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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

Napat Armartmuntree[a,b], Mariko Murata[c], Anchalee Techasen[b,d], Puangrat Yongvanit[a,b], Watcharin Loilome[a,b], Nisana Namwat[a,b], Chawalit Pairojkul[b,e], Chadamas Sakonsinsiri[a,b], Somchai Pinlaor[b,f], Raynoo Thanan[a,b,*]

[a] Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
[b] Cholangiocarcinoma Research Institute, Khon Kaen University, Khon Kaen 40002, Thailand
[c] Department of Environmental and Molecular Medicine, Mie University Graduate School of Medicine, Mie 514-8507, Japan
[d] Faculty of Associated Medical Science, Khon Kaen University, Khon Kaen 40002, Thailand
[e] Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
[f] Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

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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

* Corresponding author at: Department of Biochemistry, Khon Kaen University, Faculty of Medicine, Khon Kaen 40002, Thailand.
E-mail address: raynoo@kku.ac.th (R. Thanan).

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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.

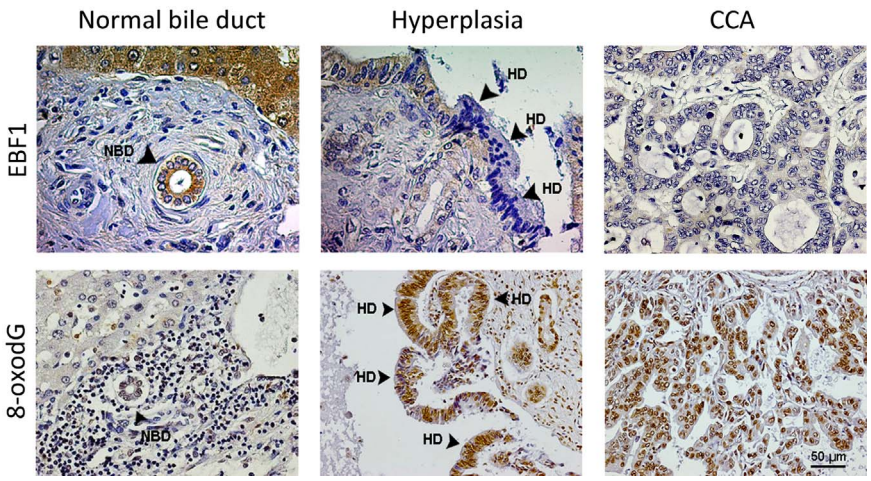

Fig. 1. Immunohistochemical analysis of EBF1 expression and 8-oxodG formation in normal bile duct of a tumor adjacent area, hyperplasia and CCA tissues. NBD = normal bile duct, HD = hyperplasia bile duct. An original magnification is 200× for all figures.

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.

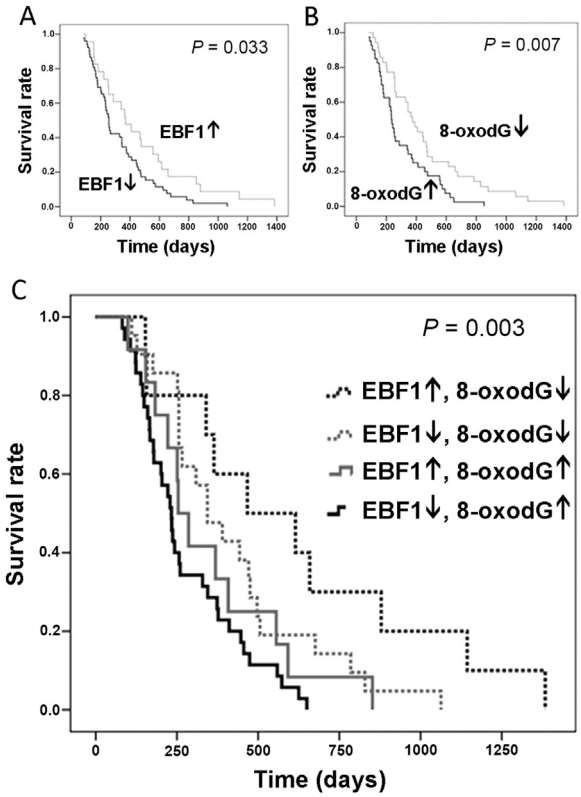

Fig. 2. Kaplan-Meier analysis of EBF1 (A), 8-oxodG formation (B), and combined EBF1 expression and 8-oxodG formation (C) with survival rate in CCA. *P*-value was analyzed by Log-rank test. EBF1↓= low EBF1 expression, EBF1↑= high EBF1 expression, 8-oxodG↓= low 8-oxodG formation, and 8-oxodG↑= high 8-oxodG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

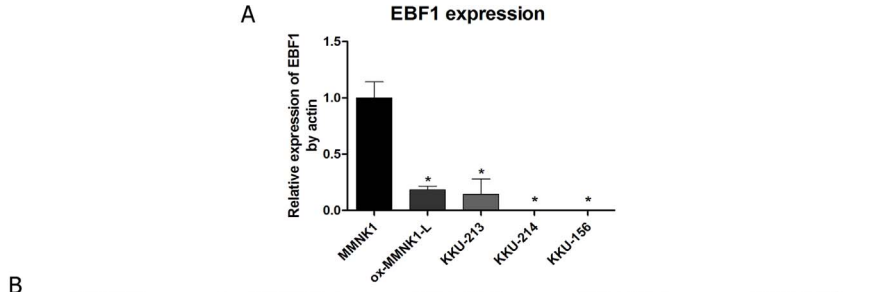

Fig. 3. (A) Relative mRNA expression levels of EBF1 was measured by real-time PCR and adjusted by β-actin mRNA expression in MMNK1, ox-MMNK1-L and CCA cell lines. The symbol asterisk (*) indicates statistical significance at $P < 0.05$. (B) Immunocytochemical analysis of EBF1 expression.

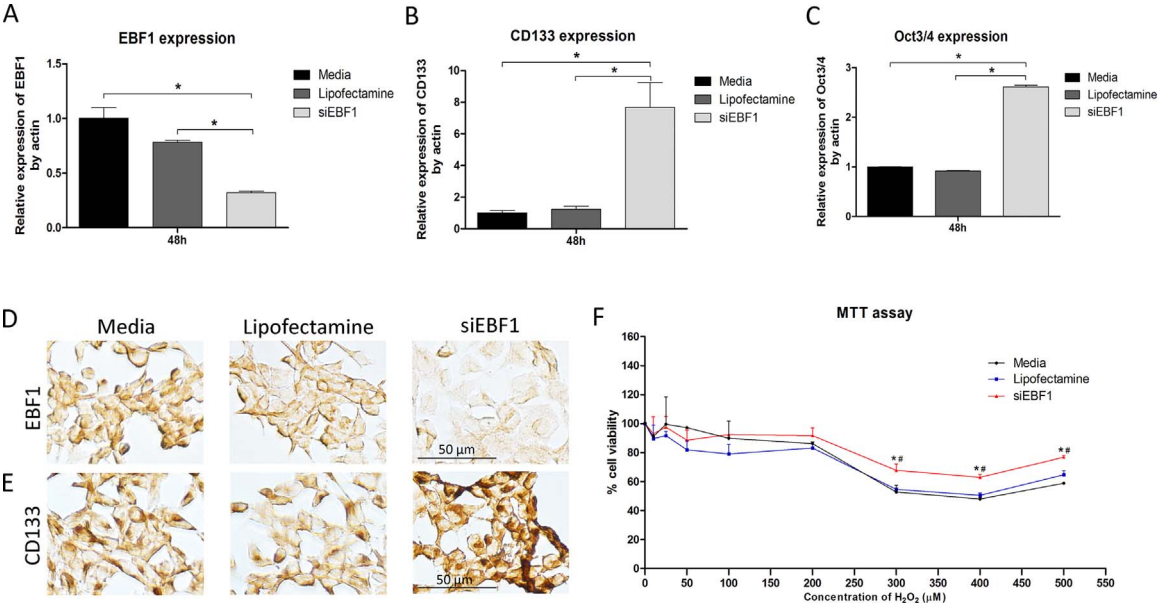

Fig. 4. Relative mRNA expression levels of EBF1 (A), CD133 (B) and Oct3/4 (C) were measured by real-time PCR and adjusted by β-actin mRNA expression. The asterisk (*) indicates statistical significance at $P < 0.05$. Protein expression levels of EBF1 (D) and CD133 (E) were detected by immunocytochemical 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

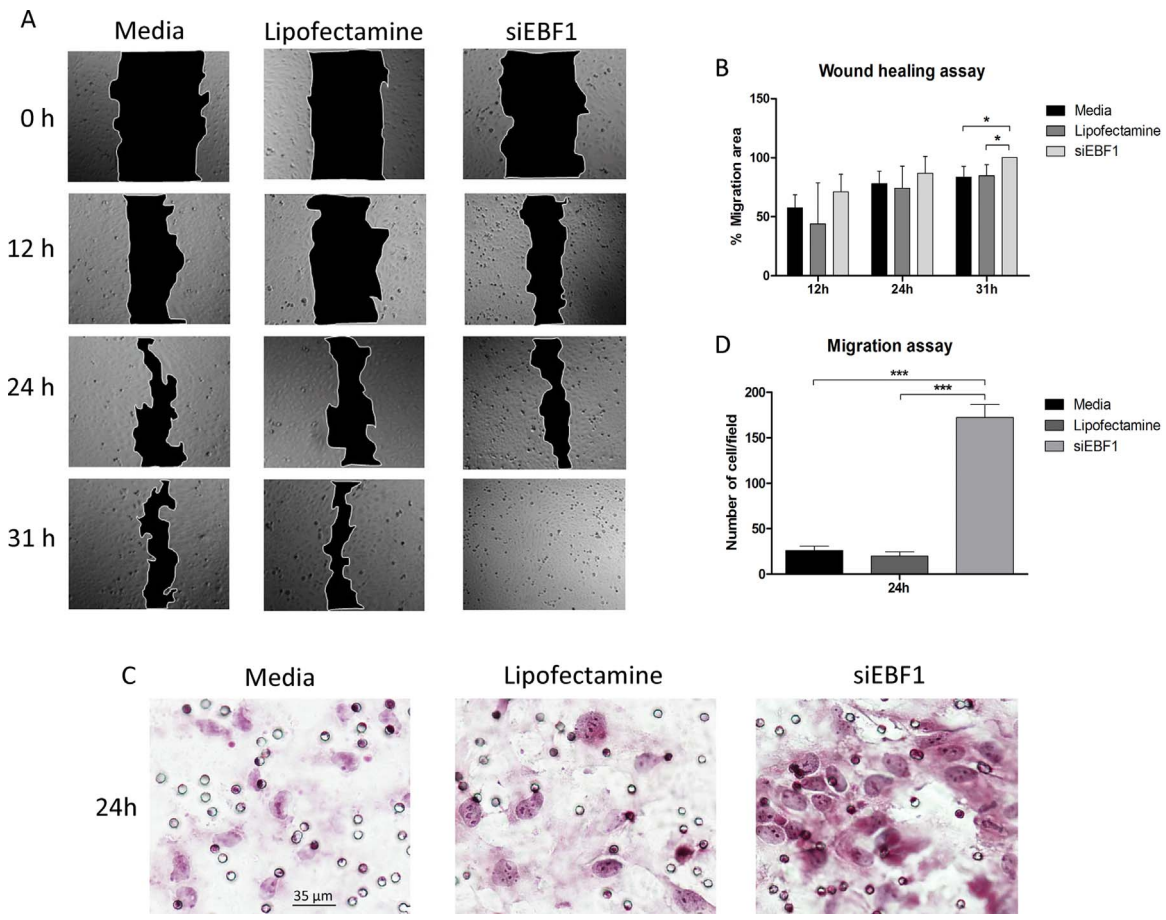

Fig. 5. (A) Wound healing assay under microscope (10×). (B) The graphical data represented the percentage of migration area determined by wound healing assay. (C) Hematoxylin-staining migrated cholangiocyte cells after 24 h treated with lipofectamine and siEBF1 using a Boyden chamber transwell consisting 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 $P < 0.001$.

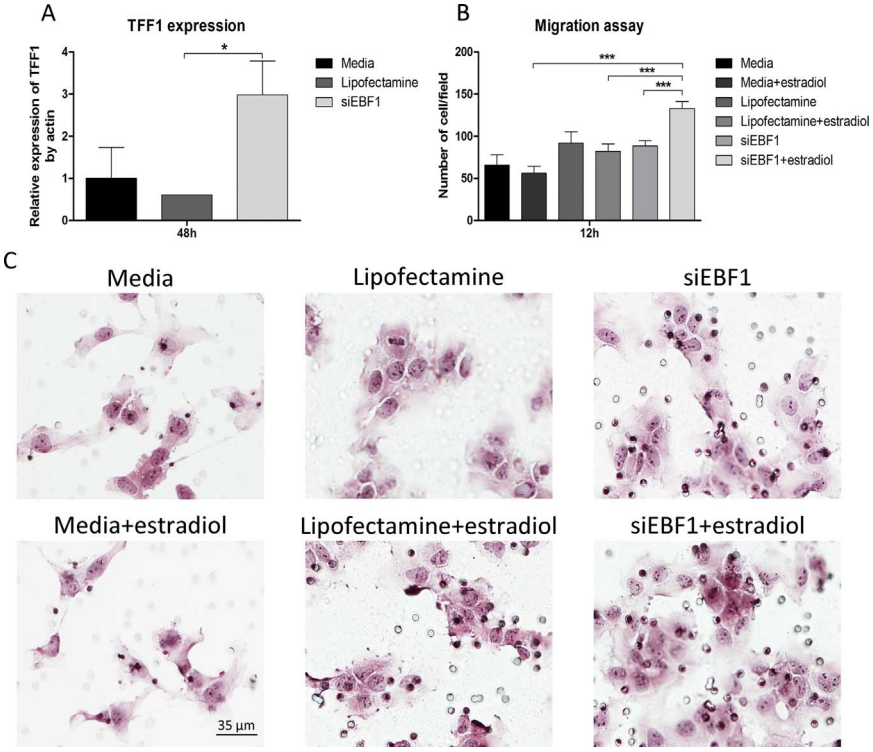

Fig. 6. (A) Relative mRNA expression levels of TFF1 was measured by real-time PCR and adjusted by β-actin mRNA expression. (B) The graphical data represent the migrated cells detected by the migration assay. The Y axis represents the number of migrating cells per field and the X axis the experimental 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.

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