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ORIGINAL ARTICLE

Effect of *Helicobacter bilis* Infection on Human Bile Duct Cancer Cells

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Abstract
Background *Helicobacter pylori* infection is known to be associated with chronic atrophic gastritis, peptic ulcers, and gastric malignancies. However, the effects of other *Helicobacter* species have not been investigated extensively. In mice, a close relationship is observed between *Helicobacter hepaticus* and hepatocellular carcinoma, and *Helicobacter* species can be found in humans, most commonly in extragastric organs. There have also been reports that *H. bilis* may be associated with biliary malignancies in humans. The effect of *H. bilis* infection on a human bile duct cancer cell line was investigated in this study.
Methods We prepared HuCCT-1, the human bile duct cancer cell line, which was cocultured with *H. bilis* and cultured alone as a control. HuCCT-1 with and without *H. bilis* were transfected with the NF-κB, E2 transcription factor (E2F), and cyclic AMP response element (CRE) luciferase vectors. The activity of NF-κB between *H. bilis* and the infected and noninfected HuCCT-1 cells was also measured by dual luciferase reporter assay. The concentration of vascular endothelial growth factor (VEGF) in the cocultured medium and control medium were measured by ELISA. To investigate the effect of *H. bilis* infection on HuCCT-1 with regard to human umbilical vein endothelial cell (HUVEC) tube formation, HUVECs and fibroblasts were cocultured in 24-well plates with and without the conditioned medium.
Results NF-κB, E2F and CRE activity, production of VEGF, and angiogenesis in *H. bilis*-infected cell lines were enhanced compared with controls.
Conclusions *H. bilis* infection in a human bile duct cancer cell line activates transcript factors such as NF-κB that stimulate production of VEGF and lead to enhancement of angiogenesis. *H. bilis* infection may play an important role in malignancies in the biliary tract.

Keywords *Helicobacter bilis* · NF-κB · VEGF · Angiogenesis

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Introduction

Helicobacter pylori infection is widely accepted as the most common cause of gastritis and is etiologically involved in gastric ulcer, duodenal ulcer, gastric adenocarcinoma [1], and gastric mucosa-associated lymphoid tissue lymphoma [2]. Other kinds of *Helicobacter* infection of extragastric organs have not been extensively investigated; however, in 1992, a high rate of liver tumors in mouse colonies was noted at the US National Cancer Institute [3]. An extensive search for the cause of these tumors revealed that the livers of the mice were infected with *Helicobacter hepaticus*, which induced chronic hepatic inflammation and subsequent liver cancer in a large population of the mouse colony. According to a previous report, *Helicobacter bilis* isolated from the bile, liver, and intestines of mice [4] showed a 97.4% similarity to *H. hepaticus* and a 93.4% similarity to *H. pylori* based on 16S rRNA sequence comparison. *H. bilis* was thus found to be closely related to *H. hepaticus*, which plays a role in hepatobiliary malignancies. *Helicobacter* was subsequently detected by PCR in the liver [5], gallbladder [6], and bile

[7] of human samples. Two reports have shown a significantly higher positive rate for *H. bilis* in Japanese and Thai patients with bile duct and gallbladder cancer [8, 9], and *H. bilis* has also been found in the bile, liver, and intestines of mice [10]. It is known to be responsible for chronic hepatitis and hepatocellular tumors in mice and may play a major role in cholesterol gallstone formation [11]. However, a direct association between *H. bilis* and bile duct cancer has not been established yet. The aim of this study was to examine the influence of *H. bilis* infection on a bile duct cancer cell line.

Methods

Bacteria

The ATCC 51630 *H. bilis* strain was used in this study. It was cultured at 37°C under microaerobic conditions in Brucella broth (Becton Dickinson, Cockeysville, MD) supplemented with 3% heat-inactivated fetal bovine serum. Bacteria were harvested from the broth culture by centrifugation and resuspended at the indicated concentrations in antibiotic-free RPMI 1640 (Sigma Chemical Co., St Louis, MO).

Cell Culture and Materials

The human bile duct cancer cell line HuCC-T1 was obtained from Health Science Research Resources Bank (Osaka, Japan). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in 5% CO_2 and 95% air.

Dual Luciferase Reporter Assay

Luciferase is a highly sensitive enzymatic reporter that can be assayed using standard luciferase detection methods. We used the pNF-κB-Luc, pE2F-Luc, and pCRE-Luc reporter vectors, which are designed for monitoring induction of NF-κB, E2F, and CRE. These three vectors were designed to measure binding of transcription factors to the enhancer, thus providing a direct measurement of activation for this pathway. These vectors contain the firefly luciferase gene from *Photinus pyralis*. In addition, we used the phRL-TK renilla luciferase reporter vector (Promega, Madison, WI) as a control. HuCCT-1 cells were seeded in a suitable tissue culture plate and incubated for 24 h. Each three semiconfluent (70–80%) cells was transfected with three vectors each, pNF-κB-Luc, pE2F-Luc, pCRE-Luc, and simultaneously transfected control vector phRL-TK for all of them, at a ratio of 30:1 for 4 h using SuperFect Transfection Reagents (Qiagen, Valencia, CA) according to the manufacturer's instructions. The medium was replaced and transfectants were further incubated for 12 h. After incubation, the medium was changed to RPMI 1640 with *H. bilis* or to RPMI 1640 alone as control. The RPMI 1640 with *H. bilis* medium was adjusted by approximately 50 MOI (multiplicity of infection). Growth medium was removed from the cultured cells and a sufficient volume of phosphate buffered saline was applied to wash the surface of the culture vessels. One hundred microliters of passive lysis buffer was then dispensed into each culture well. Culture plates were placed on an orbital shaker with gentle shaking at room temperature for 15 min. Lysate samples were cleared for 30 s by centrifugation at 10,000 rpm in a refrigerated microcentrifuge. Firefly and renilla luciferase activity in the samples was measured using a luminometer.

Enzyme-Linked Immunosorbent Assay

HuCC-T1 cells were seeded at a density of 2×10^5 cells into 24-well plates containing culture medium supplemented with 10% FCS. After cells reached subconfluency, live *H. bilis* was added at approximately 50 MOI. After 24 h of coculture, culture media were collected and centrifuged at 1,500 rpm for 5 min to remove particles, and the supernatants were frozen at −80°C until used for ELISA. The VEGF content in the culture supernatants was determined using an ELISA kit (Becton Dickinson) according to the manufacturer's instructions.

Angiogenesis Assay

For conditioned media, HuCCT-1 cells were seeded at a final density of 5×10^6 cells into a flask (80 cm^2) that contained medium with 10% FCS. After overnight culturing, the medium was changed and *H. bilis* was added at approximately 50 MOI with 2% FCS medium and then cultured for an additional 24 h. The supernatant was collected and centrifuged at 6,000 rpm for 5 min, then filtered with a 0.22-mm filter to remove particulate matter and bacteria. HUVECs basal medium and filtered bacterial-free medium were mixed at a ratio of 1:1 and used as conditioned medium. To investigate the influence of *H. bilis* infection on HuCCT-1 cells on tube formation by HUVECs, fibroblasts and HUVECs were cocultured in basal medium or conditioned medium using an angiogenesis kit (Kurabo, Osaka, Japan) according to the manufacturer's protocols [12, 13]. Briefly, HUVECs and fibroblasts were cocultured in 24-well plates with basal medium or the conditioned medium. Media were changed every 3 days and HUVECs and fibroblasts were cocultured for 11 days. HUVECs were then stained with anti-CD31 antibody to detect endothelial cells according to the manufacturer's protocols. Tube formation area was measured

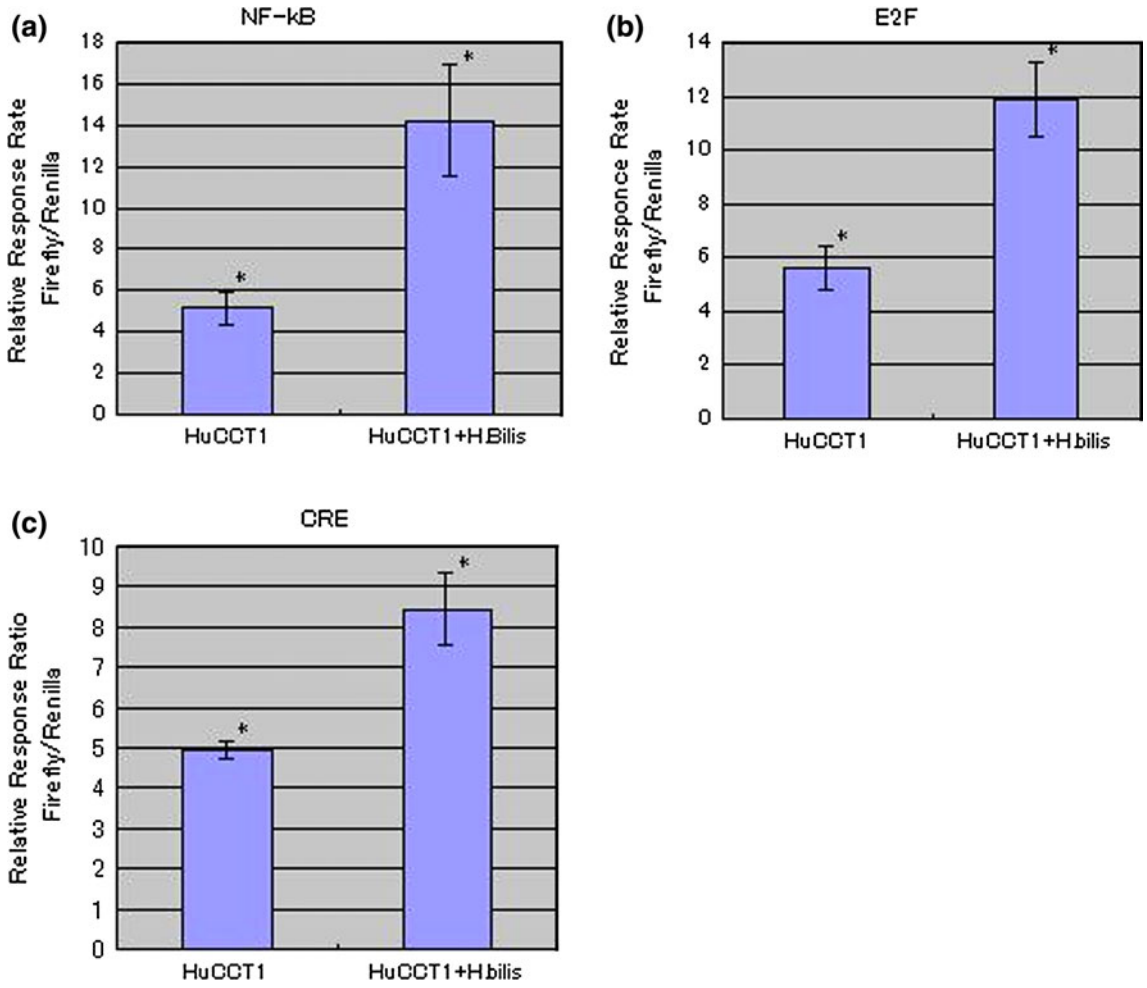

Fig. 1 Results of dual luciferase reporter assay of NF-κB, E2F, and CRE. Influence of *Helicobacter* infection on NF-κB, E2F, and CRE activity in HuCC-T1 cell lines. Cell lines were incubated with and without *H. bilis* for 24 h and the relative luciferase activity was measured by a luminometer. NF-κB, E2F, and CRE activity of *H. bilis*-infected HuCCT-1 cells was significantly higher in controls (* $P < 0.01$), and significantly greater NF-κB activity was observed in the *H. pylori*-infected HuCCT-1 cells compared with the controls (* $P < 0.01$), which was of the same level as *H. bilis*-infected HuCCT-1 cells

quantitatively over 15 different fields for each condition using an image analyzer (Kurabo).

Statistical Analysis

Differences between the two groups were evaluated using Student's *t*-test. Differences were considered statistically significant if $P < 0.05$.

Results

NF-κB, E2F, and CRE Activity Induced by *H. bilis* Infection

In the control vector and various luciferase expression vectors transfected with the HuCC-T1 cell line, relative luciferase activities were enhanced by *H. bilis* infection. NF-κB, E2F, and CRE activity was significantly higher than that in the noninfectious stimulation controls. The NF-κB baseline ratio in the HuCC-T1 cell line was approximately 5.15 (range 4.14–6.09). Infection with *H. bilis* enhanced NF-κB activity to approximately 14.2 (11.29–18.87), which was nearly a three-fold increase over the basal level. The E2F baseline ratio in the HuCC-T1 cell line was approximately 5.64 (4.95–7.11). Infection with *H. bilis* enhanced E2F activity to approximately 11.9 (9.36–13.44), which was nearly a two-fold increase over the basal level. The CRE baseline ratio in the HuCC-T1 cell line was approximately 4.94 (4.68–5.08). Infection with *H. bilis* enhanced CRE activity to approximately 8.44 (7.68–9.84), which was nearly a two-fold increase over the basal level (Fig. 1).

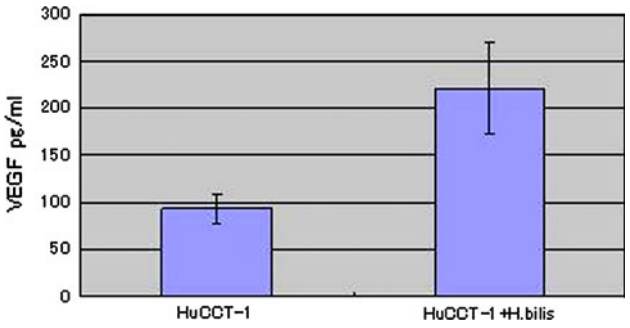

Fig. 2 Result of ELISA of VEGF. Secretion of VEGF into cultured medium by bile duct cancer cell lines determined by ELISA. Cell lines were cultured with and without *H. bilis* (control). Values are expressed as mean ± SD. Comparisons were performed by Student's *t*-test. Results show that secretion of VEGF was significantly higher in the cell line with *H. bilis* compared with the control (* $P < 0.01$)

VEGF Induced by *H. bilis* Infection

We examined the production of VEGF in the HuCC-T1 cell line cocultured with live *H. bilis*. The HuCC-T1 cell line produced a constitutively marked amount of VEGF (Fig. 2). The baseline production of VEGF in the HuCC-T1 cell line was approximately 93.6 pg/ml. *H. bilis* infection enhanced VEGF production to approximately 221 pg/ml, which was nearly a 2.4-fold increase over the basal level ($P < 0.01$). The increase in VEGF production suggests that *H. bilis* infection of the HuCC-T1 cell line enhanced NF-κB activity, which leads to VEGF gene transcription.

Angiogenesis Induced by *H. bilis* Infection

HUVECs cultured in control medium and condition media derived from *H. bilis*-infected cancer cells were stained with anti-CD31 antibody to examine tube formation. Pictures were taken of five different points per well from three wells, for a total of 15 points. The area of tube formation was measured quantitatively using an image analyzer. The baseline tube formation area for HUVECs was 51,600 pixels, and the tube formation area for the HUVECs in conditioned medium was 68,000 pixels. Thus, tube formation in HUVECs was enhanced by *H. bilis*-infected medium ($P < 0.01$) (Fig. 3a–c).

Discussion

In recent years, there has been an increase in the number of reports confirming that *H. pylori* infection is associated with not only gastric disease but also extragastric disease. For idiopathic thrombocyte purpura, *H. pylori* eradication treatment is a favorable therapeutic option for patients who are *H. pylori*-positive [13]. Considering that *H. pylori* is involved in the pathogenesis and pathophysiology of these autoimmune disorders, it might also trigger autoimmune pancreatitis through induction of autoimmunity and apoptosis [14]. As stated previously, it is probable, although not yet confirmed, that live *H. pylori* is present in extragastric

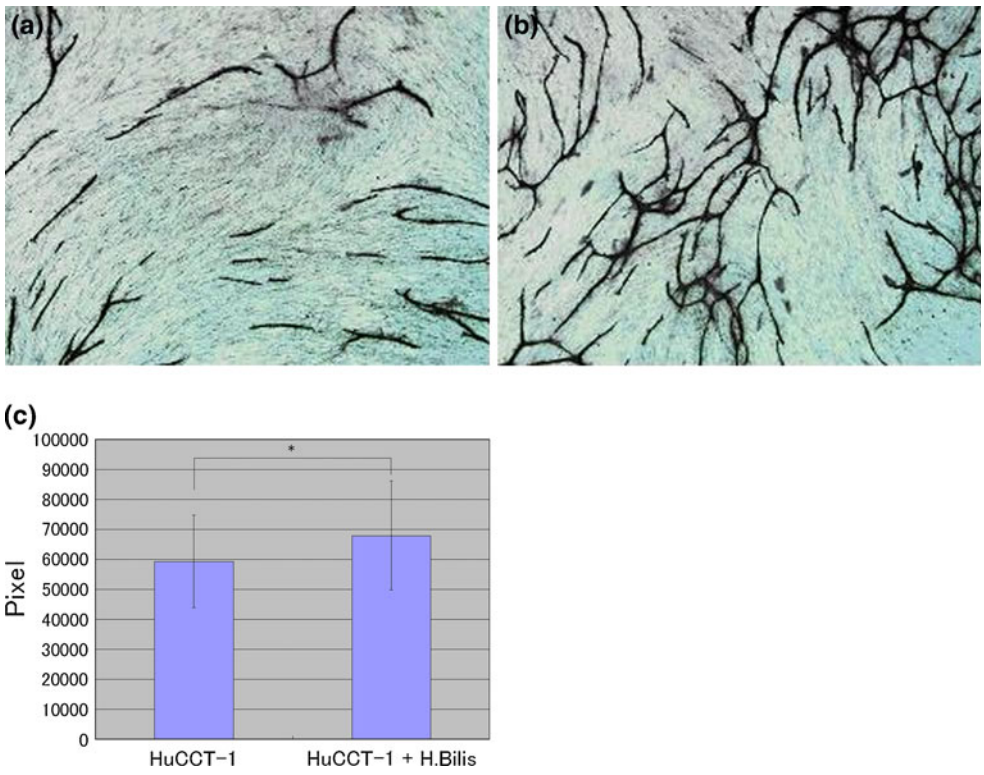

Fig. 3 Results of angiogenesis assay. HUVECs cultured with either *H. bilis*-infected HuCC-T1 medium or noninfected HuCC-T1 medium. After 11 days of culturing, cells were stained with anti-CD31 antibody. Tube formation in cultures of control medium (**a**) or *H. bilis* cocultured with HuCCT-1 medium (**b**) was statistically analyzed using the *t*-test. The results show that angiogenesis was activated by *H. bilis*-infected medium (**c**) (* $P < 0.01$)

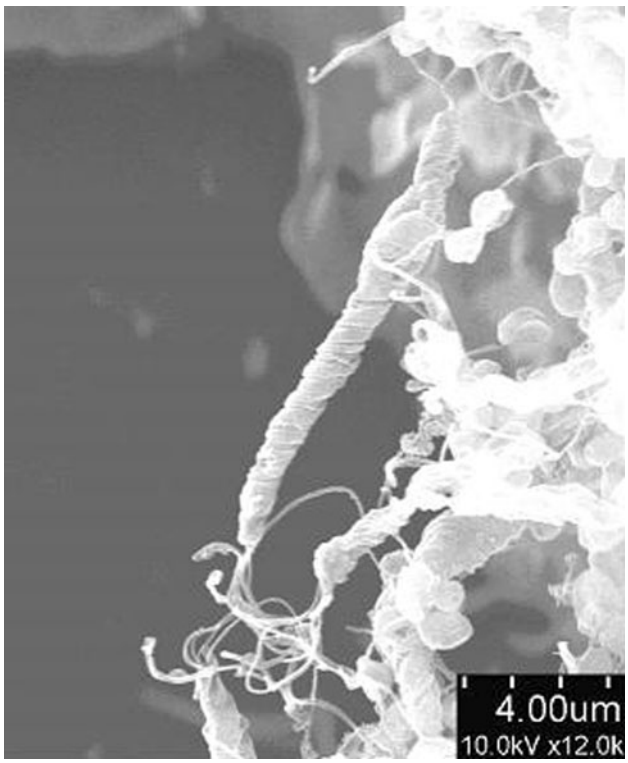

Fig. 4 The scanning electron microscopy photo of *H. bilis*. *H. bilis* coculture with HuCCT-1 cells. The cells were gently fixed using 2.5% glutalaldehyde for 1 h on ice. After two 10-min washes with phosphate buffered saline, cells were fixed through an ethanol series (50%, 70%, 80%, 85%, 90%, 95%, and 100% for 10 min each) and two 20-min *t*-butyl alcohol washes. Afterward, the samples were dried under −20°C for 3 h and coated with platinum. Scanning electron microscopy revealed that *H. bilis* has a fusiform body with multiple bipolar flagella and periplasmic fibers wrapped around the cells

organs such as the spleen, pancreas, and biliary tracts. On the other hand, *H. bilis* is bile-tolerant and commonly detected through PCR in patients with cholangio-ductal disease.

In this study, we focused on *H. bilis* as a virulent organism in humans. Although *H. bilis* is grown at 37°C in a microaerophilic atmosphere, as for other *Helicobacter* species, its shape is different from that of *H. pylori* in that it has a fusiform body with 3–14 multiple bipolar flagella and periplasmic fibers wrapped around the cell. Scanning electron microscopy confirmed the presence of these features (Fig. 4), and the images were similar to the transmission electron microscopic images obtained by Fox et al. [10]. *H. bilis* is thought to be one cause of hepatitis and colitis in mice [15]. 16S rRNA sequence comparison showed that it had a 97.4% similarity to *H. hepaticus* and a 93.4% similarity to *H. pylori*, indicating that *H. bilis* is more closely related to *H. hepaticus* [10], which is involved in hepatobiliary malignancies in mice, than to *H. pylori*. This suggests that *H. bilis* may have a system that stimulates the malignant potential of targeted cells, which is similar to that of *H. hepaticus* and *H. pylori*.

To investigate the effect of *H. bilis* on bile duct cancer cells, we focused first on NF-κB, one of the key signal transmitters for cancer cell activation. Activation of the NF-κB pathway is involved in the pathogenesis of chronic inflammatory diseases [16], and abnormalities in the NF-κB pathway are frequently seen in a variety of human cancers. NF-κB proteins are localized in the cytoplasm and are associated with a family of inhibitor proteins known as IκB-B.C, which comprise a family of proteins that share a 300-amino acid domain designated as the Rel homology domain [17, 18]. The Rel homology domain mediates DNA binding, dimerization, and nuclear transport of NF-κB proteins. NF-κB is also activated after *H. pylori* infection in gastric cancer cells [19, 20]. Additionally, it is involved in the upregulation of VEGF mRNA. It has been demonstrated that NF-κB, a critical regulator of genes involved in inflammation, is also required for VEGF gene expression [21]. *H. pylori* infection also promotes gastric epithelial cell invasion through activation of VEGF expression. These effects appear to be mediated through an NF-κB pathway, since the NF-κB inhibitor significantly attenuates the invasiveness of gastric cancer cells and expression of the VEGF protein [22, 23]. In other transcription factors, VEGF activates CRE-binding protein [24], and CRE mediate the transcription of early growth response of vascular smooth muscle cells [25]. These genes may also play an important role in angiogenesis. The E2F family, whose expression was increased by VEGF [26], plays a crucial role in the control of cell cycle and action of tumor suppressor proteins and is also a target of the transforming proteins of small DNA tumor viruses. This protein and another two members, E2F2 and E2F3, have an additional cyclin binding domain. This protein binds preferentially to retinoblastoma protein pRB in a cell-cycle dependent manner. It can mediate both cell proliferation and p53-dependent/independent apoptosis [27].

In this study, we confirmed that *H. bilis* infection activates NF-κB in bile duct carcinoma cells, thereby increasing expression of the angiogenic factor VEGF from the cells and elevating their angiogenic potential. Moreover, VEGF may elevate their angiogenic potential via CRE-binding protein, which is activated by *H. bilis* infection. This is because NF-κB and CRE activation lead to VEGF [28] gene transcription, and VEGF is one of the most important angiogenic factors. Additionally, VEGF may elevate nuclear expression of E2F that leads to bile duct carcinoma cell proliferation. We think that *H. bilis* may have some malignant potential for bile duct cancer cell lines similar to *H. hepaticus* for hepatoma of mice. To determine whether *H. bilis* is definitely a risk factor for bile duct malignancy, it is necessary to culture live *H. bilis*

directly from the bile. This study is the first step in examining the risk of *H. bilis* infection on bile duct cancer, and it may provide significant insights into the mechanism of development of cholangio-ductal tumor.

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