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SHORT COMMUNICATION

Host Response to the Presence of *Helicobacter* spp. DNA in the Liver of Patients with Chronic Liver Diseases

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Abstract

Literature data indicate an association between the presence of *Helicobacter* spp. in the liver and the development of hepatocellular carcinoma (HCC). However, the role of *H. pylori* infections in chronic liver diseases (CLD) remains controversial. The aim of this study was to detect *Helicobacter* spp. DNA in patients with CLD, and to investigate the host response to the presence of the bacterium in the liver. *Helicobacter* spp. DNA was detected in 59% samples. *H.pylori* was the most prevalent species (94%). We estimated the expression level of IL-1 and IL-8 genes. The presence of *Helicobacter* spp. did not have a significant effect on the gene expression of IL-8 and IL-1.

Key words: *Helicobacter*, interleukin 1, interleukin 8, nested-PCR

On the basis of various epidemiological studies *H. pylori* has been classified as a type I carcinogen by the Working Group of the World Health Organization International Agency for Research on Cancer in 1994 (IARC 1994). *H. pylori* is specialized in colonizing human gastric mucosa of more than 50% of world population. It can be a cause of chronic gastritis, peptic ulcers, and gastric adenocarcinoma (Wedi *et al.*, 2002). The bacterium has also been implicated in extra-gastric conditions such as ischemic heart disease, vascular and immunological disorders, halitosis, migraine, and poor growth in children (Pellicano *et al.*, 2008). Recently, *Helicobacter* spp. DNA has been found in the liver of patients with various chronic liver diseases, such as primary sclerosing cholangitis, hepatocellular carcinoma (HCC), hepatitis C virus-related chronic infection, and cirrhosis. Inflammatory disease is characterized by increased levels of pro-inflammatory cytokines, such as interleukins 1 and 8 (IL-1 and IL-8). The higher prevalence of *Helicobacter* spp. associated with more advanced stages of the liver disease supports the possibility of their role in the progression of chronic hepatitis towards cirrhosis and HCC (Pellicano *et al.*, 2008).

In this study we aimed at detecting *Helicobacter* spp. genetic material in patients with chronic liver diseases in the population of Northern Poland. Further, the host response to the presence of *Helicobacter* spp. in the liver was investigated.

The study included 27 patients suffering from different chronic liver diseases (CLD): Hepatitis B (HBV) and C (HCV) virus infections, HBV/HCV double infection, non-alcoholic steatohepatitis, non-alcoholic fatty liver disease with HCV infection, non-alcoholic fatty liver disease with HBV infection, hereditary hemochromatosis, alcoholic steatohepatitis and autoimmunohepatitis. The biopsy from each patient was halved, and used for DNA and RNA extraction, respectively.

DNA was extracted using a High Pure PCR Template Preparation Kit (Roche). Twenty-seven DNA samples with high quality and quantity were amplified. *Helicobacter* spp. DNA was detected by nested polymerase chain reaction with genus specific primers targeting *Helicobacter* spp. 16S rRNA gene. The reaction mixture for the first step contained (25 µl):100 ng of genomic DNA, 1x chelating buffer, 2.5 mM $Mg(OAc)_2$, 0.2 mM dNTP, 0.4 U of Taq DNA polymerase (Fermentas), 0.1 mg/ml of casein, 0.01% (v/v) formamide and 0.125 µM of primers: 1F and 1R (Al-Soud *et al.*, 2003). Amplification conditions for the first PCR were: 94°C for 2 min; then 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; and finally 72°C for 5 min. The reaction mixture for the second step (25 µl) contained: 1x chelating

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Table I
Results of liver function test, blood morphology and expression of interleukin 1 and 8 in liver in patients with CLD

	Helicobacter positive	*Helicobacter* negative	*Range*	P*
ALT (alanine aminotransferase) [IU/L]	80.73±107.5	83±78.8	10–409	0.45
AST (aspartate aminotransferase) [IU/L]	59.26±77.31	51.4±39.92	12–316	0.62
ALP (alkaline phosphatase) [IU/L]	97±45.72	87.6±61.5	29–212	0.42
GGTP (γ-glutamyl transpeptidase) [IU/L]	108.53±164.82	178.5±208.86	9–707	0.2
HGB (hemoglobin) [mg/dL]	13.58±1.88	0.77±0.34	0.24–2.31	0.49
Bilirubin [mg/dL]	0.94±0.61	0.86±0.44	0.24–2.31	1
Liver biopsy grading	1±0.83	0±0.52	0–3	0.13
Liver biopsy staging	0.47±0.74	0.5±1.08	0–3	0.66
IL-8 relative expression (in arbitrary units)	1.1±0.9	1.3±2.5	0–6	0.35
IL-1 relative expression (in arbitrary units)	27±66.1	16±31.4	0.12–259	0.62

P* Statistical significance was assessed by U Mann-Whitney Test

buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 U of AmpliTaq Gold polymerase, 0.25% (v/v) glycerol, 0.4% (v/v) BSA, 0.125 μM of primers: 2F (5'-AGGGAATATTGCTCAATGGG-3', designed by the authors) and 2R (Al-Soud *et al.*, 2003), and 1 μl of the first amplification step product. Amplification conditions were: 95°C for 10 min; than 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; and finally 72°C for 5 min.

Helicobacter genus-specific PCR products were sequenced, aligned and compared with the sequences from GenBank database using BLASTN 2.2.1 (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Total RNA was extracted by a RNeasy® kit (Qiagen), 100 ng of RNA was used for reverse strand cDNA synthesis according to the manufacturer's protocol (Quanti Tect® Reverse Transcription Kit, Qiagen).

Expression of IL-1 and IL-8 genes was quantified using real-time RT-PCR (LightCycler®; Roche Diagnostic). β-glucuronidase gene (GUS) was used as reference (Romanowski *et al.*, 2008).

The reaction mixture contained (20 μl): 2 μl of cDNA, 5 μM concentration of each primer, 3 mM of $MgCl_2$ and 2 μl of ready-to-use Light Cycler® DNA Master SYBR Green I (Roche Diagnostic). The polymerase was activated at 95°C for 10 min. The following cycling conditions were used in the reaction: 1 s (IL-1, IL-8) and 5 s (GUS) 95°C denaturation step, 15 s annealing at 64°C (IL-1, IL-8) and 60°C (GUS), and 20 s (IL-1, IL-8) and 10 s (GUS) extension at 72°C. Melting-curve analysis followed 45 and 50 cycles of IL-1, IL-8 and GUS genes amplification, respectively. Results were normalized with respect to the reference gene.

To determine expression levels of genes of interest in the studied samples, standard curves for IL-1 and IL-8 were generated (serial dilutions of a standard sample used at this step were as follows: 1x, 0.5x, 0.2x, 0.1x). As a standard we used a mixture of all cDNAs. Each sample was studied in three repetitions and normalized with respect to the reference gene.

The role of *H. pylori* in various gastric and duodenal diseases is well documented. In recent years *H. pylori* DNA has been identified in the bile, gallbladder and liver tissue of patients with different hepato-biliary diseases. Recently published detection rates of *Helicobacter* spp., in tissue samples collected from patients with liver cancer, varied from 0% to 100% (Avenaud *et al.*, 2000; Nilsson *et al.*, 2001; Verhoef *et al.*, 2003; Huang *et al.*, 2004; Ito *et al.*, 2004; Rocha *et al.*, 2005; Vivekanandan and Torbenson 2008). In our study we selected 16S rDNA as the target because it contains both conserved and highly variable regions and gene sequences for almost all *Helicobacter* species available in public data bases. We identified *Helicobacter* genus-specific PCR products in 16/27 (59%) of the liver specimens, which constitutes a more than 2-fold increase of the detection frequency in comparison to the data previously published by our group (Stalke *et al.*, 2005). The differences in detection level might be related to improvements in PCR reaction conditions. Previously only BSA was used to avoid the inhibitory effect of bile, whereas at present we used several PCR facilitators which improved the frequency of *Helicobacter* spp. DNA detection. Moreover, the amount of DNA per reaction, which we used as a template was 20 ng, whereas previously 5 μl of each DNA solution was added, regardless of the concentration. Additionally, in the previously published study, diluted product from the first PCR reaction was used as a template in the second step, while here, we added undiluted product. All this differences could influence detection sensitivity.

The DNA sequence of 15 out of 16 (94%) positive samples showed the highest similarity to *H. pylori* 16S rRNA gene, whereas in 1 sample (6%), *H. cetorum*-like DNA was detected. Detailed chromatogram analysis

revealed that 3 samples contained mixed DNA material. In those three samples *H. rodentium*-like DNA was identified based on double fluorescent signals in positions where the 16S rRNA genes differ for *H. pylori* and *H. rodentium*. This may indicate that in humans, different isolates of particular *Helicobacter* species may exist, like it was found in other species (Nilsson *et al.*, 2004). In our recently published results we also found *H. rodentium*-like DNA by means of denaturing gradient gel electrophoresis (DGGE) and subsequent DNA sequencing (Nakonieczna *et al.*, 2010). This *H. rodentium*-like DNA was actually the most prevalent one in the previously studied group of patients, which in comparison to the presented data was less heterogeneous.

Literature data indicate that expression levels of IL-1 and IL-8 are higher in gastric epithelial cells of *H. pylori*-infected than in uninfected patients (Backhed *et al.*, 2003). As the phenomenon of *Helicobacter* species presence in the human liver is widely discussed with respect to its participation in disease state and/or progression, we analyzed the level of IL-8 and IL-1 in the group of patients, in which *Helicobacter* DNA was detected. As a control group, patients with negative result of nested-PCR were used. There were no differences in IL-8 gene expression between *Helicobacter*-positive and *Helicobacter*-negative patients. The biopsy samples obtained from *H. pylori*-positive patients expressed about two times higher levels of IL-1, however this data was not statistically significant (Table I). Moreover, comparison of *Helicobacter* spp. positive and negative group did not show any statistically relevant differences in the functional liver test, including the levels of alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, alkaline phosphatase, hemoglobin and bilirubin (Table I). However, we did not study the status of cytotoxin associated gene A (*cagA*) in the *Helicobacter*-positive group, which might have influenced the results. It is known that *cagA*$^+$ strains are associated with enhanced secretion of interleukins, especially IL-8 (Backhed *et al.*, 2003; Rieder *et al.*, 2005). The relatively low expression level of the studied interleukins can be explained by defective immune system of investigated individuals or their general low immunity. Another interpretation might be low bacterial load and their adaptation to a special environment. Besides, the expression level of cytokines is associated with disease progression and is highly increased at the initial stage of disease. Because the investigated material originated from patients with chronic liver diseases we can suppose that levels of IL-1 and IL-8 were low.

Lack of significant differences in expression levels of studied genes may be associated with the fact that *H. pylori* possesses lipopolysaccharide (LPS) with a lower virulence compared to the typical bacterial endotoxins, such as *Escherichia coli* LPS. Furthermore, *H. pylori* adheres and is internalized into hepatocytes more efficiently than into gastric epithelial cells. It appears that *H. pylori* may survive inside hepatocytes and effectively avoid host response (Ito *et al.*, 2008).

Sequence analysis showed that *H. pylori* is the most prevalent species (94%) in the studied population. In contrast to gastric epithelial cells, the presence of *H. pylori* in the liver of patient with CLD had no influence on IL-8 and IL-1 mRNAs status as well as biochemical parameters describing liver functioning.

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