Original Paper

Serum Hepcidin and Iron Indices Affect Anemia Status Differently According to the Kidney Function of Non-Dialysis Chronic Kidney Disease Patients: Korean Cohort Study For Outcome in Patients with Chronic Kidney Disease (KNOW-CKD)

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Key Words

Transferrin saturation • Ferritin • Hepcidin • Anemia • Severity

Abstract

Background/Aims: No studies have examined the association among serum hepcidin, iron indices, or anemia status based on the kidney function of non-dialysis chronic kidney disease (CKD) patients. **Methods:** We reviewed data of 2238 patients from a large-scale multicenter prospective Korean study (2011–2016) and excluded 198 patients with missing data regarding serum hepcidin, hemoglobin, transferrin saturation (TSAT), ferritin, and usage of erythropoiesis-stimulating agents (ESA) or supplemental iron and 363 patients using ESA or supplemental iron. Finally, 1677 patients were included. **Results:** The mean patient age was 53.5 years, and 65.4% were men. TSAT and serum hepcidin were significantly associated with anemia status, whereas serum ferritin was not, regardless of anemia severity. For patients with an estimated glomerular filtration rate (eGFR) ≥ 45 mL/min/1.73 m², a 10% increase of TSAT was associated

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with hemoglobin <13 g/dL (odds ratio [OR], 0.628; 95% confidence interval [CI], 0.515-0.765; $P < 0.001$) and hemoglobin <11.5 g/dL (OR, 0.672; 95% CI, 0.476-0.950; $P = 0.024$), whereas a 10-ng/mL increase of serum hepcidin was associated with hemoglobin <11.5 g/dL (OR, 1.379; 95% CI, 1.173-1.620; $P < 0.001$) and hemoglobin <10.0 g/dL (OR, 1.360; 95% CI, 1.115-1.659; $P = 0.002$) for patients with eGFR <45 mL/min/1.73 m² according to multivariate logistic analysis. **Conclusions:** TSAT was associated with less severe anemia in early CKD patients. Serum hepcidin was associated with more severe anemia in advanced CKD patients.

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Introduction

The prevalence of anemia is higher among patients with chronic kidney disease (CKD) than among the general population [1]. Furthermore, anemia tends to be more severe in patients with more advanced CKD [1, 2]. Because anemia in CKD patients contributes to an increased risk of end-stage renal disease (ESRD), cardiovascular events, and death [3, 4], the identification of factors associated with anemia in CKD patients is important. The major factor contributing to anemia in CKD patients is erythropoietin deficiency. Erythropoietin is produced by renal interstitial fibroblasts and stimulates red blood cell (RBC) production in the bone marrow [5]. As CKD progresses, erythropoietin levels are inappropriate for the level of anemia, and normocytic, normochromic, and hypo-proliferative anemia can develop [5]. To correct erythropoietin deficiency, studies have attempted to normalize hemoglobin (Hb) levels by replacing erythropoietin [6-9], but they ultimately failed to improve the clinical outcomes [6, 7].

Recently, iron metabolism has been suggested as another factor contributing to anemia in CKD patients [10, 11]. Conventionally, transferrin saturation (TSAT) and ferritin have been used as serum iron indices [12, 13]. TSAT, which is the ratio of serum iron and total iron-binding capacity, is a marker of the available serum iron, and serum levels of ferritin, which is an intracellular iron storage protein, indirectly indicate whole-body iron storage [10, 14]. However, the effectiveness of these two iron indices for anemia in CKD patients is inconclusive [15-18]. In this regard, hepcidin may be a better biomarker of anemia in CKD patients than conventional iron indices because it is more fundamental; hepcidin lowers available serum iron levels by limiting iron efflux from whole-body iron storage [19, 20]. Although previous studies have suggested the important role of hepcidin in anemia in non-dialysis [21-28] and dialysis [29-33] CKD patients, many relied on small population samples from single centers. No studies have explored the differential associations of hepcidin and iron indices with anemia according to the severity of anemia and kidney function. Because the serum hepcidin level is affected by kidney function [20], we hypothesized that hepcidin and iron indices are differentially associated with anemia in non-dialysis CKD patients according to kidney function. We also hypothesized that because iron is sequestered by hepcidin [19], anemia associated with hepcidin is more severe than anemia associated with iron indices.

Materials and Methods

Participants

The KoreaN cohort study for Outcome in patients With Chronic Kidney Disease (KNOW-CKD) is a multicenter, prospective cohort study in Korea of 2238 non-dialysis patients with CKD stages 1-5 who were enrolled between February 2011 and January 2016. The detailed design and methods of the KNOW-CKD were previously published (NCT01630486 at <http://www.clinicaltrials.gov>) [34]. The protocol of the KNOW-CKD adhered to the principles of the Declaration of Helsinki and was approved by the institutional review board at each participating hospital, including Seoul National University Hospital, Yonsei University Severance Hospital, Kangbuk Samsung Medical Center, Seoul St. Mary's Hospital, Gil Hospital, Eulji Medical

Center, Chonnam National University Hospital, and Busan Paik Hospital. Written informed consent was obtained from all subjects. The estimated glomerular filtration rate (eGFR) was calculated by the equation of Modification of Diet in Renal Disease study formula [35]. CKD and its stages were defined using the Kidney Disease Improving Global Outcomes (KDIGO) 2012 guidelines [36].

Of the 2238 patients, 561 were excluded: 198 with missing data regarding serum hepcidin, Hb, TSAT, ferritin, and usage of erythropoiesis-stimulating agents (ESA) or supplemental iron and 363 patients who had used ESA or supplemental iron. Therefore, 1677 patients were included in the final analysis.

Serum hepcidin measurement

Serum hepcidin levels were measured at a central laboratory with competitive enzyme-linked immunosorbent assay using EIA5258 kits (DRG Diagnostics, Marburg, Germany) according to the manufacturer's instructions. The intra-assay and inter-assay coefficients of variation ranged from 2.1% to 9.9% and from 11.5% to 14.6%, respectively [20]. The detectable maximum level was 80 ng/mL; therefore, higher levels were recorded as 80 ng/mL.

Definitions

Clinical data, including detailed demographic information and baseline laboratory results, were extracted from an electronic data management system (PhactaX). Hypertension was defined as systolic blood pressure (BP) ≥ 140 mm Hg or diastolic BP ≥ 90 mmHg or treatment with anti-hypertensive drugs. Diabetes was defined as fasting glucose ≥ 126 mg/dL, or treatment with insulin or oral anti-diabetic drugs. The ever smoking status was defined as past or current smoking. High income was defined as a monthly household income of more than KRW 4.5 million (approximately USD 4000). Body mass index (BMI) was calculated as weight (kg) per square meter of height (m^2). TSAT (%) was calculated as serum iron $\times 100$ /total iron binding capacity. The study outcome was anemia severity defined by the necessity of ESA: Hb < 13.0 g/dL (ESA should not be used to increase Hb levels intentionally), Hb < 11.5 g/dL (ESA should be used to maintain Hb levels), and Hb < 10.0 g/dL (ESA should be started), as stated by the KDIGO 2012 anemia guidelines [37]. Early and advanced CKD were defined as eGFR ≥ 45 and < 45 mL/min/ $1.73 m^2$, respectively [20].

Statistical analysis

Variables are expressed as mean $\pm$ standard deviation for continuous variables with normal distribution, median (interquartile range) for continuous variables with non-normal distribution and percentage for categorical variables. The odds ratio (OR) and 95% confidence interval (CI) were analyzed using logistic regression analysis. During multivariate analysis, variables associated with anemia status during univariate logistic regression were selected as covariates, along with white blood cells and C-reactive protein. The area under the curve (AUC) and its 95% CI of the receiver-operating characteristics (ROC) curve were evaluated using R version 3.03 (2014; R Core Team) with "pROC" packages [38]. Comparisons between two AUCs were performed using the DeLong test [39]. All analyses, unless otherwise specified, were performed using SPSS version 22 (2013; IBM Corp., Armonk, NY).

Results

The mean age of the 1677 patients was 53.5 years, and 65.4% were men. The mean eGFR was 54.8 mL/min/ $1.73 m^2$, with a median urine protein-to-creatinine ratio (UPCR) of 0.4 g/g creatinine. The mean Hb level was 13.2 g/dL, with a mean TSAT of 31.9%. The median serum levels of hepcidin and ferritin were 11.8 ng/mL and 207.2 pmol/L, respectively (Table 1). The prevalence of anemia severity was 45.1% for Hb < 13.0 g/dL, 18.8% for Hb < 11.5 g/dL and 4.2% for Hb < 10.0 g/dL, respectively.

We explored the association between serum hepcidin, iron indices, and anemia severity (Table 2). Regardless of anemia severity, serum hepcidin and iron indices were significantly associated with anemia according to the univariate logistic regression analysis, which was confirmed using the ROC analysis (Fig. 1). During comparisons of AUCs, the predictive power

Table 1. Baseline characteristics of the study participants. Values are expressed as mean $\pm$ standard deviation for normally distributed continuous variables, median (interquartile range) for non-normally distributed continuous variables, and percentage for categorical variables. Except for smoking status (10.4%), income status (2.9%), high-sensitivity C-reactive protein (4.8%) and urine protein-to-creatinine ratio (1.4%), missing rates of all above variables were $<1.0\%$

Characteristics	Total (n = 1,677)
Age (years)	53.5 $\pm$ 12.4
Male sex (%)	65.4
High income (%)	23.5
Ever smoking (%)	48.8
Systolic blood pressure (mm Hg)	127.4 $\pm$ 15.9
Diastolic blood pressure (mm Hg)	77.2 $\pm$ 11.0
Hypertension (%)	95.1
Diabetes (%)	25.0
Cause of chronic kidney disease	
Diabetic nephropathy (%)	22.7
Hypertensive nephropathy (%)	32.6
Glomerular nephropathy (%)	21.2
Other (%)	23.4
Body mass index (kg/m ²)	24.7 $\pm$ 3.4
Fasting plasma glucose (mmol/L)	6.2 $\pm$ 2.2
Blood urea nitrogen (mmol/L)	9.1 $\pm$ 4.7
Creatinine (μ mol/L)	144.4 $\pm$ 83.2
Estimated glomerular filtration rate (mL/min/1.73m ²)	54.8 $\pm$ 30.2
Bilirubin (μ mol/L)	12.0 $\pm$ 5.3
Albumin (g/L)	42.0 $\pm$ 4.3
Cholesterol (mmol/L)	4.6 $\pm$ 1.0
White blood cells ($\times 10^3/\mu$ L)	6.7 $\pm$ 1.9
Hemoglobin (g/dL)	13.2 $\pm$ 1.9
Serum transferrin saturation (%)	31.9 $\pm$ 11.9
Serum hepcidin (ng/mL)	11.8 (6.2–21.5)
Serum ferritin (pmol/L)	207.2 (115.8–368.4)
High-sensitivity C-reactive protein (nmol/L)	5.7 (2.0–15.2)
Urine protein-to-creatinine ratio (g/g Creatinine)	0.4 (0.1–1.4)

Table 2. Association of serum hepcidin and iron indices with anemia severity. Hb, hemoglobin; TSAT, transferrin saturation; OR, odds ratio; CI, confidence interval. OR and its CI were calculated using logistic regression analysis. In multivariate analysis, TSAT, ferritin and hepcidin were entered into together with age, sex, income status, smoking status, body mass index, fasting plasma glucose, systolic blood pressure, estimated glomerular filtration rate, bilirubin, albumin, and urine protein-to-creatinine ratio. * per 10 % increase in TSAT, per 10 pmol/L increase in ferritin, and per 10 ng/mL increase in hepcidin

Outcomes	Factors*	Univariate		Multivariate	
		OR (95% CI)	P	OR (95% CI)	P
Hb <13.0 g/dL	TSAT (10 %)	0.660 (0.602–0.723)	<0.001	0.701 (0.610–0.807)	<0.001
	Ferritin (10 pmol/L)	0.995 (0.991–0.999)	0.016	1.001 (0.993–1.008)	0.890
	Hepcidin (10 ng/mL)	1.175 (1.100–1.255)	<0.001	1.200 (1.054–1.367)	0.006
Hb <11.5 g/dL	TSAT (10 %)	0.671 (0.595–0.756)	<0.001	0.747 (0.620–0.901)	0.002
	Ferritin (10 pmol/L)	1.001 (0.996–1.005)	0.785	0.992 (0.984–1.001)	0.072
	Hepcidin (10 ng/mL)	1.316 (1.224–1.413)	<0.001	1.403 (1.221–1.613)	<0.001
Hb <10.0 g/dL	TSAT (10 %)	0.765 (0.612–0.956)	0.019	0.691 (0.484–0.986)	0.041
	Ferritin (10 pmol/L)	1.010 (1.003–1.017)	0.006	0.999 (0.988–1.009)	0.798
	Hepcidin (10 ng/mL)	1.425 (1.283–1.583)	<0.001	1.422 (1.180–1.713)	<0.001

of serum hepcidin was inferior to those of TSAT and ferritin when the outcome was Hb <13.0 g/dL. However, as anemia severity increased, the predictive power of serum hepcidin increased, resulting in the largest serum hepcidin AUC among the three variables when the outcome was Hb <10.0 g/dL. During multivariate logistic regression analysis, after adjusting for kidney function (eGFR) and other confounders (age, sex, income status, smoking status, BMI, fasting plasma glucose, systolic blood pressure, bilirubin, UPCR, and albumin), TSAT and

Fig. 1. Receiver-operator characteristics curve analysis of serum hepcidin and iron indexes for anemia severity. AUC, area under the curve; CI, confidence interval; TSAT, transferrin saturation; Hb, hemoglobin. * $P < 0.05$ compared to ferritin and † $P < 0.05$ compared to TSAT using Delong's test.

serum hepcidin maintained statistical significance, whereas serum ferritin lost statistical significance (Table 2).

We performed a subgroup analysis according to kidney function to evaluate the association between serum hepcidin, iron indices, and anemia severity (Fig. 2). For patients with early CKD ($n = 919$), the AUC of serum hepcidin for anemia was the smallest among the three variables, regardless of anemia severity. However, for patients with advanced CKD ($n = 758$), the AUC of serum hepcidin increased as anemia severity increased, resulting in the highest AUC when the outcome was Hb < 10.0 g/dL. Similar findings were observed during multivariate logistic regression analysis after adjusting for kidney function (eGFR) and other confounders (age, sex, income status, smoking status, BMI, fasting plasma glucose, systolic blood pressure, bilirubin, UPCR, and albumin) (Table 3). For patients with early CKD, serum hepcidin was not associated with anemia, regardless of anemia severity. Among iron indices, only TSAT was significantly associated with anemia; it was particularly associated with less severe anemia (Hb < 13.0 g/dL and Hb < 11.5 g/dL). However, for patients with advanced CKD, serum hepcidin was significantly associated with anemia; it was particularly associated with more severe anemia (Hb < 11.5 g/dL and Hb < 10.0 g/dL), whereas the effects of TSAT and ferritin on anemia severity were minimal.

Fig. 2. Subgroup analysis according to the kidney function of the receiver-operator characteristics curve analysis of serum hepcidin and iron indexes for anemia severity. eGFR, estimated glomerular filtration rate; AUC, area under the curve; CI, confidence interval; TSAT, transferrin saturation; Hb, hemoglobin. Upper rows show results of patients with preserved kidney function (eGFR ≥ 45 mL/min/1.73m²; n = 919) and lower rows show results of patients with decreased kidney function (eGFR <45 mL/min/1.73m²; n = 758). * P<0.05 compared to ferritin and † P<0.05 compared to TSAT using Delong's test.

Table 3. Subgroup analysis according to the kidney function for the association of serum hepcidin and iron indices with anemia severity. Hb, hemoglobin; TSAT, transferrin saturation; Adj. OR, adjusted odds ratio; CI, confidence interval. Adj. OR and its CI were calculated using multivariate logistic regression analysis. TSAT, ferritin and hepcidin were entered into together with age, sex, income status, smoking status, body mass index, fasting plasma glucose, systolic blood pressure, estimated glomerular filtration rate, bilirubin, albumin, and urine protein-to-creatinine ratio. * per 10 % increase in TSAT, per 10 pmol/L increase in ferritin, and per 10 ng/mL increase in hepcidin

		Estimate glomerular filtration rate			
		≥ 45 mL/min/1.73m ² (n = 919)		<45 mL/min/1.73m ² (n = 758)	
Outcomes	Factors*	Adj. OR (95% CI)	P	Adj. OR (95% CI)	P
Hb < 13.0 g/dL	TSAT (10 %)	0.628 (0.515–0.765)	<0.001	0.805 (0.648–0.998)	0.048
	Ferritin (10 pmol/L)	0.997 (0.984–1.011)	0.677	1.008 (0.997–1.019)	0.179
	Hepcidin (10 ng/mL)	1.112 (0.887–1.394)	0.357	1.138 (0.946–1.369)	0.172
Hb < 11.5 g/dL	TSAT (10 %)	0.672 (0.476–0.950)	0.024	0.791 (0.626–1.001)	0.051
	Ferritin (10 pmol/L)	1.006 (0.983–1.030)	0.592	0.993 (0.984–1.003)	0.166
	Hepcidin (10 ng/mL)	1.060 (0.684–1.642)	0.794	1.379 (1.173–1.620)	<0.001
Hb < 10.0 g/dL	TSAT (10 %)	0.350 (0.116–1.058)	0.063	0.717 (0.477–1.078)	0.110
	Ferritin (10 pmol/L)	1.022 (0.970–1.077)	0.417	1.002 (0.991–1.014)	0.726
	Hepcidin (10 ng/mL)	1.293 (0.377–4.436)	0.683	1.360 (1.115–1.659)	0.002

Discussion

Iron metabolism has been suggested as a key factor contributing to anemia in CKD patients [10, 11]. Anemia guidelines for CKD patients consider that TSAT and ferritin are important markers of anemia in CKD, and they recommend iron replacement according to TSAT and ferritin serum levels [9, 37]. Hepcidin has been suggested as a good biomarker of anemia in CKD patients [21-33]. Hepcidin lowers the available serum iron levels by limiting iron efflux from the body's iron stores [19]; therefore, it is plausible that iron should be sequestered in iron stores as the serum hepcidin level increases. This may cause bone marrow iron deficiency despite sufficient iron in storage sites [40], suggesting that sufficient serum levels of TSAT and ferritin may not guarantee sufficient production of RBC when the serum hepcidin level is increased. Our study group recently reported that kidney function was one of the major determinants of the serum hepcidin level, thereby suggesting serum hepcidin as a novel uremic toxin [20]. We hypothesized that serum hepcidin and iron indices associated with anemia are affected by kidney function. Because iron sequestration by hepcidin can result in a profound decrease in RBC production [41], we also hypothesized that anemia associated with hepcidin is more severe than anemia associated with iron indices.

In this study, serum ferritin levels were not associated with anemia status. Although the serum ferritin level of CKD patients was associated with hepatosplenic iron stores [40], it was not correlated with bone marrow iron stores [15, 40, 42]. This so-called iron sequestration in CKD patients may result in a null association between serum ferritin and anemia status in our study participants. This null association between serum ferritin and anemia status was evident even in patients with early CKD, thus implying that iron sequestration in CKD patients may occur during the early stage of CKD. Hepcidin is a fundamental peptide in iron sequestration, and serum hepcidin levels in CKD patients are higher than those of healthy volunteers [43, 44]. Serum hepcidin levels also increased with CKD progression [20]. Therefore, we can hypothesize that a small increase in serum hepcidin even during early CKD may nullify the effect of serum ferritin on Hb levels.

In the current study, unlike ferritin, TSAT was significantly associated with anemia, which was in accordance with the fact that TSAT is more predictive of bone marrow iron contents [15] and the Hb response after intravenous iron replacement [45] than ferritin in CKD patients. Serum hepcidin was also significantly associated with anemia in non-dialysis CKD patients. These associations were independent from kidney function. Because of the large sample size, we could further analyze the kidney function. During the analysis, TSAT was associated with less severe anemia in patients with early CKD, whereas serum hepcidin was significantly related to more severe anemia in patients with advanced CKD. These results suggested that TSAT is a major determinant of anemia in early CKD patients, whereas serum hepcidin is a key determinant of anemia in advanced CKD patients.

We also found that serum hepcidin and iron indices were not associated with Hb <10.0 g/dL in patients with early CKD. This may imply that serum hepcidin levels in early CKD patients were not high enough to cause profound decreases in Hb [20]. In addition, decreases in TSAT and ferritin in early CKD patients may not be sufficient to result in decreases in Hb <10.0 g/dL. Therefore, clinicians should rule out other causes of anemia such as gastrointestinal bleeding [12] in patients with early CKD, when Hb levels are decreased profoundly.

We hypothesized that increased serum hepcidin levels may sequentially nullify the effects of iron indices on anemia (ferritin first and TSAT later). For early CKD patients, the serum hepcidin level may be enough to nullify the effect of ferritin on anemia, causing iron sequestration; however, this level may not be enough to diminish the effect of TSAT on anemia. This may be why only TSAT was associated with less severe anemia during early CKD. As CKD progresses, the serum hepcidin level increases, and the increased serum hepcidin level may be high enough to incapacitate the effect of TSAT on RBC production in advanced CKD patients. Very high hepcidin will sequester iron profoundly enough to cause severe

anemia. However, this new hypothesis needs to be confirmed using future experimental and human studies.

This study had several limitations. First, because the study was cross-sectional, a causal relationship could not be accurately determined, and the results need to be interpreted carefully. However, most previous studies regarding hepcidin were cross-sectional studies, because drugs specifically targeting hepcidin are not currently available for humans [14, 19]. To our knowledge, the current study used the largest sample to examine the associations between hepcidin, iron indices and anemia. Therefore, this study limitation may be acceptable. Second, we did not have data regarding the causes of anemia because KNOW-CKD was not designed specifically for anemia in CKD patients. Furthermore, there were no data from healthy volunteers and patients with ESRD on dialysis. In addition, the study was performed in a single country and involved a single ethnicity, thus limiting the generalizability of the results.

In conclusion, ferritin was not associated with anemia, regardless of its severity, or the level of kidney function. TSAT was associated with less severe anemia in patients with early CKD. However, serum hepcidin was associated with more severe anemia in patients with advanced CKD. These results need to be confirmed using future studies.

Disclosure Statement

The authors have declared that no competing interests exist.

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