Serum hepcidin-25 levels and anemia in non-dialysis chronic kidney disease patients: a cross-sectional study

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Abstract

Background. Hepcidin is a central regulator of iron homeostasis. Increased hepcidin concentrations could cause iron-restricted erythropoiesis in chronic kidney disease (CKD)-associated anemia. This cross-sectional observational study was conducted to evaluate the association between hepcidin and CKD-associated anemia in non-dialysis CKD patients.

Methods. A total of 505 non-dialysis CKD patients not treated with parenteral iron were recruited, and serum hepcidin-25 levels were measured by liquid chromatography tandem mass spectrometry. Multiple linear regression analysis was used to examine the relationship between hepcidin and glomerular filtration rate (GFR) and the relationship between hemoglobin concentration and predictors including the hepcidin level.

Results. The median hepcidin level among the 505 CKD patients was 15.4 ng/mL (interquartile range, 5.5–33.6 ng/mL). Although hepcidin level significantly increased according to the CKD stage, multivariate analysis did not reveal an association of GFR with the hepcidin level. Hepcidin level was a significant predictor of hemoglobin concentration after the adjustment for confounders, and a significant interaction between hepcidin and ferritin was found. After stratifying at the median ferritin level, 91 ng/mL, we found a negative association between hepcidin level and hemoglobin in the high-ferritin group. A trend toward a negative association between hepcidin level and mean corpuscular volume was observed in the high-ferritin group.

Conclusions. Serum hepcidin-25 levels were negatively associated with hemoglobin concentrations in non-dialysis CKD patients with sufficient iron stores. We found that ferritin modified the association between hepcidin level and hemoglobin concentration. In addition, our results confirmed that the serum hepcidin level is not associated with GFR.

Keywords: anemia; CKD; ferritin; glomerular filtration rate; hepcidin-25

Introduction

Anemia is a major complication of chronic kidney disease (CKD) [1]. Treatment of CKD-associated anemia has been dramatically advanced by the introduction of recombinant human erythropoietin (EPO). However, CKD-associated anemia can be resistant to EPO treatment [2]. In addition to EPO deficiency, inflammatory effects of the primary disease, inflammatory effects of its complications and of its treatments, and iron-restricted erythropoiesis could be involved in this pathogenesis [3]. To achieve the optimal effect of EPO treatment, adequate iron management in patients with CKD-associated anemia is essential [4, 5]. Although serum ferritin and transferrin saturation (TSAT) are commonly used as biomarkers for iron status in CKD patients, these markers are not sensitive enough to distinguish functional iron deficiency from iron overload [2].

Hepcidin, a 25-amino acid peptide primarily produced in the liver, is thought to be the central regulator of body iron metabolism [6]. Hepcidin controls the plasma iron concentration by inhibiting iron export by ferroportin from enterocytes and macrophages [7]. Therefore, increased hepcidin production leads to a decrease in plasma iron concentrations and to iron-restricted erythropoiesis [3]. Hepcidin expression is induced by iron loading [8] and by inflammation [9] and is suppressed by erythropoietic activity [3, 10]. Studies of humans with chronic infections and severe inflammatory disease have shown markedly increased levels of hepcidin, strongly suggesting that elevated hepcidin levels play a key role in the anemia of inflammation and reticuloendothelial blockade [9]. It is plausible that increased hepcidin concentrations may cause iron-restricted erythropoiesis also in CKD-associated anemia.
The purpose of this cross-sectional observational study was to evaluate the association of hepcidin and CKD-associated anemia in non-dialysis CKD patients. We also evaluated whether serum ferritin modified the association of hepcidin and CKD-associated anemia.

**Materials and methods**

**Study design**

We conducted the cross-sectional study from February 2007 through June 2007. Consecutive patients were recruited from the outpatient nephrology clinic at Osaka Medical General Center. Patients were eligible for this study if they were ≥20 years of age and had a history of CKD. Exclusion criteria included renal replacement therapy (hemodialysis, peritoneal dialysis or kidney transplant), parenteral iron therapy within 6 months preceding the period in question, and the use of erythropoiesis-stimulating agents (given as recombinant human EPO; erythropoietin). None of the patients had received EPO during the 2-week period before the study. Erythropoiesis-stimulating agents were introduced to a PLRP-S column (5 mm, 300 Å, 150 μm) after using standard laboratory techniques. Concentrations of serum hepcidin were expressed in nanograms per milliliter. The reference level of serum hepcidin in 63 healthy volunteers was 5.7 ng/mL.

**Clinical measurements**

Data on demographic characteristics and blood samples were collected at enrollment. Blood samples were immediately centrifuged, separated into aliquots and stored at −80°C for future assays. The serum levels of hepcidin-25 were quantified by liquid chromatography tandem mass spectrometry (LC-MS/MS) by a method reported previously [11]. In brief, isotopic human synthetic hepcidin (Peptide Institute, Osaka, Japan) was mixed with each sample as an internal standard. The samples were then introduced to a PLRP-S column (5 mm, 300 Å, 150 μm) after using standard laboratory techniques. Concentrations of serum hepcidin were expressed in nanograms per milliliter. The intra- and interassay coefficients of variation were <6.7% and <8.8%, respectively. The lower limit of detection was 1.0 ng/mL. The median reference level of serum hepcidin in 63 healthy volunteers was 5.7 ng/mL (interquartile range, 1.6–12.7 ng/mL).

**Statistical analysis**

Baseline characteristics were assessed with standard descriptive statistics. eGFR was examined both on a continuous scale and also categorically using the National Kidney Foundation stage system: CKD Stage 1, eGFR ≥194 × (age)−0.287 (×0.739, if female) [12]. Other serum parameters were measured by using standard laboratory techniques.

| Table 1. Clinical characteristics of 505 patients with CKD of Stages 1–5a |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | CKD Stage 1–2, n = 130 | CKD Stage 3, n = 179 | CKD Stage 4, n = 127 | CKD Stage 5, n = 69 |
| Age (years)    | 50.1 ± 17.0      | 67.1 ± 12.0      | 68.9 ± 12.8      | 68.9 ± 11.8      | <0.001 |
| Female (%)     | 54.9             | 34.6             | 46.9             | 50.7             | 0.8    |
| Serum creatinine (mg/dL) | 0.72 (0.62–0.82) | 1.20 (1.06–1.41) | 2.10 (1.82–2.40) | 4.30 (3.60–5.56) | <0.001 |
| eGFR (mL/min/1.73 m²) | 80.7 ± 15.7   | 43.3 ± 8.6       | 22.7 ± 4.2       | 10.1 ± 2.8       | <0.001 |
| Hemoglobin (g/dL) | 13.6 ± 1.5     | 12.8 ± 1.8       | 11.2 ± 1.5       | 10.5 ± 1.5       | <0.001 |
| Mean corpuscular volume (fL) | 91.1 (88.5–91.1) | 93.1 (90.3–96.2) | 93.7 (89.8–97.5) | 93.4 (90.6–96.3) | <0.001 |
| Hepcidin (ng/mL) | 15.7 (3.5–20.3) | 15.4 (6.5–29.2)  | 19.6 (6.3–43.0)  | 27.2 (11.5–68.2) | <0.001 |
| Serum ferritin (ng/mL) | 69.3 (22.0–137.5) | 89.7 (49.4–190.0) | 112.0 (54.3–211.0) | 111.0 (62.8–247.0) | <0.001 |
| TSAT (%)        | 28.8 ± 12.5      | 30.0 ± 12.1      | 29.0 ± 13.0      | 29.0 ± 13.0      | 0.3    |
| CRP (mg/dL)     | 0.05 (0.02–0.05) | 0.07 (0.04–0.17) | 0.07 (0.03–0.36) | 0.12 (0.03–0.24) | <0.001 |
| IL-6 (pg/mL)    | 0.59 (0.30–1.50) | 1.14 (0.67–2.38) | 1.93 (1.05–3.46) | 1.92 (1.17–3.54) | <0.001 |

**Causes of CKD (%)**

- Chronic glomerulonephritis: 59.4%
- Nephrosclerosis: 5.3%
- Diabetic nephropathy: 1.5%
- Polycystic kidney disease: 0.7%
- Unknown: 4.5%
- Other: 28.6%

**Medications (%)**

- EPO use: 0%
- Oral iron administration: 1.5%
- ACE Inhibitor/ARB: 47.4%
- Statins: 17.3%

**Comorbidities (%)**

- Diabetes: 8.3%
- Hypertension: 48.1%
- Coronary artery disease: 1.5%
- Congestive heart failure: 0.8%
- Cerebrovascular disease: 0.8%
- Peripheral vascular disease: 3.8%
- Chronic hepatitis: 3.8%

*Continuous variables are expressed as mean ± SD or median (interquartile range). TSAT, transferrin saturation; ACE, angiotensin-converting enzyme; ARB, angiotensin-receptor blocker.*
ferritin and examined whether ferritin modified the association between two iron parameters. We stratified the patients at the median level of hemoglobin concentration and the interaction between these parameters. To examine the relationship of eGFR with the log-normalized hepcidin level, multiple regression analyses were performed. In the multivariate models, an association of eGFR with the log-normalized hepcidin level was not observed either in the series of all patients (including EPO administration as a confounder; P = 0.8) or in the series of EPO-free patients (P = 1.0) (Table 2).

**Association between hemoglobin level and iron parameters**

To examine the ability of hepcidin, ferritin and TSAT to predict the hemoglobin concentration, we assessed the associations between the hemoglobin level and these iron parameters. Univariate analyses showed that the hemoglobin concentration was negatively and significantly correlated with the log-normalized hepcidin level (r = −0.19, P < 0.001) and was positively correlated with TSAT (r = 0.15, P = 0.001) but was not correlated with log-normalized ferritin (P = 0.4) (data not shown). Multiple linear regression analyses were performed using JMP for Windows version 5.1 (SAS Institute Inc, Cary, NC). The Jonckheere-Terpstra trend test and the Cochran-Armitage test were performed by using STATA version 10 (STATA Corp, College Station, TX).

**Results**

**Patient characteristics**

Table 1 summarizes the characteristics of patients with CKD of Stages 1–5. Causes of CKD were chronic glomerulonephritis (241/505, 47.7%), nephrosclerosis (98/505, 19.4%), diabetic nephropathy (58/505, 11.5%), polycystic kidney disease (5/505, 1%), other (80/505, 15.8%) and unknown (23/505, 4.6%). Of 505 patients, 102 (20.2%) had diabetes and 375 (74.3%) had hypertension, and up to 10% of patients had other comorbidities (coronary artery disease, 45/505, 8.9%; congestive heart disease, 24/505, 4.8%; stroke, 40/505, 7.9%; peripheral artery disease, 24/505, 4.8%; chronic hepatitis, 35/505, 6.9%).

**Hepcidin levels in CKD patients**

The median hepcidin level among the 505 CKD patients was 15.4 ng/mL (interquartile range, 5.5–33.8 ng/mL). Hepcidin levels as well as ferritin increased significantly as the CKD stages progressed (P-value for trend <0.001), whereas TSAT showed no significant trend with increasing CKD stages. As previous studies reported [13, 14], log-normalized serum hepcidin was strongly correlated with the log-normalized ferritin (r = 0.72, P < 0.001; Figure 1a). The hepcidin level significantly increased according to the CKD stage, and the log-normalized hepcidin was negatively correlated with eGFR (r = −0.27, P < 0.001) (b).
analyses showed that log-normalized hepcidin was a significant predictor of hemoglobin concentration (Table 3), both in the series of all patients as well as in the series of EPO-free patients. We assessed the effect of the interaction between log-normalized hepcidin and log-normalized ferritin, and in both series, we found a significant correlation between hemoglobin concentration and this interaction \( P \) for interaction (log hepcidin \( \times \) log ferritin) < 0.001]. Therefore, we stratified
the patients at the median ferritin level (91 ng/mL) and performed further analyses with hemoglobin concentration as the dependent variable.

Patients in the high-ferritin group had significantly lower eGFR and significantly higher hepcidin and TSAT levels and significantly higher CRP and IL-6 levels, compared with patients in the low-ferritin group (Table 4). However, there was no significant difference in hemoglobin concentration between the two groups. In the series of all patients, eight in the low-ferritin group (8/253, 3%) and seven in the high-ferritin group (7/252, 3%) were treated with oral iron. Univariate analysis showed that lower hemoglobin level was associated with older age, female gender, reduced eGFR, elevated IL-6 and EPO administration in each group (data not shown). Notably, the log-normalized hepcidin level was significantly negatively correlated with the hemoglobin concentration in the high-ferritin group (b) but was not correlated in the low-ferritin group (a). In contrast, TSAT was positively correlated with hemoglobin concentration in the low-ferritin group (c) but was not correlated in the high-ferritin group (d).

Multiple linear regression analyses of the series of all patients and the series of EPO-free patients revealed a negative association between log-normalized hepcidin level and hemoglobin concentration in the high-ferritin groups, and in contrast, a positive association in the low-ferritin groups (Table 5). As expected, in the low-ferritin groups, a lower TSAT level was significantly associated with reduced hemoglobin level. Multiple regression analysis in the EPO-free patients showed a trend for a negative association [\(\beta = -1.8\) (95% confidence interval (CI), -3.70 to -0.01), \(P = 0.05\)] between log-normalized hepcidin level and mean corpuscular volume in the high-ferritin group and a significant positive association [\(\beta = 1.4\) (95% CI, 0.36–2.38), \(P = 0.008\)] in the low-ferritin group (data not shown).

Fig. 2. Scatter plots of hemoglobin level (Hb; g/dL) versus serum hepcidin-25 (a and b) and hemoglobin level versus transferrin (TSAT; %) (c and d) in the low-ferritin group (<91 ng/mL, \(N = 253\)) (a and c) and in the high-ferritin group (≥91 ng/mL, \(N = 252\)) (b and d). Log-normalized hepcidin level was negatively correlated with hemoglobin concentration in the high-ferritin group (b) but was not correlated in the low-ferritin group (a). In contrast, TSAT was positively correlated with hemoglobin concentration in the low-ferritin group (c) but was not correlated in the high-ferritin group (d).
To examine whether the median ferritin level is valid as a cutoff, we performed sensitivity analyses. At a cutoff ferritin level of 100 ng/mL—the minimum ferritin level recommended by the 2006 National Kidney Foundation Disease Outcomes Quality Initiative [15] for non-dialysis CKD patients taking erythropoiesis-stimulating agents—the results were consistent with those of the median cutoff.

**Discussion**

To the best of our knowledge, this is the first study demonstrating that hepcidin-25 levels are negatively associated with hemoglobin concentrations in non-dialysis CKD patients with sufficient iron stores. We found that ferritin modified the association between the hepcidin level and hemoglobin concentration. In addition, although univariate analysis showed that hepcidin levels negatively correlated with eGFR, our results in multivariate analysis confirmed the results of a prior study demonstrating that the serum hepcidin level is not associated with glomerular filtration rate (GFR) after adjustment for confounders [14].

In our study, hepcidin was negatively associated with hemoglobin in non-dialysis CKD patients with sufficient iron stores. This relationship between hepcidin and hemoglobin was observed both in the series of all patients and in the series of EPO-free patients. Our results are consistent with the results of one previous study in dialysis patients [16] but inconsistent with the results of other studies in dialysis patients [17, 18] and inconsistent with studies in non-dialysis CKD patients [14, 19]. In a US study [19], multivariate analysis showed that there was no association between serum hepcidin and hemoglobin in 48 pediatric and 32 adult non-dialysis CKD patients; in a Dutch study [14], univariate analysis showed no such association in 83 non-dialysis CKD patients. These conflicting results may be attributable to differences in the iron status of the populations studied, differences in inflammatory state or sample size. The association of hepcidin and anemia could differ between patients with deficient iron stores and those with sufficient iron stores. Therefore, we performed analyses stratified according to the serum ferritin level and revealed details of how ferritin level modified the effect of hepcidin on anemia.

There are several reasons why hepcidin may be negatively associated with hemoglobin in non-dialysis CKD patients with sufficient iron stores. Firstly, hepcidin inhibits iron absorption from enterocytes and iron recycling from macrophages, leading to limited iron availability for erythropoiesis. High-ferritin levels normally indicate iron overload, but this does not necessarily mean sufficient bone marrow iron stores [20]. In our results, a trend for a negative association between hepcidin level and mean corpuscular volume was observed in the high-ferritin group, supporting the concept of iron-restricted erythropoiesis. Secondly, hepcidin is elevated in non-dialysis CKD patients [13, 14, 19], as was also shown in our patients. A previous study showed that erythropoiesis activity is required in order to suppress hepcidin production [10] and in patients with reduced erythropoiesis activity, such as those with CKD, hepcidin production might not be suppressed. Thirdly, hepcidin is upregulated by inflammation [9, 21], and CKD is an inflammatory process. However, no apparent correlation between inflammatory markers and hepcidin was observed in this population, consistent with the results of a previous study [13]. We excluded patients with active infections from the current study. Therefore, the association between hepcidin and inflammation might not have been observed in our patients. The involvement of inflammation in the association between iron availability and anemia in non-dialysis CKD patients needs to be further elucidated.

A surprising finding was that hepcidin was positively associated with hemoglobin in non-dialysis CKD patients with normal to subnormal iron stores. This relationship between hepcidin and hemoglobin was observed

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**Table 5. Predictors of hemoglobin concentration in the low- and high-ferritin groups (multivariate models)**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Low-ferritin group (ferritin &lt; 91 ng/mL), n = 253</th>
<th>High-ferritin group (ferritin ≥ 91 ng/mL), n = 252</th>
<th>EPO-free patients&lt;sup&gt;c&lt;/sup&gt;</th>
<th>High-ferritin group (ferritin ≥ 91 ng/mL), n = 188</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coefficient ± SE</td>
<td>P</td>
<td>Coefficient ± SE</td>
<td>P</td>
<td>Coefficient ± SE</td>
</tr>
<tr>
<td>Age (per 10 years)</td>
<td>−0.1 ± 0.06</td>
<td>0.1</td>
<td>−0.2 ± 0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>Female</td>
<td>−1.4 ± 0.2</td>
<td>&lt;0.001</td>
<td>−0.5 ± 0.2</td>
<td>0.02</td>
</tr>
<tr>
<td>eGFR (per 10 mL/min/1.73 m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.3 ± 0.04</td>
<td>&lt;0.001</td>
<td>0.3 ± 0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Log hepcidin [log (ng/mL)]</td>
<td>0.3 ± 0.1</td>
<td>0.02</td>
<td>−0.6 ± 0.3</td>
<td>0.02</td>
</tr>
<tr>
<td>Log IL-6 [log (pg/mL)]</td>
<td>0.2 ± 0.2</td>
<td>0.2</td>
<td>−0.7 ± 0.2</td>
<td>0.001</td>
</tr>
<tr>
<td>TSAT (%)</td>
<td>2.7 ± 0.8</td>
<td>0.001</td>
<td>−0.5 ± 0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>EPO use</td>
<td>−1.0 ± 0.3</td>
<td>&lt;0.001</td>
<td>−0.8 ± 0.2</td>
<td>0.001</td>
</tr>
<tr>
<td>EPO level (mIU/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjusted R²&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.44</td>
<td>0.54</td>
<td>0.54</td>
<td>0.42</td>
</tr>
</tbody>
</table>

<sup>a</sup>TSAT, transferrin saturation.
<sup>b</sup>Multivariate model included age, gender, eGFR, log IL-6, TSAT, EPO use and log hepcidin.
<sup>c</sup>Multivariate model included age, gender, eGFR, log IL-6, TSAT, EPO level and log hepcidin.
both in the series of all patients and in the series of EPO-free patients. We suggest two possible explanations of why hepcidin was positively associated with hemoglobin in non-dialysis CKD patients with normal to subnormal iron stores. Firstly, the median hepcidin level among the low-ferritin group was 7.0 mg/mL (2.3–17.3 ng/mL), which may not have been sufficient to act as the main regulator of systemic iron homeostasis. Secondly, the hepcidin level may reflect absolute iron deficiency in this group.

Our study confirmed a previous finding that the serum hepcidin-25 level was not associated with GFR [14]. Although several studies have demonstrated that hepcidin levels are elevated in non-dialysis CKD patients [13, 14, 19], the association between hepcidin and GFR remains to be determined. A study from England [13] using radioimmunoassay showed that serum hepcidin level was associated with eGFR in 44 CKD patients, even after adjustment for ferritin. A US study [19] using an ELISA assay showed that the hepcidin level was inversely correlated with GFR in 32 adult CKD patients. Meanwhile, a Dutch study [14] using a mass spectrometry-based assay in 83 non-dialysis patients reported that hepcidin-25 was not associated with eGFR, but rather it was mainly associated with ferritin. Our findings in 505 non-dialysis CKD patients by using LC-MS/MS assay showed that hepcidin levels were negatively correlated with eGFR in univariate analysis (Figure 1b). However, our results in multivariate analysis were consistent with the results from the Dutch study [14]. There are several possible explanations for the conflicting results. Firstly, there were differences in the hepcidin assays used. It is reported that hepcidin levels measured by various immunochemical methods vary considerably [22] and that immunochemical assays lack the selectivity to distinguish hepcidin-25 from hepcidin-20 and -22 [23]. Secondly, the differences in sample size may have affected the results of the analyses. The analyses in previous studies were performed on samples of relatively small size. Thirdly, there may have been inherent differences in the study populations. The strength of our finding that serum hepcidin-25 was not associated with GFR is enhanced by the more specific assay and larger sample size.

This study has potential limitations. Firstly, the cross-sectional nature of the present study hindered assessment of the causal relationship between hepcidin level and anemia among the CKD patients, which should be ascertained by longitudinal studies. Secondly, we did not measure hepcidin isoforms; therefore, we could not provide additional information on why different techniques may have yielded different results with respect to the association between hepcidin and GFR. Thirdly, we did not measure urinary hepcidin excretion. Fourthly, as in any observational study, we could not account for unmeasured or residual confounding.

In conclusion, hepcidin-25 levels were negatively associated with hemoglobin concentrations in non-dialysis CKD patients with sufficient iron stores. These findings need to be confirmed by longitudinal studies but may have the important clinical implication that iron stores should be taken into account when evaluating the role of hepcidin in CKD-associated anemia.

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Conflict of interest statement. None declared.

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