

# Occult and Previous Hepatitis B Virus Infection are Not Associated with Hepatocellular Carcinoma in United States Patients with Chronic Hepatitis C

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Previous studies have suggested that prior exposure to hepatitis B virus (HBV) infection may increase the risk of development of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C. The aim of this study was to compare the prevalence of previous or occult HBV infection in a cohort of hepatitis B surface antigen–negative patients with histologically advanced chronic hepatitis C in the United States who did or did not develop HCC. Stored sera from 91 patients with HCC and 182 matched controls who participated in the Hepatitis C Antiviral Long-term Treatment against Cirrhosis (HALT-C) Trial were tested for hepatitis B core antibody (anti-HBc), hepatitis B surface antibody, and HBV DNA. Frozen liver samples from 28 HCC cases and 55 controls were tested for HBV DNA by way of real-time polymerase chain reaction. Anti-HBc (as a marker of previous HBV infection) was present in the serum of 41.8% HCC cases and 45.6% controls ( $P= 0.54$ ); anti-HBc alone was present in 16.5% of HCC cases and 24.7% of controls. HBV DNA was detected in the serum of only one control subject and no patients with HCC. HBV DNA (as a marker of occult HBV infection) was detected in the livers of 10.7% of HCC cases and 23.6% of controls ( $P = 0.18$ ).

**Conclusion:** Although almost half the patients in the HALT-C Trial had serological evidence of previous HBV infection, there was no difference in prevalence of anti-HBc in serum or HBV DNA in liver between patients who did or did not develop HCC. In the United States, neither previous nor occult HBV infection is an important factor in HCC development among patients with advanced chronic hepatitis C. (HEPATOLOGY 2011;54:434-442)

**H**epatitis B virus (HBV) infection is marked by the presence of hepatitis B surface antigen (HBsAg) in serum. Clearance of HBsAg indicates recovery from infection; however, low levels of HBV DNA may persist within the liver and occasionally in the serum, indicating that infection is not

*Abbreviations:* AFP, alpha-fetoprotein; anti-HBc, hepatitis B core antibody; anti-HBs, hepatitis B surface antibody; CI, confidence interval; HALT-C, Hepatitis C Antiviral Long-term Treatment against Cirrhosis; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; OR, odds ratio; PCR, polymerase chain reaction.

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totally resolved in some patients. The presence of HBV DNA in HBsAg-negative persons has been referred to as "occult" HBV infection. It has been suggested that occult HBV infection may contribute to ongoing liver disease and the development of hepatocellular carcinoma (HCC).<sup>1-3</sup> There is evidence that previous HBV infection, marked only by the presence of antibodies to HBV—notably hepatitis B core antibody (anti-HBc) with or without hepatitis B surface antibody (anti-HBs)—may also indicate persistent HBV infection. Previous HBV infection has also been linked with progressive liver disease, particularly as a cofactor among patients with another form of underlying liver disease such as chronic hepatitis C or alcoholic liver disease.

Prior studies have shown that the prevalence of occult HBV infection is higher in countries where HBV infection is prevalent, in patients with serological markers of previous HBV infection, and in patients who have risk factors for HBV infection such as those with human immunodeficiency virus or hepatitis C virus (HCV) infection. What remains uncertain is the clinical significance of low-level, persistent HBV infection, especially among patients with another cause of liver disease. Several studies, mostly from Europe and Japan, have found a higher rate of occult HBV infection in patients with chronic HCV infection who have HCC compared with HCV-infected patients with no HCC.<sup>1-4</sup> In some of these studies, the prevalence of occult HBV infection in HCV patients with HCC was as high as 60%-70%.<sup>2-4</sup> However, data on the prevalence of occult HBV infection in HCV patients and the contribution of occult HBV to HCC in the United States are limited.

The Hepatitis C Antiviral Long-term Treatment against Cirrhosis (HALT-C) Trial prospectively followed patients with chronic HCV infection and advanced hepatic fibrosis for clinical outcomes including HCC. All the patients tested negative for HBsAg in the serum at enrollment. This large cohort provides an excellent opportunity to study the clinical significance of previous or occult HBV infection in patients with chronic HCV infection in the United States. The aims of this analysis were to compare the prevalence of previous and occult HBV infection in HALT-C patients who developed HCC and those who did not develop HCC. In addition, we assessed the demographics, risk factors for HCV infection, and laboratory and histological indicators of liver disease in HALT-C patients with and without previous and occult HBV infection.

## Patients and Methods

The design of the HALT-C Trial has been described.<sup>5,6</sup> Briefly, patients with chronic hepatitis C had to meet the following criteria for enrollment: failure to achieve sustained virological response after previous interferon treatment with or without ribavirin, presence of advanced hepatic fibrosis on liver biopsy (Ishak fibrosis score  $\geq 3$ ), no history of hepatic decompensation or HCC, and absence of defined exclusion criteria. Specifically, patients who were positive for HBsAg or human immunodeficiency virus antibody were excluded from the trial.

All patients were required to undergo a liver biopsy before enrollment. For those in whom the entry biopsy was performed subsequent to consent into the HALT-C Trial, a portion of the biopsy was snap-frozen and stored for future research after an adequate specimen was allocated for histologic assessment. The biopsy specimens were initially stored at  $-70^{\circ}\text{C}$  at the clinical sites and then sent to a central repository (SeraCare Life Sciences, Gaithersburg, MD) on dry ice. Upon arrival at the central repository, the specimens were stored in  $-70^{\circ}\text{C}$  freezers with backup generators.

All patients had been treated previously for chronic hepatitis C with one or more courses of interferon, with the most recent course being a combination of peginterferon and ribavirin. Patients who remained viremic during treatment or experienced viral breakthrough or relapse after initial response were randomized to maintenance therapy (peginterferon alfa-2a 90  $\mu\text{g}/\text{week}$ ) or to no further treatment for the next 3.5 years. Following completion of the 3.5 years of the randomized trial, all patients were invited to continue follow-up without treatment.

At entry, all patients were required to undergo ultrasound, computed tomography, or magnetic resonance imaging examination with no evidence of hepatic mass lesions suspicious for HCC and to have an alpha-fetoprotein (AFP)  $< 200$  ng/mL.

Patients were scheduled to be seen every 3 months during the 3.5 years of the randomized trial and every 6 months thereafter. At each visit, patients were evaluated clinically and blood tests were performed. Blood samples for research were collected on site and then centrifuged; sera were initially stored at  $-70^{\circ}\text{C}$  at the clinical sites and periodically sent on dry ice to SeraCare where they were stored in  $-70^{\circ}\text{C}$  freezers with backup generators. Protocol-defined ultrasound examination was performed at intervals of 6-12 months.<sup>5,6</sup> Patients with elevated or rising AFP and those with new lesions on ultrasound examination were further

evaluated by way of computed tomography or magnetic resonance imaging.

Two definitions of HCC, one for presumed and one for definite, have been published.<sup>7</sup> Definite HCC was defined by histology or a new mass on imaging with AFP levels increasing to  $\geq 1,000$  ng/mL. Presumed HCC was defined as a new mass on ultrasound in conjunction with two liver imaging studies showing a lesion with characteristics of HCC or evidence of progression on follow-up. All cases of HCC were reviewed by an Outcomes Review Panel comprised of panels of three clinical investigators.

To compare the prevalence of previous and occult HBV infection, a case-control study was performed. All HCC cases (definite or presumed) diagnosed at any time after enrollment into the HALT-C Trial were studied. Two controls were selected for each case of HCC, matched for fibrosis stratum on baseline biopsy (Ishak score 3 or 4 versus 5 or 6), treatment assignment (peginterferon versus no treatment for randomized patients), and duration of follow-up. To ensure that control subjects did not harbor early HCC, they were required to be followed for at least 12 months after the date of their matching with the HCC patient, and to not have HCC at any time during the HALT-C Trial.

Previous HBV infection was defined as the presence of anti-HBc with or without anti-HBs or HBV DNA in serum. Occult HBV infection was defined as the presence of HBV DNA in the liver.

**HBV Markers in Serum.** All patients tested negative for HBsAg in the clinical laboratory at the local HALT-C site prior to their enrollment into the HALT-C Trial. Stored serum samples were coded and sent to the clinical laboratory at University of California, Irvine, where they were tested for anti-HBc (ETI-AB-COREK PLUS; DiaSorin Inc., Stillwater, MN) and anti-HBs (ADVIA Centaur anti-HBs; Siemens Healthcare Diagnostics, Inc., Tarrytown, NY) by way of enzyme immunoassay (anti-HBc) or chemiluminescent immunoassay (anti-HBs). Serum HBV DNA was tested by way of real-time polymerase chain reaction (PCR) assay (COBAS TaqMan HBV Test; Roche Diagnostics, Indianapolis, IN) with a lower limit of quantification of 30 IU/mL and a lower limit of detection of 10 IU/mL.

**HBV DNA in Liver.** Frozen liver samples from the HCC cases and selected controls, where available, were coded and tested for the presence of HBV DNA at the University of Michigan in the laboratory of one of the authors (A. S. F. L.). DNA was extracted from liver biopsies using the QIAamp DNA mini kit (QIA-

GEN, Valencia, CA) and HBV DNA was quantified by way of real-time PCR assay, as described.<sup>8</sup> Each sample was tested in duplicate with two sets of primers and probes (Supporting Table 1), one spanning nucleotide positions 1167-1283 in the HBV polymerase gene and the other nucleotide positions 333-476 in the HBV surface gene (that overlaps with the polymerase gene). To monitor for contamination during each step, sterile double-distilled water and liver specimens from uninfected persons (liver donors who were HBsAg-negative and anti-HBc-negative with undetectable HBV DNA in serum by way of PCR assay) were used as negative controls. Each assay also included explant liver from an HBsAg-positive patient who was previously demonstrated to have detectable hepatic HBV DNA as a positive control. Quantification of  $\beta$ -actin was used to estimate the amount of genomic DNA and the number of hepatocytes in each liver sample, and the amount of HBV DNA was expressed as IU/cell. The lower limit of detection of the assay was 5 IU/mL.<sup>8</sup>

To verify reproducibility of the assay results, six of 54 samples with negative results in all four PCR reactions and two of 10 samples with positive results in all four PCR reactions were retested, and all samples showed 100% concordance on retesting. All 22 samples with positive results in one to three PCR reactions were retested, and only samples with positive results in at least six of eight PCR reactions (i.e., positive results with both sets of primers) were considered positive.

**Statistical Analyses.** Descriptive statistics were reported as number and percent or mean and standard deviation. Baseline characteristics and HBV markers of HCC cases and matched controls were compared with conditional logistic regression for matched pairs. Controls were excluded if data were not available for their case. All analyses were performed at the data coordinating center (New England Research Institutes, Watertown, MA) using SAS statistical software version 9.2 (SAS Institute, Cary, NC). A two-sided significance level of 5% was used for all analyses.

## Results

Ninety-one HCC cases (definite and presumed) and 182 matched controls were included in this study. Among the HCC cases, three were diagnosed during the lead-in phase of HALT-C, one after achieving a sustained virologic response, and the remaining 87 were nonresponders. In the latter group, HCC was diagnosed a median of 4.2 years (range, 0.2-8.5 years) after randomization. In comparing HCC cases and

**Table 1. Baseline Characteristic of HCC Cases and Controls**

	HCC (n = 91)	No HCC (n = 182)	P Value*
<b>Demographics</b>			
Age, years	52.8 (7.42)	50.3 (7.26)	<b>0.01</b>
Male sex	75.8%	73.6%	0.70
<b>Race</b>			
White	64.8%	73.1%	0.08
Hispanic	6.6%	10.4%	
Black	23.1%	15.9%	
Other	5.5%	0.6%	
<b>Viral factors</b>			
HCV genotype 1	91.2%	96.1%	0.09
Log HCV RNA (IU/mL)	6.37 (0.51)	6.47 (0.52)	0.16
Duration of HCV infection, years	30.7 (8.79)	27.7 (8.19)	0.01
<b>Laboratory tests</b>			
Platelets $\times 1,000/\text{mm}^3$	126.3 (49.2)	162.0 (61.5)	< <b>0.0001</b>
Albumin, g/dL	3.73 (0.39)	3.87 (0.45)	<b>0.02</b>
AST, U/L	112.0 (63.3)	91.3 (68.0)	<b>0.02</b>
ALT, U/L	128.2 (90.6)	110.0 (88.9)	0.13
Alkaline phosphatase, U/L	118.0 (57.7)	103.3 (50.9)	<b>0.04</b>
Total bilirubin, mg/dL	0.90 (0.44)	0.81 (0.39)	0.10
INR	1.09 (0.12)	1.04 (0.11)	<b>0.002</b>
AFP, ng/mL	27.5 (31.6)	14.4 (24.5)	<b>0.002</b>
<b>Liver histology*</b>			
Cirrhosis	56.0%	55.5%	0.99
Fibrosis	44.0%	44.5%	
<b>Treatment assignment</b>			
Maintenance peginterferon	42.5%	46.7%	0.99
Control	57.5%	53.3%	
<b>Smoking/Alcohol</b>			
Ever smoked	82.4%	75.3%	0.20
Lifetime no. of drinks	18,725 (23,756)	20,610 (36,424)	0.65
<b>Portal hypertension</b>			
Esophageal varices on endoscopy	43.5%	28.0%	<b>0.02</b>
<b>Hepatitis C risk factors</b>			
Transfusion of blood or blood products	43.3%	38.7%	0.44
Occupational exposure to human blood	24.4%	24.2%	0.92
Tattoos	28.6%	32.4%	0.51
Body piercing	0.0%	1.6%	0.99
Recreational drug use	44.0%	44.5%	0.93
Cocaine use	51.7%	56.0%	0.47

Data are expressed as the mean (SD) or as a percentage. Controls were matched on fibrosis stage, treatment assignment, and duration of follow-up. ALT, alanine aminotransferase; AST, aspartate aminotransferase; INR, international normalized ratio. Bold type indicates statistical significance.

\*Determined by way of conditional logistic regression analysis.

controls, those with HCC were older and more likely to have characteristics of more advanced liver disease, including lower platelet counts, lower serum albumin levels, and higher bilirubin levels, and more frequently had varices (Table 1). Risk factors for HCV infection, estimated duration of HCV infection, alcohol consumption, and history of smoking were not different between HCC cases and controls.

Frozen liver samples were available from 28 of 91 (30.8%) HCC cases and from 55 of 182 (30.2%) controls. Patients (HCC cases and controls) with liver samples were similar to those without liver samples regarding demographics, severity of liver disease, fibrosis stage, and treatment assignment. Among those with

liver samples, the baseline characteristics of the cases and controls were similar except for lower serum albumin levels in the HCC cases.

**HBV Markers in Serum.** Almost half of the patients had evidence of previous HBV infection; thus 38 of 91 (41.8%) HCC cases and 83 of 182 (45.6%) controls had anti-HBc detectable in their serum (Fig. 1, Table 2). Of these, 15 (16.5%) HCC cases and 45 (24.7%) controls had isolated anti-HBc (without anti-HBs). Compared with patients who were seronegative for anti-HBc, the odds ratio (OR) and 95% confidence interval (CI) for HCC development was OR 0.85 (95% CI 0.51-1.43) for those who were anti-HBc positive (with or without anti-HBs) and OR 0.63

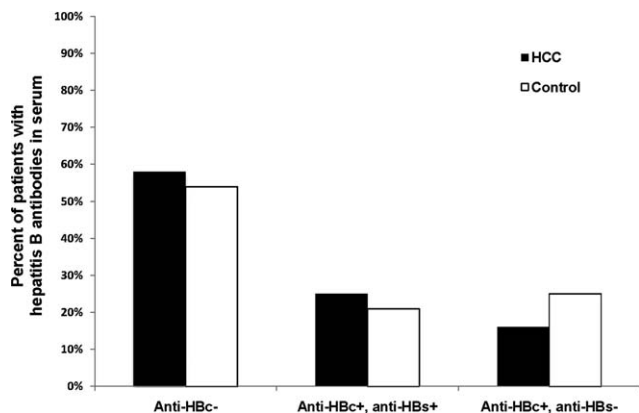


Fig. 1. Prevalence of anti-HBc and anti-HBs in the sera of HCC patients (n = 91) and matched controls (n = 182).

(95% CI 0.33-1.12) for those who were anti-HBc-alone positive. The OR was similar when only patients with definite HCC were evaluated.

HBV DNA was detected in the serum of only one (0.4%) patient. This was a control subject with isolated anti-HBc. Serum HBV DNA level was very low (<30 IU/mL), but HBV DNA was detected in the liver

**HBV DNA in Liver.** Three of 28 (10.7%) HCC cases and 13 of 55 (23.6%) controls had HBV DNA detectable in the liver (Fig. 2). Compared with the patients with no detectable HBV DNA in the liver, those with HBV DNA in the liver had a matched OR of 0.42 (95% CI 0.12-1.52;  $P = 0.18$ ) for HCC development (Table 2). The mean  $\pm$  SD HBV DNA concentrations in the cases and controls were  $0.0047 \pm 0.0056$  and  $0.0267 \pm 0.0602$  IU/cell, respectively. Two of the three (66.7%) HCC cases and eight of 13 (61.5%) controls with HBV DNA in the liver were anti-HBc-positive in serum; of these, one HCC case and four controls had isolated anti-HBc.

**Characteristics of Patients with and Without HBV Markers.** Compared with patients who were anti-HBc-negative, those who were anti-HBc-positive were more likely to have a history of injection drug

use and/or a history of snorting cocaine and less likely to have a history of blood transfusion; anti-HBc-positive patients also were shown to have lower serum albumin levels (Table 3). Patients who were anti-HBc-positive were also more likely to have a history of being tattooed and a history of body piercing and to be black than patients who were anti-HBc-negative, although these differences were not significant. Stage of hepatic fibrosis, presence of esophageal varices, and estimated duration of HCV infection were similar between those with and without anti-HBc.

Patients with and without HBV DNA in the liver were similar with regard to demographics, baseline laboratory values, fibrosis stage, presence of esophageal varices, risk factors for HCV infection, and estimated duration of HCV infection (data not shown).

## Discussion

This nested case-control study of HBsAg-negative patients from the United States with advanced chronic hepatitis C showed that neither previous (presence of anti-HBc with or without anti-HBs in serum) nor occult (detectable HBV DNA in liver) HBV infection was associated with the development of HCC. In this study, HBV DNA was detected in the livers of 11% of patients with HCC and in 24% of matched controls without HCC (HCC: OR 0.42, 95% CI 0.12-1.52;  $P = 0.18$ ). In studies from Japan and Italy, the reported frequency of HBV DNA detection in the liver of HBsAg-negative, anti-HCV-positive patients has ranged from 15%<sup>9</sup> to 49%<sup>10</sup> for patients without HCC and up to 73% among patients with HCC.<sup>11</sup> Most<sup>4,12-14</sup> but not all<sup>9,15-17</sup> studies from Asia and Europe have found that patients with hepatitis C who had detectable HBV DNA in the liver or serum had an increased risk of HCC.

In the current study, 67% of HCC patients and 62% of matched controls with HBV DNA in the liver had anti-HBc in serum, an indication of previous

Table 2. HBV Markers in Serum and Liver of HCC Cases and Controls

	HCC Cases	Controls	Odds Ratio	95% Confidence Interval	P Value
HBV antibodies in serum	n = 91	n = 182			
Anti-HBc-	53 (58%)	99 (54%)	Ref.		
Anti-HBc+, anti-HBs+/-	38 (42%)	83 (46%)	0.85	0.51-1.43	0.54
Anti-HBc+, anti-HBs-	15 (16%)	45 (25%)	0.63	0.33-1.12	0.12
Anti-HBc+, anti-HBs+	23 (25%)	38 (21%)	1.13	0.60-2.138	0.27
HBV DNA in liver	n = 28	n = 55			
HBV DNA-	25 (89%)	42 (76%)	Ref.		
HBV DNA+	3 (11%)	13 (24%)	0.42	0.12-1.52	0.18

Controls were matched 2:1 to cases for fibrosis strata on baseline biopsy, treatment assignment, and duration of follow-up. Matched odds ratios were determined by logistic regression analyses.

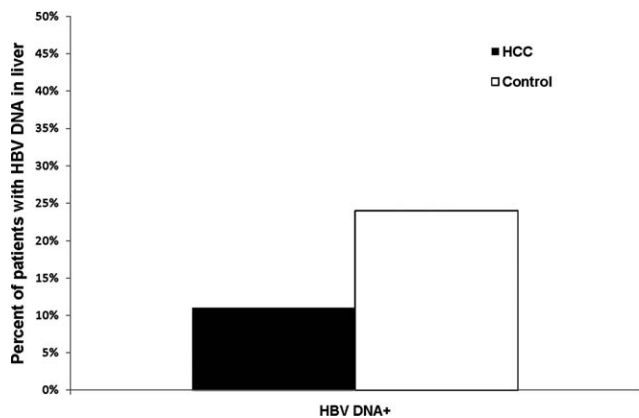


Fig. 2. Prevalence of HBV DNA in the livers of HCC patients (n = 28) and matched controls (n = 55).

HBV infection. Similarly, studies in Asia and Europe found that most but not all patients with occult HBV infection had markers of previous HBV infection in the serum.<sup>4,10-13,18,19</sup> HBV DNA was detected in the liver of 32 of 57 anti-HBc-negative patients with HCC in one study in Italy<sup>4</sup> and in three of four anti-HBc-negative patients with HCC in a study in Japan.<sup>11</sup> Several mechanisms for occult HBV infection

have been proposed. First, patients with chronic HBV infection who have undergone spontaneous loss of HBsAg often have persistent HBV DNA in the liver.<sup>20</sup> These patients are often positive for anti-HBc only. Second, patients who have recovered from acute HBV infection may have detectable HBV DNA in the liver for many years after HBsAg to anti-HBs seroconversion.<sup>21,22</sup> These patients usually are anti-HBc-positive and anti-HBs-positive. Third, patients might be chronically infected with HBV variants with decreased HBsAg production or altered HBsAg epitopes leading to false negative results in standard enzyme immunoassays for HBsAg, although this situation appears to be rare.<sup>23</sup> These patients would be positive for anti-HBc. Fourth, patients coinfectd with other hepatitis viruses such as HCV may have suppressed HBV replication as a result of viral interference.<sup>24</sup> Finally, some patients may have primary occult HBV infection, i.e., low-dose infection and only a partial induction or a total lack of humoral immunity accounting for the absence not only of HBsAg but also anti-HBc and anti-HBs.<sup>25,26</sup> This phenomenon was first described in woodchucks<sup>25</sup> and confirmed in humans.<sup>26</sup> In the latter study, anti-

**Table 3. Baseline Characteristics of Patients with and Without HBV Markers in Serum**

	Anti-HBc+ (n = 121)		Anti-HBc- (n = 152)	P Value*
	Anti-HBs- (n = 60)	Anti-HBs+ (n = 61)		
<b>Demographics</b>				
Age, years	50.4 (5.59)	51.8 (7.38)	51.1 (8.01)	0.96
Male sex	83.3%	73.8%	71.1%	0.16
<b>Race</b>				
White	70.0%	60.7%	74.3%	0.09
Hispanic	5.0%	9.8%	10.5%	
Black	23.3%	24.6%	13.8%	
Other	1.7%	4.9%	1.3%	
<b>Laboratory tests</b>				
Platelets, ×1,000/mm <sup>3</sup>	150.4 (53.5)	141.4 (47.1)	153.4 (66.7)	0.30
Albumin, g/dL	3.77 (0.44)	3.74 (0.39)	3.88 (0.44)	0.02
AST, U/L	110.2 (83.6)	91.6 (44.4)	96.1 (67.2)	0.57
ALT, U/L	126.8 (105.4)	110.2 (63.0)	114.1 (92.3)	0.69
Alkaline phosphatase, U/L	112.6 (65.4)	111.1 (54.4)	105.3 (48.0)	0.32
Total bilirubin, mg/dL	0.81 (0.35)	0.83 (0.34)	0.85 (0.46)	0.57
INR	1.07 (0.11)	1.06 (0.11)	1.05 (0.13)	0.17
AFP, ng/mL	19.0 (31.9)	23.4 (36.4)	16.8 (21.1)	0.20
Cirrhosis	60.0%	49.2%	56.6%	0.74
Maintenance peginterferon	44.1%	54.1%	42.3%	0.26
Esophageal varices	33.9%	33.3%	32.4%	0.84
<b>Risk factors</b>				
Transfusion of blood or blood products	33.9%	32.8%	45.7%	0.04
Occupational exposure to human blood	23.3%	26.2%	23.8%	0.86
Tattoos	33.3%	41.0%	26.3%	0.054
Body piercing	0.0%	4.9%	0.0%	0.09
Recreational drug use	63.3%	52.5%	33.5%	<0.0001
Cocaine use	73.3%	57.4%	46.1%	0.002

Data are expressed as the mean (SD) or as a percentage.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; INR, international normalized ratio.

\*Comparison of 121 anti-HBc+ versus 152 anti-HBc- patients.

HBc-positive patients but not anti-HBc-negative patients with occult HBV infection showed a T cell response typical of protective memory.

Nearly half of the patients in this study had anti-HBc in the serum, indicating they had been infected with HBV in the past; however, the prevalence of anti-HBc was not different between the HCC cases and the controls: 42% versus 46% (HCC: OR 0.85;  $P = 0.54$ ). Our results differ from the majority of studies from Japan and Italy, which found that HBsAg-negative patients with chronic hepatitis C who were anti-HBc-positive are at increased risk for HCC.<sup>17,27</sup> Another difference between our findings and many other studies is the low prevalence of HBV DNA in serum. Only one of 273 patients tested had detectable serum HBV DNA at a very low level and this was observed in a control patient. By contrast, detection rates of low-level DNA of up to 78% had been reported in Asian studies.<sup>9,17,19,27</sup> Although a higher percentage of patients from Asia and southern Europe were anti-HBc-positive, the difference in detection rate of HBV DNA in serum between our study and these studies cannot be explained by differences in prevalence of anti-HBc. The lower rate of HBV DNA detection in our patients who were anti-HBc-positive may be related to the fact that most of the HCC patients likely acquired HBV infection as adults and cleared HBsAg after a transient infection, whereas many of the anti-HBc-positive patients in Italy and Japan likely acquired HBV infection during childhood and did not clear HBsAg until after many years of chronic HBV infection. Several studies from Asia also reported very low rates of HBV DNA detection in serum (2%-11%), despite a high prevalence of anti-HBc.<sup>17,19</sup>

Data on occult HBV infection and HCC in the United States are limited. Hsia et al.<sup>28</sup> found HBV DNA in the livers of 17 of 31 (54.8%) HBsAg-negative North American patients with HCC; five of these patients were infected with hepatitis C and seven were anti-HBc-positive. Kannangai et al.<sup>29</sup> detected HBV DNA in the livers of three of 19 (16%) HBsAg-negative patients in the United States; only five of the 19 were infected with hepatitis C. Shetty et al.<sup>30</sup> found HBV DNA in the livers of 13 of 21 (62%) patients with HCV-related HCC and 9 of 23 (39%) patients with HCV-related cirrhosis and no HCC. An erratum published by Shetty concluded that occult HBV was not associated with HCC ( $P = 0.36$ ).<sup>31</sup> Our study differs from these three studies in that very small samples from liver biopsies rather than surgically resected tumors or explant livers were available for testing for

HBV DNA. Moreover, the baseline biopsies from our patients were obtained 0.3-9.1 years (median, 5.15 years) before the diagnosis of HCC was made, whereas liver samples in the other studies were obtained at the time of HCC diagnosis.

Our study differed from studies in Asia and Europe in several additional respects. First, the prevalence of chronic HBV infection is low in the United States compared with Asia and southern Europe. Thus, the likelihood of detecting markers of previous or occult HBV infection in our patients would be expected to be lower than in patients from Asia or southern Europe. Nevertheless, we found HBV DNA in the livers in 19% and anti-HBc in the serum in 44% of our patients. The relatively high prevalence of occult HBV and previous HBV infection in a country with low endemicity is likely related to the shared risk factors for hepatitis B and hepatitis C. In this study, the controls were carefully matched to the HCC cases in having similar stage of fibrosis on liver biopsy as well as comparable duration of follow-up with no HCC. In prior studies, the frequency of HBV DNA detection increased with more advanced liver fibrosis.<sup>16,18</sup> Thus, studies comparing patients with HCC with those with less advanced fibrosis would be expected to show a more marked difference in HBV DNA or anti-HBc detection between the two groups. Indeed, some HBsAg-negative patients with HBV DNA in the liver or anti-HBc in the serum might have been chronically infected with HBV for decades before spontaneous loss of HBsAg, and the previous chronic HBV infection may have contributed to increased risk of HCC as well as increased risk of liver fibrosis. Several studies have shown that the risk of HCC persists if HBsAg clearance occurred after age 50 or after the development of cirrhosis.<sup>20,32</sup> HBV genotypes in our patients and those in Europe or Asia may also be different.

Our study had several potential limitations. First, the number of patients with HCC was relatively small. Nonetheless, this is the largest such study in the United States with 83 liver and 273 serum samples from patients with chronic HCV infection. Second, the liver tissue and serum samples had been stored for up to 9 years before being tested. However, all the samples had been stored at  $-70^{\circ}\text{C}$  and had not been subjected to freezing/thawing previously. Although it is possible that DNA may have been degraded during long-term storage, serum antibodies should be robust, and there is no reason to expect more rapid DNA degradation in the samples from HCC patients than controls. Third, only 2-3 mm of liver tissue was generally available for HBV DNA detection. In many other

studies, surgically resected HCC and/or surrounding noncancerous liver tissue or explant liver were used for HBV DNA detection. It is possible that the HBV DNA detection rate may be higher if larger samples of liver tissue were available, but the increase in yield would have to be substantial for us to show a statistically significant difference between patients with or without HCC. Fourth, PCR amplification of DNA from liver samples was performed from only two regions of the HBV genome in this study, and both reactions must be positive for the sample to be considered as positive, whereas some of the prior studies performed PCR reactions in three or four regions of the HBV genome and considered samples with positive results in two of three or two of four regions as positive. The likelihood that our method led to a gross underdetection of HBV DNA in the liver is low, because other studies have shown that HBV DNA sequences are generally preserved, and HBV DNA detection rate is similar with primers in different regions of the HBV genome.<sup>3,4,33</sup> Fifth, although the HALT-C Trial is a prospective study, we performed a case-control study and did not test stored serum and liver samples from all patients in the study. However, the nested case control study used here is an efficient design that allows reasonable inference for the entire HALT-C cohort. Sixth, frozen liver samples were available in only 31% of HCC cases, but there was no difference between HCC cases with and without liver samples regarding demographics, severity of liver disease, fibrosis stage, treatment assignment, and risk factors for HCV infection. Finally, despite matching cases and controls for baseline fibrosis stage, the HCC cases were older and had laboratory values, suggesting more advanced liver disease.

In conclusion, patients with HCC in the HALT-C cohort did not have a higher rate of detection of anti-HBc in serum or HBV DNA in liver compared with matched controls with no HCC. Our data suggest that neither previous nor occult HBV infection is an important factor in HCC development among patients with histologically advanced chronic hepatitis C in the United States.

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