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DNA of *Helicobacter* spp. and common gut bacteria in primary liver carcinoma

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Abstract

Background and aim. Gastric and enteric *Helicobacter* species have been associated with the pathogenesis of some extragastric diseases.
Methods. We retrospectively investigated the presence of DNA of *Helicobacter* species in samples of the cancer and the surrounding tumour-free liver tissues of patients with hepatocellular carcinoma (HCC, $n = 12$) and cholangiocarcinoma (CC, $n = 13$). The patients were from an area with low liver cancer incidence and with low hepatitis B and C prevalence. Patients with a benign liver disease ($n = 24$) were included as controls. Paraffin-embedded liver samples were examined by a *Helicobacter* genus-specific PCR assay as well as group-specific PCR assays for *Enterobacteriaceae*, *Bacteroides*, *Lactobacillus* and *Enterococcus*. PCR products of positive samples were characterised by denaturing gradient gel electrophoresis (DGGE) and DNA sequencing.
Results. PCR assay detected *Helicobacter* DNA in seven of 12 (58%) and eight of 13 (62%) normal liver tissue specimens from HCC and CC patients, respectively. Two cancer samples from HCC patients were *Helicobacter*-positive but none of the CC cancers. In the control group, three of 24 (12.5%) patients with a benign liver condition were positive for *Helicobacter* species ($p < 0.01$ compared to results of tumour-free liver tissue from the cancer patients). DGGE and DNA sequence analysis showed that 90% of the detected PCR products were "*H. pylori*-like". DNA of some other enteric bacteria was detected in the liver of one cancer patient and one control (4% of all patients).
Conclusion. The presence of DNA of *Helicobacter* species in liver specimens, but not of other common gut bacteria, was associated with human hepatic carcinogenesis.

Keywords: DGGE; DNA sequencing; *Helicobacter*; Liver cancer; PCR

1. Introduction

Chronic inflammation has long been recognised as an important risk factor for various human cancers [1]. It has been estimated that 16% of the worldwide incidence of cancer can be attributed to infection with viruses, bacteria, schistosomes, and liver flukes [2]. Hepatocellular carcinoma (HCC) is regarded as a common cause of cancer related death worldwide. The incidence of HCC has a wide geographical variation, and a variety of risk factors, such as hepatitis B (HBV) and C (HCV) viruses, alcohol abuse, and aflatoxin B1 intake, have been identified [3]. However, no specific molecular and/or genetic trait linked to the development of HCC has been reported [4]. Intrahepatic cholangiocarcinoma (CC) is less prevalent than HCC. Main risk factors in a low-risk population in Denmark, were alcoholic liver disease, non-specific cirrhosis, bile duct diseases and inflammatory bowel disease. In high risk areas infestation with liver flukes is important [5].

The genus *Helicobacter* contains at least 26 formally named species [6]. Based on their primary colonization

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site, *Helicobacter* are divided into gastric and enterohepatic species. *H. pylori* persistently infects the human stomach and causes gastritis, peptic ulcer disease and may ultimately lead to the development of gastric cancer. In 1994, the International Agency for Research on Cancer classified *H. pylori* as a type 1 carcinogen [7]. Enterohepatic *Helicobacter* species, such as *H. hepaticus*, *H. bilis*, *Helicobacter* sp. flexispira and *H. cinaedi*, have been identified in the lower intestinal and biliary tract of animals, and may cause chronic inflammatory bowel and liver diseases in rodents, poultry and primates, and have been implicated in gastroenteritis, cholecystitis and some liver diseases in man [6,8,9]. The difficulty to culture *Helicobacter* species has made molecular methods such as PCR and sequencing attractive to identify these microbes in clinical specimens. Recently, *H. pylori* DNA has frequently been detected in liver tissues of patients with chronic liver diseases such as primary sclerosing cholangitis, primary biliary cirrhosis and primary liver cancer [10–14]. In addition, a high seroprevalence of antibodies to *H. pylori* and non-gastric *Helicobacter* spp. has been reported in patients with chronic liver diseases and hepatocellular carcinoma [15,16]. Despite the association of helicobacter DNA with human liver diseases and cancer, the possible role of *Helicobacter* species is debated [17].

The aim of this study was to investigate the prevalence of *Helicobacter* spp. in primary liver carcinoma in a HBV and HCV low-endemic area in southern Sweden. *Helicobacter* prevalence in primary cancer tissues was compared with benign liver diseases such as focal nodular hyperplasia (FNH) and adenoma. In addition, four groups of common endogenous enteric bacteria were analysed by PCR to test whether gut microbes translocate to a diseased liver.

2. Methods

2.1. Patients and samples

Paraffin embedded liver specimens were retrospectively collected from the Pathology department at Lund University Hospital from consecutive patients with primary liver cancer comprising subjects with HCC ($n = 13$, 7 males, mean age 63 years, range 58–73 years) and CC ($n = 13$, 6 males, mean age 58 years, range 47–73 years). A control group ($n = 24$) consisting of liver tissue specimens from consecutive patients with FNH ($n = 17$, 1 male, mean age 36 years, range 24–63 years), liver adenoma ($n = 4$, 1 male, mean age 42 years, range 32–49 years), focal fatty lesions ($n = 2$, females, mean age 36 years, range 33–40 years) and haematoma ($n = 1$, female, 32 years), was analysed as well. No signs of alcoholic liver disease or haemochromatosis were observed in any patient. There was a slight to moderate number of lymphocytes in the portal space in three HCC patients (one of which had moderate portal fibrosis) and ten CC patients. Apart from one HCC patient with cryptogenic cirrhosis, none of the remaining subjects, neither in the cancer group nor in the control group, had cirrhosis. The cases were collected between 1972 and 1992 from an area with low liver cancer incidence and low hepatitis B and C prevalence. Serology for HBV was performed on three patients, all with negative results. Tests for HCV were not performed.

Approximately 50 mg was taken from the paraffin blocks with the tip of a scalpel. By carefully comparing the blocks with the slides, it was ascertained that a pure tissue type was excised. Two samples, from the tumour as well as normal peritumorous tissue, were obtained from each HCC and CC patient. For one HCC patient with cryptogenic cirrhosis, tumour-free tissue was so sparse that it could not be taken for analysis. Similarly, tissue from the benign lesion as well as from the normal tissue surrounding the lesion was obtained from each control patient. The Research Ethics Committee at Lund University approved this study (Permit no. 588/2006).

2.2. DNA extraction

Paraffin embedded liver tissue samples were de-embedded as previously described [18]. DNA was extracted from approximately 10 mg of liver tissue as previously described [18].

2.3. Helicobacter genus-specific PCR

Amplification of *Helicobacter* DNA was performed using the primers 16S1F, 16S1R and 16S2R (Table 1) described by Goto et al. [19]. The reaction mixtures and amplification conditions were as previously described [20] with a minor modification. The reaction mixture of the first step contained 0.01% (w/v) casein and 0.01% (w/v) formamide instead of 0.4% (w/v) BSA. As a positive control, 5 μl of *H. pylori* (CCUG 17874) DNA was added to the reaction mixture, whereas 5 μl of sterile Millipore-filtered deionised water was used as a negative control. Detection of the 0.47 kb PCR product was performed as described elsewhere [20].

2.4. Amplification of non-Helicobacter bacteria

The reaction mixture and amplification conditions, except for annealing temperatures, for non-*Helicobacter* PCR assays were the same as in the first step of the semi-nested *Helicobacter* PCR [20]. The annealing temperatures and primers used for detection of *Enterobacteriaceae*, *Bacteroides*, *Lactobacillus* and *Enterococcus* are listed in Table 1. As a positive control, *Escherichia coli* (CCUG 17620), *Bacteroides fragilis* (CCUG 4856), *Lactobacillus fermentum* (strain LB3) or *Enterococcus faecalis* (CCUG 9997) DNA was used. The amplified PCR products were detected as described before [20].

2.5. DGGE analysis

Helicobacter genus-specific PCR products were amplified using the conditions described above with the exception