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Host-pathogen interaction between Helicobacter pylori and biliary cells mediated by sialic acid receptor

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KEYWORDS
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ABSTRACT
Commensalism involving H. pylori and O. viverrini may have evolved and may facilitate conveyance of the bacillus into human bile duct. The adherence between bacterial ligands and host receptor is an initial step in colonization of H. pylori in bile duct epithelium leading to disease pathogenesis. We investigated tissue adhesion of FITC-labelled H. pylori on normal, pre-cancerous and cancerous bile duct epithelium from 42 cholangiocarcinoma (CCA) cases. The results revealed that all cases exhibited different degrees of the bacterial adhesion. Quantitatively, normal and pre-cancerous bile duct epithelium showed significantly higher number of H. pylori adhesion per area than those of CCA tissue (p-value < 0.001). The mechanism by which H. pylori binds to the bile duct epithelium was then explored. A H. pylori adhesin, specifically sialic acid-binding adhesin (SabA) which recognizes sialyl-Lewis X glycan receptor was studied in human normal cholangiocytes (H69 cell line). The results revealed that the number of FITC-labelled H. pylori adhesion was significantly decreased after blocking with wheat germ agglutinin (WGA) which binds to sialic acid (p-value < 0.05) but not with Ulex eropaeus agglutinin I (UEA I) which is specific for alpha-L-fucose. Pretreatment of H69 cells with antibody to sialyl-Lewis X resulted in significantly decreased adhesion of H. pylori-in a dose dependent manner, particularly at a dilution of 1:200 (p-value < 0.005) and 1:1000 (p-value < 0.05). The results clearly show that H. pylori can colonize on the bile duct epithelium and sialyl-Lewis X may be a receptor for the adhesion.

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Introduction

Cholangiocarcinoma (CCA) is a major public health problem in Thailand and neighboring Mekong countries where the liver fluke, Opisthorchis viverrini (O. viverrini), is endemic[1, 2]. In recent years, there have been increasing evidence that the carcinogenic bacterium Helicobacter pylori (H. pylori) co-infected with the liver fluke may a play major role in cholangiocarcinogenesis[3-7]. Commensalism involving H. pylori and O. viverrini may co-evolved, given that the liver fluke has been shown to harbor the bacteria, and may facilitate conveyance of the bacteria into the bile ducts[3, 8]. Thus, the H. pylori might be transported by the migrating parasites, further adhere to the cholangiocytes, and activate cascades of signaling downstream leading to carcinogenesis in a similar way to those described for the gastric epithelium[9, 10].

Colonization of H. pylori on host epithelium requires bacterial adhesins and host cell receptors. Several adhesive factors belong to the largest of outer membrane proteins (OMPs) family of the bacterium, the Hop family. This contains blood group A antigen binding adhesin (BabA), sialic acid-binding adhesin (SabA), AlpA/B, HopZ and outer inflammatory protein (Oip)A[11], heat shock protein 60 (Hsp60), H. pylori outer membrane protein (HopZ), neutrophil-activating protein (NAP), adherence-associated proteins (AlpA and AlpB), and lacdiNAc-binding adhesin (LabA)[12, 13]. Of these, BabA mediates binding of the bacteria to Lewis B antigens[14] and related terminal fucose residues found on blood group O (H antigen), A and B antigens[15]. SabA binds to sialyl-Lewis X[16] which has been reported in biliary epithelium and CCA[17]. Wheat germ agglutinin (WGA) binds to N-acetyl-D-glucosamine and sialic acid residues of glycoproteins and glycolipids and has been described in human normal bile duct epithelium and CCA tissue[18]. UEA1 is specific for a-L-fucose which is variably found in normal bile duct epithelium[18-20]. Upon adherence of H. pylori to the human epithelium allows its colonization and activates host responses leading to inflammation and promoting carcinogenesis. However, the interaction between human cholangiocyte receptors and H. pylori adhesins have yet been described. We, therefore, aimed to investigate the adhesion between H. pylori and bile duct epithelium and its interaction with host receptors, specifically sialyl-Lewis X in human cholangiocytes.

Materials and methods

Helicobacter pylori strains

A *cagA*-positive *H. pylori* Thai isolate BT112 was cultured on brain heart infusion medium agar with 5% human blood or sheep blood, at 37°C in a microaerophilic condition for 3-5 days. The bacterial colonies were picked up, mixed, and washed in PBS prior next step of experiments. For bacterial adhesion assays, the *H. pylori* was labeled with fluorescein isothiocyanate, FITC as previously described[21]. Briefly, FITC (Thermo Scientific) diluted in dimethyl sulfoxide (10 mg/ml) was incubated with the bacterial suspension for one hour in the dark at room temperature. The bacteria were recovered by centrifugation at 3000 x g for five min, resuspended by gentle pipetting in one ml of PBS/0.05% Tween 20, and pelleted by centrifugation as above. The wash cycle was repeated three times before used.

Liver tissue specimens and tissue adhesion assay

Paraffin sections containing non-tumorous normal, precancerous and cancer tissue were obtained from 42 CCA cases who underwent surgery at Srinagarind Hospital, Khon Kaen University (HE42075). The sections were deparaffinized in xylene, hydrated in a series of alcohol and PBS for tissue adhesion assay. Adhesion of *H. pylori* to bile duct epithelium was performed in the tissue sections by using FITC-labeled bacteria as previously described[17]. Briefly, the deparaffinized tissue sections were incubated for 30 min in blocking buffer (0.2% bovine serum albumin/0.05% Tween 20, prepared in PBS). The FITC-labeled bacterial suspension was diluted in blocking buffer and 200 µl was placed on the slide and incubated for 1 hour at room temperature. Slides were subsequently washed six times with PBS prior to inspection. The stained sections were viewed and

photographed under a confocal microscope (ZIESS, LSM800). Analysis of *H. pylori* adhesion in non-tumorous normal, precancerous (dysplastic) and cancerous tissue was done in 8-10 fields of each tissue type by ImageJ software and expressed as the number of bacterial cell adhesion per square millimeter (mm^2).

Cell culture

Immortalized nontumorigenic human cholangiocyte cell line (H69) was used in this study. The H69 cholangiocytes were cultured in DMEM plus DMEM/F12 supplemented with 10% fetal bovine serum, adenine, insulin, epinephrine, T3-T, hydrocortisone and epidermal growth factor. The cells were grown until reaching 70% confluence before being subjected to bacterial adhesion assays.

Bacterial adhesion assay to H69 cholangiocytes

Binding between *H. pylori* and H69 cholangiocytes as well as blocking assays using antibody to a host receptor, sialyl-Lewis X (*Thermo Fisher* Scientific) and specific lectins wheatgerm hemagglutinin (WGA) and *Ulex europaeus* agglutinin I (UEA I) (Vector Laboratories) were performed. Briefly, H69 cells were fixed in cold 4% paraformaldehyde, washed with PBS, and incubated with increasing dilutions of sialyl-Lewis X; 1:200, 1:1000, 1:5000, 1:25,000 for 2 hours. Similarly, WGA and UEA I at a dilution of 1:200 were used. After washing with PBS, FITC labeled *H. pylori* were then added to H69 cells and incubated at 37°C for 1 hour. The cells were washed, and nuclei were stained with Hoechst *33342* dye (Sigma-Aldrich) prior to inspection and photograph under a confocal microscope (ZIESS, LSM800). Control H69 cells were parallelly performed without antibody or lection treatments. The number of bacteria binding to the H69 cells was analyzed in a total of 1,500 cells by using ImageJ software with four biological replicates.

Statistical analysis

Correlation between number of bacterial adhesions in H69 cells was analysed by one-way ANOVA. For tissue adhesion assay, non-parametric analysis using Man Whitney test was employed, given that the data were not normally distributed. Analysis and graphic generation were accomplished either using GraphPad Prism version 7 software (GraphPad, San Diego, CA) or *IBM® SPSS® Statistics V.* 26. A *p*-value < 0.05 was considered to be statistically significant.

Results

Tissue adhesion assay for H. pylori

H. pylori was detected in normal bile duct epithelium (large and medium size bile ducts), precancerous (dysplastic) epithelium, and CCA tissue in all 42 cases studied (Figure 1). The labeled bacteria were rarely seen in the small bile ducts. The number of bacterial adhesions varied greatly among the cases and different tissue types. Significant higher *H. pylori* adhesion per mm^2 was observed in non-tumorous normal (15.95 ± 10.94) and dysplastic bile duct epithelium (14.16 ± 9.861) than CCA tissue (8.636 ± 7.868) (*p*-value < 0.001) (Figure 2).

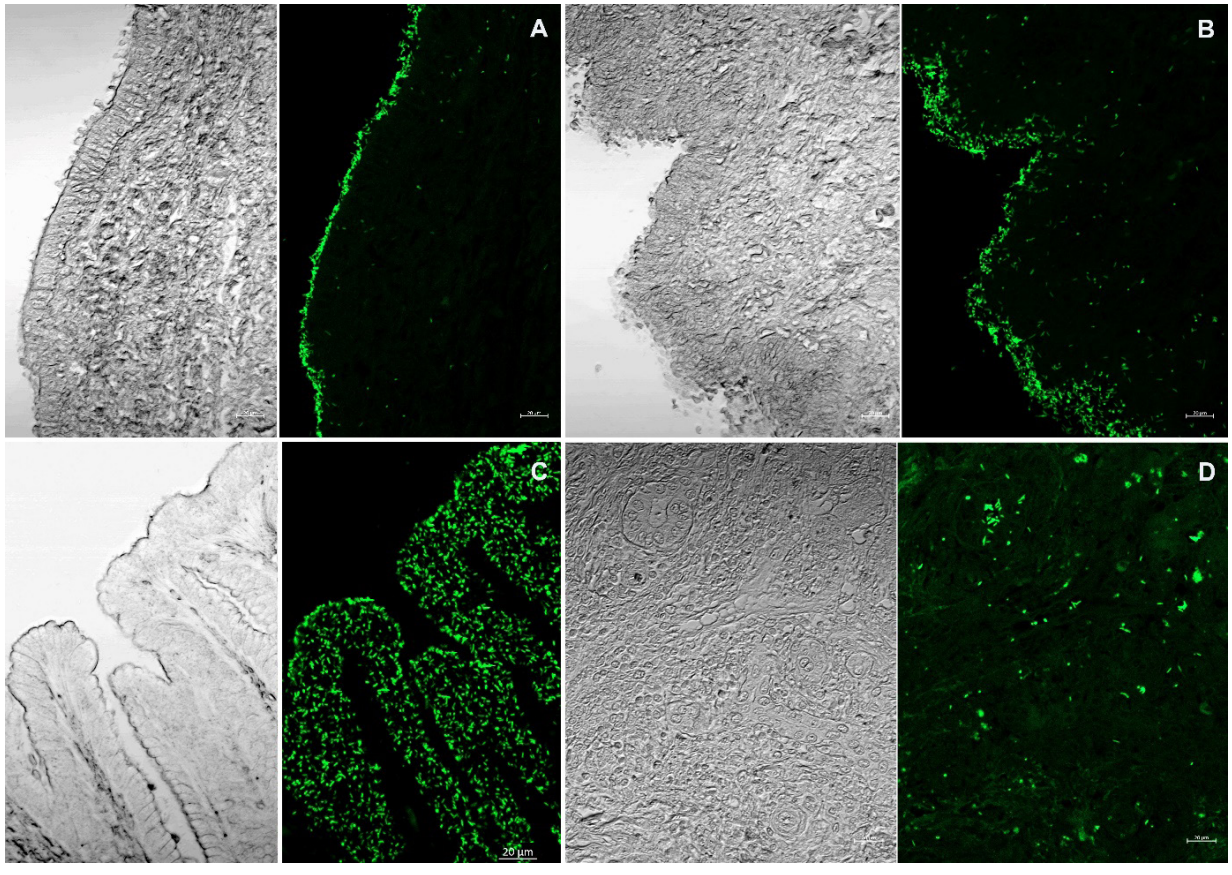

Figure 1 In situ adherence of *Helicobacter pylori*. Adhesion of *H. pylori* on normal (A), precancerous (B), and cancerous bile ducts (D). Bright and fluorescence dark field counterparts of each histologic type are shown. C = control staining on gastric epithelium. Scale bar = 20 µm.

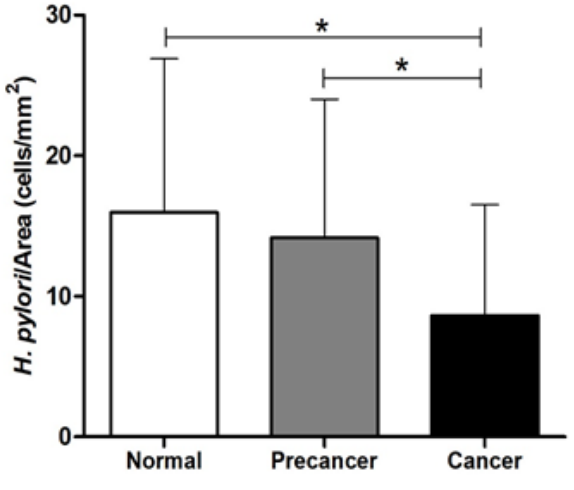

Figure 2 Number of *Helicobacter pylori* adhesion in each histologic type. Significantly higher numbers of *H. pylori* were found in non-tumorous normal and precancerous biliary epithelium than those in cancerous CCA tissue (p-value < 0.001). Precancer = pre-cancerous.

Helicobacter pylori adhesion to biliary cells via sialyl-Lewis X

To investigate specific receptors for *H. pylori* on the bile duct epithelium, H69 cholangiocytes were pretreated with WGA or UEA I lectins prior to adhesion with FITC-labelled bacteria. The results revealed that the number of *H. pylori* adhesion on H69 cells was significantly decreased after blocking with WGA (p-value < 0.05) but not with UEA I p-value > 0.05) (Figure 3A). We further investigated the role of sialyl-Lewis X receptor in *H. pylori* - cholangiocyte interaction by pretreating with different concentrations of sialyl-Lewis X antibody. The results showed a significant decreasing number of *H. pylori* adhesion correlated with the increasing dilution of sialyl-Lewis X (Figure 3 B, C, D), which implicates the essential role of sialyl-Lewis X in cholangiocyte-bacteria interaction.

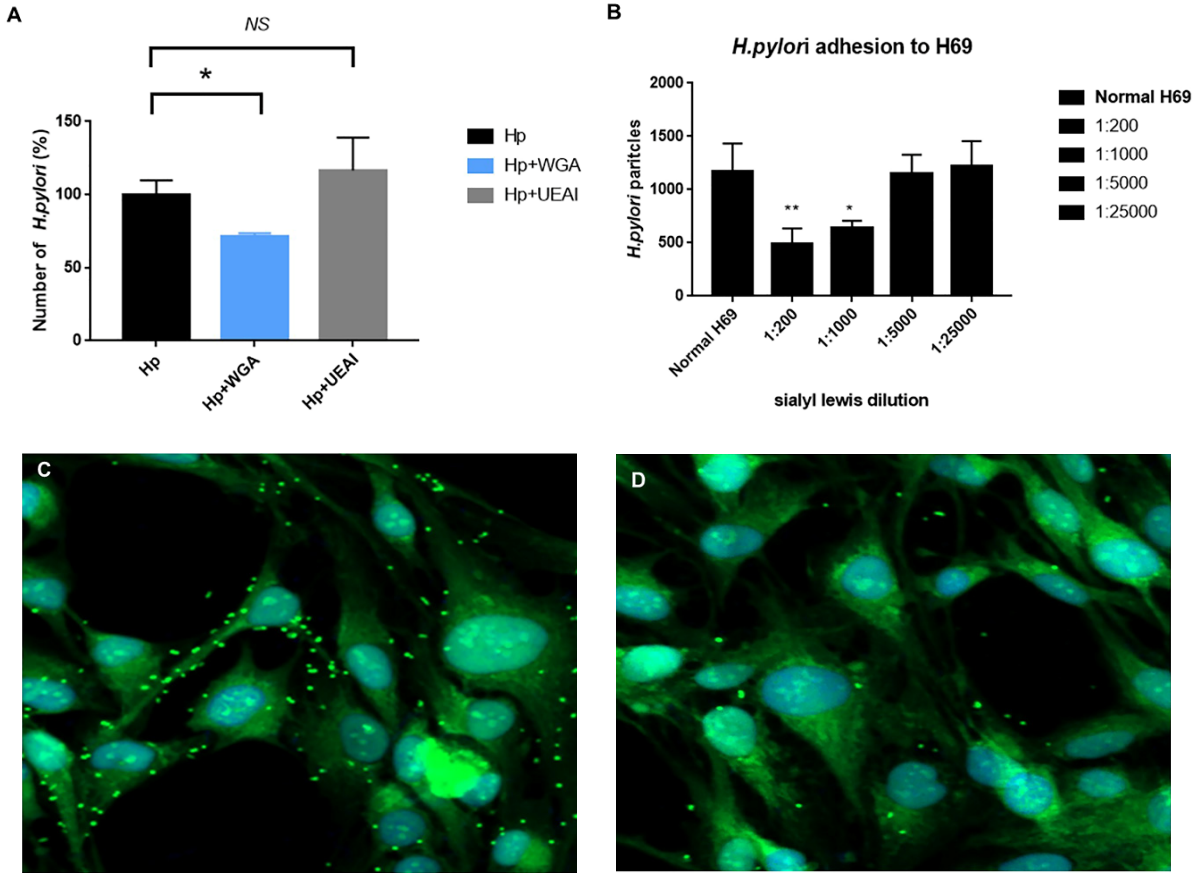

Figure 3 *Helicobacter pylori* adhesion to biliary cells via sialyl-Lewis X. Inhibition assays of *H. pylori* adhesion by WGA and UEA I lectins (A) and sialyl-Lewis X (B) showing a significant reduction of bacterial adhesion for WGA and sialyl-Lewis X but not UEA I. In addition, the adhesion reduction was in a dose-dependent manner for sialyl-Lewis X (B). Representative pictures of bacterial adhesion by confocal microscopy on control H69 cells (C) and those blocked with 1:200 sialyl-Lewis X antibody (D).

Discussion

There have been increasing evidence that *H. pylori* co-infected with *O. viverrini* are involved in the pathogenesis of opisthorchiasis, specifically periductal fibrosis both in an experimental animal model[22] and human study[4] implicating in CCA development[5]. Given that the liver fluke harbors *H. pylori*[3] in its gut and tegument[22]; upon reaching the bile ducts, the *H. pylori* has to bind to the host epithelium before colonization and induces tissue damage. Several major adhesins essential for colonization and infection establishment has been described in the gastric epithelium[13, 16, 23]. Here, for the first time, we reported the adhesion of *H. pylori* on human normal, precancerous and cancerous bile duct epithelium and the adherence to the cholangiocytes were via sialic acid-binding adhesins.

Tissue adhesion of *H. pylori* has been reported in several tissue types including epithelium of gastric tissue, esophageal submucosal glands with less binding extent in intestinal epithelium[17]. In addition, *H. pylori* does not bind to any epithelial cell populations represented in kidney, cervix, endometrium, or squamous cells[17]. No report on *H. pylori* tissue adhesion in the bile duct epithelium has been described. Our team reported adhesion of *H. pylori* to malignant biliary cell lines (KKU-100) and KKU-M156[9]. Here we demonstrated that *H. pylori* can bind to the biliary epithelium and may elicit cascades of signaling activation downstream leading to disease pathogenesis[10-12]. The higher adhesion rates to non-tumorous normal biliary epithelium than those in pre-cancerous or CCA tissue may be from the difference in *H. pylori* receptor expression in different tissue gradings. More detailed study is warranted.

Sialic acid-binding adhesin (SabA) recognizes sialyl-Lewis A and X glycan antigens[13]. SabA, one of *H. pylori* adhesin molecules, has been identified by the retagging technique based on its affinity for sialyl-Lewis X[24]. Our study showed that the bacterial adhesion on H69 cholangiocytes was significantly inhibited by blocking with sialyl-Lewis X antibody. Moreover, *H.pylori* adhesion to the H69 cells was also inhibited by WGA but not UEA I. WGA from *Triticum vulgar* non-enzymatically binds to N-acetyl-D-glucosamine and sialic acid residues of glycoproteins and glycolipids including those from *H. pylori*[25]. WGA has been described in human normal bile duct epithelium and CCA tissue[18]. UEA I is specific for a-L-fucose which is not or variably bound to normal bile duct epithelium[18, 19]. Therefore, blocking with the lectin UEA I but not WGA did not affect the adhesion of *H. pylori* on the H69 cholangiocytes even though it was slightly increased but it was not significantly different compared to controls *(p*-value > 0.05). Blocking with WGA and UEA I lectins is cost effective and has been used in several adhesion applications[26-28].

These results altogether suggest that sialyl-Lewis X might be a candidate receptor for *H. pylori* in the biliary epithelium. The interaction between the bacterium adhesins and the host cell surface receptor is involved in the initial stages of colonization. Adherence of bacteria to host cell receptors triggers cellular changes, signal transduction cascades leading to inflammation following the release of virulent factors[29], cell morphology change, EMT and cell transformation or may be reminiscent of the modified appearance of carcinoma cells after exposure *H. pylori* such as the hummingbird phenotype[30, 31].

Conclusion

Our study showed that *H. pylori* can bind to the biliary cells via sialyl-Lewis X receptor and may elicit the colonization on bile duct epithelial cells prior trigger pathogenesis in the hepatobiliary system.

Take home messages

Our study provides basis of host-pathogen interaction at initial stage in *H. pylori* infection of the biliary system. Further in-depth study on host-pathogen interactions is warranted.

Conflicts of interest

The authors declare no conflict of interest.

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