Comparative analysis of *Helicobacter* DNAs and biliary pathology in patients with and without hepatobiliary cancer

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Several *Helicobacter* species have recently been isolated from the bile and hepatobiliary systems of murine species, and are well recognized as a pathogen of the hepatobiliary disorder. This study was planned to investigate whether *Helicobacter* species possess a causative potential for human hepatobiliary disease, especially for hepatobiliary carcinogenesis. Bile and hepatobiliary tissue samples from 19 patients with hepatobiliary cancer and 19 patients with benign biliary diseases were subjected to polymerase chain reaction analyses for the detection of *Helicobacter* DNAs. Using a proliferating cell nuclear antigen (PCNA) staining technique, we also investigated the biliary epithelial cell kinetics with special reference to the presence of *Helicobacter* DNAs in the hepatobiliary system. We found that *Helicobacter* DNAs were positive in 10 (52.6%) of the 19 patients with hepatobiliary cancer. The incidence was significantly higher than that (15.7%) in the benign cases ($P = 0.03$). The PCNA labeling index in the biliary epithelium in *Helicobacter* DNA-positive patients was statistically higher than that in *Helicobacter* DNA-negative ones, regardless of whether the patient was suffering from hepatobiliary cancer and/or biliary inflammation. A close correlation between the presence of *Helicobacter* DNAs and an elevation of the PCNA labeling index in the biliary epithelium was demonstrated by multiple regression analysis. Our findings suggest that *Helicobacter* species may play a role in the pathogenesis of hepatobiliary cancer through an acceleration of biliary cell kinetics.

**Introduction**

*Helicobacter pylori* (*H.pylori*), which was initially isolated from gastric tissue (1), has been well recognized as a causative factor in the pathogenesis of chronic gastritis (2) and peptic ulcer (3,4). Furthermore, epidemiological analyses (5,6) and experimental studies (7,8) have revealed that *H.pylori* is linked to gastric carcinogenesis, and a Working Group of the World Health Organization International Agency for Research on Cancer concluded that *H.pylori* is a group 1 carcinogen in humans (9).

In recent years, *Helicobacter* DNAs have been detected in human bile and hepatobiliary tissue by polymerase chain reaction (PCR) assays (10–13). *Helicobacter* DNAs were found in bile and gallbladder tissue from Chileans with chronic cholecystitis, and they were identified as known *Helicobacter* organisms, including *Helicobacter bilis* (*H.bilis*), by phylogenetic analyses (14). *Helicobacter* DNAs were also detected in the liver specimens from patients with primary liver cancer (15). However, the pathogenicity of *Helicobacter* species in hepatobiliary carcinogenesis has not been fully elucidated.

The aim of this study was to use a molecular approach and immunohistological technique to investigate whether *Helicobacter* species have a pathological potential in hepatobiliary carcinogenesis.

**Materials and methods**

**Patients**

Between April 2000 and March 2001, 17 bile samples and 16 hepatobiliary tissue specimens were obtained from 19 Japanese patients who underwent surgical resection for hepatobiliary cancer; eight samples were from patients with extrahepatic bile duct cancer, five were from patients with intrahepatic bile duct cancer, three were from patients with gallbladder cancer, and three were from patients with cancer of the papilla of Vater. The patients ranged in age from 35 to 84 years, with a mean of 63.1 years. There were seven men and 12 women. As controls, bile and tissue samples were obtained from 19 Japanese patients without cancer; 15 of these patients had cholecystolithiasis, two had adenomyomatosis of the gallbladder, and two had gallbladder polyps. The mean age of this group was 62.7 years, with ages ranging from 32 to 91 years. Of these, eight were men and 11 were women. All the patients consented to the genetic evaluation of the collected samples.

**PCR analysis of *Helicobacter* DNAs**

**DNA extraction**

DNA was extracted from each bile sample and hepatobiliary tissues with a DNA extraction kit (ISOTISSUE, Nippon Gene, Tokyo, Japan). Approximately 50 µg of tissue sample obtained from the non-cancerous tissue was homogenized with a metallic pestle and then processed as outlined by the manufacturer. DNA pellets were dissolved in 30 µl of distilled water. Each bile sample (~1.5 ml) was pelleted by centrifugation for 10 min at 12 000 g and treated in the same way as the hepatobiliary tissue.

**PCR amplification**

All reactions were carried out in a reaction mixture (25 µl) containing 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 200 mM dNTP, 1.0 unit of Taq polymerase (Takara Shuzo, Tokyo, Japan), 100 pmol of each primer, and 1 µl of template DNA.

Four sets of primers were used to detect *Helicobacter* DNAs: the C97/C98 set of primers are specific to the 16S rRNA of *Helicobacter genera* (14), the HPUI/HPU2 set of primers are specific to the *H.pylori* urease A gene (16), the C62/C12 set of primers are specific to the 16S rRNA of *H.bilis* (17), and the B38/B39 set of primers are specific to the 16S rRNA of *Helicobacter hepaticus* (*H.hepaticus*) (18). Table I shows the nucleotide sequences of four primers, the PCR conditions, and the size of the amplified fragments.

Ten microliters of the PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light (Figure 1). All reactions were repeated more than twice, and the repeated reactions had the same results. As positive controls, the DNA extracted from *H.pylori* (ATCC 43504), *H.bilis* (ATCC 51630), and *H.hepaticus* (ATCC 51448) were subjected to PCR assays.

**Sequence analyses of PCR products**

The PCR products were gel-purified by QiaEX Gel II Extraction kit (Qiagen, Courtaboeuf, France) and analyzed by direct sequencing using an ABI 377 sequencer. Sequence comparison was performed using the Blast program (Genetics Computer Group, Madison, WI) and the GeneBank databases.
Table I. Oligonucleotide primers used for Helicobacter-specific PCR assays

<table>
<thead>
<tr>
<th>Primers</th>
<th>Specific for:</th>
<th>Sequence (5' to 3')</th>
<th>Genes</th>
<th>Cycles</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C97</td>
<td>H. genus</td>
<td>GCTATGACGGTGATCC</td>
<td>16S rRNA</td>
<td>94°(1'), 55°(0',30), 72°(1')</td>
<td>400 (14)</td>
<td></td>
</tr>
<tr>
<td>C98</td>
<td></td>
<td>GATTITACCCCTACCCA</td>
<td></td>
<td>40 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPu1</td>
<td>H. pylori</td>
<td>GCCATGTTAAATTATTGT</td>
<td>Urease A</td>
<td>94°(1'), 50°(0',30), 72°(1')</td>
<td>411 (16)</td>
<td></td>
</tr>
<tr>
<td>HPu2</td>
<td></td>
<td>CTTCTAATGTGTTCACAC</td>
<td></td>
<td>40 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C62</td>
<td>H. bilis</td>
<td>AGAACTGCTATTGAAACTCTTT</td>
<td>16S rRNA</td>
<td>94°(1'), 56°(0',30), 72°(1')</td>
<td>638 (17)</td>
<td></td>
</tr>
<tr>
<td>C12</td>
<td></td>
<td>GTTATGTGCTCTTTTGTGTT</td>
<td></td>
<td>40 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B38</td>
<td>H. hepaticus</td>
<td>CGAUAUGGAAACUGUAACUCUG</td>
<td>16S rRNA</td>
<td>94°(1'), 61°(0',30), 72°(1')</td>
<td>417 (18)</td>
<td></td>
</tr>
<tr>
<td>B39</td>
<td></td>
<td>GGGGACGUGUAAAACAG</td>
<td></td>
<td>40 cycles</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

bp: Base pairs; rRNA: ribosome RNA.

Fig. 1. Electrophoresis of DNA amplified by PCR using the C62/C12 set of primers listed in Table I. Lanes: 1, DNA extracted from cultures of H. bilis (ATCC 51630); 2–7, DNA extracted from hepatobiliary tissue of the patients with cancer; 8, negative control.

Immunohistopathological detection of Helicobacter species

Tissue samples were routinely fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE) and Giemsa. Immunohistochemical staining for H. pylori was also performed by using the indirect immunoperoxidase method. Tissue sections were cut at 4 μm, mounted on glass slides coated with 5-aminoprophyltriethoxy saline, and dewaxed in xylene. The sections were treated with microwave heating for 5 min in phosphate-buffered saline (PBS) at 500 W. After the blocking of endogenous peroxidase, the sections were incubated with rabbit anti-H. pylori polyclonal antibody (Dako, Kyoto, Japan) at a dilution of 1:50 for 2 h at room temperature, and they were then prepared for biotin-labeled secondary antibody (DAKO, Kyoto, Japan) for 1 h at room temperature. A peroxidase-labeled avidin-biotin complex with diaminobenzidine (Djin Chemical Co., Kumamoto, Japan) was used as a substrate.

Biliary inflammation and cell kinetic studies

For the evaluation of biliary inflammation and biliary epithelial cell kinetics, we prepared non-cancerous parts discrete by 2 cm from the margin of cancer. The degree of biliary inflammation was scored in accordance with the infiltration of neutrophils on HE staining, i.e. grade 0: no infiltration; grade 1: mild infiltration of neutrophils; grade 2: severe infiltration of neutrophils; and grade 3: abscess formation in the hepatobiliary system.

Proliferating cell nuclear antigen (PCNA) was used as a marker of the biliary epithelial cell kinetics. Tissue sections were treated in the same way as the immunohistochemical staining of H. pylori and were incubated with mouse monoclonal antibodies against PCNA (clone-PC 10; DAKO, Kyoto, Japan) at a dilution of 1:100. To determine the PCNA labeling index (PCNA-LI), PCNA-labeled and unlabeled cells were counted to a minimum of 1000 serial cells in the biliary epithelium. The PCNA-LI was calculated as the percentage of PCNA-labeled cells with respect to the total number of cells scored.

To elucidate the factors influencing the PCNA-LI, including the presence of Helicobacter DNAs in the hepatobiliary system, we evaluated the inflammatory score of the biliary tree, the white blood cell count, serum levels of total bilirubin and alkaline phosphatase, and the C reactive protein level in patients with hepatobiliary cancer.

Statistical analyses

We used the $\chi^2$ analysis and the Mann-Whitney U test to compare positivity in the PCR assays, the PCNA-LI, and the grade of biliary inflammation. The differences at $P < 0.05$ were considered significant. Multiple regression analysis was carried out to determine the confirmed influential factors of the PCNA-LI in patients with hepatobiliary cancer.

Results

Detection of Helicobacter DNAs

The results of the Helicobacter specific PCR assays are shown in Table II. Among the patients with hepatobiliary cancer, 6 bile samples from 17 patients and eight hepatobiliary tissue samples from 16 patients were positive for Helicobacter DNAs. In total, 10 (52.6%) of the 19 patients with cancer were judged to be positive in Helicobacter DNAs by PCR assays for bile and/or tissue. In the control group, three (15.7%) of the 19 patients were positive for Helicobacter DNAs. The difference in Helicobacter DNAs positivity between the patients with and without cancer was statistically significant ($P = 0.03$). In the 10 Helicobacter DNA-positive patients with cancer, six patients were positive for the 16S rRNA of H. bilis, four were positive for the 16S rRNA of H. hepaticus, and one was positive for H. pylori Urease A gene.

For further confirmation of PCR analyses, three products amplified using C62/C12 primers, two products amplified using B38/39 primers, and one product amplified using HPu1/HPu2 primers were subjected to sequence analysis and were found to be at least 99% identical to the corresponding Helicobacter DNA.

The PCRs for the 16S rRNA of Helicobacter genus were sensitive to all the Helicobacter species detected by HE staining, Giemsa staining, or immunohistochemical staining of H. pylori.

In addition, results proved to be positive in two patients who were judged to be negative for H. bilis, H. hepaticus, or H. pylori DNAs. One of the two patients had a cancer of papilla of the Vater. In additional sequence analyses of the products amplified using C97/98 primers in these two cases, sequence comparison showed 99% homology to Helicobacter cinaedi in the cancer patient. However, in the other patient without cancer, <94% homology to Helicobacter spp. liver 16S ribosomal DNA (GenBank accession number AF 142585) and H. bilis strains was determined.

Under a powerful microscopic examination, no evident helical bacteria were detected by HE staining, Giemsa staining, or immunohistochemical staining of H. pylori in any of the tissue sections.

Biliary inflammation and cell kinetic studies

In patients with hepatobiliary cancer, the average inflammation score was 2.11 ± 0.26 in the Helicobacter DNA-positive patients, whereas the score was 1.38 ± 0.32 in Helicobacter DNA-negative patients. The different was not statistically significant (Table III). Similarly, in the control group, the inflammation score in the Helicobacter DNA-positive and -negative patients did not differ.
PCNA-LIs of the biliary epithelium in Helicobacter DNA-positive patients with and without cancer were higher than those in Helicobacter DNA-negative patients with and without cancer, respectively, and these differences in the PCNA-LI were statistically significant. Moreover, multiple regression analysis revealed that the presence of Helicobacter DNAs and biliary pathology are associated with increased PCNA-LI of the biliary epithelium in patients with hepatobiliary cancer and benign biliary diseases.

Factors linked to increased PCNA-LI

The multiple regression analysis for influential factors of the PCNA-LI in the biliary epithelium in patients with hepatobiliary cancer demonstrated that the presence of the Helicobacter DNAs in the hepatobiliary system was the confirmed influential factor of the PCNA-LI (P = 0.041), as shown in Table IV.

Discussion

The age-specific prevalence of H. pylori infection depends on whether the person in question was born before or after 1950 in Japan (19). In the present study, therefore, special attention was paid to the distribution of comparable age-matching between the cancer group and the non-cancer group, and the ratio of persons who were born after 1950 was 3/19 in both of the two groups. In our study, Helicobacter DNAs were detected in the hepatobiliary system in patients with hepatobiliary cancer at an extremely high incidence. Furthermore, a close correlation between the presence of Helicobacter DNAs and an acceleration of the biliary epithelial cell kinetics was demonstrated. It is well established that genetic mutations in biliary carcinogenesis are facilitated by an acceleration of the biliary epithelial cell kinetics (20,21). Studies on pancreatobiliary maljunction and hepatolithiasis also revealed that an activated epithelial cell kinetics of the hepatobiliary system might be a possible factor in the occurrence of hepatobiliary cancer (21–23). Our findings, therefore, suggested that Helicobacter species, or organisms closely related to the known Helicobacter species, in the hepatobiliary system might be a pathogenic factor for the development of hepatobiliary cancer.

A relationship between gastric chronic inflammation caused by H. pylori and gastric carcinogenesis is generally accepted, because experimental studies using Mongolian gers-bils confirmed that H. pylori induces gastric cancer through chronic gastritis (7,8). In patients with hepatobiliary cancer, in the present study, the degree of biliary inflammation in the Helicobacter DNA-positive patients was higher than that in the Helicobacter-negative patients, but there was no statistical significance. Moreover, multiple regression analysis revealed that the biliary epithelial cell kinetics were affected by the presence of Helicobacter DNA, regardless of whether the biliary inflammatory reaction was mild or severe, suggesting...
that the organisms identified by our PCR analyses have a particular pathway in the hepatobiliary carcinogenesis besides the induction of biliary inflammation. Coyle et al. (24) reported that eradication of H. pylori reduced elevated mucosal levels of epidermal growth factor (EGF), and they emphasized that the over expression of EGF might be a possible pathogenic mechanism of H. pylori-induced gastric carcinogenesis. In the Helicobacter DNA-positive patients with hepatobiliary cancer, the biliary cell kinetics may be accelerated directly by the bacteria or bacterium-induced cytokines or growth factors, such as EGF, leading to the development of hepatobiliary cancer.

In our patients with hepatobiliary cancer, the 16s rDNA of H. bilis and H. hepaticus was more frequently detected in the hepatobiliary system than H. pylori Urease A gene in PCR analysis. H. bilis has been isolated from liver tissue and bile in mice, and infection by this microorganism can lead to chronic active hepatitis (17,25). H. hepaticus has also been identified by isolation from liver tissue in mice as a causative factor of chronic hepatitis and liver tumors (26–28). Considering our results and the viability of these murine Helicobacter species in bile, the organisms detected by our PCR analyses using C62/12 primers and B38/39 primers, possibly H. bilis and H. hepaticus, should be significant pathogenic colonizers in the hepatobiliary system in humans. The only patient positive in H. pylori DNA with hepatobiliary cancer had choleodochoduodenal fistula. In this case, H. pylori might be located in the biliary system because of transient immigration from the duodenum.

No helical bacteria were detected in the hepatobiliary tissue by means of immuno-histopathological analysis in the present study. There are two possible explanations for this discrepancy. First, Helicobacter organisms in the surface mucous gel layer were removed in the formalin fixed method (29). Second, the organisms detected in this study had a close similarity to Helicobacter in genetic feature but had no helical form. Meanwhile, several studies have shown that H. pylori was unable to grow in bile acids, especially unconjugated bile acids (30–32). The toxicity of unconjugated bile acids may also be related to the diffusion of culturing for the detection of Helicobacter species in the hepatobiliary system. Therefore, PCR assays appear to be a powerful approach to detecting Helicobacter species in the hepatobiliary system at present. However, isolation and characterization of these organisms should be accomplished to confirm the relationship between Helicobacter and hepatobiliary carcinogenesis.

In conclusion, the organisms detected by our PCR analyses for Helicobacter might be involved in hepatobiliary carcinogenesis. Thus, the eradication of these bacteria in the hepatobiliary system may be desirable as a chemoprevention of hepatobiliary cancer.

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References


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