Association between Hepatitis C Virus and Hepatocellular Carcinoma Using Assays Based on Structural and Nonstructural Hepatitis C Virus Peptides

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ABSTRACT

Stored sera from 181 Greek patients with hepatocellular carcinoma (HCC), 35 patients with metastatic liver cancer, and 416 hospital controls with diagnoses other than malignant neoplasm or liver disease were examined with first and second generation hepatitis C virus (HCV) enzyme immunoassays as well as with five HCV supplemental assays based on structural and nonstructural HCV peptides. Second generation HCV enzyme immunoassays were more sensitive than first generation assays. However, both assays had suboptimal specificity using the standard reactivity criterion (absorbance of sample to cutoff > 1.0). Specificity was improved by centrifugation and by using a sample’s optical density and cutoff ratio > 3.0 or supplemental assays; in this instance the prevalence of antibodies to HCV was 13.3% (24 of 181), 0 (0 of 35), and 1.4% (6 of 416) in HCC, metastatic liver cancer, and hospital controls, respectively. A similar estimation of prevalence of antibody to HCV in HCC (12.5% or 4 of 32) was obtained when the recombinant immunoblot assay, second generation, was used to screen a random sample of HCC patients. The relative risk linking HCV to HCC was estimated as 10.4 (95% confidence interval, 4.2–26.0; P < 0.0001). These data suggest that the prevalence of antibodies to HCV in HCC using stored sera has been previously overestimated even though the evidence of a causal association of HCV with HCC persists.

INTRODUCTION

Hepatocellular carcinoma HCC is one of the most common cancers worldwide with an estimated 500,000 to 1 million new cases/year and incidence rates varying from minimal to more than 100 cases/100,000 persons/year (1). The tumor is especially common in sub-Saharan Africa and certain regions of Asia. There is substantial evidence that HCC is causally related to hepatitis B virus infection, aflatoxin consumption, ethanol intake, and possibly tobacco smoking (2–4). Several other factors may be involved in the etiology of HCC but the fraction of HCC cases attributable to these factors is limited (5). Liver cirrhosis of any type, including cirrhosis associated with genetic diseases such as hemochromatosis, α1-antitrypsin deficiency, and tyrosinemia, increases the risk of HCC development (6, 7). Since non-A, non-B hepatitis is one of the main causes of cirrhosis, it has been suggested that non-A, non-B hepatitis may be associated with HCC (8, 9).

The cloning of HCV and the development of an assay for anti-HCV against the nonstructural HCV protein c100 (10, 11) led to studies which have indicated that the prevalence of anti-HCV among patients with HCC may be as high as 76% (12–31). However, the sensitivity and specificity of the anti-HCV assay based on c100, especially for stored sera, have been questioned on several lines of evidence (32). Therefore the prevalence of anti-HCV in HCC and the extent of the HCV-HCC association have not been adequately quantified.

In a large case-control study that was undertaken in Athens, Greece, and was specifically designed to explore the viral etiology of HCC (2, 3, 33), we have recently shown that only strongly anti-c100 reactive specimens are specifically associated with HCC (22). The recent advances in the serological diagnosis of HCV infection using recombinant and synthetic peptides derived from structural and nonstructural regions of the HCV genome (34, 35) provided the opportunity to further explore the specificity and strength of the association between HCV and HCC.

PATIENTS AND METHODS

Subjects. The subjects of the present study have been described in previous publications that examined the role of hepatitis B virus and other factors in the origin of HCC (3, 33). In the present analysis, study subjects were 181 patients with a final diagnosis of HCC (106 were histologically established whereas 75 were confirmed only by elevated α-fetoprotein values, ≥ 600 µg/liter) who were admitted between April 1976 and October 1984 to 9 major hospitals in Athens, Greece. Control subjects were 416 hospitalized patients with diagnoses other than malignant neoplasms or liver disease. A second group of controls consisted of 35 MLC patients who were, initially, suspected of having HCC but later shown to have metastatic cancer (all with a known primary site). Patients with MLC form a unique comparison group, since these patients share with HCC patients a disease pattern that is similar in severity and history of hospitalization. Moreover the use of MLC cases provides an unusual insight into the biological specificity of the anti-HCV assays (22, 36).

All subjects were Caucasians of Greek nationality and residence. The three patient populations were similar with respect to years of schooling (an indicator of socioeconomic status) and place of birth (urban, semiurban, or rural), but their distributions by age and gender were different.

Laboratory Methods. Sera were stored at −25°C for 7 to 15 years. However, the duration of sera storage was similar in the three groups. Hepatitis B serology was done by radiolmmunoassays in 1985 as reported previously (3). Determination of anti-HCV was undertaken in 1990 and repeated in 1991. Anti-HCV testing in 1990 was done by a first generation EIA based on the recombinant HCV peptide c100, derived from the nonstructural (NS5) region of the putative HCV genome, expressed in yeast as a SOD fusion protein (Ortho HCV Antibody ELISA Test Systems, Raritan, NJ) (11, 22, 36).

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3 The abbreviations used are: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MLC, metastatic liver cancer; SOD, superoxide dismutase; SHCVa, supplemental HCV assays; RIBA-2, second generation recombinant immunoblot assay; EIA, enzyme immunoassay(s); EIA-1, first generation EIA; EIA-2, second generation EIA; CKS, cytidine monophosphate-2-keto-3-deoxyoctonate synthetase; S/CO, absorbance of the sample to cutoff; anti-HCV, circulating antibodies to HCV.
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Because of the uncertainties surrounding HCV serology (32), anti-HCV testing was repeated in 1991 using first and second generation EIA from Abbott GmbH Diagnostica, Wiesbaden, Germany. EIA-1 (35) uses the recombinant HCV c100 solid phase antigen expressed in yeast as a SOD fusion protein. EIA-2 is based on recombinant HCV nonstructural peptides, derived from NS4 and NS3 (putative protease) regions of the HCV genome, and on a recombinant structural peptide (putative core) derived from the 5’ end region of the HCV. Accordingly, the following antigens are coated onto a polystyrene bead of the HCV EIA, second generation: (a) the c100 (NS4) expressed in yeast as a SOD fusion protein; (b) a recombinant HCV peptide EIA based on the putative core HCV protein expressed in E. coli as a CKS fusion protein containing the peptide 33c (35c) and parts of the c100 (NS4); and (c) the pHCV 34 (putative core) expressed in E. coli as a CKS fusion protein; this protein has been also designated c22-3.

The specimens were tested in duplicate by EIA-1 and EIA-2 and only repeatedly reactive samples were considered positive. In an attempt to improve the specificity of testing by EIA-1 and EIA-2 the specimens were tested as crude sera as well as after centrifugation at 5000 rpm for 20 min. Every repeatedly reactive sample with either EIA-1 or EIA-2 after centrifugation was further tested by SHCVA (Abbott Labs, North Chicago, IL). The following SHCVA were used: (a) a recombinant HCV peptide EIA based on the putative core HCV protein expressed in E. coli as CSS fusion protein (CSS-core EIA); (b) a recombinant HCV peptide EIA based on the putative protease (NS3) protein expressed in E. coli as CSS fusion protein (CSS-33c EIA); (c) a synthetic peptide EIA based on the combination of two peptides sp65/67 from the c100 (sp 65/67 EIA) (35); (d) a synthetic peptide EIA based on the synthetic peptide sp57 from the putative core (sp75 EIA); and (e) a solution-blocking or inhibition assay based on a recombinant HCV antigen expressed in E. coli as a CSS fusion protein containing 256 of the 363 amino acids of the c100 (CSS-c100) (35).

Table 1. Patterns of reactivities of screening and supplemental HCV assays

<table>
<thead>
<tr>
<th>Pattern</th>
<th>CKS-core EIA</th>
<th>CKS-33c EIA</th>
<th>sp65/67 EIA</th>
<th>sp75 EIA</th>
<th>CKS-c100 inhibition assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

EIA-1* and EIA-2, first and second generation enzyme immunoassays developed by Abbott GmbH Diagnostica, respectively; EIA, enzyme immunoassay; ND, not done.

All supplemental EIA were tested in a protocol similar to that for the EIA-1 and EIA-2 according to the manufacturer’s instructions. Every sample was considered reactive when the S/CO ratio was ≥1. The HCV inhibition assay was considered reactive when the absorbance of the sample after adding the CKS-c100 was decreased ≥50% compared with the base-line absorbance (35).

The algorithm of the anti-HCV testing by EIA-1, EIA-2, and SHCVA in order for a specimen to be considered reactive is shown in Table 1. Briefly, any sample repeatedly reactive by EIA-1 or EIA-2 was tested for CKS-core, CKS-33c, and sp65/67. Every sample reactive to CKS-33c (any combination with CKS-core and sp65/67) was considered as confirmed reactive. Samples reactive with CKS-core (only) or sp65/67 (only) were further tested by the sp75 EIA or the CKS-c100 inhibition assay, respectively, in order to confirm anti-HCV reactivity. Samples reactive to both CKS-core and sp65/67 were tested by the sp75 EIA and when this was reactive the specimen was considered confirmed reactive. If sp75 was not reactive the specimens were further tested by the CKS-c100 inhibition assay and when the percentage of neutralization was ≥50% the sample was considered confirmed reactive.

A random sample of 32 HCC patients and 13 hospital controls was also tested by RIBA-2 (Chiron, Emeryville, CA) (34). Antibody reactivity in RIBA-2 was assessed by comparison with a weak and strong IgG positive control, including a SOD negative control, and it was rated as −, ±, 1+, 2+, 3+, and 4+. A positive band was rated 1+ or greater. A sample was considered as reactive (+) when 1+ or greater reactivity existed in any of the two recombinant antigens and indeterminate when an 1+ or greater reactivity was present in one recombinant antigen. Evaluation of RIBA-2 was done blindly to the EIA-1 or EIA-2 results.

Results

Prevalence of anti-HCV by Screening and Supplemental Immunoassays. Table 2 shows the distribution of 181 patients with hepatocellular carcinoma, 35 patients with metastatic liver cancer, and 416 hospital controls by S/CO ratio in anti-HCV EIA-1 and anti-HCV EIA-2 performed before and after sera centrifugation. It is evident that the majority of samples had S/CO ratios between 1.0 and 2.99 irrespective of the group or the assay used. However, a S/CO ratio ≥3.0 clearly differentiated HCC patients from MLC and hospital controls using anti-HCV EIA-1. The same S/CO ratio plus the centrifugation step were necessary in order to eliminate some apparently nonspecific results in the MLC group according to the anti-HCV EIA-2.

All centrifuged sera found reactive for anti-HCV (S/CO ≥ 1.0) with either EIA-1 or EIA-2 were tested with SHCVA. All sera found reactive with EIA-1 but not reactive with EIA-2 were confirmed as nonreactive. Table 3 shows the results of SHCVA.

Table 2. Comparison of first and second generation EIA reactivity among HCC patients, MLC patients, and controls according to S/CO ratio

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>≤0.99</th>
<th>1.0–2.99</th>
<th>≥3.0</th>
<th>Test conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC n (%)</td>
<td>181</td>
<td>34 (18.8)</td>
<td>127 (70.2)</td>
<td>20 (11.0)</td>
<td>Anti-HCV EIA-1 before centrifugation</td>
</tr>
<tr>
<td>MLC n (%)</td>
<td>35</td>
<td>2 (5.7)</td>
<td>33 (94.3)</td>
<td>0 (0.0)</td>
<td>Anti-HCV EIA-1 before centrifugation</td>
</tr>
<tr>
<td>Controls n (%)</td>
<td>416</td>
<td>121 (29.1)</td>
<td>288 (69.2)</td>
<td>7 (1.7)</td>
<td>Anti-HCV EIA-1 before centrifugation</td>
</tr>
<tr>
<td>HCC n (%)</td>
<td>181</td>
<td>47 (26.0)</td>
<td>115 (63.5)</td>
<td>19 (10.5)</td>
<td>Anti-HCV EIA-1 after centrifugation</td>
</tr>
<tr>
<td>MLC n (%)</td>
<td>35</td>
<td>2 (5.7)</td>
<td>33 (94.3)</td>
<td>0 (0.0)</td>
<td>Anti-HCV EIA-2 before centrifugation</td>
</tr>
<tr>
<td>Controls n (%)</td>
<td>416</td>
<td>179 (43.0)</td>
<td>228 (54.8)</td>
<td>9 (2.2)</td>
<td>Anti-HCV EIA-2 before centrifugation</td>
</tr>
<tr>
<td>HCC n (%)</td>
<td>181</td>
<td>13 (7.2)</td>
<td>138 (76.2)</td>
<td>30 (16.6)</td>
<td>Anti-HCV EIA-2 after centrifugation</td>
</tr>
<tr>
<td>MLC n (%)</td>
<td>35</td>
<td>0 (0.0)</td>
<td>31 (88.6)</td>
<td>5 (9.2)</td>
<td>Anti-HCV EIA-2 after centrifugation</td>
</tr>
<tr>
<td>Controls n (%)</td>
<td>416</td>
<td>85 (20.4)</td>
<td>320 (76.9)</td>
<td>11 (2.6)</td>
<td>Anti-HCV EIA-2 after centrifugation</td>
</tr>
<tr>
<td>HCC n (%)</td>
<td>181</td>
<td>52 (28.7)</td>
<td>105 (58.0)</td>
<td>24 (13.3)</td>
<td>Anti-HCV EIA-2 after centrifugation</td>
</tr>
<tr>
<td>MLC n (%)</td>
<td>35</td>
<td>0 (0.0)</td>
<td>35 (100.0)</td>
<td>0 (0.0)</td>
<td>Anti-HCV EIA-2 after centrifugation</td>
</tr>
<tr>
<td>Controls n (%)</td>
<td>416</td>
<td>183 (44.0)</td>
<td>224 (53.8)</td>
<td>9 (2.2)</td>
<td>Anti-HCV EIA-2 after centrifugation</td>
</tr>
</tbody>
</table>

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for all sera found reactive with anti-HCV EIA-2 after centrifugation, by the level of S/CO ratio. It is evident that the use of SHCVA has not completely resolved the problem of discriminating specific from nonspecific anti-HCV reactivity in levels of S/CO ratios from 1.0 to 2.99 where anti-HCV reactive samples appeared to occur more frequently in MLC than in HCC. However, the use of a S/CO ratio ≥3.0 was sufficient not only to differentiate anti-HCV status in HCC and MLC but also to generate perfect agreement of EIA-2 and SHCVA in HCC cases.

Based on these data the prevalence of anti-HCV by EIA-2 was estimated to be 13.3% (24 of 181), 0 (0 of 35), and 1.4% (6 of 416) in HCC, MLC, and hospital controls, respectively (Table 3).

Prevalence of Anti-HCV by RIBA-2. A random sample of our sera from HCC cases and hospital controls was tested by RIBA-2. Selection of sera was blind with respect to the anti-HCV status or any other serological information. The prevalence of anti-HCV by RIBA-2 was 12.5% (4 of 32) and 0 of 13 in HCC and hospital controls, respectively. All RIBA-2 reactive samples presented S/CO ratio ≥3.0 by EIA-1 and/or EIA-2 while only 1 of 41 RIBA-2 nonreactive samples presented a S/CO ≥3.0 according to EIA-1 and/or EIA-2. No sample was found reactive to SOD or scored as indeterminate.

Magnitude of the Association of HCV and HCC. The relative risk (odds ratio) for HCC among subjects with confirmed anti-HCV reactivity, compared to anti-HCV negative subjects, was 10.4 (95% confidence interval, 4.2-26.0; P < 0.0001).

DISCUSSION

The present study allows a better identification of anti-HCV prevalence in HCC patients and controls. A serious limitation of screening HCV serology in various patient groups relates to the detection of a variable proportion of weakly reactive samples. The low specificity of the weakly reactive anti-HCV reactions in enzyme immunoassays has been attributed to various factors such as autoimmune chronic active hepatitis, rheumatoid arthritis, paraproteinemia, malaria, cross-reaction with the fusion protein SOD, heat inactivation of sera, prolonged storage, and repeat cycles of thawing and freezing (39-44). Increased γ-globulin levels have been proposed as one of main reasons of nonspecificity in HCC because of the coexisting cirrhosis. However, it has been shown that hyperglobulinemia is unlikely to account alone for the nonspecificity of anti-HCV testing in HCC cases (45). A common strategy to overcome specificity problems of the screening assays is the use of supplemental or confirmatory assays. However, the use of a battery of supplemental assays with excellent performance in other settings (35, 46) was not entirely satisfactory in this study. On the basis of this study the use of anti-HCV supplemental assays in stored sera may be limited to samples, screened by EIA-2, with S/CO ratios ≥3.0.

The association of anti-HCV with HCC has been examined earlier in several studies (12-31). On the basis of the present study it appears that the anti-HCV prevalence in HCC cases and the proportion of HCC cases attributable to HCV may be inflated in many of the studies. This was also suggested by other authors (47), on the basis of a very small study. This conclusion does not exclude the possibility that a substantial part of anti-HCV prevalence variation among HCC cases may be due to other reasons such as geographical, racial, or other differences.

In conclusion, the present analysis undertaken with second generation screening assays and a battery of supplemental tests, provides insight into the sensitivity and specificity of the currently available means of detecting chronic HCV infection and suggests that the relative risk linking anti-HCV to HCC is elevated but not overwhelmingly so. Case-control studies incorporating recent advances in the diagnosis of chronic HCV infection with polymerase chain reaction may further refine the parameters of the association by allowing a distinction between active and nonactive (past) HCV infection (48, 49).

REFERENCES

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