

Serum prohepcidin levels in chronic hepatitis C, alcoholic liver disease, and nonalcoholic fatty liver disease

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Background/Aims: Patients with various chronic liver diseases frequently have increased body iron stores. Prohepcidin is an easily measurable precursor of hepcidin, which is a key regulator of iron homeostasis. This study investigated the serum prohepcidin levels in patients with various chronic liver diseases with various etiologies. **Methods:** Serum prohepcidin levels were measured in patients with chronic hepatitis C (CH-C) ($n=28$), nonalcoholic fatty liver disease (NAFLD) ($n=24$), and alcoholic liver disease (ALD) ($n=22$), and in healthy controls ($n=25$) using commercial ELISA. Serum interleukin 6 (IL-6) levels and blood iron indices were also measured. **Results:** The serum levels of both prohepcidin and IL-6 were significantly higher in CH-C patients than in healthy controls, and there was a positive correlation between the IL-6 and prohepcidin levels ($r=0.505$, $p=0.020$). The prohepcidin levels in ALD patients did not differ from those in controls, despite their significantly elevated IL-6 levels. There was a tendency for a negative correlation between serum prohepcidin levels and transferrin saturation in ALD patients ($r=-0.420$, $p=0.051$). Neither prohepcidin nor IL-6 was significantly elevated in the NAFLD group, despite the presence of elevated serum iron and ferritin levels. **Conclusions:** The role of prohepcidin may differ in different human liver diseases. In the setting of CH-C, both the serum prohepcidin and IL-6 levels were significantly elevated and were positively correlated with each other. (**Korean J Hepatol 2010;16:288-294**)

Keywords: Prohepcidin; Hepatitis C; Fatty liver; Alcohol; IL-6

INTRODUCTION

Iron is an essential element for all living organisms, and it is required in a wide range of metabolic processes, including DNA synthesis, oxygen transport, and energy production. However, excess body iron can be harmful, in part through the generation of oxygen radicals.¹ Body iron homeostasis is tightly controlled through iron absorption in the duodenum, utilization and storage according to bone marrow needs, and internal iron replenishment.²

Various diseases arise from imbalances in iron homeostasis. Iron accumulation in the liver is common in patients with chronic liver diseases,³ especially patients with chronic hepatitis C (CH-C),⁴⁻⁷ alcoholic liver disease (ALD),⁷⁻⁹ and nonalcoholic fatty liver disease (NAFLD).^{7,10} Oxidative stress has been

proposed as a mechanism of liver injury in these diseases.

The peptide hepcidin is proposed to be the key mediator of iron metabolism and systemic distribution. It is synthesized by hepatocytes in response to both iron overload and inflammatory stimuli.¹¹⁻¹³ Hepcidin acts by down-regulating both iron absorption and iron release of enterocytes and macrophages¹⁴⁻¹⁷ in response to high iron levels and inflammatory cytokines such as interleukin 6 (IL-6).¹⁸

Despite enormous interest in the role of hepcidin, a lack of available methods for quantifying circulating hepcidin in clinical samples hampers investigation of the role of hepcidin in human disease. However, ELISA can easily be utilized to measure serum levels of prohepcidin, the precursor molecule.¹⁹ Nevertheless, it has not been confirmed if serum prohepcidin accurately

Received March 1, 2010; Revised June 29, 2010; Accepted July 21, 2010

Abbreviations: ALD, alcoholic liver disease; CH-C, chronic hepatitis C; IL-6, interleukin 6; NAFLD, nonalcoholic fatty liver disease; TS, transferrin saturation

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reflects active hepcidin or just a non-functional precursor. Currently, the data concerning serum prohepcidin levels in patients with various chronic liver diseases and in healthy controls is very limited.

The aims of the present study were to evaluate the serum prohepcidin levels in patients with CH-C, ALD, NAFLD, and healthy controls and to determine the clinical variables affecting serum prohepcidin levels, including blood iron, transferrin saturation (TS), ferritin, and IL-6.

MATERIALS AND METHODS

Subjects

Patients were enrolled at the Hepatology Department in Seoul National Bundang Hospital between December 2006 and December 2007.

The CH-C group included 28 patients with positive serum HCV RNA and anti-HCV for greater than 6 months. The ALD group included 22 patients who had consumed alcohol at least daily (80 g for men or 40 g for women) for more than 5 years, and in whom other liver diseases-viral hepatitis, drug-induced liver disease, and autoimmune and genetic liver diseases such as Wilson disease - had been excluded. The NAFLD group included 24 patients who were diagnosed using NAFLD criteria: minimal alcohol use (<20 g/day in men or <10 g/day in women), elevated aminotransferase levels, compatible ultrasonographic findings, and appropriate exclusion of alcoholic liver disease and other etiologies as above. Patients with liver cirrhosis or hepatocellular carcinoma were excluded from the study. Subjects were consecutively enrolled among those patients amenable to participation in the study. The healthy control group included 25 health-check examinees with no evidence of liver disease on laboratory or radiological examination. This study was performed with the approval of the Seoul National University Bundang Hospital Institutional Review Board (IRB). Informed consent was obtained from all subjects.

Measurement of serum iron indices

The serum iron concentration, unbound iron binding capacity, and serum ferritin concentration were measured simultaneously by spectrophotometry using the FerroZine method (TBA 200, Toshiba, Tokyo, Japan) and electrochemiluminescence immunoassay (E170, Roche, Basel, Switzerland), respectively, according to the manufacturer's instructions. Transferrin saturation

(TS, %) was calculated by dividing the serum iron level by the total iron binding capacity and multiplying the figure by 100. The cutoff levels for elevated TS and ferritin were 55% and 300 µg/ml for men and 50% and 200 µg/ml for women, respectively, based on our previous study.²⁰

Measurement of serum prohepcidin and IL-6

Serum samples were stored at -80°C and allowed to return to room temperature before analysis. Commercially available enzyme immunoassays were used to determine serum prohepcidin (Hepcidin Prohormone ELISA, DRG Instruments, Marburg, Germany) and IL-6 (Human IL-6 immunoassay, R&D Systems, Minneapolis, MN, USA) levels, according to the manufacturer's instructions. We drew a standard curve for each measurement. All serum samples were measured in duplicate, and the average values were adopted.

Statistical analysis

Continuous variables are presented as means±standard deviations. Continuous variables were compared using the one way analysis of variance that was corrected by the DUNNETT's method as multiple comparison test. Nominal data were compared using Fisher's exact test or Pearson's chi-square test, as appropriate. Simple linear regression was performed to determine the clinical correlates associated with iron overload. Statistical analyses were performed using SPSS software (SPSS 12.0K for Windows; SPSS Korea, Seoul, Korea). *P* values <0.05 were considered statistically significant.

RESULTS

Clinical features and serum iron indices of the study subjects

The clinical features and laboratory results, including serum iron indices in patients with CH-C, ALD, NAFLD, and healthy controls, are summarized in Table 1. Although the mean subject age was constant across all groups, the proportion of male patients was significantly higher in the ALD group. Serum aspartate aminotransferase level in patients with CH-C, ALD, alanine aminotransferase level in patients with CH-C, ALD, was higher aminotransferase and fasting glucose levels were higher in patients with CH-C, ALD, and NAFLD, compared to those seen in healthy controls. The serum total cholesterol level was significantly lower in the CH-C group compared to the healthy

Table 1. Comparative data for clinical features and serum iron indices in healthy controls and in patients with various liver diseases

	H-C (n=25)	CH-C (n=28)	ALD (n=22)	NAFLD (n=24)
Age (yr)	48.5±12.1	53.4±13.0	46.1±9.3	44.4±13.8
Sex (M:F)	11:14	14:14	21:1 [†]	16:8
BMI (kg/m ²)	22.34±2.67	24.19±2.74	24.38±3.07 [†]	26.63±3.21 [‡]
Diabetes mellitus, n (%)	0	1 (3.6)	3 (13.6)	3 (12.5)
AST (IU/L)	23.0±6.1	42.0±26.5*	68.8±109.8 [†]	50.4±35.6
ALT (IU/L)	19.6±8.4	54.4±41.8*	42.5±31.1	95.3±78.6 [‡]
Fasting blood glucose (mg/dL)	83.9±8.2	93.6±9.6	106.3±45.9 [†]	99.4±26.9
Total cholesterol (mg/dl)	208.7±40.1	184.9±38.5*	198.8±32.4	219.4±30.8
Hemoglobin (g/dL)	13.96±1.23	14.30±1.72	15.34±1.08 [†]	15.14±1.48 [‡]
Serum iron (µg/dL)	120.6±47.0	124.4±41.3	205.4±60.0 [†]	157.0±48.8 [‡]
TIBC (µg/dL)	334.2±37.8	348.2±54.3	367.8±38.0 [†]	361.3±51.1
TS (%)	37.0±15.9	36.8±13.8	56.6±18.0 [†]	44.4±16.3
TS >55% (men) or >50% (women), n (%)	5 (20.0%)	3 (10.7%)	12 (54.5%) [†]	6 (25.0%)
Serum ferritin (µg/dL)	115.0±82.7	157.4±168.0	322.3±402.7 [†]	283.9±309.1
Serum ferritin >300 µg/L (men) or >200 µg/L (women), n (%)	1 (4.0%)	4 (14.3%)	5 (22.7%)	8 (33.3%) [‡]

H-C, healthy controls; CH-C, chronic hepatitis C; ALD, alcoholic liver disease; NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TIBC, total iron binding capacity; TS, transferrin saturation.

* $P < 0.05$ between CH-C patients and H-C.

[†] $P < 0.05$ between ALD patients and H-C.

[‡] $P < 0.05$ between NAFLD patients and H-C.

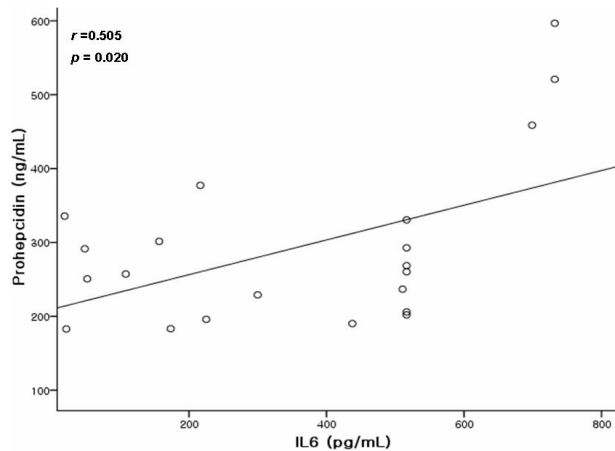


Figure 1. Positive correlation between serum prohepcidin and interleukin-6 (IL-6) levels in patients with chronic hepatitis C (CH-C).

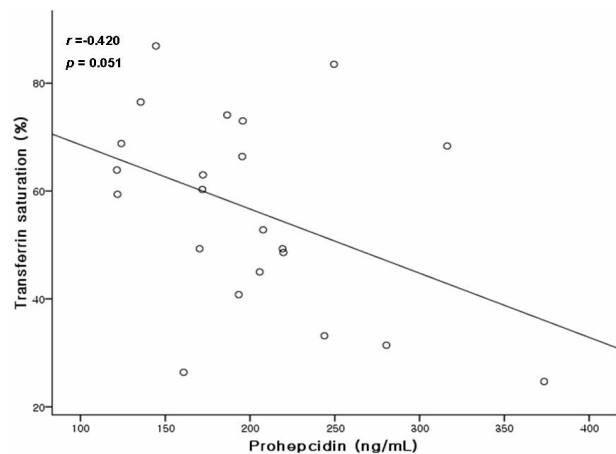


Figure 2. Negative correlation between the serum prohepcidin level and transferrin saturation (TS) in patients with alcoholic liver disease (ALD).

control group.

The mean serum TS was significantly higher in the ALD group ($p < 0.001$), and the mean serum ferritin levels were significantly higher in the ALD group ($p = 0.023$) compared to those seen in the healthy control group. Neither TS nor ferritin was significantly elevated in the CH-C group, although the serum ferritin levels of CH-C patients showed an increasing tendency compared to those of the healthy control group.

Serum prohepcidin and IL-6 levels in CH-C, ALD, NAFLD, and healthy controls

The serum levels of prohepcidin and IL-6 in the patients with CH-C, ALD, NAFLD, and healthy control patients are summarized in Table 2. Both the serum prohepcidin and IL-6 levels were significantly higher in the CH-C group than in the healthy control group ($p < 0.001$ and $p < 0.001$, respectively). The serum prohepcidin level in the ALD group was no different from

Table 2. Comparative data for prohepcidin and interleukin-6 (IL-6) levels in healthy controls and in patients with various liver diseases

	H-C (n=25)	CH-C (n=28)	ALD (n=22)	NAFLD (n=24)
Serum Pro-hepcidin (ng/mL)	184.70±60.49	308.45±116.43*	200.42±63.55	178.69±38.86
Serum IL-6 (pg/mL)	1.25±0.68 (N=15)†	358.60±242.11* (N=21)†	438.29±336.86‡ (N=7)†	0.95±0.75 (N=11)†
Serum Pro-hepcidin/Serum ferritin ratio	2.62±2.38	4.70±5.65	1.17±0.85	1.37±1.49

IL-6, Interleukin-6; H-C, healthy controls; CH-C, chronic hepatitis C; ALD, alcoholic liver disease; NAFLD, nonalcoholic fatty liver disease. BMI, Body mass index; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; TIBC, Total iron binding capacity; TS, Transferrin saturation.

* $P < 0.05$ between CH-C patients and H-C.

† IL-6 level was measured only in 15 healthy controls, 21 patients with chronic hepatitis C, 7 patients with alcoholic liver disease and 11 patients with nonalcoholic fatty liver disease.

‡ $P < 0.05$ between ALD patients and H-C.

that seen in the healthy control group, despite significantly elevated IL-6 levels (438.29±336.86 pg/ml in ALD vs. 1.25±0.68 pg/ml in healthy controls, $p < 0.001$). In the NAFLD group, neither the serum prohepcidin level nor the IL-6 level was different compared to that seen in healthy controls.

Correlation between serum prohepcidin level and other variables

A positive correlation was found between serum prohepcidin levels and serum IL-6 levels in patients with CH-C ($r = 0.505$, $p = 0.020$, Fig. 1). However, the correlation was not significant in healthy controls, ALD, or NAFLD patients.

A negative correlation tendency was found between serum prohepcidin levels and TS in patients with ALD ($r = -0.420$, $p = 0.051$, Fig. 2). However, the correlation was not statistically significant in the healthy, CH-C, or NAFLD groups. There was no significant correlation between serum prohepcidin and ferritin levels among the four groups of subjects.

Prohepcidin/ferritin ratios in CH-C, ALD, NAFLD, and healthy controls

When we compared the prohepcidin/ferritin ratios among the four subject groups, there was no significant difference in the CH-C group (4.70±5.65, $p = 0.067$), the ALD group (1.17±0.85, $p = 0.830$) and the NAFLD group (1.37±1.49, $p = 0.422$) compared to the healthy control group (2.62±2.38), although the ratio in the CH-C group and the healthy control group showed relatively higher level than others.

There was a negative correlation between the prohepcidin/ferritin ratio and TS in healthy controls ($r = -0.448$, $p = 0.025$) and in patients with CH-C ($r = -0.417$, $p = 0.030$). However, this correlation was not significant in patients with ALD or NAFLD.

DISCUSSION

In this study, we observed that both the serum prohepcidin and IL-6 levels were significantly elevated in CH-C patients compared to those in healthy controls, and both prohepcidin and IL-6 were positively correlated with each other in CH-C. ALD patients showed significantly higher serum iron and ferritin levels compared to healthy controls, but their serum prohepcidin levels were not different from those seen in healthy controls, despite the significantly elevated IL-6 levels. Although the NAFLD group showed elevated serum iron and ferritin levels, neither the prohepcidin nor the IL-6 levels were elevated. Therefore, in CH-C, prohepcidin seems to be induced by IL-6. However, in ALD, prohepcidin is not induced by IL-6, probably due to the inhibitory effect of alcohol on hepcidin. Moreover, there was a negative correlation between the prohepcidin/ferritin ratio and TS in healthy and CH-C patients, while no such significant correlation was noted in ALD and NAFLD patients. This suggests that the prohepcidin response to body iron stores is functional in normal and CH-C patients, while it might be dysfunctional in ALD and NAFLD patients. Therefore, different regulatory mechanisms of iron metabolism and different roles of prohepcidin exist in different human liver diseases.

Hepcidin is the product of the hepcidin antimicrobial peptide (HAMP) gene on human chromosome 19, and expression of hepcidin mRNA is mostly confined to the liver. The transcript encodes a precursor protein of 84 amino acids, whereas the mature circulating bioactive forms of hepcidin consist of only the carboxy-terminal portion of 20-25 amino acids, which is cysteine-rich.¹² Serum hepcidin is not easily measured due to structural containment. Conversely, serum prohepcidin can be measured using a commercialized ELISA kit. Kulaksiz et al.¹⁹ showed that the mean prohepcidin level in the serum of healthy

German volunteers (n=26) was 106.2 ng/ml. Thereafter, the serum prohepcidin levels measured by the same method were reported to be 85.1±6.1 ng/ml in female Korean college students (n=82),²¹ and to be 227±207 ng/ml in Finnish women (n=37) and 254±201 ng/ml in Finnish men (n=16),²² findings quite similar to our own findings for healthy control subjects (184.7±60.5 ng/ml).

HCV infection is associated with alterations in body iron homeostasis through a poorly understood mechanism. Nagashima et al.²³ reported that prohepcidin levels in CH-C (n=137) and HCV-related liver cirrhosis (n=37) patients were 137.3±140.2 ng/ml and 53.2±116.7 ng/ml, respectively, significantly lower than those seen in healthy controls (n=103), 448.5±200.7 ng/ml. Furthermore, hepcidin expression in the liver was negatively correlated with the total iron score in 49 patients. In their study, serum prohepcidin levels were quite high in healthy controls compared to other studies, including our results. Their findings with regard to the relationship between intrahepatic hepcidin expression and hepatic iron deposition contradicted the results of Aoki et al.,²⁴ which showed that hepcidin mRNA expression correlated with hepatic iron concentration and serum ferritin levels in liver biopsy samples obtained from chronic hepatitis C patients. Our findings contrast with those of Nagashima et al, but are compatible with the last study. Moreover, we found that the ratios of prohepcidin/ferritin in the healthy control group and in the CH-C groups were no different, and there was a negative correlation between the prohepcidin/ferritin ratio and TS in healthy controls ($r=-0.448$, $p=0.025$) and patients with CH-C ($r=0.417$, $p=0.030$); this correlation did not exist in ALD and NAFLD patients. These findings may indicate that the prohepcidin response to body iron stores is functional in healthy controls and CH-C patients, while it is dysfunctional in ALD and NAFLD patients.

Our findings related to serum IL-6 levels in CH-C patients were compatible with several previous studies. Serum IL-6 levels are significantly elevated in chronic hepatitis C patients compared to healthy controls,²⁵ and HCV induces IL-6 production by inducing Toll-like receptor 4 expression *in vitro*²⁶ or Toll-like receptor 2 expression *in vivo*.²⁷ In human liver cell cultures, as well as in mouse and human volunteer studies, IL-6 is the necessary and sufficient cytokine for the induction of hepcidin during inflammation.²⁸ Therefore, the significantly increased serum IL-6 and prohepcidin levels seen in CH-C patients in this study support the idea that HCV infection induces IL-6 expression, which in turn induces hepcidin and serum prohepcidin expression. However, a recent study in a transgenic mouse model

expressing HCV polyprotein showed a mild elevation of hepatic iron compared with nontransgenic mice, and this elevation was associated with a reduction in hepatic hepcidin mRNA expression and a reduction in serum prohepcidin protein levels.²⁹ However, this mouse model did not exhibit inflammation via the IL-6-mediated STAT2 signaling pathway, nor were cytokine changes noted in the human. Our clinical observations were in contrast to the mouse model, therefore, this model may not apply to human patients with chronic hepatitis C.

Hepcidin expression is reported to be consistently downregulated by alcohol in rat models with alcoholic liver disease and *in vitro* cell culture models.³⁰ Alcohol metabolism-mediated oxidative stress down-regulates hepcidin transcription via reduced CCAAT enhancer binding protein alpha activity and leads to increased duodenal iron transporter expression.³¹ Ohtake et al. showed that serum prohepcidin levels in ALD patients (n=47), including 8 cirrhosis patients, were significantly lower than those in 9 healthy subjects (710±540 ng/ml vs. 1,570±260 ng/ml), and the serum prohepcidin/ferritin ratios in ALD and healthy subjects were 4.8±5.8 and 13.4±7.5, respectively ($p<0.05$).³² Although the prohepcidin levels in healthy control subjects were unusually high compared to other studies, the ratio of prohepcidin/ferritin in ALD patients was significantly lower than that seen in healthy controls, which was consistent with our findings. We noted no significant difference in the serum prohepcidin levels between ALD and healthy control patients. However, serum IL-6 levels were significantly elevated in ALD patients, which was compatible with previous reports.³³ This suggests that prohepcidin was not induced in ALD, despite the elevation of IL-6.

Insulin resistance, the initial triggering factor of NAFLD, is closely related to hyperferritinemia, and hepatic iron could promote oxidative stress, the second factor in NAFLD pathogenesis and a probable downregulator of hepcidin. A recent study reported hepcidin expression in adipose tissue of severely obese patients, suggesting that severe obesity itself causes hypoferrinemia through overproduction of hepcidin in adipocytes and liver tissue, which may negate the effect of oxidative stress on hepcidin expression.³⁴ In our study, the serum prohepcidin levels in NAFLD patients were not different from those seen in healthy controls. However, the prohepcidin/ferritin ratio in NAFLD patients was significantly lower than that seen in healthy controls. This finding was similar to that in a recently reported study,³⁵ and could be explained by hyperferritinemia in NAFLD.

The basic limitation of this study was the measurement of prohepcidin rather than the active compound hepcidin, because

of the unavailability of such a measuring method. Serum prohepcidin levels have considerable interindividual variations, and commercially available ELISA kits suffer from low sensitivity, despite their high cost. Our study was also limited in that the sample sizes were small for each liver disease group, and hepatic hepcidin expression was not measured. However, the diagnostic classification of our subjects was strict, and the clinical data showed typical patterns with regard to disease. In addition, we obtained complete data for serum iron indices and other blood chemistry and clinical variables, which made it possible to analyze the prohepcidin/ferritin ratio and to determine its correlation with TS. To our knowledge, comparative studies on the serum prohepcidin levels in healthy subjects and in those with various liver diseases were limited.

In conclusion, the regulation of prohepcidin and probably hepcidin is complex and variable in the different liver diseases. In the setting of CH-C, both the serum prohepcidin and IL-6 levels were significantly elevated and had positive correlation.

Acknowledgements

The authors thank Mrs. Ji Hye Lee for her technical assistance. This study was supported in part by grants from the Korea Center for Disease Control and Prevention.

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