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Low Frequency of Helicobacter DNA in Benign and Malignant Liver Tissues from Baltimore, United States

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Abstract

Helicobacter DNA has been reported in hepatocellular carcinoma tissues in several studies from varying geographical locations, raising the possibility that helicobacter infection may contribute to the pathogenesis of hepatocellular carcinoma. Other known risk factors for hepatocellular carcinoma show significant geographical variability, but whether the same holds for helicobacter is unknown. We studied the prevalence of Helicobacter DNA in a United States cohort of HCC, where the prevalence of helicobacter infection is low in the general population. Liver tissues from 57 individuals were examined. Thirty-five individuals had paired tumor/non-tumor samples, including 21 cases of hepatocellular carcinoma, for a total of 92 samples studied. Both Helicobacter genus and Helicobacter pylori species specific PCR was performed. Helicobacter DNA was detected in 5/57 (9%) cases, all in non-neoplastic cirrhotic liver tissues from individuals with hepatitis C infection (N=4) or alcohol liver disease (N=1). Tissues from 22 hepatocellular carcinomas and 10 cholangiocarcinomas were all negative as were tissues from 8 benign primary hepatic tumors. In conclusion, Helicobacter DNA was detectable in 9% of liver tissues in this cohort, but was not found in primary benign or malignant liver tumors. These findings indicate that Helicobacter infection is unlikely to be etiologically associated with hepatocellular carcinoma in this cohort. If Helicobacter infection does contribute to the development of hepatocellular carcinoma in general, then significant regional variability must exist.

Keywords

Helicobacter; hepatocellular carcinoma; cholangiocarcinoma

Background

Helicobacter species were reported to colonize the livers of mice and cause hepatitis and hepatocellular carcinoma (HCC) in 1994 [1–3]. A subsequent report demonstrated the presence of the Helicobacter DNA in the human biliary tree [4]. Since that time, a growing body of literature has suggested a role for Helicobacter in human HCC. Specifically, individuals with HCC are more likely to have serological evidence for past Helicobacter infection [5–8] and Helicobacter DNA can be found in HCC tissues more commonly than controls [9–16]. In

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addition to HCC, Helicobacter DNA has also been reported in intrahepatic cholangiocarcinomas [9,12]. The potential role of Helicobacter infection in the development of HCC and intrahepatic cholangiocarcinomas has not been studied in the United States, where the prevalence of Helicobacter is low. Thus the aims of this study were to determine the frequency of Helicobacter DNA in benign liver tissues, primary HCC, and intrahepatic cholangiocarcinoma to better understand the role of Helicobacter infection, if any, in this region.

Materials and Methods

Liver tissues from the Johns Hopkins Hospital, Baltimore, Maryland were retrospectively studied. All tissue were from individuals who underwent liver transplantation (N=40) or surgery for a hepatic mass (N=17). Tissues were harvested at the time of surgery and stored at −80C prior to use.

Helicobacter DNA amplification

DNA was extracted from 20–25 mg of liver tissue using the QIAmp DNA mini kit (Qiagen, Valencia, CA, and USA). At least 100ng of liver DNA was used for each 25 ul PCR reaction, using primers listed in Table 1. To aide in comparing results from other studies, single round PCR was performed using primers reported by Rocho et. al. [15]. For helicobacter single round PCR assays using genus and species specific primers, 45 cycles of amplification were performed to enhance sensitivity. Hot start Taq was used and conditions were 95C for 10 minutes followed by 45 cycles of 95 C° for 20s, 55 C° for 30s, and 72 C° for 45s. A nested PCR assay was also performed on all liver samples (Table 1) with a first round of 50 cycles of 95 C° for 20s, 55 C° for 30s, and 72 C° for 45s. Using 1 ul of the first round product as template, a second round of PCR was performed for 45 cycles using the conditions of 95 C° for 20s, 55 C° for 30s, and 72 C° for 45s. In all samples, primers to the beta-catenin gene (CTNNB1) were used to ensure that extracted DNA was amplifiable.

Positive Controls

Three gastric biopsies (unrelated to the liver samples) with active chronic helicobacter pylori infection were micro-dissected from formalin fixed, paraffin embedded tissues, the DNA extracted, and the DNA used as controls to ensure that the helicobacter primers worked adequately The amplicons from one of the positive control were then cloned (Invitrogen, Carlsbad, CA) and PCR performed on 10 fold serial dilutions to determine the sensitivity of the PCR assays. In addition, for each PCR assay 10, 100, and 1000 copies of cloned helicobacter DNA were spiked into pooled liver DNA from five randomly selected liver samples and each PCR performed to investigate the performance of the primers in the setting of abundant liver genomic DNA.

Results

All sets of helicobacter primers amplified all positive control stomach biopsies. For the single round PCR assay, serial dilutions of the cloned amplicons demonstrated sensitivity down to 10 copies per reaction for primers C-97/C-98 and HPYS/HPYA and 100 copies per reaction for HS-1/HS-2 (Figure 1). Spiking the cloned helicobacter DNA into liver genomic DNA did not interfere with the assay's sensitivity (Figure 1).

Liver tissues from 57 individuals were examined. 35 individuals had paired tumor/non-tumor samples, including 21 cases of HCCs, for a total of 92 samples studied. The liver tissues were from 34 men and 23 women with an average age of 52.8±14 years. The non-neoplastic livers were cirrhotic in 40 cases, mostly from chronic HCV infection (Table 2). In 11 cases, the non-

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neoplastic livers were not cirrhotic and contained no significant inflammation or fibrosis. The HCCs were all typical HCCs and no variants such as cholangiohepataocellular carcinomas or fibrolamellar carcinomas were examined. In addition to the HCC, 10 cholangiocarcinomas and 8 benign lesions were studied (Table 2). The cholangiocarcinomas were all intrahepatic and 9/10 had a pure glandular morphology, while one had a giant cell morphology.

All cases showed very strong bands following amplification of the beta-catenin gene. However, no cases had detectable Helicobacter DNA using the single round PCR assays. Because of this surprising finding, the same samples were re-tested with a nested PCR assay and Helicobacter DNA was identified in 5/57 (9%) patients. Sequencing confirmed Helicobacter DNA in all cases. All of the positive cases were non-neoplastic tissues from cirrhotic livers, including 4 cases with HCV associated cirrhosis and one case of alcohol associated cirrhosis, giving an overall frequency of 13% in all cirrhotic livers and 19% in HCV associated liver cirrhosis. All of the remaining samples were negative by nested PCR.

Discussion

Our results demonstrate the presence of low levels of Helicobacter DNA in a small proportion of cirrhotic livers and suggest that Helicobacter infection did not play a major etiologically role in the development of HCC in this cohort. The absence of Helicobacter DNA in the 10 cases of intrahepatic cholangiocarcinoma further emphasizes that Helicobacter is unlikely to have an etiological role in the other major form of primary liver carcinomas in this cohort. These results significantly extend our understanding of the role of Helicobacter in HCC carcinogenesis by emphasizing that its role has important regional variations. Careful attention to positive controls and this laboratory's experience with amplifying low-level targets from hepatic tissues [17] suggest that inadequate assay sensitivity is unlikely to explain the lack of detectable Helicobacter DNA in primary liver carcinomas. The absence of detectable Helicobacter DNA in benign neoplasms seen in this study is overall in good agreement with those studies that found no evidence for helicobacter in benign primary hepatic tumors [11, 15].

As recently reviewed, Helicobacter may not necessarily be directly carcinogenic in the liver and may be a co-risk factor or even an innocent bystander [18]. To date, the possible association between HCC and helicobacter has been supported by a number of diverse observations which can be summarized as follows. First, based on serological studies as well as direct detection in liver tissues, Helicobacter is more common in those with cirrhosis than non-cirrhosis and, of those with cirrhosis, positive serology is found more frequently in those who have HCC [9–16,19]. Secondly, helicobacter DNA in HCC tissues has been reported from multiple geographic locations. Third, studies have reported positive helicobacter culture from liver tissues [20] and positive immunostaining for helicobacter on liver tissues [11,13,19]. Fourth, the lack of DNA from other gut organisms in HCC tissues, suggesting that helicobacter DNA detection does not represent non-specific DNA contamination from the portal circulation [13,15]. Finally, the absence or lower frequency of helicobacter in primary versus metastatic liver carcinomas suggests a more specific association with HCC [12,14,16]. Nevertheless, this link between HCC and Helicobacter is tempered by the observations of others that Helicobacter DNA was not detected in a group of 55 liver tissues [21]. Even if lack of assay sensitivity accounts for some of their findings [22], their results still strongly imply that any Helicobacter DNA that may have been present in the tested tissues was at a very low concentration. Our findings also indicate that Helicobacter DNA is absent in primary liver carcinomas and present in only 13% of cirrhotic liver tissues overall. While we did not quantitate the levels of Helicobacter DNA, the observation that sample positivity was found only with a nested PCR assay further implies very low Helicobacter DNA copy numbers.

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Environmental, genetic, or clinical management methods may explain the discrepancy between our findings and those from other geographical locations. It seems unlikely that this discrepancy is entirely explained by Helicobacter pylori prevalences in the general population alone, as studies from France [9,15], Sweden [12], and the Netherlands [16] have also identified a higher frequency of Helicobacter DNA in HCC tissues, despite have an overall low Helicobacter prevalence, similar to the United States. Further study of this question may reveal important information on the biology of helicobacter infection as well as HCC.

In conclusion, Helicobacter DNA was detectable in 13% of cirrhotic liver tissues, but was not found in any case of primary liver cancer. Helicobacter is unlikely to have played an etiological role in the development of liver cancer in this cohort.

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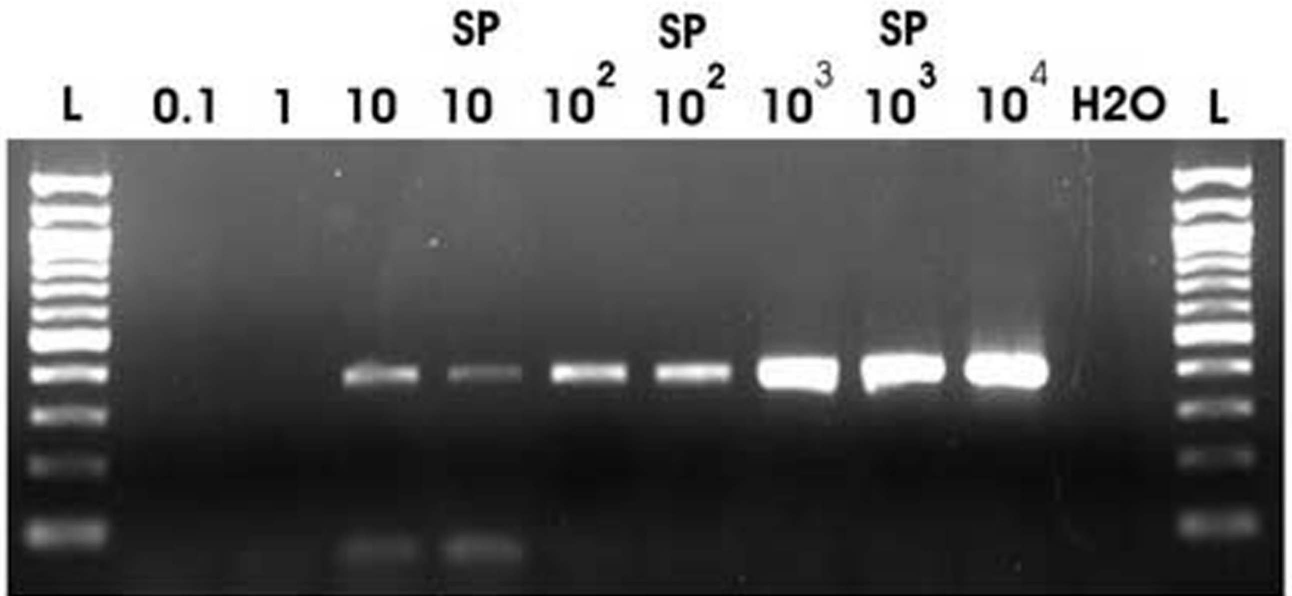

Figure 1.
For all three PCRs, the amplicon was cloned from one of the stomach positive controls, diluted, and PCR performed to determine sensitivity. Data for primer HS-1 and HS-2 are shown. The copy numbers of template per PCR reaction are shown above each lane. In addition, some of the assays were spiked with genomic DNA (designated by “SP”).

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Table 1

Primers used in PCR amplification. The non-nested primers were selected from those used by Rocho et. al.. [15] The original citation is also indicated below.

Primer	Sequence 5' to 3'	Target	Amplicon size (bp)
Helicobacter genus			
HS-1[9]	AACGATGAAGCTTCTAGCTTGCTAG	16S rDNA	400
HS-2	GTGCTTATTCGTTAGATACCGTCAT		
C97[4]	GCTATGACGGGTATCC	16S rDNA	400
C98[4]	GATTTTACCCCTACACCA		
Helicobacter pylori			
HPY S[23]	AGGTTAAGAGGATGCGTCAGTC	23S rDNA	267
HPY A[23]	CGCATGATATTCCCATTAGCAGT		
Hemi-Nested PCR			
Helicobacter genus		163 rDNA	
Outer primers[24]	TATGACGGGTATCCGGC		375
HG-1	ATTCCACCTACCTCTCCCA		
HG-2			
Inner Primers	CTGAGACACGGTCCAGACTC		293
HG-3	CAAATGCAGTTCTRYRGTTAAGC		
HG-2			
CTNNB1			
For	ATGGAACCAGACAGAAAAGC	Beta-catenin	200
Rev	GCTACTTGTTCTTGAGTGAAG		

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Table 2

Liver samples tested for Helicobacter species DNA.

Type of sample	Number Samples Tested
Non-neoplastic liver, total	***51***
Cirrhosis, total	40
HCV	21
cryptogenic	6
HBV	2
ETOH	5
Other	6
Non-cirrhotic	11
Tumors, total	***35***
Hepatocellular carcinoma, total	21
HCV	7
cryptogenic	1
HBV	2
ETOH	3
Other	4
non cirrhotic background liver	4
Cholangiocarcinoma	6
Macro-regenerative nodule	4
Focal nodular hyperplasia	2
Hepatic adenoma	1
Angiomyolipoma	1
Isolated tumors without corresponding non-neoplastic liver tissues	***6***
Hepatocellular carcinoma	1
Cholangiocarcinoma	4
Focal nodular hyperplasia	1
Total	**92**