Down-regulation of Early B Cell Factor 1 and Its Significance in Liver Fluke-associated Cholangiocarcinoma Development of Animal Model

การยับยั้ง Early B Cell Factor 1 และความสำคัญในการพัฒนามะเร็งท่อน้ำดีที่สัมพันธ์กับการติดพยาธิใบไม้ตับในสัตว์ทดลอง

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ABSTRACT

Cholangiocarcinoma (CCA) is a cancer of bile duct epithelial cells. Chronic inflammation induced oxidative stress via liver fluke (*Opisthrochris viverrini*) infection is the major cause of CCA in Thailand. We focused on Early B cell factor 1 (EBF1), which play roles in cell differentiations and tumor suppression. The expression profiles of EBF1 and its related proteins including PAX5 (EBF1 downstream) and ZFP521 (EBF1 inhibitor) in animal model of *O. viverrini*-induced CCA were detected using immunohistochemical analysis. Our results demonstrated that EBF1 and PAX5 expressions were down-regulated during CCA development whereas ZFP521 expression was up-regulated along the carcinogenesis. Therefore, down-regulation of EBF1 may play the synergistic role in *O. viverrini*-driven CCA carcinogenesis.

บทคัดย่อ

มะเร็งท่อน้ำดี เป็นมะเร็งของเซลล์เยื่อบุผนังของทางเดินท่อน้ำดี สาเหตุหลักในประเทศไทยนั้นเกิดจากการติดพยาธิใบไม้ตับซึ่งทำให้เกิดสภาวะความเครียดออกซิเดชั่นจากการอักเสบแบบเรื้อรัง โดยยีน Early B cell factor 1 หรือ EBF1 นั้นมีการรายงานพบว่าทำหน้าที่ในกระบวนการ cell differentiation ได้หลายทางและยังทำหน้าที่เป็นยีนต้านมะเร็งอีกด้วย การศึกษาครั้งนี้ได้ทำการศึกษารูปแบบการแสดงออกของ EBF1 และโปรตีนที่เกี่ยวข้องกับ EBF1 คือ PAX5 (ภายใต้การควบคุมของ EBF1) และ ZFP521 (ตัวยับยั้งของ EBF1) โดยวิธี immunohistochemistry ในสัตว์ทดลองที่เหนี่ยวนำให้เป็นมะเร็งท่อน้ำดีด้วยการติดพยาธิใบไม้ตับ ผลการศึกษาพบว่าเกิดการยับยั้งการแสดงออกของ EBF1 และ PAX5 แต่เพิ่มการแสดงออกของ ZFP521 ในขณะที่มีการพัฒนาของมะเร็งท่อน้ำดี แสดงให้เห็นว่าการยับยั้ง EBF1 อาจจะนำไปสู่การพัฒนาของมะเร็งท่อน้ำดีได้

Key Words: EBF1, *Opisthrochris viverrini*, Cholangiocarcinoma
คำสำคัญ: EBF1 พยาธิใบไม้ตับ มะเร็งท่อน้ำดี

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Introduction

Cholangiocarcinoma (CCA) is a cancer of epithelial cells in bile duct. The incidence of CCA is increasing worldwide and accounts for 3% of all gastrointestinal cancers (Khan et al. 2005). The established risk factors for CCA in western countries include primary sclerosing cholangitis, Caroli's disease and congenital choledochal cysts (Khan et al. 2002). Hepatitis C virus is the major risk of CCA in Japanese cases (Tyson et al. 2011). In contrast, CCA is highly found in Thailand, particularly among people in the northeastern region, where is an endemic area of liver fluke *(Opisthorchis viverrini)* infection. CCA incidence in Khon Kaen province is remarkably high with age-standardized incidence rate (ASRs) of 89.7 per 100,000 in males and 67.2 per 100,000 in females (Vatanasapt et al. 2002). Our previous studies strongly supported that chronic inflammation induced by *O. viverrini* infection is a major cause of CCA development through oxidative stress in animal and human models (Yongvanit et al. 2012).

Early B cell factor 1 (EBF1) is a transcription factor which is essential for the differentiation of several cell lineages including B lymphocyte development (Liao 2009), bone development and adipogenesis (Stephens 2012). EBF1 binds to DNA as a dimer in solution which can interact with p300/CBP is likely to underlie EBF-mediated gene activates many B cell regulator genes such as PAX5. In contrast, ZFP423 or ZFP521 can bind to EBF1 which will recruit the Mi-2/NuRD (nucleosome remodeling and deacetylase) complex for repression B cell regulator genes. However, down-regulation of EBF1 was investigated in leukemia and solid cancers via mutation and genetic instability suggesting its function as tumor suppressor. For example, genomics loss at chromosome 5q34 which encode EBF1 was found in breast cancer (Neve et al. 2006) and somatic missense mutation causes the amino acid substitution of arginine for glutamine at position 242 at DNA binding domain of EBF was detected in pancreatic ductal adenocarcinoma (Jones et al. 2008). In addition, several studies reported that overexpression of EBF1 inhibitors were found in many kinds of cancers such as ZFP521 in leukemia (Bond et al. 2004) and medulloblastoma (Bond et al. 2008). Furthermore, ZFP423 is also highly expressed in leukemia and nasopharyngeal carcinoma (Chung et al. 2013). These suggest that down-regulation of EBF1 and up-regulation of EBF1 inhibitors play critical roles in tumor promotion and progression.

Here, we hypothesized that oxidative stress induced by *O. viverrini* infection may suppress the expression of EBF1 consequently to suppress PAX5 (EBF1 downstream regulation) and induce the expression of ZFP521 (EBF1 inhibitor) which may involve in liver fluke-associated cholangiocarcinoma development.

Objective of this study

The aim of this study was to determine the expression profiles of EBF1, PAX5 (downstream protein of EBF1) and ZFP521 (inhibitor of EBF1) in hamster's liver tissues of *O. viverrini*-induced CCA model.

Methodology

Preparation of metacercaria

Opisthorchris viverrini metacercariaes were extracted from the naturally infected cyprinoid fish by artificial pepsin digestion as previously described

(Pinlaor et al. 2013). Cyprinoid fish was purchased from the market in Northeastern part of Thailand. Fishes were chopped into small pieces by an electrical motor. Then, they were minced; using an electrical blender, with freshly prepared of 0.25% pepsin solution and incubated at 37 °C in a shaking water bath for 1 hour to activate the digestion of the fish-muscle by the pepsin enzyme. After digestion, the digested content was filtered and washed several times. The metacercariaes of *O. viverrini* were collected and identified under a dissecting microscope. Fifty viable active cysts were fed to Syrian golden hamsters for *O. viverrini*-infected (OV) and *O. viverrini*-infected plus NDMA-treated (OV+NDMA) groups.

Animal model

The protocol of collection and study were approved by Animals Ethic Committee, Faculty of Medicine, Khon Kaen University (approval number AEKKU 23/2555). The conditions of treatment groups were defined in Table 1. The animals were sacrificed on days 21, 60, 90, and 180 post infections (n=3 per time point). For tissue sections, excised liver specimens were cut and fixed in 10% buffered-formalin for 24 h. The fixed tissues were embedded in paraffin and then serially dissected into 4 µm thick slides.

Table 1 Treatment conditions of hamsters.

Group	Treatment
(A) Untreated	untreated
(B) OV	50 infective metacercariaes
(C) NDMA	12.5 ppm in drinking water
(D) OV+NDMA*	(B)+(C) treatments

*CCA carcinogenic group.

Immunohistochemistry analysis

Immunohistochemical method was performed to determine the expression profiles of EBF1, PAX5 and ZFP521 in hamster tissues. In brief, 4 µm thick of liver sections were deparaffinized and rehydrated with xylene and stepwise decreasing concentration of ethanol. Antigen retrieval was performed by microwave (Sharp Microwave Oven, R-219, Bangkok, Thailand) treatment in 10 mM sodium citrate buffer with 0.5% tween pH 6.0 at low power setting for 10 minutes then sections were immersed for 30 minutes in 3% (v/v) hydrogen peroxide in PBS for endogenous hydrogen peroxide activity blocking. Non-specific binding was blocked by 10% skim milk in PBS for 30 min. After that, sections were washed in PBS with 0.1% tween 20 (3 times) and incubated with primary antibody at 4°C overnight. The conditions of each primary antibodies were showed in Table 2. Then the sections were washed 3 times by PBS with 0.1% tween 20, and

Table 2 Listed of primary antibodies and their conditions for immunohistochemical analysis.

Primary antibody	Final Conc. (µg/ml)	Company
1) Rabbit anti-EBF1 polyclonal antibody	2 µg/ml	Sigma chemical, USA
2) Mouse anti-PAX5 monoclonal antibody	2 µg/ml	Sigma chemical, USA
3) Rabbit anti-ZFP521 polyclonal antibody	3.33 µg/ml	Sigma chemical, USA

incubated with Dako EnVision antibody (Dako An Agilent Technologies Company, USA) for 1 h then the brown color was developed with DAB (3,

3'diaminobenzidine tetrahydrochloride) substrate kit (Vector Laboratories, Inc., Burlingame, USA) for 5 min. Mayer's haematoxylin was used for nuclear counterstaining. The sections were dehydrated with stepwise increasing concentration of ethanol and xylene. Finally, the stained sections were mounted with permount solution and reviewed under a microscope.

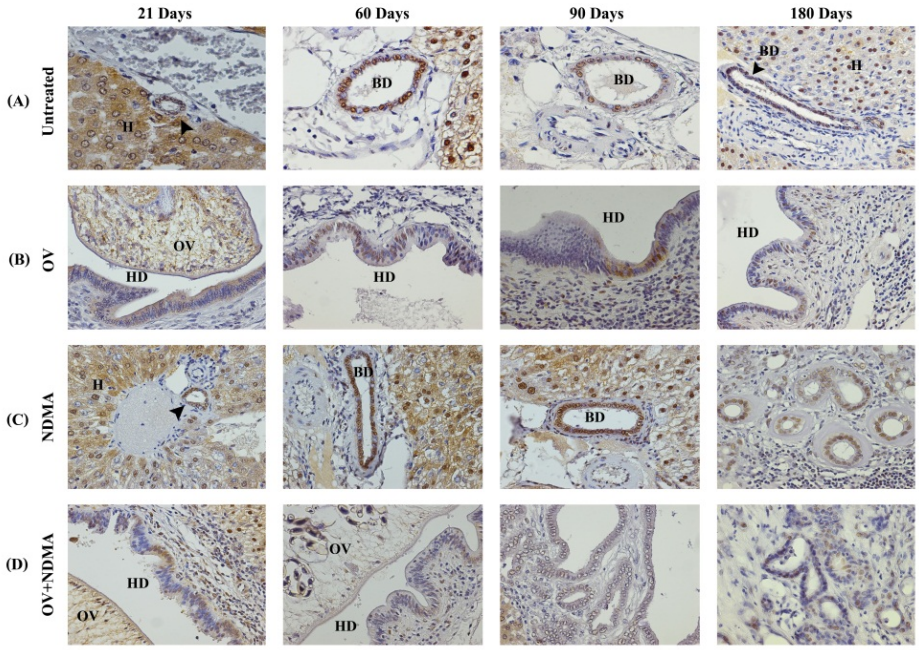

Figure 1 Immunohistochemical staining of EBF1 expression in liver sections of untreated controls (A), OV-infected (B), NDMA administration (C) and OV+NDMA treated (D) hamsters. EBF1 and nucleus immunostainings were represented in brown and blue respectively. Black arrow heads represent normal bile ducts. BD=normal bile ducts, HD=hyperplasia bile ducts, H=hepatocytes, and OV= *O. viverrini*

Immunohistochemical grading

We defined immunohistochemical grading based on intensity and frequency derived from the staining results. The staining intensity was scored as negative (0), weak (+1), moderate (+2), or strong (+3). Frequency of positive cells in the section was scored as negative (0), less than 25% (+1), 25-50% (+2), 51-75% (+3), or more than 75% (+4). IHC score (0-12) was assigned by multiplying the intensity score by the frequency score. Statistical analysis was analyzed using SPSS 13.0 software.

Results

Expression pattern of EBF1 in animal of *O. viverrini*-induced CCA carcinogenesis

Immunohistochemical staining of EBF1 in liver tissues of *O.viverrini*-induced CCA carcinogenesis of hamsters is shown in Figure 1. EBF1 was strongly localized at cytoplasm and nucleus of normal bile duct epitherium cells and hepatocytes in the livers of hamsters which were untreated control and NDMA treated groups (Figure 1A and 1C). In OV group (Figure 1B), immunoreactivity of EBF1 was decreased in hyperplasia bile ducts at 90 and 180 days after

treatment. In OV+NDMA group (Figure 1D), EBF1 expression was decreased in hyperplasia bile ducts at 21 days after treament. The EBF1 down-regulation was also detected at precancerous and CCA tumor areas at 90 and 180 days after treatments, respectively.

Expression pattern of PAX5 in animal of *O. viverrini*-induced CCA carcinogenesis

Figure 2. shows immunostaining of PAX5 (downstream of EBF1) in bile ducts and hepatocyte during carcinogenesis. PAX5 was localized in cytosol and nucleus of normal bile ducts and hepatocytes of untreated control and NDMA treated groups as shown in Figure 2A and 2C. PAX5 expression was decreased in hyperplasia bile ducts at 180 days after *O. viverrini* infection as shown in Figure 2B. The expression of PAX5 was slightly decreased during CCA carcinogenesis as shown in Figure 2D.

Expression pattern of ZFP521 in animal of *O. viverrini*-induced CCA carcinogenesis

ZFP521 expression is shown in Figure 3. The localization of ZFP521 was highly detected in cytosol and nucleus of hepatocytes and hyperplasia bile ducts and the cancer cells of CCA in OV and OV+NDMA treated groups as shown in Figure 3B and 3D. It was slightly detected at cytosol and nucleus of normal bile ducts in untreated control and NDMA treated groups as shown in Figure 3A and 3C.

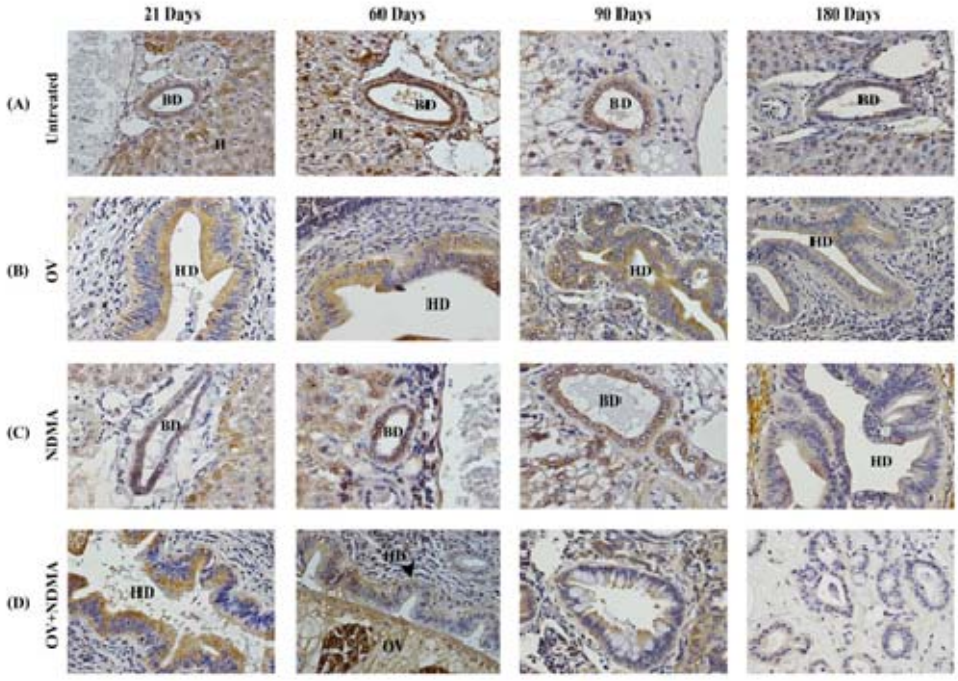

Figure 2 Immunohistochemical staining of Pax5 expression in liver sections of untreated controls (A), OV-infected (B), NDMA administration (C) and OV+NDMA treated (D) hamsters. Pax5 and nucleus immunostainings were represented in brown and in blue respectively. Black arrow heads represent normal bile ducts. BD=normal bile duct, HD=hyperplasia bile duct, H=hepatocyte, and OV= *O. viverrini*

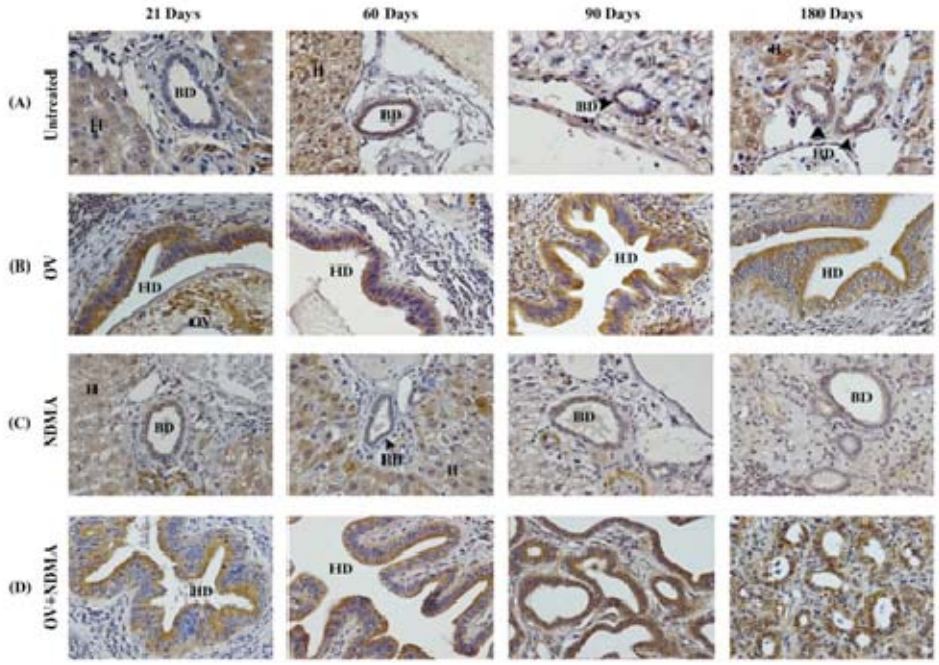

Figure 3 Immunohistochemical staining of ZFP521 expression in liver sections of untreated controls (A), OV-infected (B), NDMA administration (C) and OV+NDMA treated (D) hamsters. ZFP521 and nucleus immunostainings were represented in brown and in blue respectively. Black arrow heads represent normal bile ducts. BD=normal bile duct, HD=hyperplasia bile duct, H=hepatocyte, and OV= *O. viverrini*

Statistical analysis

Statistical analysis by Mann-Whitney U test is shown in Figure 4. EBF1 and PAX5 were significantly decreased in hyperplasia bile ducts, precancerous and the cancer cells of OV and OV+NDMA treated groups at 21, 90 and 180 days after treatments when compared with untreated control group (Figure 4A and 4B). At 90 and 180 days after OV+NDMA treatment, EBF1 was also significantly decreased when compared with NDMA treated group (Figure 4A). ZFP521 was significantly increased in hyperplasia bile ducts, precancerous and the cancer cells of OV+NDMA treated groups at 21, 90 and 180 days after treatments when compared with untreated control group (Figure 4C). At 90 and 180 days after OV and OV+NDMA treatments, ZFP521 expression was also significantly increased when compared with NDMA treated group.

Discussion and Conclusion

Our results demonstrated that EBF1 and PAX5 expressions were decreased in hyperplasia bile ducts and CCA cancer cells when compared to normal bile duct cells and they were decreased along OV + NDMA treatment, suggesting that EBF1 was down-regulated during CCA development. Thus, down-regulation of EBF1 during CCA carcinogenesis may play significant role in CCA development. Moreover, EBF1 was found to suppress in other kinds of solid cancers for instance breast cancer and pancreatic ductal adenocarcinoma in different ways via genetic instability and point mutation, suggesting its function as a tumor suppressor (Neve, Chin et al.

2006Jones, Zhang et al. 2008Liao 2009). Complete deletions of EBF1 gene in tumor cells was also found in the mono-allelic B cell progenitor acute lymphoblastic leukemia (ALL) (Mullighan et al. 2007).

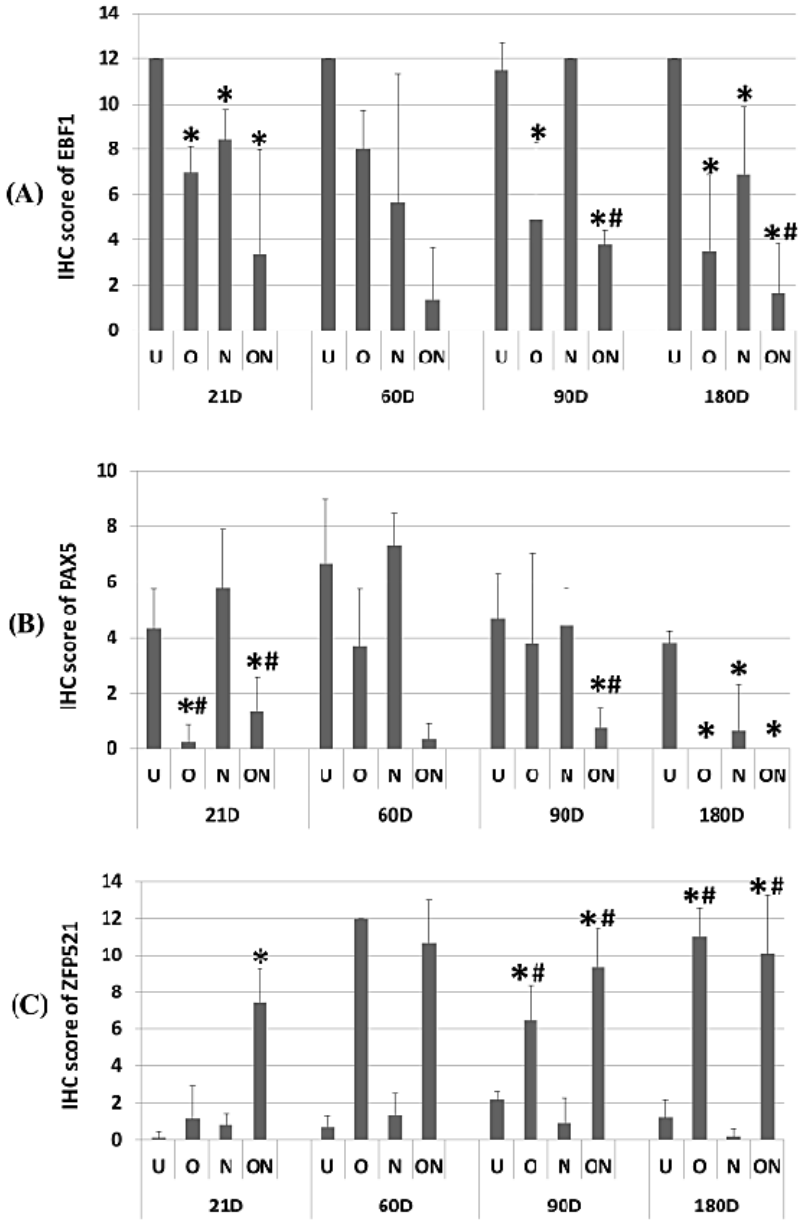

Figure 4 IHC score of EBF1 (A), PAX5 (B) and ZFP521 (C). IHC scores were calculated from normal bile ducts of untreated and NDMA treated groups whereas they were calculated from hyperplasia bile ducts of OV and OV+NDMA (21D and 60D) treated groups. At 90D and 180D after treated with OV+NDMA, the scores were calculated from precancerous and cancer cells, respectively. Statistical analysis was analyzed by Mann-Whitney U test. U= untreated control group, O= OV-infected group, N= NDMA treated group, ON= OV+NDMA treated group, * = p<0.05 compared with untreated control group (U), # = p<0.05 compared with NDMA treated group (N).

Stem cells are cells that have the ability to unlimitedly self-renewal and to generate mature cells of a particular tissue by differentiation (Reya et al. 2001). EBF1 play roles in hematopoietic stem cells differentiate into mature B-cells (Kikuchi et al. 2012). It was also involved in mesenchymal stem cell (MSC) differentiation which induces MSC differentiation into adipocytes whereas suppressed differentiation into osteocytes is occured (Stephens 2012). According to a liver development, bipotential liver stem cells could differentiate into cholangiocytes (bile ducts) and hepatocytes (Roskams 2006). Recently, Thanan *et al.* 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 (Thanan et al. 2013). The present results indicated that EBF1 was highly detected in 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 leading to increase stem cell property of the tumor initiating cells.

ZFP521 and ZFP423 are known as EBF1 inhibitors. In normal B cells, ZFP521 was expressed at low levels and ZFP423 was not expressed (Liao 2009). Interestingly, Hentges K.E. *et al.* reported that when ZFP521 and ZFP423 were overexpressed in normal B cells, it was found that these proteins can inhibit EBF1 activity leading to leukemia development (Hentges et al. 2005). Recently, Chung and coworkers studied the role of EBF1 inhibitor ZFP423 in EBV-associated nasopharyngeal carcinoma (NPCs) and their results suggested that expression of the EBF1 inhibitor protein might contribute to the transformation of NPCs (Chung, Lung et al. 2013). Thus, previous literatures suggesting that tumorigenesis can be induced by ZFP521 and ZFP423 expressions.

Interestingly, the present results demonstrated that ZFP521 expression was increased in hyperplasia bile ducts and CCA cancer cells of *O. viverrini*-induced CCA. Therefore, up-regulation of the EBF1 inhibitor (ZFP521) may also play role in CCA development. Chronic inflammation mediated by infection is a major risk factor causing carcinogenesis. Chronic *O. viverrini* infection can induce oxidative stress via reactive oxygen (ROS) and reactive nitrogen species (RNS) (Yongvanit, Pinlaor et al. 2012). ROS and RNS produced by oxidative stress can damage to all biomolecules such as DNA, protein and lipid. Pinlaor S. and coworkers demonstrated that 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-nitroguanine, biomarkers for DNA damage were highly formation in the liver of *O. viverrini*-infected hamster (Pinlaor et al. 2004). Moreover, Thanan R. and coworkers have reported that the highly formation of 8-oxodG level can be observed in *O. viverrini*-infected subjects and CCA patients (Thanan et al. 2008). Besides, oxidative stress can also mediate lipid peroxidation, consequently leading to etheno DNA adduct formations, which has been reported in both hamster and human models (Dechakhamphu et al. 2008). These indicate that oxidative damage to biomolecules

plays critical roles in CCA carcinogenesis. In addition, oxidative stress was also reported to induce the alteration of gene expressions via the inductions of mutation, genetic instability and epigenetic changes (Murata et al. 2012). Taken together, the present report and our previous studies suggested that oxidative stress which induced by *O. viverrini* infection not only induced oxidative damage to biomolecules but also may suppress the expression of EBF1 and inhibit EBF1 activity via the induction of ZFP521 expression as shown in Figure 5. Therefore, down-regulation of EBF1 may play synergistic role to *O. viverrini*-driven CCA carcinogenesis.

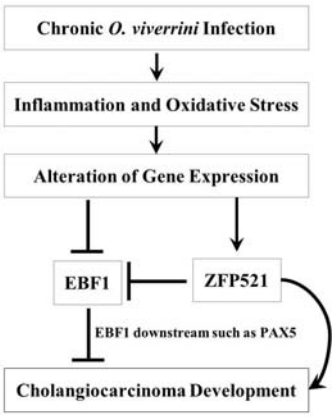

Figure 5 A possible suppression pathway of EBF1 expression via oxidative stress.

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