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Original Article

Biochemistry Section

Role of Hepcidin in causing Anaemia in Chronic Kidney Disease: A Potential Early Biomarker

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ABSTRACT

Introduction Hepcidin appears to be an emerging biomarker for anaemia in kidney damage in Chronic Kidney Disease (CKD).

Aim: To evaluate serum hepcidin level for its potential role in anaemia of CKD.

Materials and Methods: A cross-sectional study was done in 60 subjects. Out of 60 subjects, 40 were patients (without type II error) suffering with CKD admitted in Department of Nephrology, in a Tertiary Care Hospital, Kerala, India between January 2012 to June 2013 and 20 were healthy subjects assigned as controls. Blood sample (10 mL) was collected from each patient before the initiation of dialysis, assayed for serum hepcidin, serum iron, Total Iron Binding Capacity (TIBC) and serum ferritin for comparison. Statistical Package for the Social Sciences (SPSS) software version 19 was used. A p-value with >0.05 was considered statistically significant.

Results: Mean age of patients was 56.72 years and majority were males 33 (82.5%). Mean duration of years was 4.08 years ((Standard Deviation (SD) 1.95 years). Mean duration of haemodialysis twice a week was 2.12 years (SD 1.02 years). In this study, 23 (57.5%) patients were on recombinant human Erythropoietin (rhEpo) with good response, 10 (25%) without response and 7 (17.5%) were not on rhEpo. Comparison of serum hepcidin, Haemoglobin (Hb), serum iron, TIBC, serum ferritin in these CKD patients showed significant correlation with p-value <0.05.

Conclusion: In this study, serum hepcidin reflected iron status of CKD patients indicating a potential marker that can be assayed to predict anaemia related to early renal injury.

Keywords: Ferritin, Renal injury, Total iron binding capacity

INTRODUCTION

Anaemia is a usual complication in the later stages of CKD. It is known to cause symptoms such as fatigue and shortness of breath. The main feature is a relative deficit of erythropoetin leading to anaemia of CKD with complexity [1]. Hepcidin, a hepatic iron-regulatory hormone and its receptor ferroportin (cellular iron exporter), exerts a feedback mechanism for regulation which maintains optimum plasma concentrations of iron-Transferrin (TF) needed for erythropoiesis and other physiological functions, that is sufficient for iron stores, and prevents iron toxicity. In CKD, any possibility of inflammation and hampering of hepcidin clearance from plasma leads to inhibition of iron absorption from duodenum following its sequestration in macrophages. This consequence results in iron deficit, decreased synthesis of erythropoesis and also leads to resistance for endogenous synthesis as well as exogenous administration of Erythropoietin (EPO) contributing to anaemia of CKD [2]. Hepcidin is a small defensin-like peptide produced by hepatocytes. Hepcidin is known to be having antimicrobial properties and has regulator role of iron metabolism that controls both the quantity of dietary absorbed iron in the duodenum and the iron release by reticuloendothelial cells [3]. CKD is defined as any abnormalities of kidney related to its structure and or function which is present for more than three months, which has adverse effects and it may be insensitive and non specific for the cause of disease but may precede reduction in kidney function [4]. One of the studies has shown that Haemoglobin (Hb) levels tend to decrease as hepcidin levels increases [5]. Some of stimuli that induces hepcidin includes tissue iron stores, TF saturation, hypoxia and inflammation which has a potential role in CKD [6]. As hepcidin can be a better predictor for anaemia and iron status, this study was undertaken to evaluate the same.

MATERIALS AND METHODS

This cross-sectional study was done from January 2012 to June 2013 in Department of Biochemistry and Department of Nephrology, Sree Gokulam Medical College and Research Foundation, Venjaramoodu, Kerala, India. The study was carried out after approval from the Institutional Research Committee and Ethics Committee Ref. No.SGMC/IEC/92/11.

Sample Size

A sample size of 30 was sufficient for this study without type II error to estimate serum biomarker as per statistician suggestion, but a sample size of 40 was kept to increase the accuracy of the results [7].

Inclusion criteria: Forty patients with kidney disease for ≥3 months and Glomerular Filtration Rate (GFR) <60 mL/min/1.73 m^2 (stage 3 and above CKD) in the age group of 18 years and above, attending the nephrology outpatient department and on maintenance dialysis were selected as study subjects [4]. Twenty healthy subjects without any disease were included as controls so as to evaluate whether hepcidin values were within normal reference range in this population when analysed using chosen laboratory assay method [7]. All the 40 patients were undergoing haemodialysis twice a week and for a mean duration of 2.12 years with a standard deviation of 1.02 years. Haemodialysis was done with low flux polysiphone membrane for duration of four hours. The samples were taken for all the assays before the initiation of dialysis.

Exclusion criteria: Patients who were critically ill and on immunosuppressive drugs were not included. Patients with renal transplant, end stage liver disease, diagnosed non renal cause of anaemia, known evidence of bleeding, history of blood transfusion and malignancy were also excluded.

Informed written consent was taken from the study subjects. Using profoma, socio-demographic details and clinical history were collected by direct interview of study subjects. Data was also obtained from their case records and clinical laboratory reports. Blood (10 mL) was collected by venipuncture from patients before initiation of dialysis and serum was separated by centrifugation at

room temperature and used for analysis of serum hepcidin, serum iron, TIBC and serum ferritin. Serum samples for the assays were stored at -20°C until the analysis. Assay of serum hepcidin was done by Hepcidin Enzyme linked immunosorbent assay -DRG International, Inc. kit Tecan ELISA reader (reference range 13.3 to 54.4 ng/mL). Serum iron assay was done using Siemens Iron flex reagent cartridge (reference range 100 to 120 µg/dL) and serum TIBC assay was done using Siemens TIBC flex reagent catridge in Siemens Dimension RxL max fully automated biochemistry analyser (reference range 250 to 400 µg/dL) to evaluate the iron status and its binding capacity in the serum. Serum ferritin was measured by Elecsys ferritin assay using chemiluminescence immunoassay based on sandwich principle in electro Cobas e 411 fully automated Electro Chemiluminiscence Analyser (reference range 25-250 µg/L).

STATISTICALANALYSIS

The data obtained after estimation of the analytes were statistically analysed using Microsoft Excel and SPSS software version 19. Descriptive statistics and Pearson correlation were determined for comparing hepcidin, Hb, TIBC, iron, ferritin and serum ferritin level and Transferrin Saturation (TSAT) in CKD patients (n=40). A p-value <0.05 was considered statistically significant. Mann Whitney-U test was used to compare difference between serum hepcidin values in cases (n=40) with CKD and hepcidin control (n=20), as the number of controls in this study was only 20.

RESULTS

About 23 (57.5%) of the cases were in the age group 55-65 years and 13 (65 %) controls were in the age group 55-65 years. Mean age of CKD patients was 56.72±7.32 years for cases and 50.45±6.59 years for controls. About 33 (82.5%) and 13 (65%) were males in cases and controls, respectively in this study [Table/Fig-1,2].

Age group (years)	n	Percentage (%)	Minimum	Maximum	Mean±SD	Total number/ percentage
Descriptive data of cases (n=40)						
35-45	5	12.5	39	65	56.72±7.32	40/100%
45-55	12	30				
55-65	23	57.5				
Descriptive data of controls (n=20)						
25-35	1	5	32	60	50.45±6.59	20/100%
35-45	1	5				
45-55	5	25				
55-65	13	65				

[Table/Fig-1]: Descriptive data of cases and controls (n=40).

Sex	Frequency	Percentage
Distribution of cases according to sex (n=40)		
Male	33	82.5
Female	7	17.5
Distribution of controls according to sex (n=20)		
Male	13	65
Female	7	35

[Table/Fig-2]: Distribution of cases and controls according to sex (n=40).

Mean duration of CKD was 4.08 years with a standard deviation of 1.95 years. All 40 patients were undergoing haemodialysis twice a week and for a mean duration of 2.12 years with a standard deviation of 1.02 years. Mean values of serum hepcidin and indicators of iron status in CKD cases and controls are shown in [Table/Fig-3].

Serum hepcidin with TIBC and Hb when compared showed significant negative correlation whereas with serum iron, TSAT and ferritin in these CKD patients showed significant positive correlation with p-value <0.05 [Table/Fig-4].

Variable	Minimum	Maximum	Mean±SD
Cases (n=40)			
Serum hepcidin (ng/mL)	64.10	84.30	74.22±6.02
Haemoglobin (g/dL)	7.10	8.80	7.68±0.47
Serum iron (µg/dL)	73	89	79.80±4.24
TIBC (µg/dL)	331	382	358.33±15.62
Serum ferritin (µg/L)	458	496.9	477.51±9.62
Transferrin saturation (TSAT%)	19.12	26.88	22.35±2.03
Controls (n=20)			
Serum hepcidin (ng/mL)	14.3	19.2	15.710±1.52
Haemoglobin (g/dL)	13.1	15.6	14.40±0.83

[Table/Fig-3]: Mean values of serum hepcidin and indicators of iron status in CKD. cases and controls.
TIBC: Total iron binding capacity

Variables	Pearson correlation	p-value
Serum hepcidin (ng/mL) and Haemoglobin (g/dL)	-0.971	0.001
Serum hepcidin (ng/mL) and Serum iron (µg/dL)	0.731	0.001
Serum hepcidin (ng/mL) and TIBC (µg/dL)	-0.954	0.001
Serum hepcidin (ng/mL) and Serum ferritin (µg/L) Transferrin saturation (TSAT%)	0.876	0.001
Serum hepcidin (ng/mL) & Serum ferritin (µg/L)	0.969	0.001

[Table/Fig-4]: Correlation coefficient of serum hepcidin with haemoglobin, serum iron, TIBC, TSAT and serum ferritin in CKD cases (n=40).
TIBC: Total Iron Binding Capacity; TSAT: Serum ferritin level and transferrin saturation; Correlation coefficient between the parameters in cases

Out of the 40 patients, 57.5% were on rhEPO and were responding to it, 25% showed no response to rhEPO and 17.5% patients were not given rhEPO [Table/Fig-5].

Erythropoietin (Epo)	Frequency	Percentage
On Epo and responding	23	57.5
On Epo and not responding	10	25
Not on Epo	7	17.5
Total	40	100

[Table/Fig-5]: Frequency and percentage of recombinant human erythropoietin (rhEpo) responders (n=40).

Mann whitney-U test was used to compare difference between Serum hepcidin (ng/mL) (N=40) values in CKD patients and controls (N=20) as number of controls was only 20. Serum hepcidin was significantly high in CKD patients compared to healthy controls with p-value <0.0001 and degree of freedom 58 [Table/Fig-6].

Variables	Z value	p-value
Serum hepcidin (ng/mL) in CKD patients and controls	5.961	<0.0001

[Table/Fig-6]: Comparison of serum hepcidin (ng/mL) in CKD patients (n=40) and controls (n=20).

DISCUSSION

One of the complication of CKD is anaemia with reduced quality of life of patients which is known to increase morbidity and mortality with disease progression. Infact, CKD is defined as concentration of Haemoglobin (Hb) in the blood 40 patients were having CKD of stage below two times the SD of the mean Hb of the general population, corrected for age and sex [8]. In this study, all 40 patients were having CKD of stage 3 or above. Age is an independent risk factor for CKD. Majority of the patients were in the age group 55-65 years (57.5%). Out of 40 patients, 12 (30%) belonged to the age group of 45-55 years. There were only 5 (12.5%) patients who were in the age group of 35-45 years. Mean age of patients was 57.5 years with a standard deviation of 7.32 years. The age of patients showed significant positive correlation with serum hepcidin with p-value

<0.05. All 40 patients were adults above 35 years and in this study group, age and serum hepcidin values showed positive correlation. About 33 (82.5%) of the patients were males in this study and number of females were 7 (17.5%.) among cases. The mean serum hepcidin value (74.29 ng/mL) with standard deviation 6.04 ng/mL was higher in males than in females (73.94 ng/mL) with standard deviation 6.38 ng/mL in this population. In a previous study, serum hepcidin values were higher in males than females [9].

In this study mean duration of CKD in patients was 4.08 years with a standard deviation of 1.95 years. All the 40 patients were undergoing haemodialysis twice a week and for a mean duration of 2.12 years with a standard deviation of 1.02 years. Haemodialysis was done with low flux polysiphone membrane for a duration of four hours. The samples were taken for all the assays before the initiation of dialysis. Out of the 40 patients, 25% were unemployed as they could not go for work due to their illness, 37% patients were owning shops, petty business and 10% were office going, 7.5% were agricultural workers or daily workers, 12.5% were construction workers and another 7.5% were drivers. The expenditure due to the cost of dialysis sessions, investigations and medications were a financial burden to all the 40 patients, as told by them while taking history and they were also dependant on their family members for financial assistance. A single dialysis session cost amounted to 600 rupees and all 40 patients had twice a week dialysis regimen. The mean family income was 9637.50 rupees with a standard deviation of 5620.46 rupees. In this study group 30% were smokers and 70% were non smokers whereas 50% were alcoholics and rest 50% non alcoholics. Both smoking and alcoholism were personal habits in 25%. Mean values of serum hepcidin were higher in smokers and alcoholics when compared to non smokers and non alcoholics. This could lead to increased risk of cardiovascular diseases as per one of the previous studies [10].

Out of 40 patients 95% were hypertensive and 55% were diabetic. Both diabetes and hypertension were present in 50%. The two main common causes for CKD are hypertension and diabetes mellitus. In these patients also such a pattern was observed. Family history is very important as far as CKD is concerned. Family history of end stage renal disease, hereditary nephritis, polycystic kidney disease and various tubular syndromes are important in CKD patients but in this study none of the patients had family history of CKD. Hepcidin is found to have important role in anaemia of these CKD patients because its excess is the cause for dysregulation of iron metabolism. As a part of this study, relation between serum hepcidin and iron status of these patients were studied. Mean value of serum hepcidin was 74.22±6.02 ng/mL (SD) in this group of patients. Serum hepcidin level was not very high in these patients. One main reason for this could be that these patients were on maintenance dialysis.

An earlier study has revealed that hepcidin levels in haemodialysed patients were significantly correlated with eGFR but it is not considered as an independent predictor for hepcidin level in such patients [11]. Hepcidin has a role in iron regulation and anaemia of CKD. It is found to be a potential marker for iron status in CKD. It decreases absorption of iron from intestine as it down regulates ferroportin channels on the surface of iron storing cells and this is responsible for iron-restricted erythropoiesis. Lack of mobilisation of iron stores for red blood cell synthesis due to high hepcidin level is responsible for erythropoiesis stimulating agent resistance [12]. Hb levels were within normal limits in controls and was done to rule out anaemia and hence rest of the parameters like TIBC, serum iron, serum ferritin, TSAT were not done in controls as it was not necessary. The purpose of using controls in this study was to check whether serum hepcidin level were within reference range in the population of this region using this standard laboratory assay, which is not routinely used. Pearson correlation for serum hepcidin and other parameters were done only in these 40 CKD patients. In these cases haemoglobin showed mean value as 7.68 g/dL with a standard deviation 0.47 g/dL. When serum hepcidin and Hb were compared, they showed significant negative correlation with correlation coefficient as -0.971 and p-value 0.001.

Earlier studies have shown relation between serum hepcidin and Hb level. Similarly, status of iron is shown to modify hepcidin level and its association with Hb. Raised hepcidin can be a predictor and indicate the need for parenteral iron therapy and also need for higher dose of rhEPO to overcome iron-restricted erythropoiesis [13]. In this study, involving 40 CKD patients, anaemia was the common picture with increased hepcidin values and decreased Hb levels. rhEPO administration also affects erythropoiesis in CKD. Parenteral iron therapy may or may not be added in CKD management. In these CKD patients regime followed was administration of rhEPO 4000 units, weekly for one month and if no response was seen then initial dose was doubled and was given for another one month, then also if no increase in Hb level was noticed, then those patients were classified as non responders. Responders were those in whom Hb level increased by 0.5 to 2 g/dL per month. Out of the 40 patients, 57.5% patients were being given rhEPO and were responding to it, 25% showed no response to rhEPO and 17.5% patients were not given rhEPO. In present study, lower hepcidin values were noted in patients responding to rhEPO compared to patients who were not on rhEPO and not responding to it. So serum hepcidin values can be considered as a measure of EPO resistance.

One of the previous studies showed that treatment of anaemia with EPO was associated with great benefits for some patients but not all [14]. In yet another study, hepcidin assay if used as a diagnostic tool, may improve iron therapy during periods of reticuloendothelial blockage of iron transport and can prevent EPO resistance in haemodialysis patients [15].

In present study mean value of serum iron was found to be 79.80 µg/dL with standard deviation of 4.24 µg/dL and that of TIBC was 358.33 µg/dL with standard deviation of 15.62 µg/dL. Comparison of serum hepcidin and serum iron in these CKD patients showed correlation coefficient as 0.731 with significant correlation and p-value 0.001. The Pearson correlation between serum hepcidin and TIBC gave correlation coefficient as -0.954 and showed statistically significant negative correlation with p-value 0.001. It is very difficult to outline an apt explanation for serum iron values obtained in this study as all 40 patients were on parenteral iron therapy (loading dose 1 g before initiating rhEPO followed by 200 mg once a month). Serum iron was low but TIBC was not very much elevated similar to anaemia of chronic disease of any other cause. One of the previous studies has showed that lower TF level prevents the proper transportation of serum iron to the Hb sites and it may be reason for the low haemoglobin synthesis and also for the development of hypo responsiveness to EPO in some of the dialysis patients [16].

Inflammatory activation was present in these 40 CKD patients and was determined with established markers like serum ferritin, albumin and C-reactive protein evaluated in these patients. Serum ferritin is not useful in determining iron stores in CKD as inflammation also increases it. Elevated serum ferritin in these patients can be explained by inflammation present due to CKD. Mean value of serum ferritin was 477.51 µg/L with standard deviation of 9.62 µg/L. One important finding of this study was the relation between hepcidin and ferritin. Comparison of serum hepcidin and serum ferritin in these CKD patients showed significant positive correlation with p-value <0.05. Mean serum ferritin TSAT percentage was 22.35% with standard deviation of 2.03%. Comparison of serum ferritin and TSAT showed significant positive correlation with p-value <0.05. Comparison of serum hepcidin and TSAT in these CKD patients did not show significant correlation as p-value was >0.05. In this study correlation between serum ferritin, TSAT and serum

hepcidin values can be explained by inflammation and parenteral iron therapy in these patients. These findings suggest that serum hepcidin is clinically useful as a marker to determine iron stores in functional anaemia of CKD.

Limitation(s)

All the 40 patients who participated were dialysed patients. Since the clinical utility of hepcidin assay for assessing iron status of patients has been proved by this study, further studies can be done in non dialysed CKD cases also. The number of controls taken in this study was limited to 20 due to the cost of the laboratory assay kit used for serum hepcidin analysis.

CONCLUSION(S)

In this study correlation between serum hepcidin, TIBC, serum iron, serum ferritin, TSAT and Hb that indicate iron status were studied. In these 40 CKD patients, anaemia was a common feature with increased hepcidin values and decreased Hb levels. Serum iron was low but TIBC was not very high which showed that anaemia was due to CKD. All the findings showed use of serum hepcidin in assessment of iron status which is a predictor in disguise for patients with CKD.

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