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Detection of Helicobacter bilis in Bile from Thai Patients with Adenocarcinoma of Biliary Tract

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Abstract

Objectives: **There is a high incidence of the biliary tract carcinoma in the Northern part of Thailand. Almost all of them are in advanced stage. Some data support an association of bile resistant bacteria, namely *Helicobacter bilis* which may play a causative role in the development of biliary tract carcinoma. Previous study had shown that animal infected with this bacteria developed malignant liver tumor. This study was designed to search for the *Helicobacter bilis* in bile that may play a causative role for malignancy of biliary tract.**

Patients and Methods: **From July to December 1999, bile samples were collected from 40 Thai patients with benign and malignant diseases of biliary tract who underwent surgical and radiological intervention in the Faculty of Medicine, Chiang Mai University. Collection of bile sample with sterile technique was obtained by sterile needle aspiration immediately after definitive procedures were performed for treatment. Patients who had antibiotics within 7 days before bile collection were excluded. Diagnostic evaluation, surgical procedures, and pathologic examination were reviewed. The specific part of DNA sequence of *Helicobacter bilis* were identified by PCR analysis and DNA sequencing in six random positive cases.**

Results: **Twenty six cases had benign diseases and 14 cases had malignant diseases. There were positive results by PCR analysis and DNA sequencing in 79 per cent (11/14) of malignancy cases and in 38 per cent (10/26) of benign cases. The positive rates of infection between malignancy and benign groups were statistically significant differences ($p < 0.05$). Identification of positive *Helicobacter bilis* was statistically significant compared with negative *Helicobacter bilis* in the malignant biliary tract diseases at an odds ratio of 2.04 (95%CI 1.17-3.57).**

Conclusion: ***Helicobacter bilis*** **was found in human biliary tract malignancies of Thai patients. This bile resistant bacteria may be a risk factor in the pathogenesis of biliary tract malignancies.**

The liver cancer incidence was reported as high as 26.0 per 100,000 population in Thailand. Khon Kaen, in the Northeastern Thailand, has the highest incidence ratio of the bile duct cancer to primary liver cancer in the world in which more than 60 per cent were bile duct cancer.[1] Chiang Mai, in Northern Thailand also has high liver cancer incidence, that is 15.0 per 100,000, and 38.2 per cent of patients had bile duct cancer.[1] Almost all of them were in advanced stage.

Previous studies suspected that the risk of bile duct cancer might be related with exogenous N-nitroso compounds and endogenous nitrosation together with parasitic infection or other environmental factors.[2-6] Mass community infection with liver fluke, *Opisthorchis viverrini*, was reported as a suspected cause of bile duct cancer by a number of epidemiologic studies in Northeastern Thailand.[1,3,7] However, in animal study, this parasite alone has not yet been proven to cause bile duct cancer without chemical carcinogen or nitrosamine substance.

The mass treatment with Praziquantel in Northeastern Thailand has cause the prevalence of liver fluke to decline from nearly 80 per cent in 1984 to approximately 20 per cent in 1994.[7]

Tumor suppressor genes, for example p53 mutation were found in 33-35 per cent of reported cases.[1,8] Another suspected carcinogenesis of bile duct cancer was linked to Helicobacter bacteria. Some species such as *H. hepaticus* and *H. bilis* were known to induce liver tumor in A/JCr mice.[9] *H. bilis* was first identified in bile and gall bladder tissues from Chilean with chronic cholecystitis. Cultures of these organisms from human gallbladder tissue were unsuccessful. However, 5/8 *Helicobacter*- specific PCR amplicones represented *H. bilis*. Two amplicones were strains of *Flexispira rappini*, and one amplicone was a strain of *H. pullorum*.[10] In contrast, one study showed that no detectable *Helicobacter* bacteria in German patients with biliary tract diseases.[11] Germany is a country which has low incidence of gallbladder and bile duct cancer.

Thus, we suspect that *H. bilis* may be responsible for the risk of bile duct carcinoma occurrence in Thai patients who are in the endemic area. In this study, we aimed to search for *H. bilis* bacteria by *Helicobacter bilis*- specific PCR in bile and by direct DNA sequencing.

Patients and Methods

Population

Bile samples were collected from 40 Thai patients who were treated for benign and malignant biliary tract diseases from July to December 1999 in the Department of Surgery, Faculty of Medicine, Chiang Mai University. All patients' medical records were reviewed for age, sex, blood and bile culture, parasitic infection in bile, stool concentrated examination, surgical procedures, and surgical pathology. Patients who had antibiotics within 7 day before bile collection were excluded.

Three bile samples of 1.5 milliliters each, were obtained by sterile needle aspiration during operations or radiological intervention for treatment. All of collected samples were frozen at -70 ˚C, and transferred with dry ice by air to Diagnostic Division, Gene Diagnostic Research, Otsuka Assay Laboratories, Tokushima, Japan in May 2000. The laboratory technician was blinded from identification data of patients.

Helicobacter bilis DNA extraction

H. bilis DNA was extracted from each of 500 microliters bile samples with conventional method as previously described. The DNA was added to 0.1 ml of TE buffer [10mM Tris-HCl (pH 7.5), 1 mM EDTA], and the tube was placed on a rocking platform at 4˚C until the DNA had completely dissolved.

Oligonucleotide primer

The specific primer set of which homology sequences were identical with *H. bilis*, were used in the Polymerase Chain Reaction (PCR) for the amplification of the 16S ribosomal RNA in this study. The primer sequences for the region were:

HERB1-F1 (sense primer for the first PCR);
5'-GGAAAGGGGCTTTCAATAAAG-3'
HBE1-F2 (sense primer for the second PCR);
5'-GAATGAGAAATTGATGTTGTGAAG-3'
HEBI-R2 (antisense primer for the second PCR);
5'-TCTTTGGACGATAAATCGAT-3'
And HEB-R1 (antisense primer for the first PCR);
5'-GGCTGATCCTTTAGCGAAGG-3'

All oligonucleotide were obtained by chemical synthesis using the (Perseptive 8900; PE Biosystem, CA, U.S.A.) TaqMan probe, which was modified with

fluorescent, purchased from GREINER JAPAN, Inc. (Tokyo, Japan).

PCR for H. bilis detection in bile

All single and nested PCR reactions were performed in a DNA thermal cycler (Lobocycler 40, Stratagene; CA, USA, Nippon Genetics; Tokyo, Japan) for over 39 cycles with 0.5 micromilliliters primers and a Gene AmpTM Kit (Perkin-Elmer; New Jersey, USA.) under the conditions recommended by the kit supplier.

In brief, this consisted of a total volume of 50 microliters of reaction mixture containing 5 micromilliliters of extracted DNA solution, 10 mM Tris-HCl(pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% w/v gelatin, each 50 pmol of primer sets, 1.25 mM of each of the deoxynucleotides and 2.5 units of Taq DNA polymerase. Each PCR cycle consisted of denaturation at 94 ˚C for 1 minute, annealing at 60 ˚C for 1 minute, and extension at 72 ˚C for 1 minute.

Direct DNA Sequencing of H.bilis

Random PCR products of 6 amplicones which were positive for *H. bilis* were electrophoresed in 3% agarose gel and were recovered with acid-alkaline method using GFX column (Pharmacia Biotech) as a protocol described previously.[12] The sequencing reactions were performed using a Big Dye Terminal cycle sequencing kit (PE Applied Biosystem, Foster City, USA) under the conditions recommended by the kit supplier, with the protocol based on the Sanger dideoxymediated chain- termination method using Ampli Taq DNA polymerase. The recovered fragment was subjected to a template of direct sequencing, and the fragment was cycling with 5 pmol of HEB1-F2 primer and reaction mixture. The reactions were performed in a DNA thermal cycler (Lobocycler 40, Stratagene; CA, USA, Nippon Genetics; Tokyo Japan) for over 34 cycles with a total volume of 50 micromilliliters of reaction mixture. Each PCR cycle consisted of denaturation at 94 ˚C for 45 seconds, annealing at 53 ˚C for 1 minute, and extension at 72 ˚C for 1 minute.

PCR for H. pylori detection in bile

PCR procedure was the same as previously reported for detection of *H. pylori* DNA in the gastric juice.[13] All amplicones were identified by agarose gel electrophoresis and Southern blot hybridization.

Statistical analysis

Statistical analysis by qui-square was employed. A probability value of < 0.05 was considered significant. (SPSS version 9.0,on a PC, Japan)

Results

All 40 patients from whom the bile samples were collected comprised of 24 females and 16 males. There were benign biliary tract diseases in 26 patients and malignant biliary tract diseases in 14 patients. Summary of patient data were described in Table1. The results of blood and bile culture, parasitic infection in bile, and stool concentrated examination for parasite were reported as negative in all patients. Eight malignancy cases had elevated CA19-9 level. In the 26 benign biliary tract cases, 20 patients were diagnosed as calculus chronic cholecystitis with or without biliary tract stone, and 6 patients were diagnosed as acalculus chronic cholecystitis. All of the patients who had malignant biliary tract disease in this study had extrahepatic cholangiocarcinoma, except for one patient who had gallbladder cancer and the other one who had intrahepatic cholangiocarcinoma.

PCR results for H.bilis detection in bile

PCR amplicones specific for *H. bilis* 16S rRNA were identified in agarose gel electrophoresis with positive control (No. ATCC51630). DNA fragment of specific 99 base pairs was found in some of the bile samples and *H. bilis* positive control, but *H. pylori* DNA or common bacterial DNA did not appear by these PCR results with specific primer sets in all patients' bile samples (Figure 1).

H. bilis DNA was identified in 10 of 26 (38%) patients who had benign biliary tract diseases and in 11 of 14 (79%) patients who had malignat biliary tract diseases. Identification of positive *H. bilis* in patients with malignant biliary tract diseases was statistically significant when compared with negative *H. bilis* at an odds ratio of 2.04 (95%CI 1.17-3.57). There was also significant difference in positive rate of infection between malignancy and benign groups ($P< 0.05$) with the odds ratio of 5.86 (95% CI 1.31-26.33)

Direct DNA Sequencing results of H.bilis

Six of *H. bilis* PCR-positive amplicones and *H. bilis* positive control were directly sequenced with PCR

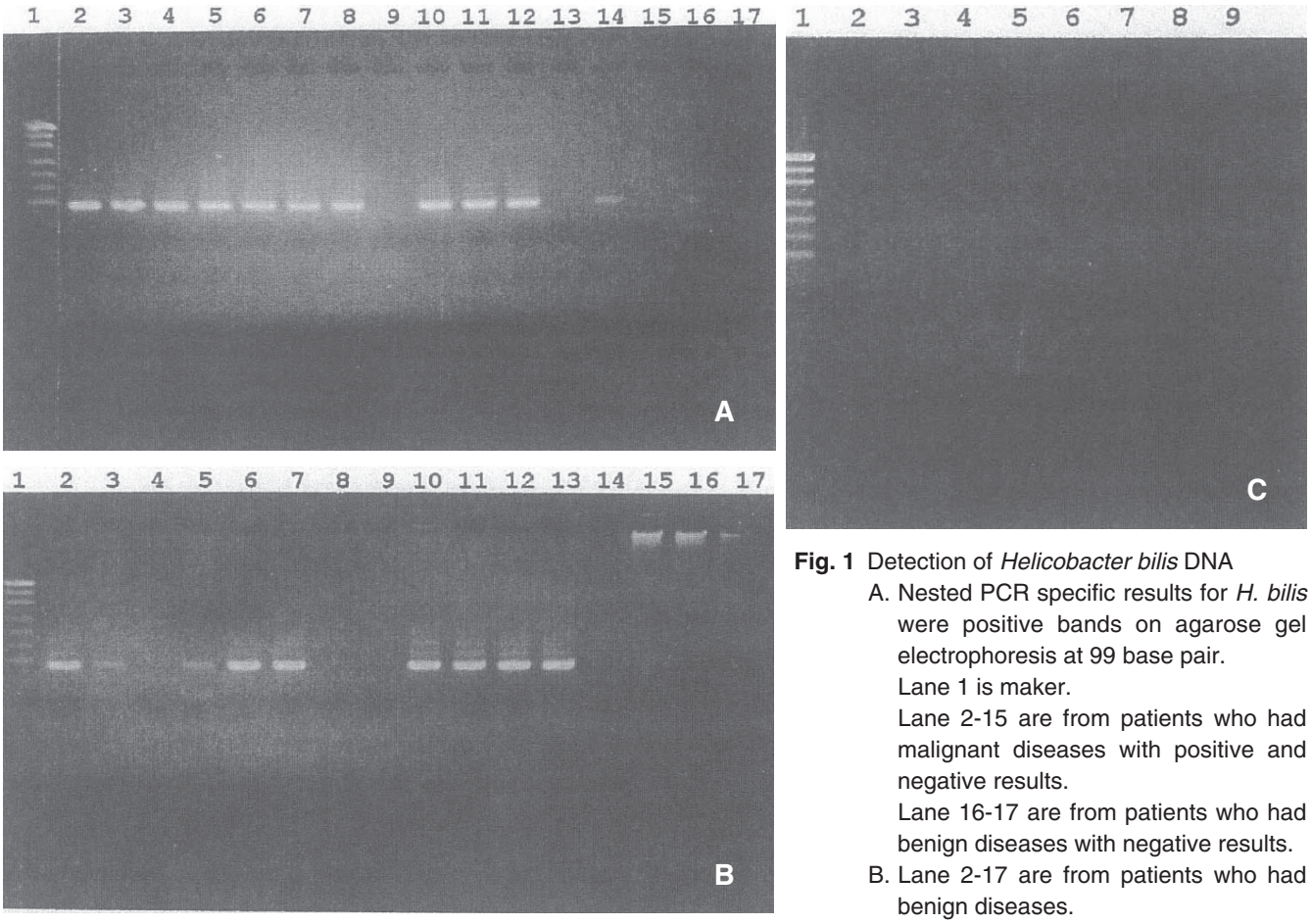

Fig. 1 Detection of *Helicobacter bilis* DNA
A. Nested PCR specific results for *H. bilis* were positive bands on agarose gel electrophoresis at 99 base pair.
Lane 1 is maker.
Lane 2-15 are from patients who had malignant diseases with positive and negative results.
Lane 16-17 are from patients who had benign diseases with negative results.
B. Lane 2-17 are from patients who had benign diseases.
Lane 3-5 have not clear positive band.
C. Lane 2-9 are all negative result from patients who had benign diseases.

primer, HEBI-2F and HEBI-2R. They all showed identical *H. bilis* specific sequence, CAATTTGT GCGGAGACTAGACTTAGTGTCTGTCGCACAAGCA AATTGCGAACT.

PCR results for H. pylori detection in bile

All bile samples were examined by PCR to detect H.pylori with a specific primer set. There was no positive result for *H. pylori* in all Thai patients' bile samples.

Discussion

This is the first report on relationship between bile resistant *Helicobacter spp.* bacteria *H. bilis* and the risk of biliary tract cancer in Thai patients. In this study, the sequence of forward primer's position on 16S rRNA of *H. bilis* was not identical to those with *H. pylori* sequence or other gram negative bacteria such as E. coli, Proteus mirabilis, K. pneumoniae, and etc., although it was close to general sequence of other animals and plants. One of the limitations in our report is the fact that *H. bilis* culture was not performed in these patients. Culture of this bacteria was successfully performed in liver tissue culture from mice in 1995 under microaerophilic conditions.[9] We believe in the reliability of genetic tests with positive control of *H. bilis* genomic DNA that was extracted from *H. bilis* strain number ATCC51630 in Gene Bank.

However, the mechanism of biliary tract carcinogenesis by *H. bilis* is unclear. There was no evidence that could suggest a causal relationship between *H. bilis* and the development of biliary tract carcinoma in this study. We suspect that chronic inflammation caused by strong urease activity and reduction of nitrate to nitrite by *H. bilis* infection may be a predisposing factor for biliary tract carcinoma. Evidence of parasitic infection or liver fluke infection was not

Table 1 Demographic and clinical characteristics of patients.

Characteristics	Cytology and/or Surgical Pathology	
	Benign	Malignancy
Number of Patients	26 (65%)	14 (35%)
Age (year), mean	51	60.5
Sex (M/F)	8/18(1:2.3)	8/6 (1.3:1)
Jaundice	6	13
CA19-9 (> 37 u/ml)	2	8
Procedure		
Laparoscopic Cholecystectomy (LC)	19	-
Open Cholecystectomy(OC) with or without CBD exploration	7	-
Exploratory Laparotomy with definite procedure for malignant diseases	-	4
Radiologic intervention (PTBD)	-	10

**PTBD = Percutaneous Transhepatic Biliary Drainage*

demonstrated in all of our patients and no one had previous history of treatment with Praziquantel or other antiparasite drugs. For the reason of bacterial overgrowth, there was no significant difference of *H. bilis* positive rate between benign biliary tract disease with and without biliary tract obstruction.

In conclusion, using *H. bilis*-specific PCR and direct sequencing method, there was a significant higher incidence of *H.bilis* infection in bile from patients with biliary tract and gall bladder cancer than bile from patients with benign biliary tract diseases. With this statistical data, we postulate that *H. bilis* infection in the bile may affect the risk of cancer in bile duct and gallbladder.

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