The Effect of Hepatitis B Virus Infection on Hepcidin Expression in Hepatitis B Patients

Xue-hua Wang¹, Pan-Pan Cheng², Fang Jiang¹, and Xiao-Yang Jiao¹

¹Department of Hematology Laboratory, The First Affiliated Hospital Of Shantou University Medical College; ²Department of Clinical Laboratory, Affiliated Hospital Of Jining Medical College, 79 Guhuai Road, Jining, Shandong, China

Abstract. Background: Hepcidin is a central regulator of iron metabolism. As hepcidin is produced mainly by hepatocytes, pathologic changes in the liver may affect hepcidin production. Abnormal hepcidin expression has been reported following liver injury, including liver cirrhosis, alcoholic liver disease, and chronic hepatitis B and hepatitis C. However, it is unclear whether there is a dose-dependent relationship between hepcidin expression and hepatitis B virus load. The aim of this study was to characterize hepcidin levels in patients with different hepatitis B virus (HBV) DNA levels. Methods: We investigated serum hepcidin levels in 71 patients with different HBV DNA loads, 10 patients with hepatocellular carcinoma (HCC), and 13 healthy individuals. The relationships between hepcidin expression and hematological/liver functional parameters, iron, and inflammatory indicators were also analyzed. Results: Serum IL-6, ferritin, and hepcidin levels were significantly higher in patients with hepatitis B and in HCC patients than in controls (P<0.05), and strong positive correlations were found between hepcidin and ferritin, AST, ALT, GGT, ALP, TBIL, IBIL, and AFU, as well as between log [hepcidin] and log [HBV], respectively. There were no significant differences in hematological parameters, including WBC, Hb, and platelets among hepatitis B patients, nor was a correlation found between hepcidin and any hematological parameters. Conclusion: Our results indicate that hepcidin expression is regulated by iron and inflammatory factors in hepatitis B infection patients, and that the virus load can affect hepcidin production.

Key words: Hepatitis B; Hepcidin; Iron; IL-6; Ferritin

Introduction

Worldwide, viral hepatitis represents a major cause of chronic liver disease and progressive liver fibrosis, eventually leading to cirrhosis and hepatocellular carcinoma (HCC) [1]. Hepatitis B virus (HBV) is a hepato-tropic, non-cytopathic DNA virus that causes acute and chronic hepatitis. Virus replication is associated with enhanced cellular metabolism, leading to increased iron bioavailability accompanied by viral expansion [2]. The liver is the principal site of iron storage. Iron accumulation in the liver is common in patients with chronic liver diseases; the accumulation acts as an activator of hepatic stellate cells, stimulating the production of collagens and other extracellular matrix proteins and leading to fibrosis [3]. Viral infections that disrupt liver function can be accompanied by changes in iron homeostasis, and iron loading of this organ can exacerbate chronic viral disease.

Recent studies have revealed that the liver plays an important role in iron homeostasis by secreting a peptide hormone named hepcidin. Hepcidin synthesis, which occurs predominantly in the liver hepatocytes, is integral to maintaining iron homeostasis in the body [4,5]. Hepcidin binds to the cellular iron export channel ferroportin to cause ferroportin internalization and degradation [6], thereby decreasing iron efflux from enterocytes and macrophages into plasma [7,8]. Hepcidin expression is up-regulated by excess iron or inflammation, whereas increased erythropoiesis and reduced iron stores all down-regulate hepcidin expression [9].
Under chronic inflammatory conditions, the excessive production of cytokines by macrophages and T-lymphocytes, as well as inflammatory cytokines, particularly IL-6, play a central role in hepcidin production [10]. The binding of IL-6 to its receptor results in phosphorylation of the intracellular signaling molecule STAT-3 (Signal Transducer and Activator of Transcription 3). Phospho-STAT-3 dimerizes and is then translocated to the nucleus, where it interacts with a characterized IL-6 response element in the hepcidin promoter to up-regulate hepcidin expression [11].

Knowledge of HBV-DNA viral load is useful for disease prognosis, as well as for determining infectivity, evaluating indications for treatment, assessing response to treatment, identifying emergence of resistance, and diagnosing occult HBV [12]. There was a dose-response relationship between viral load and risk of cirrhosis and HCC. A significantly high risk of cirrhosis and HCC was found in HBV carriers with viral levels of >10^4 copies/ml, and HBV carriers with viral levels of >10^5 copies/ml had more than a 5-fold increased risk of cirrhosis and HCC, compared to those with viral levels of ≤10^4 copies/ml [13]. However, HBV itself is not directly cytopathic. The host immune response plays a pivotal role in HBV-related liver diseases [14]. The clinical outcome of HBV infection is strongly dependent on host immune responses, including a strong and multi-specific T-cell and cytokine response [15]. During viral infection, cytokines also play an important role in both viral clearance and tissue damage mechanisms. Viruses may interfere with the normal function of this complex cytokine network to avoid destruction. IL-6 levels have been reported as elevated, compared to normal subjects, in patients with chronic hepatitis B (CHB), cirrhosis, and HCC.
and HCC [16-18]. There were reports that the levels of serum IL-6 represented the best marker of HBV-related clinical progression as compared with other cytokines such as IL-10, IL-12 and IFN-γ [19]. In particular, IL-6 plays a central role in hepcidin production [10]. Therefore, we anticipated that HBV-mediated elevations in IL-6 would result in the stimulation of hepcidin production.

Recently, research has shown hepcidin regulation disturbances in animal models of liver injury, including liver cirrhosis, alcoholic liver disease, and chronic hepatitis C [20–22]. However, in CHB patients, the association between HBV-DNA viral load and hepcidin expression is unclear. We investigated whether hepatitis B viral load affects hepcidin expression, and assessed the relationship between hepcidin and iron/inflammatory parameters under hepatitis B virus infection. In this study, we determined serum levels of hepcidin in patients with different HBV loads; liver enzymes, iron, and inflammation indicators were also analyzed.

Materials and methods

This study was approved by the Ethics Committee at the Shantou University Medical College and informed consent was obtained from each subject prior to the start of our study. 71 patients had a diagnosis of CHB, as determined by positive HBV surface antigen, HBV DNA load, and anti-HCV negativity. Participants with other chronic hepatic conditions, including Wilson’s disease, autoimmune hepatitis, hemochromatosis, alcoholic/toxic hepatitis, and concomitant HCV/HBV infections were excluded. Meanwhile 10 patients with HCC and 13 healthy individuals were selected as controls.

Using two types of vacuum containers (one with ethylenediaminetetraacetic acid, or EDTA, the other without anticoagulant), blood samples were obtained in the morning for hematological and biochemical tests after overnight fasting. Blood in EDTA was analyzed by an automatic cell counter.
(COULTER LH 750 Analyzer) for determination of the complete blood count. Blood without anticoagulant was kept at room temperature for two hours to ensure serum separation. During each follow-up examination, WBC, Hb, platelets, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) (upper limit of normal: 40 U/L, 40 U/L, and 17.1 µmol/mL, respectively), gamma glutamyl transpeptidase (gamma-GGT), total bilirubin (TB), indirect bilirubin (IB), direct bilirubin (DB), alkaline phosphatase (ALP), total protein (TP), albumin (Alb), monoamine oxidase (MAO), and α-L-fucosidase (AFU) were measured, as well as at the time of entry the study.

Serum ferritin concentrations were determined by immunoassay. Serum IL-6, HBV surface antigen, and hepcidin levels were determined using ELISA (Rapidbio Lab, Calabasas, California, USA for IL-6, Sym-Bio Life-Science, Shanghai, China for HBV surface antigen, and Uscn Life Science Inc, Wuhan, China for hepcidin) according to the manufacturer’s instructions.

HBV virus detection with Ultra Sensitive RT-PCR. HBV viral load was measured with a time-resolved fluorescence assay, reagents for which were purchased from Sym-Bio Life-Science, Shanghai, China for HBV surface antigen, and Uscn Life Science Inc, Wuhan, China for hepcidin according to the manufacturer’s instructions.

Statistical analyses. All data were given as mean ± standard deviation [mean ± SD (median)]. Qualitative data were tested with a corrected Yates’ chi-square test. The Wilcoxon test was used to assess differences between the various parameters of hepatitis B patients. Calculations for statistical differences between groups were carried out with the Student t test or by a non-parametric Kruskal-Wallis test. Associations among the various parameters in the patient groups were calculated using a non-parametric Spearman rank correlation. A P value ≤0.05 was considered as statistically significant.

Results

In this study, we divided HBV patients into A, B, and C groups according to the HBV DNA levels (group A: viral levels of 10^2 and 10^3 copies/ml; group B: viral levels of 10^4 and 10^5 copies/ml; group C: viral levels of 10^6 and 10^7 copies/ml). Group D included all patients with HCC. We did not find any significant differences in hematological parameters (including WBC, Hb, and Plt) between HBV patients, HCC patients, and controls. Similar results were also found for biochemical indicators, including TP, ALB, MAO and AFU. The clinical characteristics of the study population are presented in Table 1.

Liver enzymes, IL-6, ferritin, and hepcidin levels in each group. AST levels in HBV patient groups (1-3) were 1.44-, 2.18-, and 9.36-fold higher than in the control (P=0.004, <0.001, <0.001, respectively). In HCC, the AST level was 2.15-fold higher than in the control (P=0.007). ALT levels in HBV and HCC patients were 0.91-, 2.19-, 13.75-, and 20.79-fold higher than in the control (P=0.006 (A), 0.001 (B), 0.001 (C), and 0.016 (D), respectively). Similar results were also found in ALP levels when groups A-D were compared to the control (P=0.002 (A), 0.017(B), 0.001(C), and 0.007 (D), respectively). GGT levels in the three HBV subgroups were elevated compared to controls (P=0.033 (A), =0.002(B), <0.001(C)). We did not find any significant difference in LDH between the HBV groups and the control (P=0.201(A), 0.492(B), and 0.057(C), respectively). LDH levels in group D were 0.53-fold higher than in the control (P=0.022). We then put all the HBV patients into one group to avoid statistical error and imprecision resulting from a small sample size. In the aggregate HBV group, we found AST and ALT increased with the HBV DNA load (R=0.423, P<0.001 and R=0.430, P<0.001, respectively) (Figure 1).

IL-6 levels were 12.29-, 9.62-, 12.64-, and 8.71-fold higher in all three HBV groups and the HCC group than in the control (P<0.001(A), =0.039(B), <0.001(C), and P=0.001(D), respectively). Serum ferritin levels in both HBV and HCC were elevated significantly when compared with the control (P<0.001 for all groups), and no significant difference in serum ferritin concentrations was found between the HBV groups (P=0.462). Patients with HCC had much higher ferritin levels than patients in any of the HBV groups (P<0.001, <0.001, =0.045).
Serum hepcidin levels in HBV patient groups were 1.40-, 1.82-, and 1.88-fold higher than in the control (P<0.001 for all HBV groups). Increased hepcidin levels correlated with increased HBV DNA levels, and a positive correlation was found between log [hepcidin] and log [HBV] (R=0.383, P=0.001, Figure 2). The hepcidin levels in the HCC group were also above those of the control (P<0.001) and HBV group A (P=0.044), though we did not find a significant difference in hepcidin levels between HCC patients and HBV groups B or C (P=0.685, P=0.668).

Correlation between HBV DNA level and liver function. To evaluate to what extent elevated HBV DNA levels may interfere with liver function, we analyzed the association between virus load (log [HBV] DNA) and liver function indicators. Positive correlations were found between log [HBV] DNA levels and AST, ALT, GGT, TBIL, IBIL, and AFU (R=0.423, P<0.001; R=0.430, P<0.001; R=0.280, P=0.022; R=0.319, P=0.009; R=0.254, P=0.040; R=0.296, P=0.015; respectively, Figure 1).

Correlation between hepcidin, ferritin and liver function. In our study, serum hepcidin was not found to be associated with hematological indicators (Table 2). To examine whether elevated serum hepcidin may be partly attributable to impaired liver function or to iron metabolism disturbances, we investigated the correlations between hepcidin and liver biochemical parameters, as well as between hepcidin and ferritin. Positive correlations were found between hepcidin and ferritin in HBV groups (group A: R=0.848, P<0.001; group B: R=0.702, P<0.001; group C: R=0.632, P<0.004), in the HCC group (R=0.806, P<0.005), and in the control group (R=0.740, P<0.004). Positive correlations were also found between hepcidin and AST, ALT, GGT, ALP, TBIL, IBIL, and AFU (R=0.390, P=0.004; R=0.420, P=0.002; R=0.502, P<0.001; R=0.354, P=0.009; R=0.506, P<0.001; R=0.517, P<0.001; and R=0.329, P=0.003; respectively) in hepatitis patients. After converting the order of magnitude of the virus levels to log [HBV], we found that a positive correlation existed between log [hepcidin] and log [HBV](R=0.383, P=0.001, Figure 2).

Discussion

Hepcidin expression in the setting of chronic viral hepatitis has been extensively studied, but remains controversial. Aoki and Nagashima reported that hepatic hepcidin expression was elevated in response to iron overload in patients with chronic hepatitis C, and that serum prohepcidin positively correlated with ferritin levels in HBV patients [20, 23]. However, other studies demonstrated that hepcidin expression was down-regulated in liver cirrhosis and HCV, with serum prohepcidin and ferritin levels being negatively correlated in HCV patients [21, 24]. More specifically, Jaroszewicz et al. recently reported that serum prohepcidin concentration was lower in liver cirrhosis, possibly as a result of impaired liver function [25]. Previous reports demonstrated that the transcriptional activation of hepcidin was abrogated in response to transforming growth factor-β, one of the key profibrogenic molecules involved in chronic liver damage [26].

Chronic HBV infections are associated with increased production of ROS within the liver, which can function as a component of signal transduction cascades by activating transcription factors including STAT-3, the pathway activating hepcidin transcription in the hepatocytes [27-29]. IL-6 activity has been shown to be significantly enhanced during acute exacerbation of CHB, and plays an important role in elevating hepcidin production [10]. In our study, significantly increased IL-6 levels were found in patients with both CHB and HCC. Accordingly, serum hepcidin levels were also elevated in CHB patients, supporting the notion that elevated IL-6 might stimulate hepcidin expression during HBV infection. However, we did not find any quantitative correlation between IL-6 and hepcidin, possibly due to the complexity of immune response in hepatitis B, where panels of cytokines are expressed, a setting very different from the controlled application of single cytokine or LPS during an in vitro experiment, instead reflecting the net effects of several agonistic and antagonistic cytokines toward a specific target. Thus, the potential effect of a single immune effective molecule found in experiments may be masked in this in vivo setting. A thorough
The Effect of Hepatitis B Virus Infection

Clinical investigation would then have administered an IL-6 inhibitor to see its effect on hepcidin levels. Hepcidin is gradually being accepted as an important systemic immune response mediator. Inflammation and elevated iron stores are two major stimuli for hepcidin secretion. As the liver is the major source of hepcidin production [29], we examined whether its inflammatory status can affect hepcidin production. In hepatitis, ALT and AST become elevated with the progression of liver disease, likely as a result of direct hepatocellular damage and membrane leakage. In our CHB patients, ALT and AST levels were elevated, as was HBV virus load, indicating our patients were in the active phase of CHB. We found that ALT and AST levels correlated positively with serum hepcidin levels, whereas no correlation was found between TP/Alb and hepcidin, indicating that hepcidin production may be regulated mainly by hepatocyte inflammation. How hepcidin expression relates to ALT and AST needs further investigation.

Hyperferritinemia is a frequent phenomenon in patients with chronic liver disease, whatever the etiology of the underlying damage [30]. In HBV-infected patients with CHB, chronic hepatic inflammation seems to be responsible for hepatic iron accumulation. However, it is unclear whether the HBV infection itself has a direct influence on hepatic iron accumulation, since it has been previously demonstrated that inflammatory cytokines enhance TfR1-mediated iron uptake by hepatocytes [31]. On the other hand, serum ferritin could be elevated as an acute phase reaction during the necro-inflammatory process of CHB. In this study, we found significantly elevated ferritin levels in CHB patients, a result, hepcidin expression was regulated by their iron overload. Our findings of elevated ferritin in CHB patients, and a strong positive correlation between serum hepcidin and ferritin, suggest a feedback mechanism whereby hepcidin is up-regulated in the liver in response to elevated iron stores and acts as a signal to down-regulate iron absorption and decrease iron storage. This is consistent with known hepcidin functions.

### Table 2. Adjusted linear regression analyses for hepcidin levels in the HBV groups

<table>
<thead>
<tr>
<th></th>
<th>Beta</th>
<th>Std. Error</th>
<th>T</th>
<th>P</th>
<th>Tolerance</th>
<th>VIF</th>
<th>95% Confidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6</td>
<td>-0.269</td>
<td>3.484</td>
<td>-2.273</td>
<td>.031</td>
<td>.995</td>
<td>1.006</td>
<td>-15.066</td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.869</td>
<td>0.150</td>
<td>4.666</td>
<td>.001</td>
<td>.136</td>
<td>7.371</td>
<td>-367</td>
</tr>
<tr>
<td>HBV</td>
<td>-0.455</td>
<td>0.000</td>
<td>-2.079</td>
<td>.064</td>
<td>.098</td>
<td>10.176</td>
<td>.000</td>
</tr>
<tr>
<td>LogHBV</td>
<td>0.190</td>
<td>35.708</td>
<td>1.406</td>
<td>.190</td>
<td>.259</td>
<td>3.865</td>
<td>-29.354</td>
</tr>
<tr>
<td>WBC</td>
<td>0.191</td>
<td>22.558</td>
<td>1.522</td>
<td>.159</td>
<td>.298</td>
<td>3.354</td>
<td>-15.921</td>
</tr>
<tr>
<td>Hb</td>
<td>-0.079</td>
<td>3.496</td>
<td>-3.65</td>
<td>.723</td>
<td>.101</td>
<td>9.919</td>
<td>-9.065</td>
</tr>
<tr>
<td>Plt</td>
<td>0.182</td>
<td>0.888</td>
<td>1.123</td>
<td>.288</td>
<td>.179</td>
<td>5.585</td>
<td>-9.981</td>
</tr>
<tr>
<td>MPV</td>
<td>0.086</td>
<td>40.941</td>
<td>0.721</td>
<td>.487</td>
<td>.328</td>
<td>3.044</td>
<td>-61.694</td>
</tr>
<tr>
<td>LDH</td>
<td>-0.323</td>
<td>2.114</td>
<td>-0.863</td>
<td>.408</td>
<td>.034</td>
<td>29.755</td>
<td>-6.535</td>
</tr>
<tr>
<td>AST</td>
<td>0.150</td>
<td>1.548</td>
<td>0.205</td>
<td>.841</td>
<td>.009</td>
<td>114.132</td>
<td>-3.131</td>
</tr>
<tr>
<td>ALT</td>
<td>-0.162</td>
<td>0.525</td>
<td>-0.447</td>
<td>.665</td>
<td>.036</td>
<td>28.129</td>
<td>-1.404</td>
</tr>
<tr>
<td>GGT</td>
<td>0.563</td>
<td>2.208</td>
<td>1.637</td>
<td>.133</td>
<td>.040</td>
<td>25.148</td>
<td>-1.306</td>
</tr>
<tr>
<td>ALP</td>
<td>-0.002</td>
<td>1.494</td>
<td>-0.017</td>
<td>.987</td>
<td>.284</td>
<td>3.521</td>
<td>-3.355</td>
</tr>
<tr>
<td>TP</td>
<td>-0.070</td>
<td>8.043</td>
<td>-0.393</td>
<td>.702</td>
<td>.150</td>
<td>6.686</td>
<td>-21.084</td>
</tr>
<tr>
<td>ALB</td>
<td>-0.078</td>
<td>17.818</td>
<td>-0.286</td>
<td>.781</td>
<td>.064</td>
<td>15.663</td>
<td>-44.799</td>
</tr>
<tr>
<td>DBIL</td>
<td>-0.184</td>
<td>19.795</td>
<td>-0.466</td>
<td>.651</td>
<td>.030</td>
<td>33.143</td>
<td>-53.324</td>
</tr>
<tr>
<td>IBIL</td>
<td>0.196</td>
<td>16.088</td>
<td>0.657</td>
<td>.526</td>
<td>.053</td>
<td>18.962</td>
<td>-25.273</td>
</tr>
<tr>
<td>MAO</td>
<td>0.204</td>
<td>63.850</td>
<td>1.523</td>
<td>.159</td>
<td>.261</td>
<td>3.825</td>
<td>-45.041</td>
</tr>
<tr>
<td>AFU</td>
<td>0.149</td>
<td>7.648</td>
<td>1.061</td>
<td>.314</td>
<td>.240</td>
<td>4.169</td>
<td>-8.924</td>
</tr>
</tbody>
</table>

Beta: Beta co-efficient; 95%CI: 95 percent confidence interval, SE: standard error
showing that when serum ferritin elevates, hepcidin is up-regulated, leading to decreased intestinal iron absorption for maintenance of iron homeostasis. It is also supported by findings indicating that hepcidin expression is regulated by iron stores, even in the face of chronic injuries [32]. We found positive correlations between serum hepcidin and ferritin in HCC patients, indicating that hepcidin remains regulated by iron status in HCC. Elevated hepcidin may result from an immune response that

Figure 2. Correlations between hepcidin and liver enzymes, HBV copies in HBV patients. Correlation coefficient of nonparametric Spearman rank. The regression line and the 95% confidence interval are plotted if significance existed. Positive correlations were found between hepcidin and AST, ALT, GGT, ALP, TBL, IBL, and AFU, respectively in hepatitis patients. After translating order of magnitude of the virus levels to log [HBV], a positive correlation existed between log [hepcidin] and log [HBV].
withholds iron against pathogens or cancer cells [33]. Higher hepcidin levels favor iron depletion from blood but accumulation in reticuloendothelial cells, where it may further stimulate an immune response and exacerbate the disease [34]. A drastic reduction in hepcidin expression within the iron-depleted tumorous lesions could have physiological consequences resulting from the high proliferation rate of the tumor cells outpacing the availability of iron [35]. Therefore, hepcidin evolved as a method of host defense, decreasing the iron available to invading pathogens and malignant cells in an effort to hinder their reproducing [36].

In our cohort, hepcidin expression may be up-regulated by high serum levels of IL-6 and ferritin during the course of HBV infection, and positive correlations were found between liver enzyme and hepcidin, indicating that hepcidin is closely related to the inflammatory and iron status of CHB patients. Sequential determination of serum hepcidin, rather than one-time point detection alone, may offer more benefits for monitoring the prognosis of CHB patients.

One limitation of the current study is that we were unable to precisely define the disease phase of the patients in our cohort. We just analyzed the serum hepcidin at one time point rather than for the disease duration of CHB. In advanced stages such as cirrhosis, it remains to be determined whether hepcidin was further decreased by impaired protein synthesis due to a markedly reduced functional hepatic mass.

Acknowledgements

This study was funded by the Medical Research Foundation of Guangdong Province (NO B2011220), Science and Technology Program of Guangdong Province, China (NO2010B031600321, NO2012B031800217). We also thank Dr. Stanley Lin for his review of the manuscript.

References
