Hepatitis C Virus, Alcoholic Cirrhosis, and Hepatocellular Carcinoma

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Abstract
We undertook a retrospective study to determine the prevalence of hepatitis C virus (HCV) and hepatitis B virus (HBV) infection in 81 Caucasian patients with confirmed hepatocellular carcinoma (HCC). Besides HBV and HCV serological markers, HCV RNA and HBV DNA were detected in serum and liver tissue by polymerase chain reaction. Overall, HCV RNA was found in 20 cases (25%), HBV DNA in 21 patients (26%), and coinfection in 3 patients (3%). HCV RNA in liver tissue was not found without virus in serum, whereas HBV DNA was found in the liver tissue of one patient without viremia. In an additional analysis, 32 patients with HCC and alcoholic cirrhosis (HCC-AC) were compared to 35 cases with AC without HCC and 35 cases with alcoholic hepatitis. The prevalence of HCV RNA in HCC-AC (19%) was significantly higher than in the other groups (AC, 3%; alcoholic hepatitis, 0%). HBV DNA was present in 19% of HCC-AC as compared to 3% of AC and 0% of alcoholic hepatitis. We conclude that the form of HCC in 50% of the patients in a Western European country is related to chronic viral hepatitis. Our data obtained from a group of patients having alcoholic liver disease with or without HCC suggest that the prevalence of HCV RNA or HBV DNA in these populations increases with the severity of hepatic injury.

Introduction
Primary HCC is one of the most common malignancies in the world, with an estimated 500,000–1 million new cases a year. It is particularly common in certain regions of Asia and sub-Saharan Africa (1), where the annual incidence ranges up to 500 cases/100,000 population. The main rea-
alone and 35 patients with alcoholic hepatitis consecutively admitted to our hospital were analyzed. The diagnosis of liver cirrhosis was based on clinical examination, ultrasonography (i.e., inhomogeneous liver structure, rarefied hepatic veins), detection of esophageal varices upon endoscopy without portal vein thrombosis, and typical laboratory evidence of hepatic dysfunction (i.e., thrombocytopenia, decrease in serum albumin and prothrombin time, and increase in immunoglobulines). Diagnosis of alcoholic hepatitis was based on history of high alcohol intake, detection of fatty liver upon ultrasonography, and elevated serum transaminases (aspartate aminotransferase/alanine aminotransferase ratio, > 2) after excluding other known causes of liver disease (i.e., autoimmune disease, hyperlipidemia, or diabetes mellitus). All patients with alcoholic liver disease reported a high daily alcohol intake (males, >80 g/day; females, >50 g/day).

**Table 1** Clinical characteristics of 81 patients with HCC and a subset of 32 patients with underlying alcoholic liver disease in comparison with alcoholic cirrhosis alone and alcoholic hepatitis

<table>
<thead>
<tr>
<th>Factor</th>
<th>Patients with alcoholic liver disease</th>
<th>HCC (n = 32)</th>
<th>Cirrhosis (n = 35)</th>
<th>AH* (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ± SD</td>
<td>58 ± 10.5</td>
<td>57 ± 7.7</td>
<td>53 ± 12.2</td>
<td>47 ± 14.7</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>66 (80)</td>
<td>30 (93)</td>
<td>30 (84)</td>
<td>30 (84)</td>
</tr>
<tr>
<td>AFP* &gt; 5</td>
<td>9 (11)</td>
<td>2 (6)</td>
<td>29 (83)</td>
<td>32 (91)</td>
</tr>
<tr>
<td>AFP 5-10</td>
<td>8 (10)</td>
<td>1 (3)</td>
<td>6 (17)</td>
<td>3 (9)</td>
</tr>
<tr>
<td>AFP &lt; 10</td>
<td>64 (79)</td>
<td>29 (91)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No cirrhosis</td>
<td>4 (5)</td>
<td>0</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>Child-Pugh A</td>
<td>23 (28)</td>
<td>9 (28)</td>
<td>10 (29)</td>
<td>0</td>
</tr>
<tr>
<td>Child-Pugh B</td>
<td>19 (24)</td>
<td>5 (16)</td>
<td>6 (17)</td>
<td>0</td>
</tr>
<tr>
<td>Child-Pugh C</td>
<td>35 (43)</td>
<td>18 (56)</td>
<td>9 (28)</td>
<td>10 (29)</td>
</tr>
<tr>
<td>Previous blood transfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Increased alcohol intake (males, <80 g/day; females, <50 g/day).
* n = 81.
* AH, alcoholic hepatitis.
* AFP, α-fetoprotein (mg/l).

Viral Markers. HBsAg, HBeAg, anti-HBs, anti-HBc, and anti-HBe were detected with commercially available ELISAs (Sorin, Düsseldorf, FRG). Antibodies to HCV were analyzed by a second generation anti-HCV ELISA (Abbott Laboratories, Wiesbaden, FRG) which employs three different synthetic proteins derived from the HCV structural (core) and nonstructural region (NS3 and NS4) (15). Reactive serum samples were confirmed with a second generation recombinant immunoblot assay (RIBA, Ortho Diagnostics, Neckargemünd, FRG). This assay uses the same antigens as the ELISA blotted separately on nitrocellulose, which enables us to detect antibodies more specifically (15). Hepatitis D virus infection was serologically excluded in all HBsAg-positive patients by testing for anti-HDV antibodies (Abbott, Wiesbaden, FRG). Since HBV or HCV infection may be present without serological viral markers, all samples were tested for viral nucleic acids (HCV RNA and HBV DNA). To avoid bias, all tests were done under code.

HBV DNA Extraction from Serum and PCR. HBV DNA may be detected in serum as a marker of viremia. HBV DNA was extracted from serum by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. The amplification of HBV DNA by PCR was carried out to obtain the highest possible sensitivity. The primers used correspond to a highly conserved core region of HBV (HBV-1, position 35–58; HBV-2, position 357–380). PCR products were subjected to agarose gel electrophoresis and visualized by UV light after ethidium bromide staining. To confirm HBV specificity, the amplified products were analyzed by Southern blot hybridization using a digoxigenin-labeled probe encompassing the entire HBV genome.

HCV RNA Extraction from Serum and RT-PCR. HCV RNA is currently the only marker for the presence of virus in serum. HCV RNA was detected by "nested" PCR essentially as described by Farci et al. (16). In brief, nucleic acids were extracted from serum by the guanidinium-phenolchloroform method. HCV RNA was reverse transcribed to complementary DNA using the outer antisense primer. The resulting complementary DNA was amplified by PCR. To enhance sensitivity and ascertain specificity of the resulting PCR product, a second round of amplification was performed using a second pair of primers located within the nucleic acid sequence framed by the "outer" primers. The primers were derived from the highly conserved 5' nontranslated region and the amino terminal part of the core sequence of HCV. The outer primers were 5'-GGAACCTT-GACGTCTCTGTTGGGC-3' (position 394–414, antisense) and 5'-TGGGGGCGACACTCCACCAT-3' (position 14–33, sense). The inner primers were 5'-TGCTTCTACGCAGA-AAGGCCTCAG-3' (position 59–82, sense) and 5'-GTCCT- CATGGTGACGGTTCAG-3' (position 326–349, antisense). Amplified products were visualized under UV light after 2% agarose gel electrophoresis.

HCV RNA and HBV DNA Extraction from Liver Tissue. Approximately 0.1–0.2 g of tumorous, nonnecrotic liver tissue specimens were homogenized in 500 μl of buffer 1 (4 M guanidinium-isothiocyanat-0.5% sarcosyl-50 μl lithiumcitrate, pH 6.8–0.1 M β-mercaptoethanol), and then 50 μl of buffer 2 (1 M Tris, pH 8.0–0.1 M EDTA-10% sodium dodecyl sulfate) was added and nucleic acids were extracted by phenol-chloroform, precipitated in 100% ethanol, and resuspended in H2O. Reverse transcription and PCR were done as outlined for the detection of HCV RNA or HBV DNA in serum, respectively.

Precautions and Controls. Recommended procedures for avoiding contamination were strictly followed to avoid false positive results (17). Appropriate positive and negative controls were always included. All samples were tested at least twice, and most tests were repeated three times to confirm the validity of the results.

Statistical Analysis. The results are given as mean ± SD. Fisher's exact test was used to determine the two-tailed statistical significance of differences between proportions in 2 × 2 tables. A P value ≤ 0.05 was used as an indicator for statistical significance. All calculations were performed using the SAS statistical package.

Results

Prevalence of HBV and HCV Infection in Serum of Patients with HCC. HBsAg was detected in 23 of the 81 patients tested (28%); five of these were positive for HBeAg. Antibodies to HBs or HBe were present in 18 (23%) HBsAg-negative patients; 40 (49%) patients with HCC had no HBV serological marker. Anti-HCV was found by ELISA in 21 (26%) patients; anti-HCV was confirmed by RIBA in 17
that none HBsAg and anti-HBc negative in serum and had tissue tumorous liver tissue. HBV DNA was found in the liver Anti-HBs and/or was found in 20 (25%) cases as detected by amplification was found anti-HCV negative and HBsAg positive the other was negative for all serological HBV markers. The Cirrhosis with and without HCC and with Alcoholic Hepatitis. A subset of HCV RNA in tumorous and additionally analyzed non- circulating HCV RNA in serum tested repeatedly negative for HCV RNA in serum. One patient positive for HCV RNA in serum by nested PCR is depicted in Table 3.

Correlation of HBV DNA and HCV RNA in Serum and Liver Tissue of Patients with HCC. Cancerous liver tissue specimens were available from 15 patients. HCV sequences in liver tissue were detected in six cases (40%) that tested positive for HCV RNA in serum. One patient positive for circulating HCV RNA in serum tested repeatedly negative for HCV RNA in tumorous and no additional analysis of non- tumorous liver tissue. HBV DNA was found in the liver tissue of seven patients (46.6%); six of those patients were positive for circulating HBV sequences. The remaining patient tested HBsAg and anti-HBc negative in serum and had concomitant HCV RNA in liver tissue and serum.

In summary, either hepatitis B or hepatitis C viremia was present in 38 patients (47%). Coinfection based on PCR results was observed in three patients (4%). It is noteworthy that none of these three patients exhibited HBsAg and anti- HCV positivity simultaneously. Two patients tested anti- HCV positive; one patient was solely anti-HBc positive and the other was negative for all serological HBV markers. The third patient was found anti-HCV negative and HBsAg positive. Of the remaining 43 patients, 16 had antibodies to HBC and/or HBs. No viral marker was found in 27 (33%) patients; 12 of these patients had alcoholic cirrhosis, 14 patients had cirrhosis without known identifiable origin or risk factor, and one patient had HCC detected in a histologically normal liver. Circulating HBV or HCV sequences were not observed in any of the four noncirrhotic patients.

Prevalence of Viral Markers in Patients with Alcoholic Cirrhosis with and without HCC and with Alcoholic Hepatitis. A subset of 32 patients with HCC and underlying alcoholic cirrhosis was compared to 35 patients with alcoholic cirrhosis alone, and to 35 patients with alcoholic hepatitis. Severity of liver disease was not significantly different in the HCC and the cirrhotic groups as assessed by both the Child-Pugh classification and the requirement for blood transfusions due to complications of liver cirrhosis (Table 1). No significant difference of anti-HCV ELISA positivity prevailed between cases with alcoholic cirrhosis and HCC and cirrhosis alone (seven (22%) and five (14%), respectively), but all but one patient with alcoholic hepatitis were negative with the anti-HCV ELISA (P < 0.05). However, as shown in Table 4, anti-HCV ELISA seropositivity was not confirmed in patients with alcoholic cirrhosis alone or alcoholic hepatitis as indicated by negative testing by RIBA and HCV RNA. In contrast, 6 of 7 (86%) anti-HCV ELISA-positive patients with HCC were confirmed by RIBA and HCV RNA in serum.

Six (19%) patients with HCC were positive for circulating HCV RNA as compared to one patient (3%) with alcoholic cirrhosis alone (who tested negative with anti-HCV ELISA) (P < 0.05). HCV RNA was not observed in patients with alcoholic hepatitis (P < 0.01). As shown in Table 4, HBV DNA was present in six cases with HCC (19%) as compared to one patient (3%) with alcoholic cirrhosis alone (P < 0.05) and none in the alcoholic hepatitis group (P < 0.01).

Discussion

Our study shows that 47% of 81 patients with HCC are infected with either HBV or HCV. HCV and HBV were each present in 25%, while coinfection occurred in 3% of patients. The prevalence of HBV or HCV in patients with HCC is lower than the nearly 90% reported recently from Spain (18). In the Spanish study, 36% had HBV DNA and 62% had HCV RNA in serum. Fourteen % of Spanish patients have had blood transfusions as an identifiable potential source of HCV infection, suggesting that most of the cases were due to community-acquired hepatitis. On the basis of studies in blood donors with first generation anti-HCV ELISA, a higher rate of anti-HCV seropositivity was reported from Spain (0.9%) than from Germany (0.4%) (19–20). Although this assay has limited specificity, it suggests a higher frequency of HCV infection in the general population of Spain and consequently in patients with HCC.

Of the 15 patients with HCC from whom liver tissue was available for examination, 6 had HCV RNA in tumorous liver tissue and correlating HCV RNA in serum. One additional case was found HCV RNA positive in serum but negative in tumorous and nontumor liver tissue. Therefore, testing for HCV sequences in cancerous tissue did not increase the number of HCV-related HCCs in our study. These findings are in line with other reports showing that genomic HCV RNA in cancerous liver tissue was detected almost exclusively in the presence of circulating HCV particles (21–22). Testing liver tissue for the presence of HCV RNA could not differentiate between tissue-derived and serum-derived HCV. Sheu et al. (22) argued that presence of negative HCV RNA strands as a replicative intermediate suggests the presence of HCV in hepatocytes. However, the replication of HCV in lymphocytes has been shown (23), and detection of HCV RNA by PCR might be derived from infected lymphocytes infiltrating the liver. To fully understand HCV infection in HCC, additional studies with the in situ hybridization technique will be necessary to visualize HCV within the morphological context.

HBV DNA sequences were detected in liver from 7 of 15 cases (47%). One of these cases tested negative for serological HBV markers and circulating HBV DNA. The

<table>
<thead>
<tr>
<th>HBV status</th>
<th>Anti-HCV positive (ELISA)</th>
<th>Anti-HCV positive (RIBA)</th>
<th>Anti-HCV negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg positive</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td>22 (27)</td>
<td>23 (28)</td>
</tr>
<tr>
<td>Anti-HBs and/or anti-HBc positive</td>
<td>7 (9)</td>
<td>6 (7)</td>
<td>11 (14)</td>
<td>18 (23)</td>
</tr>
<tr>
<td>HBV negative*</td>
<td>13 (16)</td>
<td>10 (12)</td>
<td>27 (33)</td>
<td>40 (49)</td>
</tr>
<tr>
<td>Total</td>
<td>21 (26)</td>
<td>17 (21)</td>
<td>60 (74)</td>
<td>81 (100)</td>
</tr>
</tbody>
</table>

* Second generation anti-HCV ELISA.
* No serological marker of hepatitis B virus infection.
Table 3  Relationship of serological markers of HBV and HCV infection and combined results of PCR testing in serum and liver tissue of 81 patients with HCC

<table>
<thead>
<tr>
<th>Anti-HCV ELISA</th>
<th>Serological HBV markers</th>
<th>n (%) of total</th>
<th>HCV RNA (% of group)</th>
<th>HBV DNA (% of group)</th>
<th>HCV RNA and HBV DNA (% of group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HCV positive</td>
<td>HBsAg (+)*a</td>
<td>1 (1)</td>
<td>1 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>(n = 21)</td>
<td>HBsAg (-)/anti-HBc (+)</td>
<td>7 (9)</td>
<td>6 (86)</td>
<td>1 (14)</td>
<td>1 (14)</td>
</tr>
<tr>
<td></td>
<td>HBV (−)</td>
<td>13 (16)</td>
<td>11 (85)</td>
<td>1 (8)</td>
<td>1 (8)</td>
</tr>
<tr>
<td>Anti-HCV negative</td>
<td>HBsAg (+)</td>
<td>22 (27)</td>
<td>1 (4)</td>
<td>18 (82)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>(n = 60)</td>
<td>HBsAg (-)/anti-HBc (+)</td>
<td>11 (14)</td>
<td>0 (0)</td>
<td>1 (9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>HBV (−)</td>
<td>27 (33)</td>
<td>1 (4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total (%)</td>
<td>81</td>
<td>20 (25)</td>
<td>21 (26)</td>
<td>3 (4)</td>
<td></td>
</tr>
</tbody>
</table>

*a +, positive; −, negative; HBV(−), all HBV serological markers negative.

Table 4  Prevalence of serum viral markers in patients with alcoholic liver disease with and without hepatocellular carcinoma

<table>
<thead>
<tr>
<th>anti-HCV</th>
<th>ELISA n (%)</th>
<th>RIBA n (%)</th>
<th>HCV RNA n (%)</th>
<th>HBsAg n (%)</th>
<th>anti-HBc n (%)</th>
<th>HBV DNA n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl-HCC* n = 32</td>
<td>7 (22)</td>
<td>6 (19)</td>
<td>6 (19)</td>
<td>7 (22)</td>
<td>10 (31)</td>
<td>6 (19)</td>
</tr>
<tr>
<td>Cln = 35</td>
<td>5 (14)</td>
<td>0</td>
<td>1 (3)</td>
<td>1 (3)</td>
<td>8 (23)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>AHn = 35</td>
<td>1 (3)</td>
<td>0</td>
<td>0</td>
<td>2 (6)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*a Cl-HCC, HCC with underlying cirrhosis.

References

Hepatitis C virus, alcoholic cirrhosis, and hepatocellular carcinoma.

T Goeser, H M Müller, C Solbach, et al.