Helicobacter Species Ribosomal DNA Recovered from the Liver Tissue of Chinese Patients with Primary Hepatocellular Carcinoma

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Using polymerase chain reaction (PCR), we examined 16S ribosomal DNA (rDNA) of *Helicobacter* species in liver tissue specimens obtained from 15 patients with hepatocellular carcinoma. Sixty percent (9 of 15) of these specimens were found to be positive for *Helicobacter* species. Four 16S rDNA fragments from positive PCR samples were directly sequenced. By sequence comparison, all were found to be 99% identical to the 16S rRNA of *Helicobacter pylori*.

Helicobacter pylori has been widely recognized as a pathogen in the etiology of chronic antral gastritis and peptic ulceration. *H. pylori* also is linked to cancer and is considered to be a class I carcinogen because it induces a chronic gastric inflammation and because it may play an important role in the development of gastric malignancies. In addition to having been found in the gastrointestinal tract, *Helicobacter* species have been identified in the intestinal tract, liver, and bile ducts of animals, and they have also been found to play a pathological role in enterohepatic diseases in animals and humans [1]. *Helicobacter hepaticus,* an example of such species, was discovered in 1992 and was first isolated from a colony of A/JCr mice; it has been reported to be an agent that causes hepatic cancer in rodents. Recently, several separate research groups detected such *Helicobacter* organisms as *H. pylori, H. pullorum, H. bilis,* and *Helicobacter* species flexispira in the bile, gall bladder, or liver tissue of patients with primary sclerosing cholangitis, primary biliary cirrhosis, or primary liver carcinoma [2, 3]. These reports suggested that *Helicobacter* organisms, including *H. pylori,* may play a role in the development of hepatobiliary diseases in humans, similar to the role that they play in the development of such diseases in animals.

Hepatocellular carcinoma (HCC) is one of the major cancers in the world, with an estimated 500,000 to 1 million new cases occurring each year. With regard to incidence, HCC ranks fifth among all cancers, and rates of incidence vary considerably by area or by country. The regions where the risk for HCC is high are sub-Saharan Africa, China, and Southeast Asia. Of all the newly reported cases of HCC in the world each year, 45% are found among individuals in mainland China, and HCC is the second leading cause of death due to cancer in China. Although dietary exposure to aflatoxin B1 (AFB1) is one of the major risk factors for HCC, chronic infection with hepatitis B virus (HBV) is by far the most important risk factor for HCC in China. Chronic infection with hepatitis C virus (HCV) is believed to play a relatively minor role in the development of HCC in China [4]. However, no recognized risk factors are found in a significant proportion of patients with primary liver carcinoma. It is interesting to hypothesize that *Helicobacter* organisms are also a risk factor for HCC. We therefore initiated a study to ascertain whether *Helicobacter* species could be identified in the hepatic tissue of Chinese patients with primary liver carcinoma.

Liver tissue specimens obtained from 28 patients who underwent hepatic surgery or cholecystectomy were studied. Of these 28 patients, 15 (12 men and 3 women) had HCC. The other 13 patients (8 men and 5 women), who did not have a malignancy, were considered to be control subjects; 5 of these 13 patients had cholelithiasis, 4 had hepatic hemangioma, 3 had hepatic focal nodular hyperplasia, and 1 had hepatic adenoma. Diagnosis of hepatobiliary disease was made on the basis of results of histopathological testing done at our hospital. Characteristics of patients with HCC are summarized in table 1. Fresh tissue specimens from the malignant liver and the nonmalignant liver were immediately ground and seeded on Columbia agar plates for culture [5] and were stored at −80°C for PCR analysis. The specimens from both the malignant liver and the nonmalignant liver were later fixed in formalin and embedded in paraffin for staining. The tissue was homogenized, and DNA was extracted using the standard protocol. For PCR, *Helicobacter* genus–specific primers were used to amplify a 400-bp fragment of the 16S rRNA gene of *Helicobacter* species [2]. Southern blot analysis was performed using a probe generated by amplification of *H. pylori* strain ATCC (American Type Cul-

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Table 1. Characteristics of patients with primary hepatocellular carcinoma.

Patient	Sex	Age, years	Liver pathology finding	Serological markers		*Helicobacter* PCR result
				HBV-M	Anti-HCV	
1	F	63	Hepatoadenocarcinoma	−	−	+
2	M	50	HCC and cirrhosis	HBsAg+, HBeAb+, HBcAb+	ND	−
3	F	20	HCC	HBsAg+, HBeAb+, HBcAb	−	−
4	M	37	HCC	HBsAg+ and HBcAb+	−	+
5	M	42	HCC	HBsAg+, HBeAb+, HBcAb+	−	+
6	M	37	HCC and cirrhosis	HBsAg+ and HBcAb+	ND	+
7	M	64	HCC	HBsAb+	ND	+
8	M	35	HCC	HBeAb+	ND	+
9	M	58	HCC and cirrhosis	HBsAg+, HBeAb+, HBcAb	−	+
10	M	42	HCC and cirrhosis	HBsAg+, HBeAb+, HBcAb+	ND	+
11	F	33	HCC and cirrhosis	HBsAg+ and HBcAb+	ND	+
12	M	50	HCC and cirrhosis	HBsAg+, HBeAb+, HBcAb+	−	−
13	M	34	HCC and cirrhosis	HBsAg+ and HBcAb+	−	−
14	M	28	HCC and cirrhosis	HBsAg+, HBeAb+, HBcAb+	−	−
15	M	50	HCC and cirrhosis	HBsAg+ and HBcAb+	−	−

NOTE. HBcAb, antibody to hepatitis B core antigen; HBeAb, antibody to hepatitis B e antigen; HBsAb, antibody to hepatitis B surface antigen; HBsAg, hepatitis B surface antigen; HBV-M, hepatitis B virus marker; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ND, not done.

ture Collection) 49503 with C_{97}/C_{98} primers to confirm the amplicons [2], which were of bacterial 16S rRNA origin rather than nonspecific human genomic origin. The amplicons were sequenced directly using the BLAST (Basic Local Alignment Search Tool) program in GenBank.

No bacteria were cultured under microaerobic conditions, and no spiral or curved bacteria were observed using silver stains for all specimens. Sixty percent (9 of 15) of the liver samples from patients with HCC were found to be positive for *Helicobacter* species by PCR analysis with use of *Helicobacter* genus–specific primers C_{97}/C_{98}, whereas none of the 13 liver samples from patients without HCC were found to have positive PCR results by use of the same primers (table 2 and figure 1). Each of the 9 amplicons was also found to be positive by Southern blot hybridization with a digoxygenin-labeled probe. Four 16S rDNA fragments from positive PCR samples were directly sequenced. By sequence comparison, all were found to be 99% identical to the 16S rRNA of *H. pylori* (GenBank accession number AF302106) and *Helicobacter* species "liver" 3 (GenBank accession number AF142585).

Our finding, together with the findings of previously published reports [2, 3], further demonstrated that *Helicobacter* colonization of the liver tissue is present in patients with HCC. To explore the causes of chronic liver diseases, including HCC, without known risk factors, many studies were performed that focused on looking for new hepatic viruses. Although more studies are required to clarify the causal relationship between infection with *Helicobacter* species and the development of HCC, the findings of the present study do provide evidence that *Helicobacter* species are probably novel infectious agents associated with HCC. Reports of the isolation of *Helicobacter* species from bile and the gallbladder [1–3] and a report of *Helicobacter* bacteremia [6] strongly suggest that different *Helicobacter* species may spread to the liver via the bile duct tree or a bloodborne route.

Infection with several viruses has been recognized as a risk factor for many cancers in humans. However, reports of an association of bacterial infection with cancers are rare. China has a huge population; HBV infection is present in >10% of

Table 2. Results of PCR for the detection of *Helicobacter* species and results of DNA sequence comparison.

Group	No. of positive PCR results/ no. of patients tested	16S rDNA sequence alignment	
		No. (strain type)	GenBank accession no.
Patients with HCC	9/15	2 *(Helicobacter pylori)*	AF302106
Patients without HCC	0/13	2 (*Helicobacter* species "liver" 3)	AF142585

NOTE. HCC, hepatocellular carcinoma

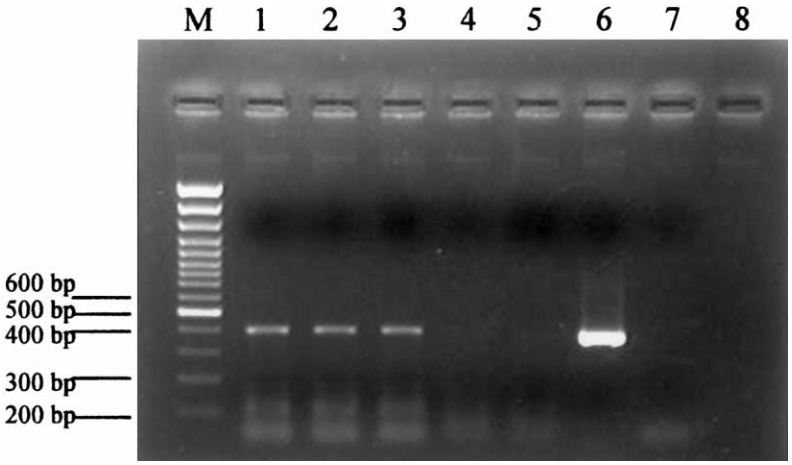

Figure 1. PCR products of the 400-bp fragments obtained by *Helicobacter* gene–specific amplification. *Lane M,* DNA marker; *lanes 1–3,* positive samples from patients with hepatocellular carcinoma; *lanes 4* and *5,* negative samples from patients without hepatocellular carcinoma; *lane 6,* positive control (*Helicobacter pylori* strain ATCC [American Type Culture Collection] 49503); *lane 7,* negative control (TE buffer); and *lane 8,* bacterial control *(Campylobacter jejuni).*

unselected people in China and is the primary risk factor for HCC [4]. Also, the prevalence of *H. pylori* infection is ~50% among individuals in China [7]. Therefore, exploration of a pathological role of *Helicobacter* colonization in the liver tissue of patients with HCC is quite important and valuable.

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