Analysis of occult hepatitis B virus infection in liver tissue of HIV patients with chronic hepatitis C

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Objective: Current data on the prevalence of occult hepatitis B virus (HBV) infection in HIV-positive individuals conflict. As occult HBV infection could have an impact on the outcome of liver disease in HIV-positive patients, we investigated a large number of HIV-positive/HBV-surface-antigen (HBsAg) negative subjects with hepatitis C virus (HCV) infection by using the ‘gold standard’ approach for occult HBV detection—analysis of liver DNA extracts.

Methods: The presence or absence of HBV DNA was determined by PCR testing of four different viral genomic regions in DNA extracts of needle liver biopsy specimens of 101 HBsAg negative individuals with HIV/HCV co-infection. HBV genotyping was performed by sequencing analysis of the preS-S gene in occult HBV isolates from 18 cases.

Results: Occult HBV infection was diagnosed in 42 of the 101 cases (41%). No clinically relevant difference was found between occult HBV-positive and -negative patients. HBV genotype D and A were detected, respectively, in 11 (61%) and 7 (39%) of 18 cases analysed.

Conclusions: Occult HBV infection frequently occurs in HIV/HCV co-infected patients indicating the importance of performing prospective studies able to clarify its clinical impact in these patients. HBV genotype A is highly prevalent in HIV-infected subjects with occult HBV infection in a similar way to HBsAg/HIV-positive individuals.

Keywords: chronic hepatitis, liver DNA analysis, occult HBV genotype, occult HBV infection, viral hepatitis co-infection

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Introduction

Hepatitis B virus (HBV) genomes may persist indefinitely in the liver of infected individuals even when the HBV surface antigen (HBsAg) has been cleared [1,2]. The molecular basis of this peculiar form of chronic viral infection — termed occult HBV infection — is related to the viral covalently-closed-circular DNA (HBV cccDNA), a long-lived HBV replicative intermediate that persists in the hepatocyte nuclei as a stable chromatinized episome and that serves as a template for gene transcription [3,4]. Much recent evidence has suggested that occult HBV infection may have a considerable clinical relevance in several categories of patients, particularly when a hepatitis C virus (HCV) infection co-exists [5–10]. In fact, occult HBV might negatively influence the response to interferon therapy [11–13] and probably favours or accelerates liver fibrosis progression in chronic hepatitis C infection [14–19]. Moreover, substantial data indicate that occult HBV infection is a major risk factor for hepatocellular carcinoma development in HCV patients, as it seems to maintain all of the potential pro-oncogenic properties commonly ascribed to the overt (HBsAg-positive) HBV infection [20–23]. The presence of occult HBV infection has been investigated quite extensively in HIV-positive individuals, but the results obtained differ considerably between studies. The published prevalence ranges from 0% to 89% [24,25], with a considerable number of other studies reporting results between those two extremes [26–34]. These discrepancies appear to be dependent mainly on the different sensitivities and specificities of the assays used in the various studies [10,35], all of which — in any case — limited testing for occult HBV to patient serum. Considering that viraemia is very low or undetectable in occult HBV infection even when sensitive methods are used, and assuming that the viral cccDNA reservoir is in hepatocytes, liver tissue extracts will provide the best evaluation of occult HBV prevalence in a defined set of patients [6,10].

We investigated the presence of HBV DNA in liver tissue from a large series of HBsAg-negative patients with HIV/HCV coinfection. In addition, we performed genotyping of the occult HBV isolates in consideration of the recently suggested epidemiological and clinical relevance of different HBV genotypes in HIV/HBsAg-positive patients.

Patients and methods

Patients

We studied liver specimens from 101 males : females, 81 : 20; mean age (years ± SD), 36.4 ± 7.25] HBsAg-negative HIV carriers with chronic HCV infection documented by both anti-HCV and HCV RNA-positive status. The cases were selected retrospectively from among the HIV/HCV-positive individuals attending four Italian Infectious Diseases Divisions — located in different geographic areas of the country — in the years 2001–2005. Selection was based on the following criteria: (i) percutaneous needle liver biopsy performed in all cases before starting any therapeutic treatment for HCV-related liver disease; (ii) availability of liver specimens for molecular analysis; (iii) documented complete set of serum HBV markers at the time of liver biopsy. Histological evaluation showed minimal or non-specific changes in 44 cases, mild chronic hepatitis in 35, severe chronic hepatitis in 13, and cirrhosis in 7 cases. Seventy nine of the 101 patients were positive for antibodies to HBV core antigen (anti-HBc) and 43 of them were also positive for antibodies to HBsAg (anti-HBs); the remaining 22 cases were negative for all HBV markers. The HCV genotype was available in 83 cases, 39 of whom belonged to genotype 1, 4 to genotype 2, 31 to genotype 3 and 9 to genotype 4. Seventy four individuals were and 27 were not on HAART at the time of liver biopsy. HAART treatment included lamivudine in 39 of the 74 cases, and no other antiviral capable of inhibiting HBV reverse transcriptase had been taken by any of the patients.

A portion of the liver specimens from 51 patients was formalin fixed and paraffin embedded for histological examination and the remainder was frozen at −80°C immediately after needle biopsy. The tissue specimens from the remaining 50 cases were formalin fixed and paraffin embedded in their entirety. The study protocol was performed according to the principles of the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital of Messina. Informed consent was also obtained from all patients.

HBV DNA analyses

DNA was extracted from the 51 frozen liver specimens [16,20] and from the paraffin embedded liver tissues according to standard procedures [36,37]. Briefly, two of 10-μm thick (1.5–2 cm long) sections were cut from each specimen and homogenized in 1 ml of xylene, kept at room temperature for 5 min and centrifuged at 14 000 rpm for 5 min. The supernatant was discarded, and the process repeated until all of the paraffin was removed. The pellet was washed twice with 1 ml absolute ethanol to remove residual xylene. Each change was preceded by vortexing and centrifugation at 14 000 rpm for 5 min at room temperature. Tissue samples were washed twice with distilled water to remove residual ethanol. Samples were treated with proteinase K (800 μg/ml) and incubated overnight at 50°C. After extraction with phenol/chloroform, nucleic acids were precipitated in 2 vols pure cold ethanol plus 1/10 volume of 3 M sodium acetate and 1 μg/ml glycogen. Nucleic acids were then resuspended and digested with pancreatic ribonuclease (100 μg/ml) followed by extraction with phenol/chloroform, and reprecipitation in pure cold ethanol.
DNA was resuspended in 50 µl 10 mmol/l Tris–HCl (pH 7.4), 1 mmol/LEDTA and its concentration was determined by spectrophotometry at 260 nm.

Occult HBV infection was assayed as previously described [16,20]. All liver DNA extracts were analysed for the presence of HBV genomes by performing four different in-house nested PCR amplifications to detect preS-S (S), precore-core (C), Pol, and X viral region, respectively. As we reported [16,20] and as also recommended by others [5,6], we considered the cases that showed positivity in at least two different viral genomic regions as HBV DNA positive. In consideration of the DNA degradation occurring in tissue after formalin fixation and paraffin embedding, we used sets of oligonucleotide primers for HBV detection that amplified small portions of viral genomic regions. These primers were complementary to conserved regions of HBV at the following 5'→3' positions, numbering from the EcoR1 restriction site of the HBV genotype D: core region, HBV5, 2021–2040; HBV8, 2385–2365; HBV7, 2048–2066; HBV29, 2289–2270; pol region, HBV9, 2414–2433; HBV3, 2951–2932; HBV11, 2424–2440; HBV28, 2834–2815; preS-S region, HBV30, 246–266; HBV25, 860–840; HBV26, 458–475; HBV2, 690–670; X region, HBV27, 1100–1120; HBV14, 1628–1608; HBV13, 1266–1286; HBV16, 1540–1521. Appropriate negative controls were included in each PCR, and direct sequencing of all amplified HBV sequences confirmed the specificity of the reactions. Finally, HBV genotyping was performed by sequencing analysis of the pre-S/S genomic region of isolates from 18 occult HBV-positive cases for which frozen liver tissues were available [38]. In fact, the partial DNA degradation occurring in formalin/paraffin treated specimens cannot allow amplification and sequencing of genomic regions as large as the HBV preS-S protein coding gene.

Table 1. Demographic, histological and clinical/virological characteristics of 101 HIV/HCV co-infected patients according to occult HBV status.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Occult positive 42/101</th>
<th>Occult negative 59/101</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male : female</td>
<td>36/6</td>
<td>45/14</td>
<td>NS</td>
</tr>
<tr>
<td>Mean age (years ± SD)</td>
<td>35.92 ± 6.91</td>
<td>36.81 ± 7.52</td>
<td>NS</td>
</tr>
<tr>
<td>Histologic diagnosis (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal changes</td>
<td>19</td>
<td>25</td>
<td>NS</td>
</tr>
<tr>
<td>Mild chronic hepatitis</td>
<td>15</td>
<td>20</td>
<td>NS</td>
</tr>
<tr>
<td>Severe chronic hepatitis</td>
<td>4</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>4</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>HBV genotypea (n)</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Anti-HBc ± anti-HBs positive</td>
<td>35</td>
<td>44</td>
<td>NS</td>
</tr>
<tr>
<td>HBV serum markers negative</td>
<td>7</td>
<td>15</td>
<td>NS</td>
</tr>
<tr>
<td>Alanine aminotransferase (unit/L ± SD)</td>
<td>147.07 ± 96.4</td>
<td>143.68 ± 105.98</td>
<td>NS</td>
</tr>
<tr>
<td>HAART (yes : no)</td>
<td>30/11</td>
<td>43/15</td>
<td>NS</td>
</tr>
<tr>
<td>Lamivudine (yes : no)</td>
<td>15/17</td>
<td>24/26</td>
<td>NS</td>
</tr>
</tbody>
</table>

aAvailable in 83 cases.

NS, Non-significant.

**Statistical evaluations**

Statistic analyses were evaluated using the Student’s t test and the χ-squared method.

**Results**

On the basis of the above mentioned criteria, we revealed occult HBV infection in 42 of the 101 cases investigated (41%) (Table 1). In particular, eight cases were positive for all four HBV genomic regions examined (S, Core, Pol, X); 14 cases were positive for three genomic regions (five for Core, Pol, X; four for Core, Pol, S; and five for Pol, S, X); 20 were positive for two genomic regions (six for Pol, X; six for Core, X; two for Core, S; and six for Core, Pol). No statistically significant difference was found between occult-positive and occult-negative cases in terms of age, sex, grade of histological damage, HCV genotype, presence of anti-HBV antibodies, alanine aminotransferase values, or HAART including or not including lamivudine (Fig. 1) (Table 1). HBV genotyping performed by sequencing analysis on isolates from 18 patients showed that 11 cases (61.1%) were infected with genotype D and 7 (38.9%) with genotype A of the HBV.

**Discussion**

There is evidence that occult HBV infection may have a relevant clinical impact in HIV patients. In fact, in these individuals the occult virus may reactivate inducing overt hepatitis B [39–41], and it seems to be capable of provoking frequent episodes of aminotransferase flares [27]. Moreover, considering that occult HBV infection is a factor that worsens the clinical outcome in HCV...
patients [6,10] it might be hypothesized that it is involved in the severe course of the liver disease frequently observed in cases with HIV/HCV co-infection [42]. A careful evaluation of the ‘occult HBV infection finding’ in HIV patients thus appears to be of importance. By means of the methods generally considered the gold standard for investigating occult HBV status, we found that >40% of Italian HIV patients with HCV-related liver disease are occult HBV carriers. This prevalence appears to be slightly higher than found previously in individuals with chronic hepatitis C and without HIV co-infection coming from the same geographic area (about one-third of these cases) [16,20]. In the present study, no significant difference was observed between occult HBV-positive and negative subjects. In particular, these two categories were very similar in terms of liver disease. However, it must be considered that both the young age and the low grade of liver histological damage of the great majority of the population examined do not allow a reliable evaluation of the clinical impact of occult HBV on HCV/HIV co-infected individuals, and only a prospective evaluation over time of these patients can clarify this fundamental issue. In accordance with previous reports, we found that a certain number of occult carriers were negative for all HBV serum markers. At the same time, we found that a certain number of occult carriers were fundamental issue. In accordance with previous reports, this latter result might reflect a true resolved infection with subsequent clearance of the virus, but we cannot rule out the possibilities of defective needle liver biopsy sampling or extra-hepatic reservoir districts of the occult viruses. Of note, genotyping analysis of the HBV isolates revealed that 39% of the patients examined were infected with genotype A viruses, whereas we recently observed that almost all the occult HBV strains from HIV-negative patients belong to genotype D which is the most common in the Mediterranean basin [38]. Interestingly, HBV genotype A was found to be highly prevalent in HIV-positive individuals with overt HBV co-infection in several geographic areas including Italy [43–46] confirming that in these subjects HBV infection often occurs in adulthood and is likely to be related to their lifestyle more than to intrafamilial transmission in infancy.

In conclusion, occult HBV infection is a frequent finding in cases with HIV/HCV co-infection making the virological scenario characterizing this category of patients even more complex than formerly believed. In this context, prospective studies – methodologically correct also from the diagnostic point of view – are needed to clarify the possible role exerted by this cryptic infection on the outcome of the liver disease and development of hepatocellular carcinoma.

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**References**

Frequent chronic hepatitis B virus infection in HIV-infected patients: a systematic review of the epidemiologic evidence.


Oncogene 2006; 5:2099–2101.


