HEPATOLOGY

Liver iron deposits in hepatitis B patients: Association with severity of liver disease but not with hemochromatosis gene mutations

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Abstract
Background and Aims: Iron deposits in the liver and abnormalities in serum iron biochemistry are frequently observed in patients with chronic liver diseases, but data for patients with hepatitis B virus (HBV) infection are scarce. Moreover, the role of HFE mutations in iron deposits in this condition remains unknown. The aim of the present study was to determine the prevalence of serum iron biochemical abnormalities and iron deposits in the liver of chronic HBV patients, and to evaluate the consequences for the activity and severity of liver disease. Additionally, we studied the role of HFE gene mutations in iron deposits.

Methods: Eighty-one male non-cirrhotic HBV patients were studied. Serum iron biochemistry, liver enzymes and C282Y/H63D mutations were investigated. Liver biopsies were scored for necroinflammatory activity (histological activity index [HAI]), fibrosis and iron deposits.

Results: Elevated transferrin saturation (TS) was found in 27.1% of patients and liver iron deposits in 48.7%; these deposits were mild in 68.4% and moderate in 31.6%. Patients with liver iron deposits exhibited significantly higher scores for HAI and fibrosis than those without iron deposits. HFE mutations were identified in 23.4% of patients (14 H63D heterozygotes, four H63D homozygotes, one compound mutation). No difference in the prevalence of C282Y and H63D mutations was observed between HBV patients (1.2% and 23.4%, respectively) and the general population (4.1% and 27.8%, respectively). No association was detected between HFE mutations and elevated TS or liver iron deposits.

Conclusions: Elevated TS and liver iron deposits were frequent in non-cirrhotic HBV patients. Iron deposits were mainly mild and associated with higher activity and severity of liver disease, but not with HFE mutations.

Key words: chronic hepatitis, hepatitis B, HFE mutations, liver iron deposits, severity of liver disease.

INTRODUCTION

Iron deposits in the liver and abnormalities in serum iron biochemistry are frequently observed in patients with chronic liver diseases, but data for patients with hepatitis B virus (HBV) infection are scarce. Abnormalities in serum iron biochemistry and liver iron deposits have been described in HBV patients, and the serum levels of iron and ferritin are elevated in up to 36% and 30%, respectively, of male patients with chronic hepatitis including HBV infection. In the majority of cases the deposits of iron in the liver of patients with chronic viral hepatitis B and C are mild.3,4

It has been observed that patients with hereditary hemochromatosis (HH), which is the most common genetic disease affecting white European persons, show a significantly higher prevalence of serological markers for HBV infection, particularly antibodies to hepatitis B core antigen (HBcAg).5 This association may suggest a synergy between HH and viral infection and that the
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genetic disease could contribute to the liver overload in these patients.

Recently, two mutations in the HFE gene on chromosome 6 were found to be associated with HH.6–8 The mutation most clearly associated with HH is 845 G → A (C282Y).6,7 The relationship with HH of the second mutation (187 C → G, H63D) is less obvious, and appears to result in hemochromatosis in some patients when co-inherited with the C282Y mutation.6–7 An association between mutations of the HFE gene and iron overload has been reported in patients with non-alcoholic steatohepatitis,10 porphyria cutanea tarda11,12 and chronic hepatitis C,13,14 but the role of these mutations in HBV infection remains unknown.

The aims of the present study were to determine the prevalence of serum iron biochemistry abnormalities and liver iron deposits in patients with chronic HBV infection and to evaluate the consequences of these for the activity and severity of liver disease. Additionally, we studied the role of C282Y and H63D mutations in the determination of iron stores.

METHODS

Eighty-one male patients aged 18–60 years (mean ± SD, 34 ± 11 years) with chronic HBV infection were prospectively included in the study. No patient had received antiviral treatment for hepatitis B at the time of the study. Patients receiving regular blood transfusions, as well as heavy alcohol users (≥60 g ethanol/day) were excluded from the study. Informed consent was obtained from all participating individuals, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki, 1975, and was approved by the local Ethics Committee.

Serum concentrations of iron and ferritin, and transferrin saturation (TS) were determined in all patients. Serum levels of aminotransferases (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) and γ-glutamyltransferase (GGT) were measured on at least three different occasions over a period of 6 months and the mean value was calculated.

All patients had been seropositive for hepatitis B surface antigen and for immunoglobulin (Ig)G antibodies to HBcAg (EIA; Abbott Laboratories, Chicago, IL, USA) for at least 6 months before the study. Serum samples were also tested for hepatitis B e antigen (HBeAg) and the corresponding antibodies using a solid-phase commercial radioimmunoassay (Abbott Laboratories). Patients coinfected with hepatitis C virus (HCV; IgG antibody to recombinant HCV antigens using second-generation ELISA assay, Abbott Laboratories) or HIV (antibodies to HIV, ELISA Abbott, USA) were excluded from the study.

Serum HBV-DNA was quantified (Amplificor HBV Monitor, Roche, Branchburg, NJ, USA) to evaluate viral replication in patients with elevated serum ALT levels in the absence of the other markers of viral replication, HBeAg in serum or HBcAg in liver. There was evidence of virus replication in 33 patients.

A liver biopsy was available for analysis in 41 cases, and this was carried out based on routine criteria for diagnosis or clinical management of liver disease. Specimens were fixed in 10% formol-saline and processed using standard paraffin techniques. Routine stains included HE, staining for connective tissue and Perls’ staining for iron. Slides were scored for necroinflammatory activity (histological activity index [HAI]) and stage using the scoring system of Knodell et al.15 as modified by Desmet et al.16 Iron deposits in 39 patients were assessed and scored on the basis of both quantity and cellular and lobular location using the scoring system of Sciot et al.17 Accordingly, iron deposits were graded from 0 to 4 as follows: in hepatocytes (grade 0; no iron; grade 1: fine hemosiderin granules/local distribution; grade 2: fine hemosiderin granules/(at least) zonal distribution; grade 3: coarse hemosiderin granules/zonal distribution; grade 4: diffuse coarse hemosiderin granules), in Kupffer cells (grade 0; no iron; grade 1: occasional fine hemosiderin granules in some Kupffer cells; grade 2: fine hemosiderin granules in most Kupffer cells; grade 3: coarse hemosiderin granules in hypertrophic Kupffer cells; grade 4: coarse hemosiderin granules in almost all Kupffer cells), and in portal tracts and septa (grade 0: no iron; grade 1: trace of fine hemosiderin granules; grade 2: scattered fine hemosiderin granules; grade 3: coarse clumping of hemosiderin granules; grade 4: massive deposits). Scores obtained from hepatocytes were multiplied by a coefficient of 3, and those from Kupffer cells and portal tracts/septa by a coefficient of 1. The total score was obtained by adding the scores of hepatocytes, Kupffer cells and portal tracts/septa. The total iron score was subdivided into four grades: grade I (score 1–5), grade II (score 6–10), grade III (score 11–15) and grade IV (score 16–20). Liver biopsies were scored by an examiner blinded to the results of mutation analysis.

Abnormal serum iron biochemistry was considered to be present when TS was higher than the upper limit of normal (40%) and liver iron deposits were considered to be present when hemosiderin granules were observed after Perls’ staining.

Genomic DNA was extracted from peripheral blood leukocytes using standard methods.18 DNA analysis was performed using polymerase chain reaction (PCR) amplification followed by restriction–enzyme digestion with RsaI (for C282Y mutation analysis) and BclI (for H63D mutation analysis). Primers and PCR conditions have been described previously.18 DNA samples from patients with HBV infection were analyzed for the presence of both mutations. The frequency of the mutations was compared with that observed in 278 healthy subjects from the same geographic area.14

Statistical analysis

Data concerning mean patient age were compared using Student’s t-test, while the other data were analyzed using the Mann–Whitney U-test and Fisher’s non-parametric test. The hypotheses were two-tailed. Values are given as mean ± 1 SD and median, and P < 0.05 was considered significant.
RESULTS

Serum iron biochemistry

Elevated TS was found in 22/81 (27.1%) patients, four of whom had elevated ferritin levels. Table 1 shows the age of the patient at the beginning of follow up, serum levels of iron, TS, ferritin and liver enzymes, the presence of gene HFE mutations, the frequency of patients with HBV replication, and HAI and liver fibrosis scores in patients with and without elevated TS. No difference was observed between patients with and without elevated TS regarding the age of the patient at the beginning of follow up, ALT, AST and GGT levels. Ferritin levels were significantly higher in patients with elevated TS (P = 0.0009). No association was detected between elevated TS and detection of HBV replication, HAI or liver fibrosis scores.

Liver iron deposits

Perls’ staining showed liver iron deposits in 19/39 (48.7%) patients (Table 1). The intensity of the deposit was mild (grade I) in 68.4% and moderate (grade II) in 31.6%. No difference was observed between patients with and without liver iron deposits regarding age when starting follow up. No association was found between liver iron deposits and the detection of viral replication. There was no difference in ALT, AST, GGT, TS and ferritin levels between patients with and without liver iron deposits. No difference was observed comparing patients with moderate liver iron deposits with those with absent or mild liver iron deposits regarding serum levels of AST (94 ± 55 and 56 ± 28 U/L, respectively, P = 0.06), ALT (125 ± 70 and 92 ± 54 U/L, respectively, P = 0.23), and GGT (58 ± 41 and 34 ± 20 U/L, respectively, P = 0.12).

The distribution of the deposit was hepaticellular in nine (47.3%), macrophagic in six (31.5%) and diffuse (hepatocellular and macrophagic) in four (21.2%) patients. Significantly higher scores for HAI (13.5 ± 1.3 vs 9.3 ± 3.5, P = 0.018) and a trend towards higher scores for fibrosis (2.5 ± 0.6 vs 1.7 ± 0.6, P = 0.062) were observed in patients in whom the iron distribution in the liver was diffuse compared with those in whom the distribution was hepatocytic or macrophagic.

A liver biopsy carried out in 15/22 patients with elevated TS showed liver iron deposits in eight (53.3%) cases. Twenty-four of 59 patients without elevated TS were submitted to liver biopsy and liver iron deposits were observed in 11 (45.8%) cases. The positive predictive value for TS was 0.53, the negative predictive value 0.54, sensitivity 42% and specificity 65%.

Significantly higher scores for HAI (P = 0.01) and for liver fibrosis (P = 0.05) were observed in patients with liver iron deposits compared with patients without liver iron deposits (Table 1; Fig. 1). In addition, patients with moderate liver iron deposits had significantly higher HAI (12.8 ± 3.2 and 7.3 ± 3.7, respectively, P = 0.001) and liver fibrosis (2.3 ± 0.8 and 1.5 ± 0.6, respectively, P = 0.02) scores compared with those with absent or mild liver iron deposits.

HFE mutations

HFE mutations were identified in 19/81 (23.4%) patients, 14 were heterozygous H63D, four homozygous H63D and one had a compound mutation. No difference was observed in the prevalence of C282Y and H63D mutations between chronic HBV patients (1.2% and 23.4%, respectively) and the general population (4.1% and 27.8%, respectively). No association was found between the presence of HFE mutations and elevated TS (P = 1.0) or liver iron deposits (P = 0.48). No patients with a homozygous C282Y mutation were found. There was only one heterozygote for C282Y and this patient also had the H63D mutation (compound heterozygote); furthermore, this patient exhibited elevated TS and liver fibrosis scores (hepatocellular and macrophagic) in four (21.2%) patients. Significantly higher scores for HAI (13.5 ± 1.3 vs 9.3 ± 3.5, P = 0.018) and a trend towards higher scores for fibrosis (2.5 ± 0.6 vs 1.7 ± 0.6, P = 0.062) were observed in patients in whom the iron distribution in the liver was diffuse compared with those in whom the distribution was hepatocytic or macrophagic.

Table 1 Age at the beginning of follow up, biochemical findings (mean ± SD), presence of HFE mutations, percentage of patients with hepatitis B virus (HBV) replication and histological activity index (HAI) and fibrosis scores in liver in male non-cirrhotic HBV patients with and without elevated serum transferrin saturation (TS), and with and without liver iron deposits.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Elevated TS</th>
<th>Liver iron deposits</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n = 22)</td>
<td>No (n = 59)</td>
<td></td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>36.7 ± 12.4</td>
<td>32.9 ± 10.3</td>
<td>0.17</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>78 ± 65</td>
<td>60 ± 60</td>
<td>0.12</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>50 ± 36</td>
<td>44 ± 43</td>
<td>0.28</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>299 ± 233</td>
<td>151 ± 86</td>
<td>0.0009</td>
</tr>
<tr>
<td>TS (%)</td>
<td>51 ± 8.9</td>
<td>27.7 ± 7.8</td>
<td>–</td>
</tr>
<tr>
<td>HFE mutation (n; %)</td>
<td>5/22 (22.7)</td>
<td>14/59 (23.7)</td>
<td>1.00</td>
</tr>
<tr>
<td>HBV replication (%)</td>
<td>52.4</td>
<td>40.7</td>
<td>0.31</td>
</tr>
<tr>
<td>HAI score (mean ± SD)</td>
<td>8.5 ± 3.6</td>
<td>8.6 ± 4.3</td>
<td>0.80</td>
</tr>
<tr>
<td>Liver fibrosis scores (mean ± SD)</td>
<td>1.7 ± 0.5</td>
<td>1.6 ± 0.7</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Normal range: ALT = 10–44 U/L; AST = 10–34 U/L; GGT = 11–50 U/L; ferritin = 18–370 ng/mL. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transpeptidase.
iron deposits, an HAI score of 7 and grade 2 fibrosis, without markers of HBV replication.

**DISCUSSION**

We observed elevated TS in 27.1% of male chronic HBV patients, a figure that falls between those reported by other authors studying patients with viral hepatitis, that is 18%\(^1\) and 32.7%.\(^{13}\) Liver iron deposits were observed in 48.7% of the patients submitted to a biopsy. When we examined the presence of iron deposits in the liver, which is regarded as the gold standard for iron overload, the sensitivity of TS was as low as 42% and the specificity was 65%. These findings are similar to those described in the literature, which show low specificity for biochemical parameters for the evaluation of iron overload and that only a minority of the patients with biochemical indicators of iron overload also have high hepatic iron concentrations.\(^3\) In the present study four patients with high levels of TS were submitted to a biopsy that revealed iron deposits in the liver.

The data available in the literature regarding iron overload in patients with HBV infection are scarce.\(^{13,19}\) Ludwig et al. observed high rates of liver staining (64.7%) and increased liver iron concentration (47.1%) in 17 cirrhotic HBV patients; however, they included only patients with cirrhosis, a condition considered to be a cause of iron overload per se.\(^{19}\) In a study by Piperno et al. only a very small number of patients had HBV.\(^{13}\) The rate of liver iron deposits observed in the present study was similar to that described in patients with liver disease secondary to various etiologies. In contrast to hepatitis B, several studies have evaluated iron metabolism in patients with chronic hepatitis C, among whom mild to moderate iron overload is common. Elevated transferrin saturation and serum ferritin is found in 18–35% of patients, with an increased liver iron concentration in 10–36% of patients.\(^3,13\) The difficulty in comparing different studies arises from the lack of uniform criteria used in the definition of iron stores. Quantitative methods yield false-negative results for samples containing mild iron deposits with a focal distribution\(^{19}\) or when the samples are small and contain extensive fibrosis.\(^{20}\) The semiquantitative method using Perls’ staining to evaluate the iron deposits also has limitations, because a wide variation in hepatic iron concentration can be observed in samples classified as exhibiting a mild iron overload using this method.\(^{20}\) In contrast, a study using both methods in patients with chronic viral hepatitis showed a significant correlation between hepatic iron grade and hepatic iron concentration.\(^{21}\) In the present study we used the semiquantitative method. Although it has limitations, we believe that this method allowed us to reach a conclusion about the presence or absence of iron deposits. To determine the influence of the intensity of the deposits it would have been better if we had associated a quantitative method.

No severe iron deposits were observed in any of our patients and the great majority presented only mild deposits. Similar results were reported by Hézode et al., who found severe iron deposits in only 1% of patients with chronic hepatitis C infection.\(^4\) These data were reinforced by the study of Riggio et al., who reported a hepatic iron index \(>1.9\) in only 12.5% of patients with chronic hepatitis C.\(^{22}\)

Our data showed that iron distribution was mainly in the hepatocytes in most patients. This finding was similar to those described in another study that evaluated chronic hepatitis C infection.\(^4\) In our study patients with diffuse iron deposits showed higher HAI scores and a tendency to higher fibrosis scores compared with patients with iron deposits in hepatocytes or macrophages. Hézode et al., studying the association between the iron distribution in the liver and the severity of chronic hepatitis, reported higher degrees of inflammation and the presence of cirrhosis in patients with diffuse or macrophagic distribution of iron deposits compared with those with hepatocytic distribution.\(^4\)

In the present study no association was found between the presence of \(HFE\) mutations and elevated TS or liver iron deposits. The association of these mutations and iron overload has been demonstrated in patients with porphyria cutanea tarda,\(^{12,23}\) chronic hepatitis C\(^{14,20}\) and non-alcoholic steatohepatitis.\(^{24}\) However, the role of these mutations in HBV patients has not been previously evaluated. We observed a low frequency of \(C282Y\) mutation (1.2%) in our patients compared with American, Australian and English studies (11–14%).\(^{11,23,25}\) In a previous study of HCV patients,\(^4\) we also found a lower frequency of \(C282Y\) mutation (4.4%) compared with these studies. However, this frequency is not unexpected if we consider that HH is a disease strongly associated with Caucasians and that in Brazil racial admixture is a very common event. The
frequency of the \(H63D\) mutation obtained in the present study (23.4\%) was similar to that reported by other authors (24.8–26.8\%).\(^{11,23,25}\) As expected, there was no difference in the frequency of both \(HFE\) mutations between HBV patients and the general population from the same geographic area.\(^{14}\) The only patient carrying the \(C282Y\) mutation was a compound heterozygote exhibiting iron overload both in serum and the liver (grade II) and with no indication of HBV replication. The compound mutation has been related to iron overload in a small percentage of patients.\(^{6,7,9}\) Thus, our results support the idea that iron deposits in the liver are common in chronic hepatitis, but that \(HFE\) mutations do not play an important role in the determination of such deposits.

Our results show that patients with liver iron deposits had higher degrees of necroinflammatory lesion and fibrosis in the liver. In a previous study with HCV patients, we observed higher scores for necroinflammation and fibrosis in the liver and body iron overload in \(HFE\) carriers.\(^{19}\) Similarly, Smith et al. observed an association between heterozygosity for \(HFE\) and fibrosis in patients with hepatitis C.\(^{20}\) In contrast, Beinker et al. found an association between liver iron overload and the severity of liver fibrosis in patients with HCV, but not in those with HBV infection.\(^{26}\) Our results reinforce the hypothesis that even a small amount of iron may increase hepatic liver injury in chronic hepatitis patients. This is in agreement with evidence suggesting iron as a comorbid factor in chronic viral hepatitis.\(^{27}\)

The pathophysiological mechanisms involved in iron deposits in the liver in chronic viral hepatitis are not clear. It has not been defined if iron deposit is a reflection of hepatocellular injury or if it contributes to liver damage.\(^{28}\) The cellular damage caused by viral infection can result in phagocytosis of the injured hepatocytes by Kupffer cells.\(^{29}\) Iron can cause an increase in the formation of reactive oxygen intermediates that lead to lipid peroxidation with subsequent oxidative damage.\(^{30}\) Iron can also affect the immune cellular response.\(^{31}\) Studies have shown that iron status might affect the response rate to interferon (IFN) therapy in patients with chronic viral hepatitis.\(^{27}\) Studies of HCV patients have shown that liver iron deposits are associated with a reduced response to IFN\(^{32}\) and that iron depletion treatment leads to a reduction in serum liver enzymes\(^{33}\) and an increase in the response rates to IFN therapy.\(^{34}\) Moreover, long-term maintenance of iron depletion by phlebotomy in non-responders to antiviral treatment has been shown to prevent the progression of liver fibrosis in HCV patients.\(^{35}\) Although most studies have evaluated patients with chronic hepatitis C, similar results were observed in the few studies involving chronic hepatitis B patients. van Thiel et al. observed a higher hepatic iron concentration in chronic hepatitis B patients who were non-responders to IFN.\(^{36}\) In addition, Bayraktar et al. have shown that desferrioxamine infusion to achieve a normal serum ferritin level enhanced the likelihood of the response to IFN in patients with chronic hepatitis B.\(^{37}\) Iron depletion by phlebotomy has not been used in hepatitis B, but our results support the idea that it may have a similar adjunctive therapeutic effect to that seen in hepatitis C.

In the present study no association was observed between the serum ferritin levels and the presence of liver iron deposits and HBV replication. There is no clear evidence of the influence of iron overload on HBV replication. Deugnier et al. demonstrated an increase of HBV infection markers in patients with genetic hemochromatosis, suggesting that iron overload may facilitate viral replication in hepatocytes or may alter the host response to the virus.\(^{5}\) This subject remains unclear. Taken together, our data show that serum iron biochemistry abnormalities and liver iron deposits are frequent in male non-cirrhotic HBV patients. The iron deposits were mild in the majority of cases and were associated with higher activity and severity of liver disease. No association was found between serum iron biochemistry abnormalities or liver iron deposits and \(HFE\) mutations.

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