdown-CCA cell line (KKU-100). Cell proliferation and colony assays were significantly reduced in the ZNF423-knockdown-CCA cells compared to the control cells. Taken together, this study demonstrated that oxidative stress induces ZNF423 expression leading to CCA cell proliferation. Therefore, ZNF423 is an oxidative stress targeted gene and it acts as oncogene in CCA.

Keywords:

Zinc finger protein 423, ZNF423, Oxidative stress, Cholangiocarcinoma, Cancer progression

1. Introduction

Cholangiocarcinoma (CCA), a malignancy of bile duct epithelial cells, has the highest incidence in the northeast of Thailand [1,2]. The major risk factor in this endemic area is the infection of liver fluke also known as *Opisthorchis viverrini* (Ov) [3]. Ov-infection can cause chronic inflammation via contribute to overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) resulting in oxidative stress. Oxidative stress is a condition that disturbance in the balance between oxidant and anti-oxidant systems. This condition can causes DNA damage via the formation of 8-oxo-2'-deoxyguanosine (8-oxodG), thus it can use as a marker for DNA damage [4,5,6]. Moreover, oxidative stress could alter gene expression profiling of tumor suppressor and oncogene genes leading to cancer promotion/progression of many tumor types including CCA [7]. On the contrary, the oxidative stress-targeted gene expression in CCA progression is under-estimated.

Zinc finger protein 423 (ZNF423) is a transcription factor which belongs to the family of Krüppel-like C_2H_2 zinc finger proteins. This protein contains 30 domains that are known as binding of DNA and proteins [8] and is implicated in numerous developmental pathways, notably neurogenesis and adipogenesis [9]. The ZNF423 causes neurogenesis by activating early B-cell factor 1 (EBF1) and Notch pathways [10,11]. ZNF423 promotes adipogenesis via peroxisome proliferator-activated receptor γ (PPARγ) pathway [12,13]. Besides, this protein acts as an important transcriptional modulator in several types of cancer, including leukemia [14,15], nasopharyngeal carcinoma (NPC) [16], neuroblastoma [17] and breast cancer [18,19]. ZNF423 plays different roles in various cell types depend on its downstream interaction. In leukemia and NPC, ZNF423 plays role as oncogene to drive progression of these cancer types [14,15,16]. On the other hand, ZNF423 act as tumor suppressor in neuroblastoma and breast cancer [17,19].

The aforementioned results enable us to assess the roles of ZNF423 in tumorigenic properties and the association between ZNF423 and oxidative stress in CCA. A hypothesis was formulated that the ZNF423 might be over-expressed in CCA by oxidative stress. Furthermore, a high expression level of ZNF423 potentially enhances tumorigenic of the cancer cells, which in turn can lead to CCA progression. This is the first functional study of ZNF423 in CCA. Firstly, we design to detect the expression patterns of ZNF423 in MMNK1 and CCA cell line using quantitative real-time PCR and immunocytochemical analysis. Likewise, ZNF423 expression and the formation of 8-oxodG in CCA tissues will be performed using immunohistochemistry analysis. In addition, H_2O_2 treatment in MMNK1 and CCA cell lines will be studied to evaluate the relationship between oxidative stress and ZNF423 expression. Moreover, the functions of ZNF423 in relation to tumorigenic will be examined using small interfering RNA (siRNA) technique.

2. Materials and methods

2.1 Paraffin-embedded CCA tissues

The 75 cases paraffin-embedded CCA tissues were recruited from biobank of Cholangiocarcinoma Research Institute, Khon Kaen University, Thailand. The protocol of tissue collection was already approved by the Khon Kaen University Ethics Committee for Human Research (HE571283).

2.2 Human cell lines and cell culture

CCA cell line which is KKU-100 (poorly differentiated adenocarcinoma), will be obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank. The immortalized cholangiocyte cell line, MMNK1 was established and characterized at Okayama University [20]. Human CCA cell line (KKU-100) and MMNK1 cell line were cultured in Ham's F-12 (Gibco®, Life technologies, NY, USA) which supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Life technologies TM). The media were changed once every two to three days. For all cell lines were incubated in a humidified incubator with 5% CO_2 and 95% relative humidity at 37 °C.

2.3 Immunohistochemistry (IHC)

The expression patterns of ZNF423 and the formation of 8-oxodG in CCA tissues were determined by IHC. Then, IHC protocol was performed follow by the previous study [21]. Rabbit anti-human ZNF423 (1:100; Abcam, MA, USA) and mouse anti-8-oxodG

(1:200; Japan Institute for the Control of Aging, Shizuoka, Japan) were used as primary antibody. The stained areas were analyzed using a light microscope. Immunostaining levels were assessed by calculating a total immunostaining index (IHC score) as the result of a frequency and intensity score. The proportion scores were depicted the estimated amount of positive stained cancer cells (0 = none; 1 = 1-25%; 2 = 26–50%; 3= 51–75%; 4 = >75%). The intensity score represented the estimated staining intensity of positive brown to dark-brown colors (0 = negative staining; 1 = weak; 2 = moderate; 3 = strong). The IHC scores was ascertained by multiplying of the frequency score and intensity score. The IHC score were ranged from 0 to 12. In this study, low and high expressions were distinguish using cutoff of the mean of IHC scores.

2.4 Immunocytochemistry (ICC)

The expression patterns of ZNF423 protein were determined in cell line by ICC. MMNK1 cells (6 $x10^4$ cells/well) and CCA cell (3 $x10^4$ cells/well) were seeded into 48-well plates overnight. Then, ICC protocol was performed follow by the previous study [22]. Rabbit anti-ZNF423 antibody (1:50; Abcam, MA, USA) was used as a primary antibody.

2.5 RNA extraction from CCA cell lines and reverse transcription

Total RNA was extracted from approximately $1x10^6$ cells using TRIZOL® reagent (Invitrogen, CA). The RNA solution was kept in -70 °C until used. A RNA sample was reversed into complementary DNA (cDNA) using High-capacity cDNA Reverse

Transcription Kit (Applied Biosystems, USA). The protocols of RNA extraction and reverse transcription were subjected following manufacturer's instructions with a little modified [21].

2.7 Qualitative real-time PCR (qPCR)

The mRNA expression levels of ZNF423 in all CCA cell lines were determined by qPCR method. cDNA was amplified using a gene specific Taqman probes (Hs00323880_m1 ZNF423 and Hs99999903_m1 *β*-actin, Thermo Fisher Scientific Inc). qPCR was performed on an ABI 6000 Real-time PCR system (Applied Biosystems, USA). The cycle threshold (Ct) values was calculated using delta-delta Ct ($2^{-\Delta Ct}$) method. In this study, *β*-actin was used as an internal control.

2.8 Treatment of MMNK1 with H_2O_2

MMNK1 cells ($1x10^5$ cells/well) were plated into 6-well plates. After that, cells were treated with 0, 25, 50 and 100 µM of H_2O_2 (Merck, Germany). The treated cells were suspended in Ham'S F-12 media (Invitrogen, CA, USA) and incubated at 37 °C in 95% relative humidified air with 5% CO_2 for 24, 48 and 72 h. After treatment with H_2O_2, cells were washed with PBS. Next, cells were subjected to RNA extraction using of TRIZOL® reagent (Life technology, USA).

2.9 Knockdown ZNF423 using siRNA

The ZNF423-siRNA was designed by GE HealthHcare Dharmacon (ON-TARGETplus Human ZNF423 siRNAs; cat no. L-012907-00-0005). CCA cells ($9x10^4$ cells/well) were seeded into 6-well plates and transfected with 50 nM siRNA against ZNF423 according to the manufacturer's protocol using lipofectamine® RNAiMax (Invitrogen, USA). Then, the transfected cells were incubated for 72 h at 37 °C in 95% relative humidified air with 5% CO_2. After ZNF423 knockdown, cell proliferation and colony forming ability were determined. The cells transfected with non-targeting control siRNAs (scramble) were used as the control experiments.

2.10 Cell proliferation assay

Cell proliferation was determined by Sulforhodamine B (SRB) assay. Cells were trypsinized and seeded at 2 $x10^3$ cells per well into 96-well flat-bottom microliters plates (triplicate wells per condition) and incubated for 24, 48 and 72 h at 37 °C in 95% relative humidified air with 5% CO_2. Then, 10% Trichloroacetic acid (TCA) was added and incubated at 4 °C for 1 h. TCA-treated cells were stained with 0.4% (w/v) SRB in 1% (v/v) acetic acid for 45 min and washed 3 times with 1% (v/v) acetic acid to remove the unbound stained. The plates were left to dry and the protein-bound stained was solubilized with 200 µl of 10 mM Tris-base (pH 10.5) for 60 min on a shaker. The absorbance was measured at 540 nm using a microplate leader (Tecan AustriaGmbH, Austria).

2.11 Clonogenic assay

Cell forming colonies were investigated by clonogenic assay. Cells were trypsinized and seeded at $2x10^2$ cells in 2,000 ul of Ham's F-12 media into 6-well plates. After that, cells were incubated for 14 days at 37 °C in 95% relative humidified air with 5% CO_2. The media were changed once every two days. The colonies were washed with 1 ml of PBS for 5 min and fixed with 1 ml of 4% paraformaldehyde in PBS for 20 min. Next, the fixed colonies were washed with 1 ml of PBS for 5 min and stained with 1 ml of 0.5% crystal violet diluted in distilled water for 10 min. Then, the dye was removed and rinsed plate with tap water. Finally, the colonies were dried and counted the number of colony using ImageJ free software.

3. Statistical analysis

In this study, statistical analysis was analyzed by SPSS Statistic software version 17.0 (IBM Cooperation, USA). The survival analysis was analyzed using Kaplan-Meier estimate with Log-rank test. The correlation between ZNF423 and 8-oxodG in all tissues were determined by Pearson's correlation coefficients test and Chi-square test. Moreover, the mRNA expression levels of ZNF423 in cell lines were estimated by student *t*-test. *P*-value < 0.05 was classified into statistically significant.

4. Results

4.1 The expression patterns of ZNF423 and the formation of 8-oxodG in CCA tissues

The protein expression levels of ZNF423 in CCA tissues (n=75) were determined using IHC. The results showed that ZNF423 was over-expressed in CCA tissues compared

to normal bile ducts (p<0.001; graph not shown). Using medium value for the cutoff point, 41% (31/75) of CCA tissues showed high ZNF423 expression pattern as shown in Figure 1A. Moreover, 47% (35/75) of CCA tissues showed high 8-oxodG detection pattern as shown in Figure 1B. The ZNF423 expression patterns in CCA tissues were significantly positive correlated with the 8-oxodG detection patterns as shown in Table 1. Taken together, CCA patients who had high ZNF423 expression and high 8-oxodG detection were significantly correlated with poor prognosis (p =0.047 analyzed by Kaplan-Meier estimate with Log-rank test) as shown in Figure 1C. These data revealed that the over-expression of ZNF423 in CCA may induced by oxidative stress consequently to CCA progression with poor prognosis.

4.2 The expressions of ZNF423 and its correlation with clinico-parameter in tissues of CCA patients

Paraffin-embedded human CCA tissues were obtained from 75 intrahepatic CCA patients. These CCA tissues contained male 69% (52/75) and female 31% (23/75). The median of age was 57.8 years. Moreover, CCA metastasis stages were divided into metastasis stage 56% (42/75) and non-metastasis stage 44% (33/75). In this study, histological types of CCA patients were classified into 53% (40/75) tubular type and 47% (35/75) papillary type. Nevertheless, ZNF423 expression had no correlation with metastasis stages, histology types, gender and age (Table 1).

4.3 Oxidative stress induced up-regulation of ZNF423 in cholangiocyte cell line

To assess the effect of oxidative stress to expression of ZNF423, MMNK1 (low ZNF423 expressed cell line) was treated with H_2O_2 in various concentrations including 0, 25, 50 and 100 µM for 24, 48 and 72 h. The result indicated that mRNA expression levels of ZNF423 were significantly increased with dose and time dependents especially at 48 and 72 h after treatments (Figure 2). These finding strongly demonstrated that over-expression of ZNF423 can be induced by reactive oxygen species (ROS).

4.4 ZNF423 induced proliferation and colony forming abilities of CCA cell line

To evaluate the role of ZNF423 in CCA cell line, the baseline expression of ZNF423 was examined in MMNK1 and KKU-100 as shown in Figure S1. The ZNF423 high expressed CCA cell line (KKU-100) was selected for ZNF423 knockdown using specific siRNA. The siRNA effectively decreased mRNA expression level of ZNF423 to approximately 70% (Figure 3A) at 72 h after knockdown. After knocked down, proliferation and colony forming ability of the cell lines were measured using cell proliferation assay and clonogenic assay. The result showed that ZNF423-knockdown-KKU100 cells were significantly decreased cells proliferation ($p<0.001$) (Figure 3B) and colony formation abilities compared to the control cells ($p<0.01$) (Figure 3C-D). Our finding suggested that ZNF423 had oncogenic properties to drive proliferation and colony formation of CCA cell line.

5. Discussion

The previously, an oxidative stress induces DNA damage marker (8-oxodG) formation was detected in CCA tissues and the highly formation of 8-oxodG was correlated with poor prognosis of the CCA patients [21,23]. In this study, ZNF423 was highlighted to be one of an oxidative stress targeted genes which play significant roles in CCA promotion and progression. Because ZNF423 expression could be induced by H_2O_2 in the cholangiocyte cell line. H_2O_2 was reported to be an oxidative stress inducing factor via increasing of superoxide anion ($O_2^{\bullet-}$) generation through NADPH oxidase activity [24,25,26]. Moreover, the expression patterns of ZNF423 in CCA tissues were also correlated with an oxidative stress formation marker (8-oxodG). In addition, ZNF423 act as a CCA progression factor via the induction of cell proliferation and colony formation activities. Notably, this is novel synergistic between oxidative stress and ZNF423 in cancers.

Oxidative stress induced proliferation in several cancer types such as colorectal cancer [27,28]. This condition triggered cell proliferation through Akt pathway via up-regulated β-catenin and cyclin D1 [27]. Moreover, oxidative stress also induced cell proliferation rate of the cholangiocyte cell line (MMNK1), which could be involved in CCA promotion [29]. Then, we further investigated the functions of ZNF423 in CCA progression. The results revealed that ZNF423 induced proliferation and colonies formation of CCA cells. Furthermore, the function of ZNF423 was studied in other cancers especially in leukemia such as chronic myelogenous leukemia (CML), ZNF423 induced proliferation of CML cell line and developed CML into late severe stage of this cancer type [15]. Thus, we assumed that ZNF423 play important roles in CCA promotion and

progression via the increasing of cell proliferation rate of cholangiocyte and CCA cells induced by oxidative stress.

ZNF423 can interacts with all retinoic acid receptor (RAR) types especially retinoid X receptor (RXR) including α, β and γ [30]. Recently, the retinoid X receptor α (RXRα) was reported to play crucial role in CCA. This gene acts as oncogene to enhance growth of CCA cell lines [31]. It activated CCA cell cycle progression through Wnt/β-catenin pathway via up-regulated cyclin D1. Moreover, RXRα play role to drive proliferation of CCA cell lines through NF-κB signaling pathway by increase proliferating cell nuclear antigen (PCNA) and p21 [31]. In addition, RXRγ was overexpressed in CCA and act as oncogene to promote proliferation of CCA cell via the Akt/NF-κB and Wnt/β-catenin pathways [32]. Therefore, ZNF423 may interacts with RXRs leading to CCA proliferation through Wnt/β-catenin and Akt/NF-κB signaling pathway [31]. In conclusion, ZNF423 was an oxidative stress related gene and played role as oncogene to drive CCA genesis. Essentially, the ZNF423 and its related genes might be a novel target therapy in CCA towards to benefit of the patients with this disease.

6. Acknowledgments

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7. References

[1] J. Bridgewater, P.R. Galle, S.A. Khan, J.M. Llovet, J.W. Park, T. Patel, T.M. Pawlik, G. J. Gores, Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma, J Hepatol 60 (2014) 1268-1289.

[2] T.P. Hennedige, W.T. Neo, S.K. Venkatesh, Imaging of malignancies of the biliary tract- an update, Cancer Imaging 14 (2014) 14.

[3] N. Kaewpitoon, S.J. Kaewpitoon, P. Pengsaa, B. Sripa, Opisthorchis viverrini: the carcinogenic human liver fluke, World J Gastroenterol 14 (2008) 666-674.

[4] S. Inoue, S. Kawanishi, Oxidative DNA damage induced by simultaneous generation of nitric oxide and superoxide, FEBS Lett 371 (1995) 86-88.

[5] S. Pinlaor, Y. Hiraku, N. Ma, P. Yongvanit, R. Semba, S. Oikawa, M. Murata, B. Sripa, P. Sithithaworn, S. Kawanishi, Mechanism of NO-mediated oxidative and nitrative DNA damage in hamsters infected with Opisthorchis viverrini: a model of inflammation-mediated carcinogenesis, Nitric Oxide 11 (2004) 175-183.

[6] R. Thanan, M. Murata, S. Pinlaor, P. Sithithaworn, N. Khuntikeo, W. Tangkanakul, Y. Hiraku, S. Oikawa, P. Yongvanit, S. Kawanishi, Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine in patients with parasite infection and effect of antiparasitic drug in relation to cholangiocarcinogenesis, Cancer Epidemiol Biomarkers Prev 17 (2008) 518-524.

[7] A. Leone, M.S. Roca, C. Ciardiello, S. Costantini, A. Budillon, Oxidative Stress Gene Expression Profile Correlates with Cancer Patient Poor Prognosis: Identification of

303 Crucial Pathways Might Select Novel Therapeutic Approaches, Oxid Med Cell
304 Longev 2017 (2017) 2597581.
305 [8] J. Turner, M. Crossley, Mammalian Kruppel-like transcription factors: more than just
306 a pretty finger, Trends Biochem Sci 24 (1999) 236-240.
307 [9] L. Harder, A.C. Puller, M.A. Horstmann, ZNF423: Transcriptional modulation in
308 development and cancer, Mol Cell Oncol 1 (2014) e969655.
309 [10] A. Hata, J. Seoane, G. Lagna, E. Montalvo, A. Hemmati-Brivanlou, J. Massague,
310 OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad
311 and Olf signaling pathways, Cell 100 (2000) 229-240.
312 [11] G. Masserdotti, A. Badaloni, Y.S. Green, L. Croci, V. Barili, G. Bergamini, M.L.
313 Vetter, G.G. Consalez, ZFP423 coordinates Notch and bone morphogenetic protein
314 signaling, selectively up-regulating Hes5 gene expression, J Biol Chem 285 (2010)
315 30814-30824.
316 [12] O.A. MacDougald, M.D. Lane, Transcriptional regulation of gene expression during
317 adipocyte differentiation, Annu Rev Biochem 64 (1995) 345-373.
318 [13] Q.Y. Yang, J.F. Liang, C.J. Rogers, J.X. Zhao, M.J. Zhu, M. Du, Maternal obesity
319 induces epigenetic modifications to facilitate Zfp423 expression and enhance
320 adipogenic differentiation in fetal mice, Diabetes 62 (2013) 3727-3735.
321 [14] L. Harder, G. Eschenburg, A. Zech, N. Kriebitzsch, B. Otto, T. Streichert, A.S.
322 Behlich, K. Dierck, B. Klingler, A. Hansen, M. Stanulla, M. Zimmermann, E.
323 Kremmer, C. Stocking, M.A. Horstmann, Aberrant ZNF423 impedes B cell

differentiation and is linked to adverse outcome of ETV6-RUNX1 negative B precursor acute lymphoblastic leukemia, J Exp Med 210 (2013) 2289-2304.

[15] K. Miyazaki, N. Yamasaki, H. Oda, T. Kuwata, Y. Kanno, M. Miyazaki, Y. Komeno, J. Kitaura, Z. Honda, S. Warming, N.A. Jenkins, N.G. Copeland, T. Kitamura, T. Nakamura, H. Honda, Enhanced expression of p210BCR/ABL and aberrant expression of Zfp423/ZNF423 induce blast crisis of chronic myelogenous leukemia, Blood 113 (2009) 4702-4710.

[16] G.T. Chung, R.W. Lung, A.B. Hui, K.Y. Yip, J.K. Woo, C. Chow, C.Y. Tong, S.D. Lee, J.W. Yuen, S.W. Lun, K.K. Tso, N. Wong, S.W. Tsao, T.T. Yip, P. Busson, H. Kim, J.S. Seo, B. O'Sullivan, F.F. Liu, K.F. To, K.W. Lo, Identification of a recurrent transforming UBR5-ZNF423 fusion gene in EBV-associated nasopharyngeal carcinoma, J Pathol 231 (2013) 158-167.

[17] S. Huang, J. Laoukili, M.T. Epping, J. Koster, M. Holzel, B.A. Westerman, W. Nijkamp, A. Hata, S. Asgharzadeh, R.C. Seeger, R. Versteeg, R.L. Beijersbergen, R. Bernards, ZNF423 is critically required for retinoic acid-induced differentiation and is a marker of neuroblastoma outcome, Cancer Cell 15 (2009) 328-340.

[18] H.M. Bond, S. Scicchitano, E. Chiarella, N. Amodio, V. Lucchino, A. Aloisio, Y. Montalcini, M. Mesuraca, G. Morrone, ZNF423: A New Player in Estrogen Receptor-Positive Breast Cancer, Front Endocrinol (Lausanne) 9 (2018) 255.

[19] J.N. Ingle, M. Liu, D.L. Wickerham, D.J. Schaid, L. Wang, T. Mushiroda, M. Kubo, J.P. Costantino, V.G. Vogel, S. Paik, M.P. Goetz, M.M. Ames, G.D. Jenkins, A. Batzler, E.E. Carlson, D.A. Flockhart, N. Wolmark, Y. Nakamura, R.M.

Weinshilboum, Selective estrogen receptor modulators and pharmacogenomic variation in ZNF423 regulation of BRCA1 expression: individualized breast cancer prevention, Cancer Discov 3 (2013) 812-825.

[20] M. Maruyama, N. Kobayashi, K.A. Westerman, M. Sakaguchi, J.E. Allain, T. Totsugawa, T. Okitsu, T. Fukazawa, A. Weber, D.B. Stolz, P. Leboulch, N. Tanaka, Establishment of a highly differentiated immortalized human cholangiocyte cell line with SV40T and hTERT, Transplantation 77 (2004) 446-451.

[21] N. Armartmuntree, M. Murata, A. Techasen, P. Yongvanit, W. Loilome, N. Namwat, C. Pairojkul, C. Sakonsinsiri, S. Pinlaor, R. Thanan, Prolonged oxidative stress down-regulates Early B cell factor 1 with inhibition of its tumor suppressive function against cholangiocarcinoma genesis, Redox Biol 14 (2018) 637-644.

[22] W. Kaewlert, C. Sakonsinsiri, N. Namwat, K. Sawanyawisuth, P. Ungarreevittaya, N. Khuntikeo, N. Armartmuntree, R. Thanan, The Importance of CYP19A1 in Estrogen Receptor-Positive Cholangiocarcinoma, Horm Cancer 9 (2018) 408-419.

[23] R. Thanan, S. Oikawa, Y. Hiraku, S. Ohnishi, N. Ma, S. Pinlaor, P. Yongvanit, S. Kawanishi, M. Murata, Oxidative stress and its significant roles in neurodegenerative diseases and cancer, Int J Mol Sci 16 (2014) 193-217.

[24] J.S. Beckman, T.W. Beckman, J. Chen, P.A. Marshall, B.A. Freeman, Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide, Proc Natl Acad Sci U S A 87 (1990) 1620-1624.

[25] C.H. Coyle, K.N. Kader, Mechanisms of H2O2-induced oxidative stress in endothelial cells exposed to physiologic shear stress, ASAIO J 53 (2007) 17-22.

[26] W.A. Pryor, G.L. Squadrito, The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide, Am J Physiol 268 (1995) L699-722.

[27] K.A. Kang, K.C. Kim, S.C. Bae, J.W. Hyun, Oxidative stress induces proliferation of colorectal cancer cells by inhibiting RUNX3 and activating the Akt signaling pathway, Int J Oncol 43 (2013) 1511-1516.

[28] S. Reuter, S.C. Gupta, M.M. Chaturvedi, B.B. Aggarwal, Oxidative stress, inflammation, and cancer: how are they linked?, Free Radic Biol Med 49 (2010) 1603-1616.

[29] R. Thanan, A. Techasen, B. Hou, W. Jamnongkan, N. Armartmuntree, P. Yongvanit, M. Murata, Development and characterization of a hydrogen peroxide-resistant cholangiocyte cell line: A novel model of oxidative stress- related cholangiocarcinoma genesis, Biochem Biophys Res Commun 464 (2015) 182-188.

[30] H. Gronemeyer, A. Zelent, Fingering modulators of retinoic acid signaling identifies new prognostic marker for neuroblastoma, Cancer Cell 15 (2009) 249-251.

[31] G.L. Huang, W. Zhang, H.Y. Ren, X.Y. Shen, Q.X. Chen, D.Y. Shen, Retinoid X receptor alpha enhances human cholangiocarcinoma growth through simultaneous activation of Wnt/beta-catenin and nuclear factor-kappaB pathways, Cancer Sci 106 (2015) 1515-1523.

[32] G.L. Huang, Q. Luo, G. Rui, W. Zhang, Q.Y. Zhang, Q.X. Chen, D.Y. Shen, Oncogenic activity of retinoic acid receptor gamma is exhibited through activation of the Akt/NF-kappaB and Wnt/beta-catenin pathways in cholangiocarcinoma, Mol Cell Biol 33 (2013) 3416-3425.

Figure legends

Figure 1 Immunohistochemical analysis ZNF423 (A) and 8-oxodG (B) in normal bile duct (NBD) of tumor adjacent area and CCA. NBD was indicated by the arrows. All figures are 200x original magnification. Scale bar is equal to 50 µm. (C) The expression of ZNF423 and the formation of 8-oxodG with survival rate in CCA analyzed by Kaplan-Meier with log-rank test analyses.

Figure 2 Relative mRNA expression levels of ZNF423 after treatment with H_2O_2 in various concentrations (0, 25, 50 and 100 µM) for 24, 48 and 72 h. Significance was calculated by Student's *t* test compared with untreated condition (*p<0.05, ** p<0.01, ***, p < 0.001). mRNA expression levels of ZNF423 were normalized by β-actin.

Figure 3 Functional analysis of ZNF423 using specific siRNA in CCA cell line. (A) mRNA expression level of ZNF423 after knockdown experiments in KKU-100 were determined using qPCR. mRNA levels of ZNF423 were normalized by β-actin. (B) Relative cell densities analyzed by SRB assay. (C) Clonogenic assay was stained with crystal violet after cell reseeding for 14 days. (D) Graphical represented the relative number of colonies (%). *P*-values were calculated by Student's *t* test compared with scramble condition (*p<0.05, **p<0.01, and ***p<0.001).

412

413 **Supplementary Figure legend**

414 Figure S1 Baseline expression of ZNF423 in KKU-100 and MMNK1 cell line.
415 (A) Quantitative real- time PCR analysis of ZNF423 mRNA level in
416 MMNK1 and KKU- 100 cell lines adjusted by β- actin. (B)
417 Immunocytochemical analysis of ZNF423 protein level in MMNK1 and
418 KKU-100 cell lines. Scale bar is equal to 100 µm.

419

420 **List of Table**

421 Table 1 The correlation of ZNF423 expression in CCA tissues with clicino-
422 pathological data of CCA patients and 8-oxodG formation.

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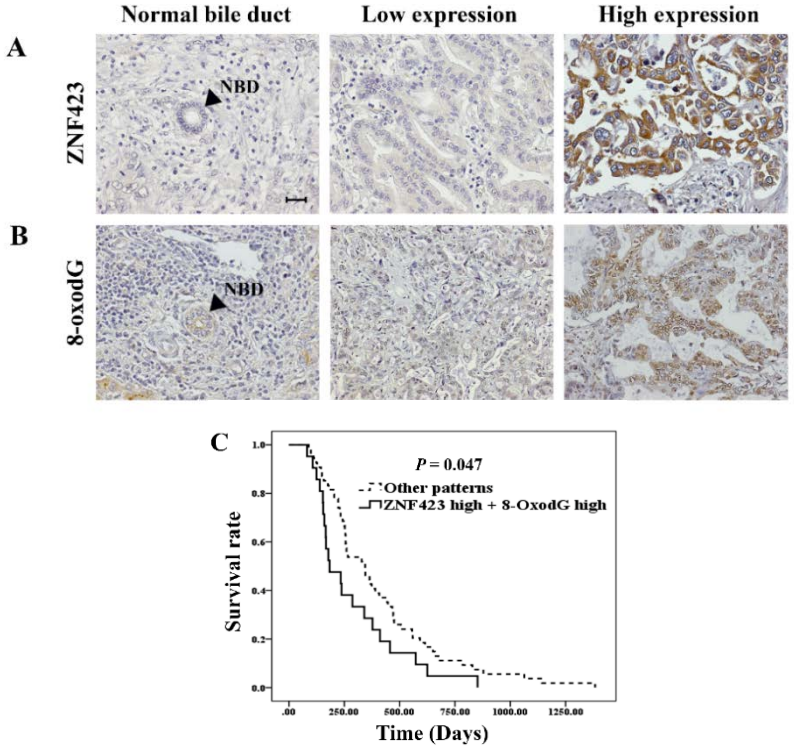
Figure 1



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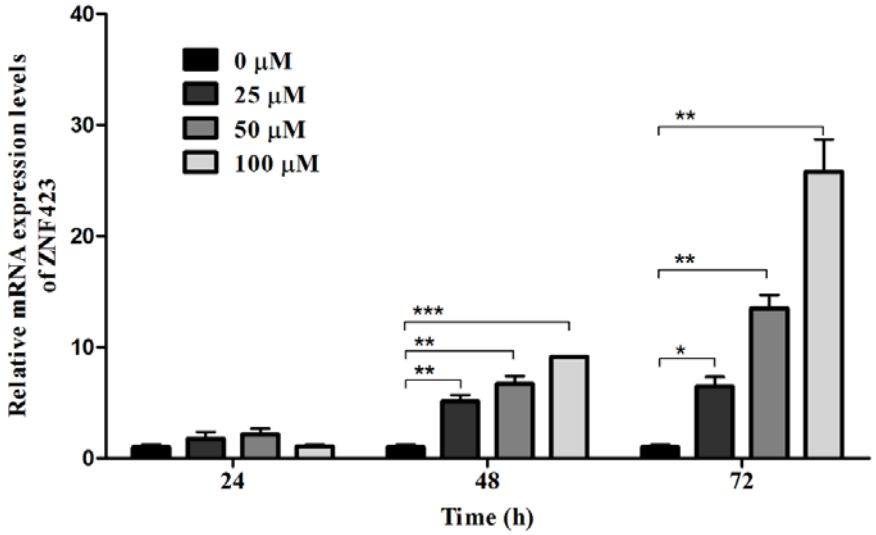
445 **Figure 2**



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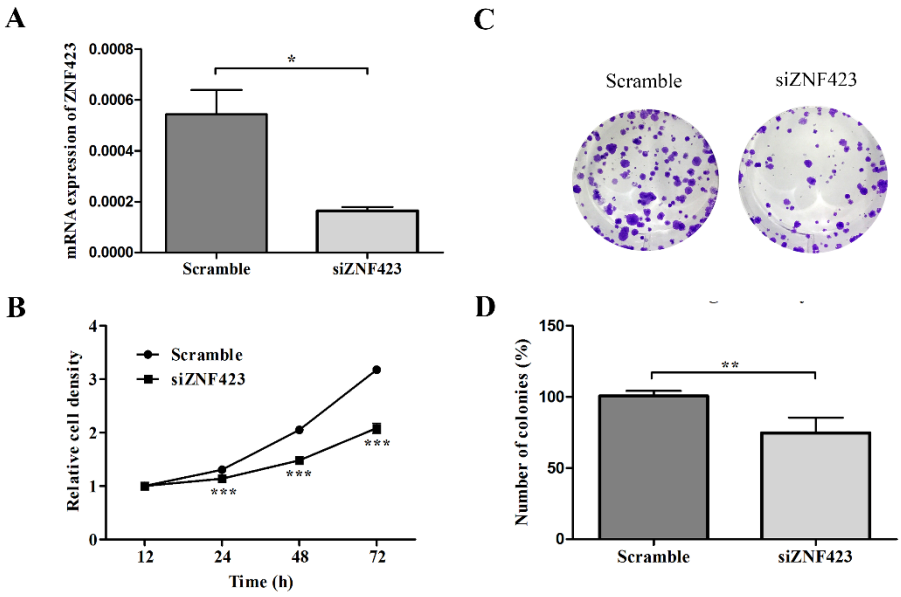
450 of ZNF423 were normalized by β-actin.

451

Figure 3



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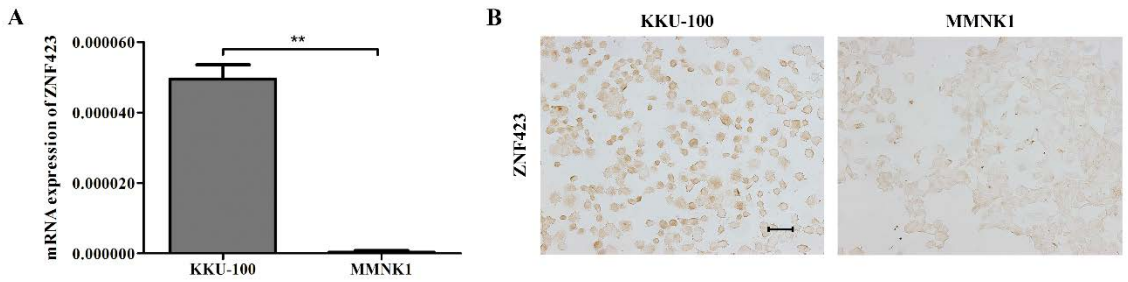
463 **Supplementary Figure**



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468 KKU-100 cell lines. Scale bar is equal to 100 µm.

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474 **Table 1** The correlation of ZNF423 expression in CCA tissues with clicino-

475 pathological data of CCA patients and 8-oxodG formation.

Variables	ZNF423		*P*-value
	Low	High	
Survival day (median(min-max))	297.5 (89-1384)	260 (82-852)	0.101
Metastasis			
- Non-metastasis	20	13	
- Metastasis	24	18	0.474
Histology			
- Tubular	24	16	
- Papillary	20	15	0.493
Gender			
- male	29	23	
- female	15	8	0.306
Age			
- <57.8	23	13	
- >57.8	21	18	0.259
Age (mean±SD)	56.97±7.72	59.03±6.81	0.228
8-oxodG			
- Low	26	9	
- High	18	22	0.009**

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ลำดับ	วันที่ได้รับรางวัล	รางวัล	ผู้ให้รางวัล
1	11 ตุลาคม 2559	รางวัลนักวิจัยดีเด่นด้านปรีคลินิก (อายุไม่เกิน 40 ปี) อ.ดร.เรณู ทานันท์ (หัวหน้าโครงการ)	คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น
2	2 สิงหาคม 2560	ผลงานวิจัยที่ได้รับการอ้างอิงสูงสุด ในปี ค.ศ.2016 ประเภท Review article ผลงานเรื่อง “Oxidative stress and its significant roles in neurodegenerative diseases and cancer” อ.ดร.เรณู ทานันท์ (หัวหน้าโครงการ)	คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น
3	2 สิงหาคม 2560	ผลงานวิจัยที่ได้รับการอ้างอิงสูงสุด ในปี ค.ศ.2016 ประเภท Original article ผลงานเรื่อง “Inflammation-related DNA damage and expression of CD133 and Oct3/4 in cholangiocarcinoma patients with poor prognosis” อ.ดร.เรณู ทานันท์ (หัวหน้าโครงการ)	คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น
4	1 สิงหาคม 2560	รับรางวัลการนำเสนอผลงานวิจัยระดับดี แบบโปสเตอร์ ระดับปริญญาเอก “Oxidative stress and DNA hypermethylation suppress Early B cell factor 1 expression in cholangiocarcinoma in relation with tumor progression and prognosis” นางสาวนภัส อามาตย์มุลตรี (นักศึกษาปริญญาเอก)	ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น
5	1 สิงหาคม 2560	รางวัลการนำเสนอผลงานวิจัยระดับดีเด่น แบบโปสเตอร์ ระดับปริญญาโท “The expressions of CYP19A1 and estrogen receptors in cholangiocarcinoma tissues” นางสาววลีพร แก้วเลิศ (นักศึกษาปริญญาโท)	ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น
6	1 ธันวาคม 2560	รางวัลการนำเสนอผลงานวิจัย ระดับดีเด่น ประเภทพัฒนางานวิทยาศาสตร์และห้องปฏิบัติการด้านมะเร็งท่อน้ำดี “DNA hypermethylation suppresses Early B cell factor 1 expression in cholangiocarcinoma in relation with tumor progression and prognosis” นางสาวนภัส อามาตย์มุลตรี (นักศึกษาปริญญาเอก)	สถาบันมะเร็งท่อน้ำดี มหาวิทยาลัยขอนแก่น
7	22 มิถุนายน 2561	รับรางวัลการนำเสนอผลงานวิจัยระดับดีเด่น แบบโปสเตอร์ “Upregulated CYP19A1 expression promotes cell proliferation and migration in male cholangiocarcinoma patients” นางสาววลีพร แก้วเลิศ (นักศึกษาปริญญาโท)	The 6th International Conference on Biochemistry and Molecular Biology (BMB 2018)
8	26 กันยายน 2561	Travel grant อ.ดร.เรณู ทานันท์ (หัวหน้าโครงการ)	The 77th Annual Meeting of the Japanese Cancer Association commitee


๒๔๘๕
คณะแพทยศาสตร์
รางวัลผลงานวิจัยที่ได้รับการอ้างอิงสูงสุด ในปี ค.ศ. 2016
ประเภท Original article
อาจารย์เรณู ทานันท์ และคณะฯ
ผลงานวิจัย เรื่อง Inflammation-related DNA damage and expression of
CD133 and Oct3/4 in cholangiocarcinoma patients with poor prognosis.
ตีพิมพ์ใน Free Radical Biology and Medicine 2013; 65: 1464-1472.
ให้ไว้ ณ วันที่ ๒ สิงหาคม พ.ศ. ๒๕๖๐
(รองศาสตราจารย์ชาญชัย พานทองวิริยะกุล)
คณบดีคณะแพทยศาสตร์


๒๕๑๕
คณะแพทยศาสตร์
รางวัลผลงานวิจัยที่ได้รับการอ้างอิงสูงสุด ในปี ค.ศ. 2016
ประเภท Review article
อาจารย์เรณู ทานันท์ และคณะฯ
ผลงานวิจัย เรื่อง Oxidative stress and its significant roles
in neurodegenerative diseases and cancer.
ตีพิมพ์ใน International Journal of Molecular Sciences 2014; 16(1): 193-217.
ให้ไว้ ณ วันที่ ๒ สิงหาคม พ.ศ. ๒๕๖๐
(รองศาสตราจารย์ชาญชัย พานทองวิริยะกุล)
คณบดีคณะแพทยศาสตร์


คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น
ขอมอบโล่เกียรติยศแด่
อาจารย์เรณู ทานันท์ และคณะฯ
รางวัลที่ 1 ผลงานวิจัยดีเด่น ประจำปี 2558
ประเภท Biomedical research (นักวิจัยอายุไม่เกิน 40 ปี)
ผลงานวิจัย Oxidative stress and its significant roles
in neurodegenerative diseases and cancer.
ตีพิมพ์ใน Int. J. Mol. Sci. 2014; 16: 193-217.
ให้ไว้ ณ วันที่ 6 ตุลาคม พ.ศ. 2558
(รองศาสตราจารย์ชาญชัย พานทองวิริยะกุล)
คณบดีคณะแพทยศาสตร์

KHON KAEN UNIVERSITY kku.ac.th

raynoo thanan <raynoo@kku.ac.th>

[JCA2018] Notice Regarding Presentation at the 77th Annual Meeting of the JCA 30102

1 message

JCA2018 Abstract <jca2018-ab@congre.co.jp> Wed, Aug 1, 2018 at 6:01 PM
Reply-To: jca2018-ab@congre.co.jp
To: raynoo@kku.ac.th

Dear Dr. Raynoo Thanan [30102],

Thank you very much for your abstract submission to 77th Annual Meeting of the JCA.
It is our pleasure to inform you that your abstract has been accepted by the Program Committee of the meeting. We would like to inform and ask you to present in the English Oral Sessions.
We appreciate your kind understanding.
Your presentation is scheduled in the program is as follows. Again, we appreciate your contribution to the 77th Annual Meeting of JCA and are looking forward to seeing you in Osaka, Japan.

Abstract Registration No.:30102
Abstract Title: The importance of aromatase, an estrogen biosynthesis enzyme, in cholangiocarcinoma progression
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~ Presentation Information ~
Your Abstract No.: E-2104
Presentation Style: English Oral Sessions
Session Title: E14-12 Biliary tract cancer
Session Date & Time: Sept. 28 (Fri.) 15:30-16:45
Venue : Room 8
---
http://www.congre.co.jp/jca2018/index_en.php

Masaki Mori
(Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University)

Best Regards,

Secretariat of the 77th Annual Meeting of the Japanese Cancer Association:
<jca2018-ab@congre.co.jp>

ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น
ขอมอบเกียรติบัตรนี้ให้ไว้เพื่อแสดงว่า

นางสาวนภัส อามาตย์มุลตรี

ได้รับรางวัลการนำเสนอผลงานวิจัยระดับดี
แบบโปสเตอร์ ระดับปริญญาเอก

ในการประชุมวิชาการ 25 ปี "ชีวเคมีทางการแพทย์" สู่
"ชีวเคมีทางการแพทย์และชีววิทยาโมเลกุล" ในวันที่ 1 สิงหาคม 2560
ณ ห้องบรรยาย 4 อาคารเตรียมวิทยาศาสตร์คลินิก คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

ให้ไว้ ณ วันที่ 1 สิงหาคม 2560

.....
(ผู้ช่วยศาสตราจารย์อุบล ชาอ่อน)
ประธานหลักสูตรบัณฑิตศึกษาภาควิชาชีวเคมี

.....
(รองศาสตราจารย์รศนา วงศ์รัตนชีวิน)
หัวหน้าภาควิชาชีวเคมี

โครงการแก้ไขปัญหาโรคพยาธิใบไม้ตับและมะเร็งท่อน้ำดีในภาคตะวันออกเฉียงเหนือ(CASCAP)
มหาวิทยาลัยขอนแก่น สถาบันวิจัยมะเร็งท่อน้ำดี และ มูลนิธิมะเร็งท่อน้ำดี
ขอมอบใบประกาศเกียรติคุณฉบับนี้เพื่อแสดงว่า

คุณนภัส อามาตย์มุลตรี

ได้รับรางวัลการนำเสนอผลงานวิจัย ระดับดีเด่น
ประเภทพัฒนางานวิทยาศาสตร์และห้องปฏิบัติการด้านมะเร็งท่อน้ำดี

การประชุมวิชาการประจำปี ๒๕๖๐ โครงการแก้ไขปัญหาสาธารณสุขและการศึกษาในภาคตะวันออกเฉียงเหนือ
เพื่อลดความเหลื่อมล้ำทางสังคมมหาวิทยาลัยขอนแก่น
จากการวิจัย สู่นวัตกรรมเพื่อลดความเหลื่อมล้ำทางสังคม และขยายผลสู่การพัฒนาอย่างยั่งยืน
ให้ไว้ ณ วันที่ ๑ ธันวาคม ๒๕๖๐ โรงแรมอวานี ขอนแก่น โฮเทลแอนด์ คอนเวนชั่น เซ็นเตอร์ จังหวัดขอนแก่น

รองศาสตราจารย์ นพ.ถนอม ขันตีแก้ว
หัวหน้าโครงการแก้ไขปัญหาโรคพยาธิใบไม้ตับและมะเร็งท่อน้ำดี
ในภาคตะวันออกเฉียงเหนือ

รองศาสตราจารย์ ดร.ภิญโญชัย ไตรรัตนศิริชัย
อธิการบดี
มหาวิทยาลัยขอนแก่น

Biochemistry and Molecular Biology Section of the Science Society of Thailand Under the Patronage of His Majesty the King
and
Department of Biochemistry, Faculty of Science
Chulalongkorn University

Confer this

CERTIFICATE OF OUTSTANDING POSTER PRESENTATION

upon

Waleeporn Kaewlert

who presented during the 6th International Conference on Biochemistry and Molecular Biology (BMB 2018)
"Networking in Molecular Biosciences towards Creativity and Innovation"

June 20-22, 2018 at Rayong Resort, Rayong, Thailand

Associate Professor Dr. Tuangporn Suthiphongchai
Chair of the Biochemistry and Molecular Biology Section

Assistant Professor Dr. Kanoktip Packdibamrung
Chairperson of BMB 2018

ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น
ขอมอบเกียรติบัตรนี้ให้ไว้เพื่อแสดงว่า

นางสาววลีพร แก้วเลิศ

ได้รับรางวัลการนำเสนอผลงานวิจัยระดับดีเด่น
แบบโปสเตอร์ ระดับปริญญาโท

ในการประชุมวิชาการ 25 ปี "ชีวเคมีทางการแพทย์" สู่
"ชีวเคมีทางการแพทย์และชีววิทยาโมเลกุล" ในวันที่ 1 สิงหาคม 2560
ณ ห้องบรรยาย 4 อาคารเตรียมวิทยาศาสตร์คลินิก คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

ให้ไว้ ณ วันที่ 1 สิงหาคม 2560

(ผู้ช่วยศาสตราจารย์อุบล ชาอ่อน)
ประธานหลักสูตรบัณฑิตศึกษาภาควิชาชีวเคมี

(รองศาสตราจารย์รศนา วงศ์รัตนชีวิน)
หัวหน้าภาควิชาชีวเคมี

คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

ขอมอบเกียรติบัตรนี้ให้ไว้เพื่อแสดงว่า

นางสาววลีพร แก้วเลิศ

ได้รับรางวัลที่ 1

การประกวดผลงานทางวิชาการ Poster Presentation

กลุ่ม นักศึกษาบัณฑิตศึกษา

เรื่อง Expressions of FOXA1, CYP19A1, TFF1 and Estrogen Receptors in Cholangiocarcinoma Tissues

ในงานประชุมวิชาการครั้งที่ 32 ประจำปี 2559 ระหว่างวันที่ 11-13 ตุลาคม 2559

ณ ห้องบรรยาย 1-4 อาคารเตรียมวิทยาศาสตร์คลินิก คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

ให้ไว้ ณ วันที่ 13 ตุลาคม 2559

(รองศาสตราจารย์สุรพล วีระศิริ)

ประธานคณะกรรมการจัดประชุมวิชาการ ครั้งที่ 32 ประจำปี 2559

-นำเสนอผลงานแบบ Oral presentation

1. ผลงานเรื่อง “Oxidative stress suppresses early B cell factor 1 (EBF1) expression and inhibits its tumor suppressive function against cholangiocarcinoma genesis” ในงานประชุมวิชาการ The 6th International Conference on Biochemistry and Molecular Biology (BMB 2018) “Networking in Molecular Biosciences towards Creativity and Innovation” ณ โรงแรมระยองรีสอร์ท จ.ระยอง ประเทศไทย ระหว่างวันที่ 20-22 มิถุนายน 2561 โดย อ.ดร.เรณู ทานันท์ (หัวหน้าโครงการ)

2. ผลงาน เรื่อง “The importance of aromatase, an estrogen biosynthesis enzyme, in cholangiocarcinoma progression” ณ งานประชุมวิชาการ The 77th Annual Meeting of the Japanese Cancer Association (JCA 2018) ณ Osaka International Convention Center & RIHGA Royal Hotel Osaka ประเทศญี่ปุ่น ระหว่างวันที่ 26-28 กันยายน พ.ศ 2561 โดย อ.ดร.เรณู ทานันท์ (หัวหน้าโครงการ)

-นำเสนอผลงานแบบ Poster presentation

1. ผลงานเรื่อง “Oxidative stress suppresses early B cell factor 1 (EBF1) expression and inhibits its tumor suppressive function against cholangiocarcinoma genesis” ในงานประชุมวิชาการ ประชุมนักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว. ครั้งที่ 17 (TRF-OHEC Annual Congress 2018) ณ โรงแรมเดอะรีเจ้นท์ ชะอำ บีช รีสอร์ท จังหวัดเพชรบุรี ประเทศไทย ระหว่างวันที่ 10-11 มกราคม 2561 โดย อ.ดร.เรณู ทานันท์ (หัวหน้าโครงการ)

2. ผลงานเรื่อง “Oxidative stress and DNA hypermethylation suppress Early B cell factor 1 expression in cholangiocarcinoma in relation with tumor progression and prognosis” ในงานประชุมวิชาการ 25 ปี “ชีวเคมีทางการแพทย์” สู่ “ชีวเคมีทางการแพทย์และชีววิทยาโมเลกุล” ณ ห้องบรรยาย 4 อาคารเตรียมวิทยาศาสตร์คลินิก คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ณ วันที่ 1 สิงหาคม 2560 โดย นางสาวนภัส อามาตย์มุลตรี

3. ผลงานเรื่อง “DNA hypermethylation suppresses Early B cell factor 1 expression in cholangiocarcinoma in relation with tumor progression and prognosis” ในงานประชุมวิชาการประจำปี 2560 โครงการแก้ไขปัญหาโรคพยาธิใบไม้ตับและมะเร็งท่อน้ำดีในภาคตะวันออกเฉียงเหนือ (CASCAP) มหาวิทยาลัยขอนแก่น สถาบันวิจัยมะเร็งท่อน้ำดี และมูลนิธิมะเร็งท่อน้ำดี ณ โรงแรมอวานี ขอนแก่น โฮเทลแอนด์ คอนเวนชั่น เซ็นเตอร์ จังหวัดขอนแก่น ระหว่างวันที่ 30 พฤศจิกายน - 1 ธันวาคม 2560 โดย นางสาวนภัส อามาตย์มุลตรี

4. ผลงานเรื่อง “The expressions of CYP19A1 and estrogen receptors in cholangiocarcinoma tissues” ในงานประชุมวิชาการ 25 ปี “ชีวเคมีทางการแพทย์” สู่ “ชีวเคมีทางการแพทย์และชีววิทยาโมเลกุล” ณ ห้องบรรยาย 4 อาคารเตรียมวิทยาศาสตร์คลินิก คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ในวันที่ 1 สิงหาคม 2560 โดย นางสาววลีพร แก้วเลิศ

5. ผลงานเรื่อง “Overexpression of CYP19A1 induces cell migration and metastasis of cholangiocarcinoma cells” ในงานประชุมวิชาการประจำปี 2560 โครงการแก้ไขปัญหาโรคพยาธิใบไม้ตับและมะเร็งท่อน้ำดีในภาคตะวันออกเฉียงเหนือ (CASCAP) มหาวิทยาลัยขอนแก่น สถาบันวิจัยมะเร็งท่อน้ำดี และมูลนิธิมะเร็งท่อน้ำดี ณ โรงแรมอวานี ขอนแก่น โฮเทลแอนด์ คอนเวนชั่น เซ็นเตอร์ จังหวัดขอนแก่น ระหว่างวันที่ 30 พฤศจิกายน - 1 ธันวาคม 2560 โดย นางสาววลีพร แก้วเลิศ

6. ผลงานเรื่อง “Upregulated CYP19A1 expression promotes cell proliferation and migration in male cholangiocarcinoma patients” ในงานประชุมวิชาการ The 6th International Conference on Biochemistry and Molecular Biology (BMB 2018) ณ โรงแรมระยองรีสอร์ท จ.ระยอง ประเทศไทย ระหว่างวันที่ 20 – 22 มิถุนายน 2561 โดย นางสาววลีพร แก้วเลิศ

7. ผลงานเรื่อง “Oxidative stress down-regulates Early B cell factor 1 resulting in cholangiocarcinoma genesis” ณ งานประชุมวิชาการ The 77th Annual Meeting of the Japanese Cancer Association (JCA 2018) ณ Osaka International Convention Center & RIHGA Royal Hotel Osaka ประเทศญี่ปุ่น ระหว่างวันที่ 26-28 กันยายน พ.ศ 2561 โดย นางสาวนภัส อามาตย์มุลตรี