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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.