OCCULT HEPATITIS B VIRUS INFECTION IN PATIENTS WITH CHRONIC LIVER DISEASE DUE TO HEPATITIS C VIRUS AND HEPATOCELLULAR CARCINOMA IN BRAZIL

Fernanda BRANCO¹, Angelo Alves de MATTOS¹, Gabriela Perdomo CORAL¹, Bart VANDERBORGHT², Diogo Edele SANTOS¹, Paulo FRANÇA² and Cláudio ALEXANDRE¹

ABSTRACT – Background: The prevalence and consequences of occult HBV infection in patients with chronic liver disease by HCV remain unknown. Aims: To evaluate the prevalence of occult HBV infection in a population of HCV-infected patients with hepatocellular carcinoma. Methods: The serum samples were tested for HBV DNA by nested PCR and liver tissue analysis was carried out using the immunohistochemical technique of 66 HBsAg-negative patients: 26 patients with chronic hepatitis by HCV (group 1), 20 with hepatocellular carcinoma related to chronic infection by HCV (group 2) and 20 with negative viral markers for hepatitis B and C (control group). Results: Occult HBV infection was diagnosed in the liver tissue of 9/46 (19.5%) HCV-infected patients. Prevalence of occult B infection was evaluated in the HCV-infected patients with and without hepatocellular carcinoma, and there were seven (77.7%) of whom from group 2, conferring a 35% prevalence of this group. No serum sample was positive for HBV DNA in the three groups. Conclusion: Occult infection B is frequently detected in liver tissue of HCV-infected patients, especially in cases of hepatocellular carcinoma. However large studies are needed to confirm that co-infection could determine a worse progress of chronic liver disease in this population.HEADINGS – Carcinoma, hepatocellular. Hepatitis B virus. Hepatitis C virus. Liver diseases.

INTRODUCTION

Hepatitis B and C viruses (HBV and HCV) are the main etiological agents of chronic hepatitis related to the emergence of liver cirrhosis and hepatocellular carcinoma (HCC)¹⁴, ²⁶.

Despite careful investigations into the etiological factors for chronic liver disease, in 5%-10% of cases no etiological factor is detected²⁷. However, this index may be smaller if molecular tests are performed in search of occult infection with HBV in this population²⁵, ²⁶.

By occult infection with HBV it is meant an infection in which there is positivity for the viral DNA in the serum or liver tissue, as diagnosed by hybridization techniques or PCR or by the presence of HBV antigens in liver tissue by immunohistochemistry, despite absence of HBsAg in the serum. Described many years ago, even before the finding of HCV⁶, ¹⁹, the infection’s true prevalence, physiopathogeny and clinical impact are still a matter of debate¹, ², ⁵, ¹¹, ¹³, ¹⁵.

The prevalence of co-infection, is about 15%-30%, and 40%-50% when serum and liver tissue are tested, respectively⁷, ⁸, ¹³, ¹⁵, ³⁶ and differs with sensibility of test used.

The importance of an association between HBV and HCV lies in the fact that although HBV viral load in occult infection is often low, a greater likelihood of progression to cirrhosis and to HCC is observed when these patients are compared with those infected only with HCV⁷, ⁹, ³⁶, ³⁷.
Identification of the HBV genome has been reported in liver tumors of patients who are HCV-positive and HBsAg-negative in the serum\(^{32, 34, 37, 38}\). The rate of occult infection in HCV-positive patients with HCC can be as high as 76%\(^{241}\).

The presence of viral DNA integration in the cells of non-tumoral liver tissue\(^{34, 37}\) in patients with HCC suggests that the genomic integration precedes the development of neoplasia. Thus, chronic infection with HBV may be correlated with the emergence of HCC even in the absence of liver cirrhosis\(^{38}\).

The consequences of occult HBV infection in patients with chronic liver disease by HCV remain unknown. Despite evidence that co-infection may accelerate the progress to liver disease\(^{9, 41}\), be related to the emergence of HCC\(^{34, 37, 38}\), and adversely influence the response to HCV treatment\(^{7, 9, 13, 41}\), other studies show that occult infection does not interfere with the natural history of the disorder in this population\(^{32, 15, 16, 22}\).

The present study was designed to evaluate the importance of occult HBV in patients with chronic infection by HCV and hepatocellular carcinoma in Brazil.

**METHODS**

From February 2003 to May 2004, serum and liver tissue samples from 66 patients, coming from the “Complexo Hospitalar Santa Casa de Porto Alegre”, in Porto Alegre, RS, Brazil, were prospectively assessed.

The patients were divided in three groups: group 1 – 26 consecutive patients with chronic infection by HCV, who were in pre-treatment evaluation in the Hepatology Unit or during hospitalization for liver transplantation; group 2 – 20 patients with chronic infection by HCV and HCC, evaluated consecutively at admission for liver transplantation, surgical resection of tumor, or liver nodule biopsy, and control group – 20 patients with negative hepatitis B and C viral markers in the serum, selected for cholecystectomy.

The patients in groups 1 and 2 did not present concomitant causes of chronic liver disease. Autoimmune hepatitis, hemochromatosis, alpha-1-antitrypsin deficiency, glycogen storage disease were excluded by negative results of anti-nuclear, anti-smooth muscle and anti-mitochondrial antibody and by normal values of α-1 antitrypsin, transferrin saturation and ceruloplasmin. The patients included in the study had no history of alcohol abuse, did not use potentially hepatotoxic drugs, and were not HIV-infected (measured by third-generation enzyme immunoassay, Cobas, Roche).

Inclusion criteria to select patients of group 2 were the following: two dynamic imaging techniques (computerized tomography and magnetic resonance imaging) showing focal liver lesion with arterial hypervascularization, or one of these positive imaging method associated with alpha-fetoprotein level above 400 ng/mL\(^{39}\). The diagnosis of HCC was confirmed by histological analysis in all cases.

Blood samples were collected at the same time of liver biopsy collection, in patients of groups 1 and 2, by biopsy with ultrasonographic-guided and wedge-shaped needle during hepatectomy or liver transplantation and, in group 3, by wedge biopsy during cholecystectomy.

**Serum viral markers**

HBsAg, total anti-HBc and anti-HBs were measured in duplicate by electrochemoluminescence (Elecsys 2010-Roche). Anti-HCV was detected by amplified electrochemoluminescence using a third generation technique (Vitros Eci, Johnson & Johnson) as recommended by the manufacturers.

**Qualitative analysis of HCV by RT-PCR**

The HCV Amplicor\(^{®}\) test of viral genome amplification was used (Roche Diagnostics Systems, Inc., Branchburg, NJ, USA) using reverse transcription polymerase chain reaction as recommended by the manufacturers and detection limit of 100 copies/mL.

**Quantitative analysis of HCV by RT-PCR**

Viremia was determined using the Amplicor HCV Monitor\(^{®}\) test, version 2.0 (Roche Molecular System Inc., Branchburg, NJ, USA). The detection limit of the test was 600 IU/mL\(^{31}\).

**HCV genotyping**

HCV genotyping was determined as described by McOMISH et al.\(^{29}\), using the product of RT-PCR in restriction enzyme assay by the restriction fragment length polymorphism (RFLP) technique.

**HBV DNA by nested PCR**

It was performed in the Laboratory of Molecular Biology of the Hepatology Service of the “Hospital Universitário Clementino Fraga Filho” of the Federal University of Rio de Janeiro, RJ, Brazil using the “in house” technique. DNA was extracted with Qiamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). B and C domains of polymerase was amplified in each DNA sample. In the first step an Outer PCR was a product of amplification, and in second assay an Inner PCR was the product of amplification. Limit of test detection was 200 copies/mL.

**Viral markers in liver tissue**

HBV antigens (HBsAg and HBcAg) were identified through primary antibodies (goat-anti hepatitis B surface antigen and rabbit-anti hepatitis B core antigen) and secondary antibody LSAB plus System (Dako). The immunologic signal of the presence of antigens was detected by a system based on streptavidin-biotin complex HRP (Dako). Positive and negative controls were used in each session\(^{12, 17}\).

**Histopathological analysis**

The samples were classified according to Metavir’s classification\(^{5}\) staging was defined as F0 (no fibrosis), F1 (fibrosis limited to portal tract), F2 (fibrosis with septa), F3 (severe fibrosis) and F4 (fibrosis with septa delimiting nodules). HCC staging was performed according to the Japanese classification\(^{28}\).

The analysis was carried out in all samples by a pathologist unaware of the status of occult B virus infection.

**Ethical considerations**

All patients or their family members signed an informed consent before inclusion in the protocol. The project was approved.
by the Ethical Committee of the “Complexo Hospitalar Santa Casa de Porto Alegre”.

**Statistical analysis**

The data were stored in the MS Access 2000 program and analyzed with resources of the SPSS 10.0 system (Statistical Package for Social Science).

The categorical variables were analyzed by Fisher’s exact test with the complementary resource of adjusted residues analysis.

The quantitative variables were analyzed by ANOVA with a complementary resource for Tukey’s multiple comparisons.

The level of significance used was 5%.

**RESULTS**

**Characteristics of the patients**

Characteristics of the patients are shown in Table 1.

Concerning laboratory tests, while patients of group 1 were similar to those of the control group as regards liver function tests (bilirubin, prothrombin time and albumin), they showed higher levels of aminotransferases and lower levels of platelets. When bilirubin, prothrombin time and albumin levels of groups 1 and 2 were compared, chronic liver disease was observed to be more severe in group 2.

In 17 of 26 patients with chronic liver disease by HCV, there was evidence of one or both risk factors, and in 9/17 (52.9%) such factors occurred more than 20 years ago. In the 20 patients in the group with HCC, risk factors were present in 15 patients, in 14 of whom they occurred more than 20 years ago ($P = 0.018$).

**Detection of HBV markers**

The HBV DNA was not detected in the serum of any of the 66 patients evaluated.

Occult B infection was diagnosed by immunohistochemistry through the detection of HBV antigens (HBsAg and/or HBcAg) in liver tissue in 9/46 (19.5%) HCV-infected patients and in 1/20 (5%) patients of the control group. The HBsAg was detected in isolation in liver tissue of 7/46 HCV-infected patients, and in 2 cases in association with HBcAg. No patient presented HBcAg in isolation in tissue. In the patient of the control group, both markers were detected.

As we analyzed the prevalence of occult infection in the HCV-infected population, we found that of the nine positive patients, seven (77.7%) were in group 2, OR = 6.46 ($P = 0.029$).

These results confer to occult B infection a prevalence of 35% in patients with HCV and HCC (Table 2).

**TABLE 1. Characteristics of the patients**

<table>
<thead>
<tr>
<th>Variables</th>
<th>HCV n=26 (%)</th>
<th>HCV-HCC n=20 (%)</th>
<th>Control n=20 (%)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age*</td>
<td>49.6 ±10.8</td>
<td>56.6 ±11.6</td>
<td>51.9 ±18.2</td>
<td>0.234</td>
</tr>
<tr>
<td>Gender**</td>
<td></td>
<td></td>
<td></td>
<td>0.062</td>
</tr>
<tr>
<td>Male</td>
<td>11 (42.3)</td>
<td>14 (70)</td>
<td>7 (35)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>15 (57.7)</td>
<td>6 (30)</td>
<td>13 (65)</td>
<td></td>
</tr>
<tr>
<td>Transfusion**</td>
<td>15 (53.8)</td>
<td>13 (65)</td>
<td>4 (20)</td>
<td>0.008</td>
</tr>
<tr>
<td>Drugs **</td>
<td>3 (11.5)</td>
<td>3 (13)</td>
<td>0 (0.0)</td>
<td>-</td>
</tr>
<tr>
<td>Laboratory tests*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>82.69 ±50.46</td>
<td>111.50 ±80.11</td>
<td>34.15 ±11.68</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>66.92 ±37.19</td>
<td>128.40 ±95.81</td>
<td>27.75 ±16.58</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BT (mg/dL)</td>
<td>1.108 ±1.294</td>
<td>1.960 ±1.268</td>
<td>0.720 ±0.438</td>
<td>0.002</td>
</tr>
<tr>
<td>BD (mg/dL)</td>
<td>0.312 ±0.414</td>
<td>0.735 ±0.656</td>
<td>0.230 ±0.266</td>
<td>0.002</td>
</tr>
<tr>
<td>FA (U/L)</td>
<td>85.42 ±29.28</td>
<td>122.95 ±70.45</td>
<td>99.10 ±58.86</td>
<td>0.053</td>
</tr>
<tr>
<td>TP (%)</td>
<td>85.26 ±13.64</td>
<td>72.10 ±12.67</td>
<td>86.4 ±12.52</td>
<td>0.005</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.26 ±0.577</td>
<td>3.53 ±0.75</td>
<td>4.04 ±0.60</td>
<td>0.001</td>
</tr>
<tr>
<td>Platelets (/mm$^3$)</td>
<td>198.840 ± 87.986</td>
<td>112.350 ±63.499</td>
<td>256.850 ±59.129</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**TABLE 2. Comparison of groups concerning immunohistochemistry**

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Immunohistochemistry (HBsAg and/or HBcAg)</th>
<th>OR (CI 95%)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>7</td>
<td>13</td>
<td>1 + 2</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>1</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

The presence of HBV antigens was tested in tumor tissue in 15 cases and in the non-tumor tissue in 20. The HBsAg was present in the tissue surrounding the tumor in seven cases, six in isolation and one concomitantly to HBcAg. In tumor tissue, among the 15 cases evaluated the HBsAg was detected in 5
cases, 4 of which in isolation and 1 in association with HBcAg. No patient presented antigen in the tumor without showing it in the surrounding tissue (Figure 1).

When the 46 HCV-infected patients with or without occult B infection were compared regarding biochemical parameters, greater liver dysfunction was observed in the co-infected group (Table 3).

When HCV-infected patients with and without co-infection were compared as regards probable time of HCV infection, 9 patients were found to be co-infected, 8 of whom evidencing the risk factors (blood transfusion and/or IV drug use), and in 7 they occurred more than 20 years ago (87.5%), as compared to 8 of the 24 patients (33.3%) without co-infection and with risk factors more than 20 years ago. Therefore, the length of HCV infection was significantly greater in patients with occult B infection than in those without it ($P = 0.013$).

Among the nine HCV-infected patients co-infected with occult HBV, six (66.6%) had markers of previous HBV infection. Five out of seven (71.4%) patients with HCC and occult B infection also presented these markers in the serum (two anti-HBc-positive and three anti-HBc and anti-HBs-positive), and in two cases no marker of previous exposure to HBV was found.

**HCV and occult infection**

HCV genotyping was assessed in 44 patients. Genotype 1 was present in 20 cases and genotypes 2 or 3 in 24 cases. Of the eight patients with HCV and occult B infection evaluated, genotype 1 was detected in four cases (50%) ($P = 1.0$). Of the 39 cases, mean viral load was 569.871 UI/mL (SD = 608.143 and median = 311.000 UI/mL) in patients without occult B infection and 222.293 UI/mL (SD = 325.403 and median = 117.118) $P = 0.119$ in patients with occult B infection.

Of the nine HCV-infected patients with occult B infection, eight showed a higher degree of activity (A2-A3), without attaining, though, a statistically significant difference as compared to patients without this type of infection ($P = 0.23$). Concerning liver fibrosis, all nine patients presented fibrosis degree between F3-F4, which was statistically significant as compared to the other patients without the infection ($P = 0.007$) (Table 4).

<table>
<thead>
<tr>
<th>TABLE 4. Comparison of degree of activity/fibrosis regarding immunohistochemistry (HBsAg and/or HBcAg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metavir (n=46)</td>
</tr>
<tr>
<td>Degree of activity*</td>
</tr>
<tr>
<td>A0-A1</td>
</tr>
<tr>
<td>A2-A3</td>
</tr>
<tr>
<td>Degree of fibrosis*</td>
</tr>
<tr>
<td>F0-F1-F2</td>
</tr>
<tr>
<td>F3-F4</td>
</tr>
</tbody>
</table>

*Variables appear as frequencies [percentage], Fisher’s exact test used

Of the seven patients with occult B infection in group 2, the tumor was well or moderately differentiated in three patient and poorly differentiated in other three. One case was not evaluated because of the presence of tumor necrosis. Among the 13 patients without occult infection, 6 presented well or moderately differentiated HCC, and 4, poorly differentiated. Two cases could not be evaluated due to lack of viable cells secondary to complete necrosis of the tumor following alcoholization bouts.

Of the 18 patients evaluated, the mean tumor diameter in the cases of occult HBV (2.74 cm) did not show a statistically significant difference ($P = 0.96$) as compared to patients with HCC without this infection (2.70 cm).
DISSCUSSION

In this study, we have investigated the prevalence of occult HBV infection in a population of HCV-infected patients at different stages of the disease, from chronic hepatitis to hepatocellular carcinoma, and we compared the findings with those of patients without chronic liver disease and without markers of previous exposure to B or C viruses.

The absence of HBV DNA in the serum of the 66 patients evaluated could be accounted for by the low viremia present in patients with occult B infection\(^{(4)}\). Other factors to be considered are: the finding, in a longitudinal study\(^{(9)}\), that viremia may in some cases occur intermittently, greater HBV DNA concentration in liver tissue samples as compared to serum samples\(^{(2, 6, 9, 23, 37)}\) and the geographical distribution of HBV infection, which is low in our community (0.4%)\(^{(39)}\).

In contrast, HBV DNA has been found in serum by PCR in 6.7% to 52%\(^{(13, 15, 16)}\) of HBsAg-negative patients with chronic HCV infection, in 5% to 76%\(^{(7, 10, 30, 31)}\) of patients with chronic liver disease of no defined etiology, and in 0%\(^{(15)}\) to 15%\(^{(21)}\) of healthy donors.

The prevalence of occult B infection in HCV-infected patients in the present study (19.5%) was above the one reported by some authors\(^{(9, 12)}\) and below the percentages reported by others\(^{(21, 28, 36, 38)}\), which can be as high as 76%\(^{(24)}\). It should be highlighted that the latter studies used PCR for viral DNA detection, a technique which is more sensitive than immunohistochemistry\(^{(4, 12)}\). It is not well established if failure to detect HBV antigens by immunohistochemistry in a few patients with occult B infection\(^{(9, 28)}\) is solely related to low viral replication, or if variations in samples size and the presence of viral mutations may play a role in these results.

In the present study, detection of HBsAg in liver tissue was more frequent than detection of HBeAg, suggesting that most patients were not in the replicative viral phase, which would be reflected by lower viremia. This finding is in agreement with other studies\(^{(20, 30, 31)}\) demonstrating that HBsAg is more sensitive than HBeAg in liver tissue.

Growing evidence suggest a high prevalence of occult B infection in HCV-infected patients with HCC\(^{(34)}\). When HCV-positive patients with and without HCC are compared, the prevalence of occult infection is 48% to 76% in the first group and 30%-40% in the second\(^{(9, 24, 34)}\).

The greater prevalence in this study of HBV antigens detected in patients with HCC could be ascribed to the role HBV may play in the hepatocarcinogenesis of patients with chronic HCV infection. This pathogenic synergism has been reported\(^{(37)}\).

In 4 of the 10 (40%) patients evaluated here (3 patients with HCV and 1 patient of the control group), occult B infection was diagnosed in the absence of any serologic marker of B virus. The explanation for this finding may be in the fact that patients with occult B infection would be infected with low levels of viral particles incapable of stimulating the immune system to form its antibodies\(^{(20)}\), or that, following acute infection patients present a progressive decline of these markers until their becoming undetectable in the serum\(^{(35)}\). Another hypothesis is the existence of mutating HBV strains which do not express neither antigens nor antibodies in the blood of this population of patients\(^{(28, 32)}\).

HCV mean viral load in patients with occult B infection was lower than in patients without occult infection in this study. MARISCAL et al.\(^{(28)}\) suggesting that the presence of HBV inhibits HCV replication. Nevertheless, the findings about HCV viral load in the presence of HBV reported in the literature are conflicting\(^{(9, 12, 13, 40)}\).

HCV patients co-infected with occult HBV presented biochemical parameters as well as a greater degree of liver fibrosis which suggest advanced stage of liver disease, as compared with patients solely infected with HCV. This finding is in keeping with the suggestion that HBV co-infection is associated with a more expressive clinical evolution leading faster to end stage liver disease and HCC developments. Nevertheless, in our study it can not be ruled out that the observed differences are due to the longer length of disease, thereby HCV patients are older and presented a longer length of infection as evaluated by the presence of the risk factors of blood transfusion and intravenous drug use.

In conclusion, it can be inferred that occult infection is a fact in our community, particularly in patients with HCV-related HCC. However, this is a cross-sectional study with a relatively small number of patients, therefore large studies are needed to confirm that co-infection could determine a worse progress of chronic liver disease in this population.
REFERENCES


