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ORIGINAL ARTICLE

Helicobacter pylori in Thai patients with cholangiocarcinoma and its association with biliary inflammation and proliferation

Wongwarut Boonyanugomol[1,5], Chariya Chomvarin[1,5], Banchob Sripa[2,5], Vajarabhongsa Bhudhisawasdi[3,5], Narong Khuntikeo[3,5], Chariya Hahnvajanawong[1,5] & Amporn Chamsuwan[4]

[1]Department of Microbiology, [2]Department of Pathology, [3]Department of Surgery, [4]Department of Forensic Medicine and [5]Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

Abstract

Objectives: To investigate whether *Helicobacter* spp. infection and the *cagA* of *H. pylori* are associated with hepatobiliary pathology, specifically biliary inflammation, cell proliferation and cholangiocarcinoma (CCA).

Methods: *Helicobacter* species including *H. pylori*, *H. bilis* and *H. hepaticus* were detected in the specimens using the polymerase chain reaction (PCR). Biliary inflammation of the liver and gallbladders was semi-quantitatively graded on hematoxylin and eosin (H&E)-stained slides. Biliary proliferation was evaluated by immunohistochemistry using the Ki-67-labelling index.

Results: *Helicobacter pylori* was found in 66.7%, 41.5% and 25.0% of the patients in the CCA, cholelithiasis and control groups ($P < 0.05$), respectively. By comparison, *H. bilis* was found in 14.9% and 9.4% of the patients with CCA and cholelithiasis, respectively ($P > 0.05$), and was absent in the control group. The *cagA* gene of *H. pylori* was detected in 36.2% and 9.1% of the patients with CCA and cholelithiasis, respectively ($P < 0.05$). Among patients with CCA, cell inflammation and proliferation in the liver and gallbladder were significantly higher among those DNA *H. pylori* positive than negative.

Conclusions: The present findings suggest that *H. pylori*, especially the *cagA*-positive strains, may be involved in the pathogenesis of hepatobiliary diseases, especially CCA through enhanced biliary cell inflammation and proliferation.

Keywords

Helicobacter pylori, *H. bilis*, *cagA*, hepatobiliary diseases, cholangiocarcinoma, inflammation, proliferation

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Correspondence

Chariya Chomvarin, Department of Microbiology and Liver fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand. Tel: 66 43 363808. Fax: 66 43 348385. E-mail: chariya@kku.ac.th

Introduction

Cholangiocarcinoma (CCA), a subtype of primary liver cancer arising from bile duct epithelial cells, is a rare malignant tumour worldwide but it is highly prevalent in Asian countries, particularly in Thailand.[1] Both epidemiological and experimental evidence have implicated the liver flukes, *Opisthorchis viverrini* and *Clonorchis sinensis*, as important carcinogenic agents in the development of CCA.[2] However, only a small proportion of liver fluke-infected patients develop CCA.[3] Other factors, such as bacterial infection in the hepatobiliary tract, may therefore synergistically trigger tumour development. In this regard, it has been postulated that *Helicobacter* spp. may be involved in the development of CCA.[4]

Helicobacter spp. (including *H. pylori*, *H. hepaticus* and *H. bilis*) have been found in the bile,[5] gallbladder[6] and liver tissue[7] of patients with hepatobiliary diseases. *Helicobacter pylori* is a well-recognized causative factor of gastrointestinal diseases, and has been strongly linked to the development of gastric adenocarcinoma.[8] Previous experimental studies showed that *H. bilis* and *H. hepaticus* were found in the bile and hepatobiliary tissues of patients with biliary tract diseases using a polymerase chain reaction (PCR) assay.[5,9] *Helicobacter pylori* has frequently been detected in patients with liver carcinoma and CCA;[7,10–12] however,

there were only small numbers of CCA cases in these studies and the results are still under debate.[13] In order to increase the understanding of the association and pathogenesis of *Helicobacter* spp. infection and the development of hepatobiliary disease including CCA, we performed a systemic investigation of the *Helicobacter* infection and its associated pathology in a large number of benign and malignant hepatobiliary diseases and normal controls.

Materials and methods

Patients and specimens

Bile samples were collected from 140 patients including 87 with CCA (the malignant group) and 53 with cholelithiasis (the benign group) who underwent surgery at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. From the 87 patients with CCA, liver ($n = 21$) and gallbladder tissues ($n = 44$) were additionally collected. From the 53 patients with cholelithiasis, an additional 35 gallbladders and 16 gallstones were collected. The bile samples from the normal control group were collected from 16 autopsied cases. Patients with gastrointestinal diseases and hepatitis virus infection were excluded. The human ethics committee at our institution reviewed and approved the present study (HE 450525). All of the patients provided informed consent before participating in the study.

DNA extraction

Approximately 50 mg each of gallbladder tissue, liver tissue (cancerous and non-cancerous) and gallstone were transferred to respective sterile normal saline solution under cold conditions. Each of the specimens was homogenized with a glass tissue grinder in 500 µl of lysis buffer. As for the bile specimens, 2 ml of bile sample were diluted with 1 volume of sterile phosphate-buffered saline (PBS). After centrifugation at 13 000 × *g*, the bile pellet was re-suspended in 500 µl of lysis buffer.

DNA was extracted using a genomic DNA purification kit (Puregene; Gentra System Inc., Minneapolis, MN, USA), according to the manufacturer's instructions. Briefly, the bile pellet – or ground sample in lysis buffer – was treated with 4 µl of proteinaseK (20 mg/ml) for 3 h at 55°C. The lysate was incubated at 98°C for 10 min; then 4 µl of RNaseA (25 mg/ml) was added to the cell lysate and incubated at 37°C for 1 h. Then, 300 µl of protein precipitation solution was added and centrifuged at 13 000 × *g* for 5 min. The supernatant was collected and precipitated by 2 volumes of absolute ethanol and centrifuged at 13 000 × *g* for 5 min. After washing the DNA pellet with 70% ethanol, the DNA was dried and suspended in TE buffer and stored at −20°C until needed.

As for the bile samples, after precipitation by two volumes of absolute ethanol, the DNA pellet was purified using a column and washed twice with a washing buffer. Finally, the DNA was eluted with 50 µl of elution buffer. The DNA was stored at −20°C until needed.

Detection of *Helicobacter* spp., *H. pylori*, *H. bilis*, *H. hepaticus* and *cag*A by PCR assay

Primer sequences for the detection of *Helicobacter* spp.; *H. pylori*, *H. bilis*, *H. hepaticus* and *cag*A gene were designed as previously described[10,14–16] with slight modifications (Table 1).

All reactions were carried out in a 50-µl solution containing 500 ng from the hepatobiliary specimens. The reaction mixture contained 1× PCR buffer (RBC; Bioscience, Taipei, Taiwan), 0.2 mM dNTP, 1.5 mM $MgCl_{2+}$, 0.2 µM primers and 1.25 U of *Taq* DNA polymerase (RBC, Bioscience, Taipei, Taiwan). PCR was performed in an automated thermocycle (GeneAmp, PCR 2400; PerkinElmer, Waltham MA, USA). The amplified product was identified by electrophoresis in 1.5% agarose gel. The DNA was stained with ethidium bromide and visualized under a UV illuminator.

Bacterial culture

For the *Helicobacter* spp. cultivation, the ground tissue and 100 µl of bile from each respective sample was inoculated onto the Belo Horizonte medium and Columbia blood agar supplemented with 10% human whole blood cell, 10 mg/ml of vancomycin, 5 mg/ml of trimethoprim, 5 mg/ml of cefsulodin and 5 mg/ml of amphotericin B. The media was incubated under microaerophilic conditions at 37°C for up to 2 weeks. For the other bacterial cultivations, 100 µl of ground tissue or bile sample was inoculated onto a blood agar base and MacConkey agar. The plates were incubated at 37°C for 24–48 h. The colonies that grew on the plates were identified using biochemical tests.[17]

Biliary inflammation

Evaluation of inflammation was performed according to Fukuda *et al.*[5] The paraffin-embedded tissues of the gallbladder and liver tissue were cut onto glass slides coated with 5-aminoprophyltriethoxy saline and stained with hematoxylin and eosin (H&E). Inflammatory cell infiltration along the portal triads and gallbladder mucosa were semi-quantitatively graded: 0 = unremarkable (0–25% of mononuclear cell infiltration), 1 = mild chronic inflammation (25–50% of mononuclear infiltration), 2 = moderate chronic inflammation (50–75% of mononuclear cell infiltration) and 3 = severe chronic inflammation with lymphoid aggregation (>75% of mononuclear cell infiltration). All tissue sections were examined in a blinded fashion without knowledge of the clinical data or PCR results at the time of the assessment.

Biliary proliferation

Biliary cell proliferation was determined by immunohistochemistry using Ki-67. Tissue sections were incubated overnight with mouse monoclonal anti-Ki-67 (Dako, Glostrup Denmark, Denmark) at dilutions of 1 : 100 at 4°C. After incubation with the primary antibody, the tissue section was incubated with goat anti-mouse conjugated with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The reaction was visualized using diami-

Table 1 Primer sequences and PCR conditions for amplification of *Helicobacter* spp.

Gene specific for	Genes	Primer sequences	PCR condition	PCR size	Ref.
Helicobacter spp.	16S rRNA (nested PCR)	OF-ATTAGTGGCGCACGGGTGAGTAA	94°C 30 s, 55°C 30 s, 72°C 1.5 min (35 cycle)	1300	10
		OR-TTTAGCATCCCGACTTAAGGC			
		IF-GAACCTTACCTAGGCTTGACATTG	94°C 30 s, 60°C 30 s, 72°C 30 s (35 cycle)	480	
		IR-GGTGAGTACAAGACCCGGGAA			
H. pylori	*ureA* (nested PCR)	OF-GCTAATGGTAAATTAGTTCCTGG	94°C 30 s, 62°C 30 s, 72°C 30 s (40 cycle)	411	14
		OR-CTCCTTAATTGTTTTTACATAGTTG			
		IF-AGTTCCTGGTGAGTTGTTCTTAA	94°C 30 s, 59°C 30 s, 72°C 30 s (40 cycle)	350	
		IR-AACCACGCTCTTTAGCTCTGTC			
H. pylori	*cagA* (nested PCR)	OF-AGACAACTTGAGCGAGAAAG	94°C 30 s, 55°C 30 s, 72°C 30 s (40 cycle)	320	15
		OR-TATTGGGATTCTTGGAGGGG			
		OF-AGACAACTTGAGCGAGAAAG	94°C 30 s, 57°C 30 s, 72°C 30 s (40 cycle)	307	
		IR-GGAGGCGTTGGTGTATTTGA			
H. bilis	16S rRNA (nested PCR)	OF-CTATGACGGGTATOCGGC	98°C 10 s, 55°C 30 s, 72°C 1 min (35 cycle)	718	16
		OR-CTCACGACACGAGCTGAC			
		IF-CAGAACTGCATTTGAAACTAC	98°C 10 s, 55°C 30 s, 72°C 30 s (35 cycle)	418	
		IR-AAGCTCTGGCAAGCCAGC			
H. hepaticus	16S rRNA (nested PCR)	OF-CTATGACGGGTATOCGGC	98°C 10 s, 55°C 30 s, 72°C 1 min (35 cycle)	718	16
		OR-CTCACGACACGAGCTGAC			
		IF-GAAACTGTTACTCTG	98°C 10 s, 55°C 30 s, 72°C 30 s (35 cycle)	405	
		IR-TCAAGCTCCCCGAAGGG			

PCR, polymerase chain reaction.

nobenzidine as a substrate. The Ki-67 protein expression was analyzed by calculation of the percentage of Ki-67 labelled biliary cells, with respect to the total number of biliary epithelial cells (labelling index, LI).

Statistical analysis

The χ^2 or Fisher's exact test was used for statistical analysis of the *H. pylori* infection, virulence genes and patient groups. Associations between biliary inflammation, Ki-67-LI and the disease groups were analyzed using the Student's *t*-test. Differences at $P < 0.05$ were considered statistically significant.

Results

Detection of *Helicobacter* spp. (*H. pylori*, *H. bilis* and *H. hepaticus*) by PCR

Helicobacter spp. was detected in the bile samples of CCA patients (71.3%) significantly more often than in patients with cholelithiasis (47.2%) or the control group (25%). The total number of *H. pylori* found in the CCA patients (66.7%) was significantly greater than in patients with cholelithiasis (41.5%) or the control group (25.0%). While the respective total number of *H. bilis* found in patients with CCA and cholelithiasis was 14.9% and 9.4%, none was found in the control group. Similarly, no PCR product from *H. hepaticus* was found in either the malignant or the benign group.

Each species of *Helicobacter* was analyzed. We found that *H. pylori* infection alone was detected in significantly more CCA patients (58.6%) than in patients with cholelithiasis (22.6%) or the control group (25%) (Table 2). By comparison, *H. bilis* infection alone was detected in 3.4% of patients with CCA and 5.7% with cholelithiasis (no significant difference between groups). A mixed infection of *H. pylori* and *H. bilis* was detected in 8% and 1.9% of CCA and cholelithiasis, respectively (Table 2).

In order to confirm that the major *Helicobacter* spp. found was *H. pylori*, we performed DNA sequencing analysis for 16srRNA *Helicobacter* spp. The results showed that the amplicons, which were randomly sequenced, were at least 98% similar to the 16S rRNA gene of *H. pylori* (GeneBank accession number CP001680.1 and AY364440.1).

For other hepatobiliary specimens, the gallbladder, liver tissues and gallstone were also tested for *Helicobacter* spp. The result showed that the *H. pylori* was detected significantly more frequently in the gallbladder of the malignant group than in the benign group ($P < 0.05$). In addition, *H. pylori* was detected in the liver tissues and gallstones of patients with CCA and cholelithiasis, respectively. The presence of *H. bilis* was also detected but the number was lower than *H. pylori* in both the malignant and benign group (data not shown).

Table 2 *Helicobacter* spp. and other bacteria detected in bile samples of hepatobiliary diseases

Diseases	Number of *Helicobacter* positive (%)					No. of other bacteria[b] positive (%)	No. of bacterial negative (%)
	H. pylori	*H. bilis*	*H. pylori* and *H. bilis*	*Helicobacter* spp.[a] and other bacteria	Total No. of *Helicobacter* spp.		
CCA ($n = 87$)	48*† (58.6)	3 (3.4)	7 (8.0)	4 (4.6)	**62*†(71.3)**	3 (3.4)	22 (25.3)
Cholelithiasis ($n = 53$)	12 (22.6)	3 (5.7)	1 (1.9)	9‡ (17.0)	**25 (47.2)**	12‡§ (22.6)	16 (30.2)
Control ($n = 16$)	4 (25.0)	0 (0)	0 (0)	0 (0)	**4 (25.0)**	1 (6.25)	10 (62.5)

*Statistical significance between CCA and cholelithiasis ($P < 0.05$).
†Statistical significance between CCA and control ($P < 0.05$).
‡Statistical significance between cholelithiasis and CCA ($P < 0.05$).
§Statistical significance between cholelithiasis and control ($P < 0.05$).
[a]*Helicobacter* spp. such as *H. pylori* and/or *H. bilis*.
[b]Other bacteria was found in bile samples, gallbladder tissues and stones samples, but was not found in liver tissues.
CCA, cholangiocarcinoma.

Table 3 *Helicobacter pylori cag*A positive in bile samples of hepatobiliary diseases

Diseases	Total No. (%) of *H. pylori* PCR positive[a]	No. (%)of *cagA* gene positive
CCA ($n = 87$)	58*† (66.7)	21*† (36.2)
Cholelithiasis ($n = 53$)	22 (41.5)	2 (9.1)
Control ($n = 16$)	4 (25.0)	0 (0)

*Statistical significance between CCA and cholelithiasis ($P < 0.05$).
†Statistical significance between CCA and control ($P < 0.05$).
[a]Total number of *H. pylori* PCR positive was *H. pylori* infection alone and mixed infection of *H. pylori* with *H. bilis* or other bacteria.
CCA, cholangiocarcinoma.

Detection of *H. pylori cag*A gene by PCR

All of the *H. pylori* PCR positive samples were further analyzed for the presence of the *cag*A gene. The *cag*A gene was detected in 36.2% and 9.1% of the CCA and cholelithiasis patients, respectively. The difference between the two groups was statistically significant ($P < 0.05$), and no PCR positive *cag*A gene was found in the control group (Table 3).

Bacterial culture

After prolonged incubation of up to 2 weeks under microaerophilic conditions, no *Helicobacter* spp. from the hepatobiliary specimens were grown. The other bacteria found included *Escherichia coli*, *Klebsiella* spp., *Acinetobacter anitratum*, *Citrobacter freundii*, *Enterobacter* spp., *Pseudomonas aeruginosa* and *Staphylococcus aureus*. In patients with cholelithiasis, we found that the types and numbers of other bacteria were significantly higher than in the patients with CCA or in the control group (Table 2).

Biliary inflammation

In the liver and gallbladder tissues of CCA patients, the average inflammatory grade for *H. pylori*-DNA-positive tissues was significantly higher than for patients with *H. pylori*-DNA-negative tissues: In the latter cases the inflammatory grade was similar to the gallbladder tissues of patients with cholelithiasis (Table 4). When we evaluated the inflammatory grade of non-infected tissues and *H. bilis*-PCR-positive tissue, the result was not significantly different for either the patients with CCA or cholelithiasis. In a mixed infection of *H. pylori* and *H. bilis* in the liver tissues, the grade of inflammation was significantly higher than in non-infected liver tissues (Table 4).

Furthermore, we found that gallbladder tissues of patients with cholelithiasis infected with other bacteria (or with a multiple infection of *Helicobacter* spp. and other bacteria) showed a significantly higher inflammatory grade than that of the non-infected gallbladder ($P < 0.05$). Biliary inflammation determined by H&E staining is presented in Figs 1a, c and 2a, c.

Biliary proliferation

The liver and gallbladder tissues of CCA patients had a significantly higher average Ki-67-LI in the *H. pylori*-PCR-positive tissues than in the non-infected tissues. By comparison, among the average Ki-67-LI in *H. bilis*-PCR-positive tissues and non-infected tissues was not significantly different (Table 5). Interestingly, CCA patients with a mixed infection (of *H. pylori*- and *H. bilis*-PCR-positive) showed a significantly higher level of Ki-67-LI than non-infected tissues in either the liver or gallbladder tissues. By contrast, there was no significant difference in the average Ki-67-LI between infections and non-infections in the gallbladder tissues of patients with cholelithiasis. The biliary proliferation as determined by immunohistochemistry of Ki-67 is presented in Figs 1b, d and 2b, d.

Discussion

The association between *Helicobacter* spp. infection and hepatobiliary diseases has been reported but its roles in the pathogenesis are still under debate.[9,10,13] The present study demonstrates a significantly higher frequency of *Helicobacter* spp., especially *H. pylori* in the bile from CCA patients than in those with cholelithiasis and the controls, as reported in patients with hepatocellular carcinoma and CCA.[7,12,13] This suggests that *H. pylori* infection may be associated with the severity of hepatobiliary diseases. Chronic cholestasis is one of the key predisposing factors

Table 4 Evaluation of inflammation in liver and gallbladder tissues infected with *Helicobacter pylori, H. bilis* or other bacteria compared with non-infected tissues

Diseases	Tissues collected	Average of inflammatory grade					
		Non-infection	*H. pylori*+	*H. bilis*+	*H. pylori* and *H. bilis*+	Other bacteria+	*Helicobacter* spp. and other bacteria+
CCA	Liver ($n = 21$)	1.17 ± 0.41 ($n = 6$)	2.33 ± 0.87* ($n = 9$)	1.00 ($n = 1$)	2.40 ± 0.89* ($n = 5$)	ND	ND
	Gallbladder ($n = 44$)	1.00 ± 0.67 ($n = 10$)	2.20 ± 0.77* ($n = 17$)	1.00 ± 1.00 ($n = 3$)	1.86 ± 1.35 ($n = 7$)	1.00 ± 0.58 ($n = 2$)	1.60 ± 0.55 ($n = 5$)
Cholelithiasis	Gallbladder ($n = 35$)	1.13 ± 0.83 ($n = 15$)	2.40 ± 0.55* ($n = 5$)	1.67 ± 1.53 ($n = 3$)	2.00 ($n = 1$)	2.20 ± 0.84* ($n = 5$)	2.17 ± 0.75* ($n = 6$)

*P-value < 0.05 was considered a statistically significant difference between the non-infected and infected tissues.
ND, not determined (other bacteria were not found in liver tissues).

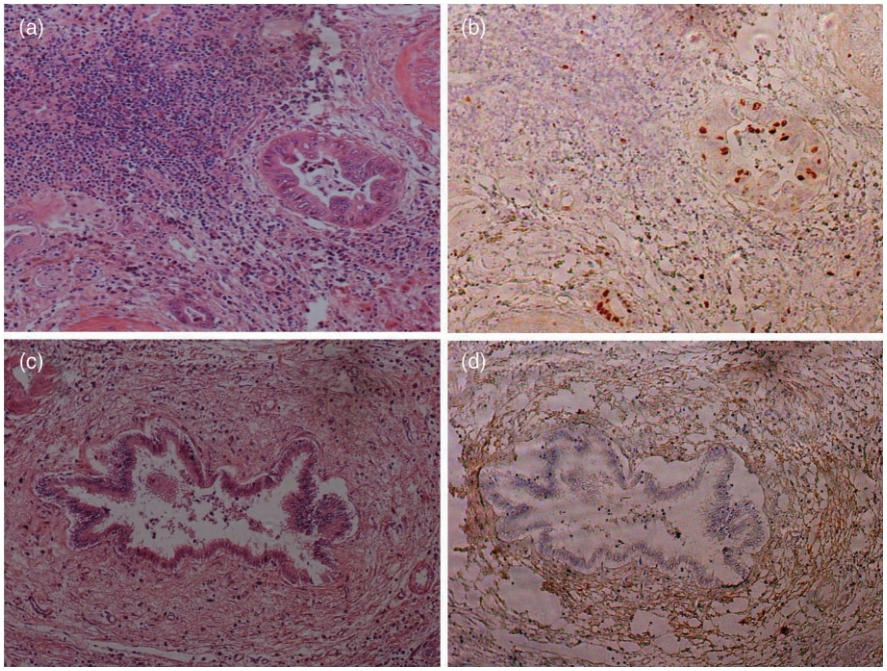

Figure 1 Evaluation of biliary inflammation by hematoxylin and eosin (H&E) staining and proliferation by immunohistochemistry staining with *Helicobacter pylori*-PCR-positive and -negative in liver tissues (200× magnification). (a) Severe chronic inflammation of the bile duct in *H. pylori* PCR-positive liver tissue. (b) High score of average Ki-67-LI of the bile duct in *H. pylori*-PCR-positive liver tissue. (c) Low grade inflammation (unremarkable) of bile duct in *H. pylori*-PCR-negative liver tissue. (d) Low score of Ki-67-LI of the bile duct in *H. pylori*-PCR-negative liver tissue

to the development of CCA.[18] Interestingly, although, cholestasis is commonly found in bile duct obstruction caused by CCA but it is not common in cholelithiasis.[19] Previous studies have shown that chronic cholestasis can induce lowering of the bile pH,[20] creating conditions that favour *H. pylori* survival.[21] Therefore, the present study suggests that *H. pylori* can survive in the bile and can be detected more in patients with CCA than those with cholelithiasis and control subjects.

We found that the *H. pylori* was the predominant *Helicobacter* species in the present study which agrees with previous reports.[22,23] Additionally, we found very few *H. bilis* in hepatobiliary specimens and no significant difference between *H. bilis* and the disease groups suggesting *H. bilis* might play a minor role in the pathogenesis of CCA. Our results agree with Huang and colleagues who reported that *H. pylori* was only found in liver tissues of hepatocellular carcinoma patients but was undetectable in the control group, according to PCR and DNA sequencing.[7] The present findings, however, disagree with Fukuda *et al*. who showed that *H. bilis* was frequently found in hepatobiliary cancer patients in Japan, whereas *H. pylori* was found in small numbers.[5] This may be explained by the variation in *Helicobacter* spp. across regions.[22]

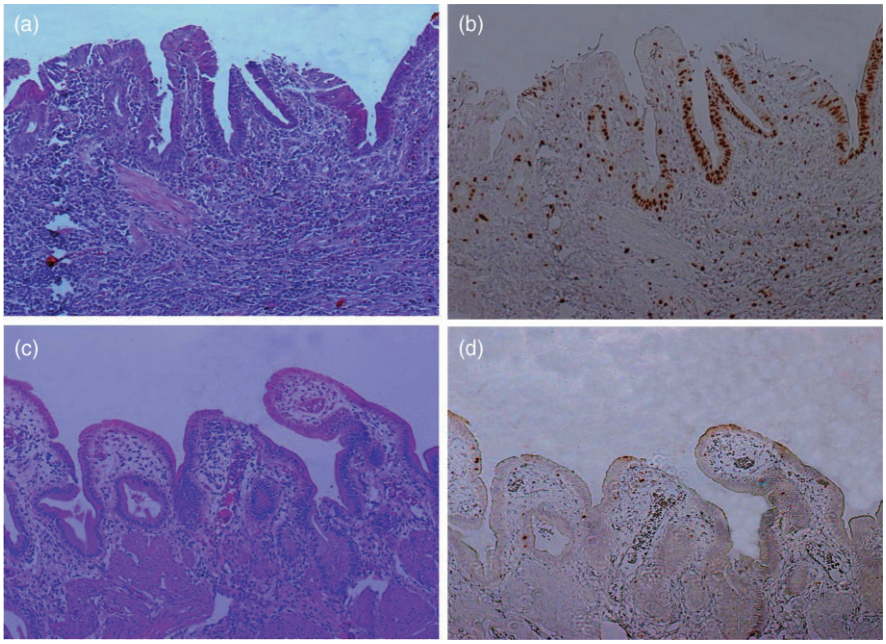

Figure 2 Evaluation of biliary cell inflammation by hematoxylin and eosin (H&E) staining and proliferation by immunohistochemistry staining with *Helicobacter pylori*-PCR-positive and -negative gallbladder tissues (200× magnification). (a) Severe chronic inflammation of *H. pylori*-PCR-positive gallbladder tissue. (b) High score of average Ki-67-LI of *H. pylori*-PCR-positive gallbladder tissue. (c) Mild chronic inflammation of *H. pylori*-PCR-negative gallbladder tissue. (d) Low score of Ki-67-LI of *H. pylori*-PCR-negative gallbladder tissue

Table 5 Evaluation cell proliferation in liver and gallbladder tissues infected with *Helicobacter pylori* or *H. bilis* compared with non-infected tissues

Diseases	Tissues collected	Average of the Ki-67 labelling index					
		Non-infection	*H. pylori*+	*H. bilis*+	*H. pylori* and *H. bilis*+	Other bacteria+	*Helicobacter* spp. and other bacteria+
CCA	Liver ($n = 21$)	4.50 ± 3.62 ($n = 6$)	13.44 ± 9.15* ($n = 9$)	5.00 ($n = 1$)	14.83 ± 10.85* ($n = 5$)	ND	ND
	Gallbladder ($n = 44$)	5.50 ± 3.95 ($n = 10$)	14.25 ± 10.24* ($n = 17$)	1.33 ± 2.31 ($n = 3$)	14.71 ± 12.27 ($n = 7$)	5.00 ± 7.07 ($n = 2$)	6.60 ± 8.10 ($n = 5$)
Cholelithiasis	Gallbladder ($n = 35$)	9.73 ± 13.57 ($n = 15$)	12.80 ± 14.18 ($n = 5$)	2.67 ± 2.52 ($n = 3$)	5.00 ($n = 1$)	2.00 ± 2.12 ($n = 5$)	10.33 ± 6.59 ($n = 6$)

*P-value < 0.05 was considered a statistically significant difference between the non-infected and infected tissues.
ND, not determined (other bacteria was not found in liver tissues).

In the present study, the DNA of the *H. pylori cagA*-positive strains was detected more frequently among patients with CCA than patients with cholelithiasis and was undetectable in the control group. This is important because the *cag*A gene is an important virulence gene in the *H. pylori* strain, which is known to induce severe inflammation associated with gastric cancer.[24] Based on our previous finding *in vitro*[25] and this present study, we suggest that *H. pylori-cag*A-positive strains may be associated with the carcinogenesis of CCA.

It is generally accepted that there is a relationship between chronic inflammation of the stomach caused by *H. pylori* and gastric carcinogenesis.[26] Chronic inflammation is a major cause of oxidative DNA damage by stimulation of NO production, leading to a high risk of cancer development.[27] In the present study, patients with CCA and *H. pylori*-PCR-positive liver tissue presented with a significantly higher inflammatory grade at the portal zone around the bile ducts than patients with a non-infected liver. Similarly, the gallbladder mucosa in both malignant and benign groups exhibited a statistically significant, higher level of mononuclear cell infiltration in the *H. pylori*-PCR-positive samples than the negative ones. Moreover, we found that co-infection of *H. pylori* with other bacteria resulted in significantly more aggressive inflammation (i.e. severe chronic inflammation) in the gallbladder mucosa of the cholelithiasis patients than in those with

CCA, indicating multiple bacterial infections may be associated with the progression of gallstone formation in patients with cholelithiasis.

Ki-67 has been used for the determination of cellular proliferative activity.[28] In inflammation, several inflammatory cytokines can induce cell proliferation, oxidative DNA damage and survival.[29] In patients with CCA, a high level of inflammation at the periportal area and gallbladder mucosa parallels a significantly higher cell proliferative index in the *H. pylori*-PCR-positive samples compared with the negative ones. This suggests that *H. pylori* may have a role in the development of bile duct cancer through the induction of cell inflammation and biliary cell proliferation. This result agrees with our previous *in vitro* study[25] and Fukuda *et al*. who demonstrated that the proliferating cell nuclear antigen labelling index (PCNA-LI) in the biliary epithelium of *Helicobacter* DNA-positive patients was significantly higher than in *Helicobacter*-DNA-negative ones.[5]

As with a previous report on an animal model, a very low level of apoptotic cells was detected (by TUNEL assay) compared with marked proliferation (by PCNA) in *H. pylori*-infected mouse livers.[30] We also studied apoptosis in both liver and gallbladder tissues using caspase-3-LI and very few apoptotic biliary cells were detected (Data not shown). Similar to our previous *in vitro* report, a low number of *H. pylori* stimulated biliary cell proliferation rather than apoptosis.[25]

Although a few previous studies have reported on the detection of *Helicobacter* spp. or *H. pylori*-like spiral bacteria in hepatobiliary samples,[7,31] all were not proven by bacterial cultivation.[7,32] Similarly, we were unable to detect *Helicobacter* spp. in hepatobiliary tissues by cultivation or immunohistochemical staining (data not shown) as with previous reports.[5,7,33] The inability to detect these by cultivation may be as a result of (i) the small number of these bacteria in hepatobiliary specimens[7] and/or (ii) the unfavourable environment in the biliary tract which may cause transformation of *Helicobacter* into its non-culturable coccoid form.[34,35] A previous study showed that the coccoid form of *H. pylori* has persistent urease activity, so it is able to adhere to the epithelial cell line and this persistence caused disease in mice; suggesting that the coccoid form of *H. pylori* possesses some pathogenicity.[36] Studies in animal models, however, showed that *H. pylori* promoted hepatic fibrosis and was involved in the development of hepatocellular carcinoma.[30,33] On comparing our previous findings *in vitro*[25] with the present study, we suggest that even although there may be a very small number of *H. pylori* in the hepatobiliary tract, they still play a role in the development of CCA.

In addition, we observed that Enterobacteriaceae was the most frequently found family, which agrees with other reports.[37] Other bacteria were found most often in patients with benign hepatobiliary disease rather than in those with malignancies, suggesting a possible role of other bacteria in the pathogenesis of cholelithiasis. These results indicate that *H. pylori* may have a potential role in hepatobiliary diseases greater than other bacteria, particularly in CCA.

In conclusion, we investigated the presence of *Helicobacter* spp. especially *H. pylori* with a concurrent pathological study on biliary inflammation and proliferation. The results suggest that *H. pylori* and *cag*A may be involved in the development of CCA. Thus, the eradication of *Helicobacter* spp., and in particular *H. pylori*, may be useful for the treatment of CCA. Notwithstanding, it is possible that the high frequency of *Helicobacter* infection among CCA patients may result from cholestasis.[19,20,38] In future studies, therefore, the cultivation of *Helicobacter* spp. from hepatobiliary samples should be improved and animal models to clarify the pathogenesis of *Helicobacter* spp. in the hepatobiliary diseases should be studied in depth.

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Conflicts of interest

We declare that we have no conflicts of interest.

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