Jpn. J. Cancer Res. **93**, 842–847, July 2002

Association between *Helicobacter bilis* in Bile and Biliary Tract Malignancies: *H. bilis* in Bile from Japanese and Thai Patients with Benign and Malignant Diseases in the Biliary Tract

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Japan and Thailand have high incidences of bile duct carcinoma and gallstones. The presence of *Helicobacter bilis* (*H. bilis*) detected by polymerase chain reaction (PCR) and 16S rRNA analysis in bile samples from Chileans with chronic cholecystitis was reported. The association between *H. bilis* in bile and biliary tract malignancies has not been investigated, and therefore the aim of this study is to determine whether malignant diseases of the biliary tract are associated with the presence of *H. bilis* in bile samples obtained from two high-risk populations. Bile samples from 45 Japanese and 40 Thai patients were subjected to PCR analysis using *H. bilis*-specific primers, and six of the *H. bilis* amplicons were sequenced. Thirteen out of 15 (87%) Japanese and 11 out of 14 (79%) Thai patients with bile duct or gallbladder cancer tested positive for the presence of *H. bilis* in their bile. Eight out of 16 (50%) Japanese and 10 out of 26 (38%) Thai patients with gallstone and/or cholecystitis tested positive for *H. bilis*. Only 4 out of 14 (29%) subjects without biliary disease tested positive for *H. bilis* among the Japanese. Bile duct and gallbladder cancer showed significantly higher positive rates for *H. bilis* than did the non-biliary diseases among the Japanese ($P<0.01$) and the odds ratios for bile duct or gallbladder cancer with *H. bilis* in comparison with gallstone and/or cholecystitis were 6.50 (95%CI 1.09–38.63) in the Japanese and 5.86 (1.31–26.33) in the Thai patients. In conclusion, *H. bilis* infection in bile was associated with biliary tract and gallbladder cancers in two high risk populations, Japanese and Thai.

Key words: *Helicobacter bilis* — Bile — Biliary tract — Malignancy

Cancers in the extra-hepatic bile duct and gallbladder have a substantial incidence in Japanese and Thai populations.[1,2] A relationship between infection with the liver fluke, *Opisthorchis viverrini*, and bile duct cancer has been documented in Thailand.[1] However, this infection is not found in Japan; instead, exogenous N-nitroso compounds and endogenous nitrosation are suspected to play a role in carcinogenesis.[1]

Previously, Fox *et al.* used *Helicobacter*-specific 16S rRNA primers to identify the presence of *Helicobacter* species in 13 out of 23 bile samples and 9 out of 23 gallbladder tissues from Chilean patients with chronic cholecystitis.[3] The organisms could not be cultured, but the sequencing of 8 polymerase chain reaction (PCR)-amplicons revealed the presence of 5 strains of *Helicobacter bilis* (*H. bilis*), 2 strains of "*Flexispira rappini*" and one of *H. pullorum*.[3]

Enterohepatic *Helicobacter* species are bile-resistant[4] and in mice, *H. bilis* is gram-negative with a fusiform to slightly spiraled shape measuring 0.5 by 4–5 μm, with 3–14 sheathed flagella; it has been identified in the liver, bile and lower intestine.[5] *H. bilis* in bile causes chronic active hepatitis[3] and inflammatory bowel diseases in mice[6] and rats[7] and experimental infections with the organism have fulfilled Koch's postulates.

In this study, we analyzed whether *H. bilis* was present in human bile samples using PCR in bile samples from patients with biliary tract and gallbladder cancers, gallstone/cholecystitis patients and patients with non-biliary diseases, using *H. bilis*-specific PCR and sequencing.

MATERIALS AND METHODS

Patients Using sterile tubes, bile samples were obtained from Japanese patients (30 men and 15 women; ages, 24–83; mean age 65.2) who underwent a laparoscopic cholecystectomy or cholecystectomy (n=16), percutaneous transhepatic cholangiodrainage (PTCD) (n=20), or endoscopic retrograde cholangiopancreatography (ERCP) (n=9) in the First Department of Surgery, Nippon Medical School,

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Tokyo between 1999 and 2000. In Thailand, bile samples were obtained by sterile needle aspiration from 40 patients (16 men; ages, 22–78; mean age 54.4) who underwent a cholecystectomy (n=26) or pancreaticoduodenectomy (n=14) in the Department of Surgery, Faculty of Medicine, Chiang Mai University, Thailand in 1999. Antibiotics were not used for at least one week prior to the bile sampling in all patients. *Opisthorchis viverrini* infections were not detected in any of the Thai bile samples. All 45 Japanese and 40 Thai patients from whom bile samples were collected were analyzed and diagnosed by pathologists of each country. In Japan, 16 cases exhibited benign biliary diseases (gallstones [n=14], or chronic cholecystitis without gallstones [n=2]), and 15 cases exhibited malignant biliary diseases (common bile duct cancer [n=13] and peripapillary cancer [n=2]). Of the 8 cases with pancreatic diseases, 3 were benign (1 pancreas cyst and 2 acute pancreatitis) and 5 had pancreatic cancer. Six cases were non-biliary-pancreas diseases (perforation of a peptic ulcer [n=3] and gastric cancer [n=3]). In Thailand, 26 cases exhibited benign biliary diseases (gallstones [n=20] and chronic cholecystitis without gallstones [n=6]) and 14 exhibited malignant diseases (common bile duct cancer [n=12], gallbladder cancer [n=1] and peripapillary cancer [n=1]).

The study was approved by the institutional review boards of Nippon Medical School and permitted by Chiang Mai University. All bile samples were frozen at −70°C after collection, and the Thai samples were placed on dry ice and transferred to Tokyo by air.

DNA extraction and amplification of *H. bilis* DNA by PCR DNA was extracted from the bile samples as follows. The bile samples (100 μl) were placed in 1.5-ml tubes and centrifuged at 8000 rpm for 10 min; the pellet was suspended in Tris/HCl (pH 8.0) and 10 m*M* ethylenediaminetetraacetic acid (EDTA) buffer (400 μl each), and then 45 μl of 10% sodium dodecyl sulfate (SDS; pH 7.2), 50 μg of proteinase K, and 20 μg of RNAse were added. The tubes were incubated at 37°C for more than 3 h. Next, 450 μl of phenol-Tris buffer was added, and the tubes were centrifuged at 14 000 rpm for 5 min to obtain the supernatant. Four hundred and fifty microliters of phenol-chloroform was added, the tubes were centrifuged, and 450 μl of chloroform was added to the supernatant, followed by centrifugation. Thirty-five microliters of a solution of sodium acetate (3 *M*, pH 5.2) and 850 μl of 100% ethanol were added to the supernatant, then 500 μl of 80% ethanol was added and the mixture was centrifuged at 14 000 rpm for 5 min. The resulting pellet was dried and diluted with TE buffer (10 m*M* Tris, 1 m*M* EDTA).

A specific primer set with sequences that are identical to selected *H. bilis* 16S ribosomal RNA sequences (culture collection: ATCC51630, GenBank accession no.: U18766; ATCC51631, U18767; ATCC51632, U18768) was utilized. *H. bilis*-specific primer pairs HEBI-F1 and HEBI-R1 were used. The primers have the following sequences: HEBI-F1 5′-GGAAAGGGGCTTTCAATAAAG-3′ (forward), HEBI-R1 5′-GGCTGATCCTTTAGCGAAGG-3′ (reverse). Nested PCR was performed using the primer pairs, HEBI-F2 and HEBI-R2, which are located between HEBI-F1 and HEBI-R1. The primers have the following sequences: HEBI-F2 5′-GAATGAGAAATTGATGTTGTGAAG-3′ (forward), HEBI-R2 5′-TCTTTGGACGATAAATCGAT-3′ (reverse). All oligonucleotides were obtained by chemical synthesis using the PerSeptive 8900 (PE Biosystems, Foster City, CA). TaqMan probes, that had been modified with a fluorescent tag (purchased from Greiner Japan, Inc., Tokyo). Five microliters of the DNA preparation was added to a 50-μl reaction mixture containing 50 μM of each primer, 5 μl of 10× PCR buffer, 2.5 units of *Taq* polymerase in 0.2 μl of buffer, 200 μM each of the four deoxynucleotides, and 34.8 μl of ddH_2O. The preparations were denatured at 94°C for 1 min, annealed at 55°C for 2 min, and elongated at 72°C for 3 min (1 cycle), then denatured at 94°C for 45 s, annealed at 55°C for 1 min, and elongated at 72°C for 1 min (39 cycles), and finally incubated at 72°C for 10 min. The amplified products were analyzed by electrophoresis on a 1.2% agarose gel and stained with ethidium bromide. *H. bilis* genomic DNA (ATCC51630), kindly provided by one of the co-authors (J. G. Fox), was used as a positive control.

Sequencing of *H. bilis* DNA The 2nd PCR products of the *H. bilis*-specific DNA were purified using a "GFX" PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ). Direct sequencing was then performed using the specific primers and a Cycle Sequencing Core Kit (Perkin-Elmer, Chiba) with an automated DNA sequencer (ABI Prism TM377, Applied Biosystems, Foster City, CA). The sequencing procedure was performed at least twice on both the sense and anti-sense strands.

Specificity of PCR for *H. bilis* A PCR assay using the first primers (HEBI-F1 and R1), and the second primers (HEBI-F2 and R2) and 2% agarose gel electrophoresis with a positive control of *H. bilis* genomic DNA (ATCC 51630) were applied to the genomic DNA of the following 18 organisms: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Proteus mirabilis*, *Proteus vulgaris*, *Escherichia coli*, *Citrobacter freundii*, *Salmonella typhi*, *Salmonella enteritidis*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Enterobacter cloacae*, *Candida albicans*, *Bacillus subtilis*, *Campylobacter jejuni*, *Helicobacter mustelae*, *Helicobacter pylori* (*H. pylori*) NCTC11638 and, *H. pylori* clinical isolates: TK1003, TK1023 and TK1047.

DNA extraction and amplification of *H. pylori* DNA by PCR Seventy-eight of the 85 bile samples underwent

PCR analysis for *H. pylori*, and 7 bile samples were not tested because of an insufficient volume of bile. The PCR procedure was the same as that previously reported for the detection of *H. pylori* DNA in gastric juice[8] except for the DNA extraction. In this study, DNA was extracted using a "GeneReleaser" Kit (Bioventures, Inc., Murfreesboro, TN). After PCR amplification, the PCR amplicons were identified by agarose gel electrophoresis and Southern blot hybridization.

Statistics Statistical analyses were performed using the χ^2 test and the two-tailed Fisher's exact test with Yates' correction. A probability value of <0.05 was considered significant. We calculated the unadjusted odds ratios using the SPSS software system for the PC (SPSS 7.5J for Windows, SPSS, Inc., Tokyo).

RESULTS

***H. bilis* sequence analysis** PCR amplicons specific for *H. bilis* were identified using agarose gel electrophoresis and an *H. bilis*-positive control (ATCC51630). A DNA fragment of 99 base pairs was identified in the bile samples and the *H. bilis*-positive control using primer pairs HEBI-F2 and HEBI-R2 (Fig. 1). Six *H. bilis* PCR-positive samples and an *H. bilis*-positive control were directly sequenced from the PCR-amplicons to verify the identity of *H. bilis*. All 6 amplicons and the positive control exhibited the identical *H. bilis*-specific sequence (CAA TTT-GTGCGGA GACTAGACTT AGTGTCTGTC GCACAA-GCAA ATTGCGAACT CATCGAT). Homology research comparing the identified sequence with the GenBank nucleic acid sequence database produced a high score of 149 bits for *H. bilis* U18766, U18767, AF054570, AF054571 and AF054572, followed by 141–101 bits for *H. bilis* U51873, U18768, AF252626 and other *Helicobacter* species (AF047851, AF072333, AJ011431, AF225546). Homology with all other known organisms was lower than 48 bits.

Specificity of PCR for *H. bilis* Nested PCR assay using the first primers (HEB1-F1 and R-1), and the second primers (HEB1-F2 and R2) and gel electrophoresis produced negative results for the genomic DNA of the 18 organisms representing normal flora or pathogens of the human gastrointestinal tract.

***H. bilis* DNA in bile by PCR** Thirteen out of 15 (87%) of the Japanese patients and 11 out of 14 (79%) of Thai patients with bile duct or gallbladder cancer tested positive for *H. bilis* in their bile (Table I). Eight out of 16 (50%) of the Japanese patients and 10 out of 26 (38%) of the Thai patients with gallstones and/or cholecystitis tested positive for *H. bilis*. In the Japanese patients, only 4/14 (29%) of those with non-biliary diseases tested positive for *H. bilis* (2/8 [25%] with pancreatic cancer and pancreatitis, and 2/6 [33%] of the patients with non-biliary-pancreatic diseases). Bile duct and gallbladder cancer showed significantly higher positive rates for *H. bilis* than did the non-biliary diseases in Japanese (13/15 vs. 4/14, $P<0.01$) and than did the benign biliary diseases in Thais (11/14 vs. 10/26, $P<0.05$). The odds ratios for bile duct or gallbladder cancer with *H. bilis* in comparison with gallstones and/or

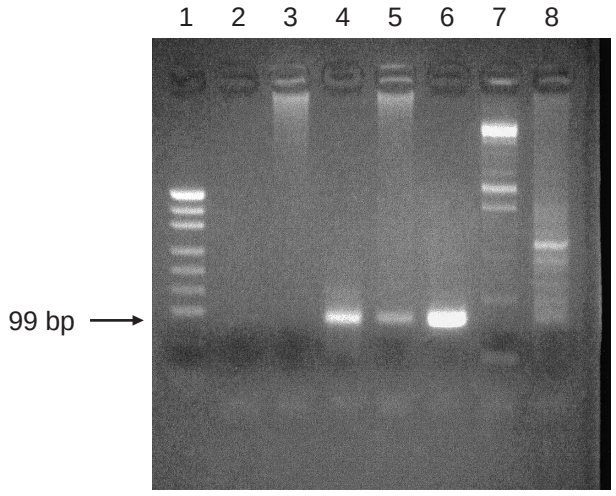

Fig. 1. Nested PCR specific for *H. bilis* and agarose gel electrophoresis. Marker (lane 1), *H. pylori*-positive saliva (lane 2), *H. bilis*-negative bile sample (lane 3), *H. bilis*-positive bile sample (lane 4), *H. bilis*-positive bile sample (lane 5), *H. bilis*-positive control (ATCC51630) (lane 6), *H. pylori*-positive control (lane 7), and *H. pylori*-positive control (lane 8).

Table I. Identification of *Helicobacter bilis* in Bile Samples from Japanese and Thai Patients with Benign and Malignant Diseases of the Biliary Tract or Non-biliary Diseases

Population	Diseases	Positive *H. bilis* PCR (Positive/total number) (%)	Odds ratio (95%CI)
Japanese	Non-biliary diseases	4/14 (29)	
	Gallstone and/or cholecystitis	8/16 (50)	1.0
	Bile duct or gallbladder cancer	13/15 (87)[a]	6.50 (1.09–38.63)
Thai	Gallstone and/or cholecystitis	10/26 (38)	1.0
	Bile duct or gallbladder cancer	11/14 (79)[b]	5.86 (1.31–26.33)

a) The two-sided P value is <0.01 for non-biliary diseases by Fisher's exact test.
b) The two-sided P value is <0.05 for gallstone and/or cholecystitis by Fisher's exact test.

cholecystitis were 6.50 (95%CI 1.09−38.63) in Japanese patients and 5.86 (1.31−26.33) in Thai patients. Infection with *H. bilis* resulted in an increased risk of cancer in the biliary tract and gallbladder at an odds ratio of 6.40 (95%CI 2.05−20.03, $P=0.001$) compared to that of benign biliary diseases using combined data from Japan and Thailand (82.8% for malignancy vs. 42.9% for benign).

***H. pylori* DNA in bile by PCR** The results of the 78 bile samples examined for *H. pylori* using PCR and *H. pylori*-specific primer pairs showed all but one sample was negative for *H. pylori* (Table II). The *H. pylori*-positive sample was an *H. bilis*-negative bile sample from a Japanese gallstone patient and none of the bile samples showed co-infection with *H. bilis* and *H. pylori*.

DISCUSSION

Helicobacter species are known to colonize the gastrointestinal tract of humans and animals.[4] The presence of *H. pylori* in the stomach is considered the most important etiological factor in the formation of peptic ulcers and gastric malignancies in humans.[9] Two classes of *Helicobacter* species have been identified, i.e. gastric and enterohepatic *Helicobacter* spp. *H. pylori* belongs to the gastric class, and *H. bilis* is an enterohepatic *Helicobacter* sp.[4] *H. bilis* was originally identified in the diseased livers and intestines of aged inbred mice.[5] Recently, *H. bilis* has been isolated from the stomach of dogs and gerbils, the cecal contents of gerbils, and the feces of cats.[4] In animals, *H. hepaticus*, *H. bilis* and *H. canis* have been associated with biliary tract and hepatic inflammation, and *H. hepaticus* infection is known to induce multifocal necrotic hepatitis and markedly increased risk of hepatic carcinoma in A/JCr mice.[4, 10]

In human, *H. bilis* was first identified in the bile and gallbladder tissue in Chilean patients with chronic cholecystitis. Fox and co-workers[3] identified the presence of *H. bilis* by PCR and sequencing of DNA from bile and gallbladder tissue of Chilean patients with cholecystitis; however, cultures of *H. bilis* were unsuccessful. Thus, the role of bile-resistant *Helicobacter* spp. in the pathogenesis of biliary tract cancer has become a topic of great interest to bacteriologists and gastroenterologists.[11] Here, we report the first PCR evidence of *H. bilis* in bile samples from humans with malignant diseases of the biliary tract and gallbladder.

The aim of this study was to determine the incidence of *H. bilis* DNA in bile samples of patients from countries with high incidences of hepatobiliary malignancy and to clarify the frequencies of *H. bilis* positivity in patients with malignant and benign biliary diseases or non-biliary diseases. Our results indicate that 38−50% of bile samples from patients with benign diseases and 79−87% of samples from patients with malignant diseases of the biliary tract and gallbladder were positive for *H. bilis* DNA in both Japanese and Thai patients. The odds ratios for bile duct or gallbladder cancer with *H. bilis* in comparison with gallstone and/or cholecystitis were 6.50 (95%CI 1.09−38.63) for Japanese and 5.86 (1.31−26.33) for Thai patients. Only 29% of patients with non-biliary diseases exhibited *H. bilis* positivity in Japanese, and the presence of *H. bilis* in the bile increased the risk of bile duct or gallbladder cancer.

Some authors have detected *H. pylori* in the bile using PCR and have suggested a possible association with cholecystitis.[12, 13] However, we tested the bile samples for *H. pylori* DNA and found only one bile sample (1.3%) that was *H. pylori*-positive. Since bile inhibits the growth of *H. pylori in vitro*[14] and *H. pylori* has difficulty surviving in bile *in vivo*,[15] our results seem reasonable. Our data suggest that *H. pylori* infection is not a risk factor for biliary malignancy. A previous study by Fox and colleagues[3] showed that 5/8 (63%) amplicons detected by PCR in human bile were identified by sequencing as *H. bilis*; two other strains were identified as '*Flexispira rappini*,' and one strain was identified as *H. pullorum*. We did not examine for the presence of other *Helicobacter* species in this study because *H. bilis* is thought to be the most prevalent *Helicobacter* species in bile of multiple animal hosts, including human.[3, 4, 6, 7]

One of the limitations of this study was the fact that *H. bilis* cultures were not obtained. Further study by bacteriologists is necessary to determine the optimal conditions for growing *H. bilis in vitro*. However, we believe that the positive *H. bilis* results in this study are reliable because (1) we used an authorized positive control for *H. bilis* genomic DNA that was extracted from *H. bilis* strain ATCC51630, (2) DNA direct sequencing was used to examine 6 amplicons and these amplicons showed the highest homology with *H. bilis*, and (3) 18 species of bacteria known to colonize the human gastrointestinal tract produced negative PCR results.

Rudi *et al.*[16] reported that *Helicobacter* species were not detected by PCR in bile from German patients with

Table II. No Co-infection of *Helicobacter bilis* and *Helicobacter pylori* in Bile from Japanese and Thai Patients with Malignant and Benign Diseases of the Biliary Tract or Non-biliary Diseases

H. bilis and *H. pylori*	Number of patients (%)
H. bilis− & *H. pylori*−	29 (40.8)
H. bilis− & *H. pylori*+	1 (1.4)
H. bilis+ & *H. pylori*−	41 (57.8)
H. bilis+ & *H. pylori*+	0 (0)
Total	71 (100)

− negative, + positive.

biliary diseases. Germany has a low incidence of bile duct and gallbladder cancer, so they assumed that the discrepancy between their results and those of Fox *et al.*[3] could be explained by regional differences in the distribution of bile-resistant *Helicobacter* species. There is a possibility that geographic regional differences in biliary malignancy between low-risk areas, like Germany, and high-risk areas, like Japan and Thailand,[2] may account for the absence and presence of *H. bilis*, respectively. Indeed, the first report identifying the presence of *H. bilis* in bile[3] was made in Chile, where the incidence and mortality rate of biliary tract malignancies are the highest in the world. As Fox and colleagues pointed out,[3] a difference of approximately 30 times between high-risk populations and low-risk populations suggests the presence of strong environmental factors and/or host-environmental interactions in biliary tract carcinogenesis.

Blaser explained the findings of *Helicobacter* DNA in the biliary tract of patients with cholecystitis in terms of several possible hypotheses[11]; (1) the finding may be artificial, but he considered this unlikely, (2) *Helicobacter* species may represent normal biota (flora) of the biliary tract of humans and have no obvious pathogenetic role, (3) *Helicobacter* species may represent normal bowel biota that secondarily colonize damaged tissue, (4) *Helicobacter* species may represent transient bowel organisms that secondarily colonize previously injured tissue in a manner analogous to the role of *Salmonella typhi* in the higher risk of biliary tract cancers in typhoid carriers, (5) the *Helicobacter* species may be normal inhabitants of the biliary tract but contribute to increased risk of disease, analogous to the increased risk of ulcer disease with *H. pylori* colonization of the stomach and (6) the *Helicobacter* species may be newly introduced high-grade pathogens that play a strong role in disease pathogenesis, analogous to the role of *Mycobacterium tuberculosis* in causing chronic inflammatory pulmonary disease. Combined infections of *H. bilis* and other bacteria known to colonize the human gastrointestinal tract are presumably possible in the biliary tract.

The mechanism of biliary tract carcinogenesis by *H. bilis* infection is unclear.[11] Experimentally, *H. bilis* induced typhlitis, colitis,[6, 7] and hepatitis[5] in mice. Biochemically, *H. bilis* is oxidase, urease and catalase-positive, reduces nitrate to nitrite and is resistant to nalidixic acid and cephalothin.[5] Chronic inflammation by *H. bilis* infection along with tissue injury from the bile may be at least partly responsible for human biliary tract carcinogenesis. A relation between *Opisthorchis viverrini* infection and cholangiocarcinoma has been reported in Thailand,[1] but not in Japan. Gallstones are also known to increase the risk of gallbladder cancer. No evidence of a causal relationship between *H. bilis* and biliary tract malignancy was presented in this study, but *H. bilis* may have a pathogenic role in biliary malignancy.

In conclusion, we found *H. bilis* at a significantly higher incidence in bile from biliary tract and gallbladder cancer patients than in patients with gallstones/cholecystitis or pancreatic diseases using *H. bilis*-specific PCR and sequencing. We postulate that *H. bilis* infection in the bile may affect the risk of cancer in the bile duct and gallbladder.

ACKNOWLEDGMENTS

The authors would like to thank staff of the First Department of Surgery, Nippon Medical School Hospital, Tokyo and the Department of Surgery, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand for their help and for collecting samples. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare, Japan, and grants CA 26731 and CA 67529 from the National Cancer Institute (JGF), USA.

(Received March 4, 2002/Revised April 18, 2002/Accepted April 25, 2002)

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