

Original Research Article

Quantification of serum hepcidin as a potential biomarker in diagnosis of iron deficiency anaemia

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ABSTRACT

Background: Iron deficiency (ID) is the most prevalent nutritional deficiency and the most common cause of anemia in the world. Present study aims to detect the serum hepcidin and ferritin levels in iron deficiency induced anaemia's and compare and correlate the values of serum hepcidin levels with their Serum ferritin levels and IL-6 levels.

Methods: A total of 94 individuals were enrolled in the study. Sample for hematological evaluations were collected and estimation was carried out for biomarker estimation by ELISA method(s) using specified kit(s) procured commercially. The statistical evaluation was done using SPSS version 24.0. Analysis of variance (ANOVA) and Pearson's correlation tests were used to compare the variables and to see the correlation between the different variables.

Results: Serum Hepcidin, a marker of iron deficiency anemia is significantly low in patients with IDA (33.23 ± 12.46 ng/mL) than in normal with p-value < 0.001 which is highly significant. In present study, we determined the cut off points differentiating IDA from healthy group which was ≤ 34.55 ; with AUC 0.845 ($P < 0.0001$), 95% confidence interval was 0.755 to 0.911, and sensitivity was 98.33 % and specificity 52.94%. These cut off points had strong confidence interval and valuable predictive potential.

Conclusions: Serum Hepcidin can be used as a simple and cost effective diagnostic marker for identification of anemia.

Keywords: Anemia of chronic disease, Iron deficiency anemia, Serum hepcidin, Serum ferritin, IL-6

INTRODUCTION

Iron deficiency anemia is the most common cause of anaemia in the world affecting more than 30% of world population. The World Health Organization (WHO) recently have highlighted its concern over anaemia and the way the condition is so often disregarded, significantly affecting the quality of life of millions. The two most commons causes are: iron deficiency (IDA), accounting for 50% of all cases of anaemia and anaemia of chronic disease (ACD), also known as anaemia of inflammation.¹ Iron Deficiency is a significant public health associated with reduced quality of life in adults and affects motor and mental development in children.

Iron is essential for proper cell differentiation, cell growth and in the development of immune system. Iron deficiency has effects on endocrine and neurotransmitters as well. Thereby, needing a prompt early diagnosis to reduce the complications and morbidities related to iron deficiency anemia. Iron homeostasis is regulated by two main mechanisms: an intracellular mechanism dependent on the amount of iron available and a systemic mechanism in which hepcidin plays a crucial role. Hepcidin has evolved as the primary regulator of iron homeostasis and its role has been demonstrated in recent studies and there is enormous interest in quantifying circulating hepcidin levels in clinical samples. The available methods for iron deficiency anemia diagnosis

have their limitations as in the case of ferritin as marker it has excessive high values in chronic infections, malignancies, hepatitis, and hyperthyroidism. It is relatively less sensitive in detecting an early iron deficiency.²⁻⁵ Similarly, bone marrow aspiration for iron studies is an invasive procedure, reportedly with non-patient compliance, tedious, involves high skill and requires aseptic condition hence cannot frequently be used as a method of choice for the detection of iron deficient anemia.⁶⁻¹¹ Therefore, hepcidin a peptide and a regulator of iron metabolism which is expressed in iron deficiency and which has sensitive method of estimation i.e. immunoassay (ELISA based) was used as a marker in the present study. Present study aims to detect the Serum hepcidin and ferritin levels in Iron Deficiency Induced Anaemia's, compare and correlate the values of Serum hepcidin levels, Serum ferritin levels and IL-6 levels.

METHODS

This is a prospective analytical case control study. Patients attending the outpatient and inpatient department of the INDEX hospital were included during study period of two year from June 2014 to June 2016. Appropriate prior consent was taken from the patients and clinical details were recorded in a proforma. All investigations were done in the pathology and clinical biochemistry laboratory of INDEX Hospital, Indore. Sample for complete blood count and biomarkers analysis were collected, coded and processed on a SYSMEX X-800i auto analyzer for hematological parameters and on ELAN 30S ELISA plate reader for Serum Hpcidin, Ferritin, IL-6 levels analysis. A total of 94 patients were evaluated, those full filling the inclusion criteria and allocated 2 groups according to their RBC's indices, peripheral smear examination and serum ferritin values.

Control (Non-anaemic) were those with Hb gm% and RBCs indices includes within normal Range to their respective reference age, sex and normal ferritin levels and a normal peripheral blood smear diagnosis. IDA group comprised of cases with Hb gm%, RBC indices lower than the normal range to their respective reference

age and sex and a hypochromic picture on peripheral blood smear. The biomarkers levels for both groups were analyzed, studied and compared. Patients already on iron supplements, blood transfusion in recent months and below 5 years of age (to exclude congenital conditions like thalassemia, haemochromatosis) were excluded from the study. The obtained data was tabulated using MS Excel to create a master chart. The power of study was kept at 99% and level of significance (α) at 5%. "Analysis of Variance (ANOVA)" was done to calculate the p value to compare the difference of mean of the study groups together. Post Hoc Turkey's test" was also applied for comparison of difference of mean in two study groups. The p value was calculated for each parameter and p value <0.05 was considered to be significant. 95% CI was also calculated. "Pearson's Correlation Coefficient (r)" was also done to find the correlation between serum hepcidin and serum ferritin, serum IL6 in studied groups. X-Y scatter plots and other graphical representation of data were done with appropriate plots and charts as needed depending on data type and distribution. AUC under ROC curves was calculated for serum hepcidin as a potential diagnostic test for detection of IDA in studied groups using Statistical Package for Social Sciences (SPSS) version 24.0 for windows and Medcalc Software.

RESULTS

The hematological parameters are summarized in Table 1 for group IDA and normal. The concentrations of Hb as well as RBC counts was recorded to be low in case of IDA patients as compared to control group. The average RBC count for IDA, and normal group was $3.49 \pm 0.69 \times 10^6/\mu\text{L}$, and $4.46 \pm 0.21 \times 10^6/\mu\text{L}$ respectively. Similarly, the % Hb estimated was 5.86 ± 1.54 g/dL, and 13.01 ± 0.69 g/dL in these groups respectively. The findings agree with the reported results of Cheng et al indicating that invariably in anaemia the blood hemoglobin levels decreased. Further, it was noticed that in IDA group the morphological appearance of RBC was hypochromic and microcytic. The MCV for IDA was 59.55 ± 7.53 fl and MCH value (16.71 ± 2.71 pg).

Table 1: Comparison of hematological parameters between normal group and IDA groups.

Parameter	Normal group	95% CI	IDA group	95% CI	P value	Remark
WBC count ($10^3/\mu\text{L}$)	8.15±2.22	(7.3806, 8.9347)	7.35±2.52	(6.7055, 8.0115)	0.839	NS
RBC count ($10^6/\mu\text{L}$)	4.46±0.21	(4.3862, 4.5343)	3.49±0.69	(3.3148, 3.6724)	0.000	S
Hb gm/dl	13.01±0.69	(12.7696, 13.2539)	5.86±1.54	(5.4659, 6.2640)	0.000	S
MCV (fL)	84.66±2.65	(83.7354, 85.5881)	59.55±5.63	(58.0913, 61.0120)	0.000	S
MCH (pg)	29.40±1.34	(28.9284, 29.8715)	16.71±2.71	(16.0123, 17.4143)	0.000	S
MCHC (g/dL)	34.73±1.16	(34.3314, 35.1450)	28.02±2.91	(27.2579, 28.7654)	0.000	S

*Value represents (Mean ± SD).

The comparative findings of the biochemical markers (Serum ferritin, serum hepcidin, and serum IL6) of normal and IDA group is reported in Table 2. In present study, the serum ferritin measured in normal group was 65.67±16.76 ng/mL while in case of IDA it was significantly reduced to an average concentration 13.23±5.53 ng/mL, the calculated P value suggested significant difference (P≤0.001). The average serum hepcidin measured in patients of normal group was 54.06±15.46 ng/mL while in case of IDA. It was significantly low and reduced to an average concentration 33.23±12.46 ng/mL, the calculated P Value suggested a significant difference (P≤0.05). In present study, the average serum IL-6 measured in normal group was 10.53±2.91ng/mL while in case of IDA it was significantly reduced to an average concentration 7.62±3.51ng/mL.

The calculated P value suggested a significant difference (P≤0.05). In present study, Kim et al has also reported that hepcidin has direct relationship with serum iron and resultant anaemia. Furthermore, no obvious correlation existed between serum hepcidin concentration, RBC counts, Hb %, and WBC count in present study. This significant decrease could be attributed to suppressive effect of deficient store of iron and iron deficient erythropoiesis. Thus, the findings in the present study coordinate well with the findings of Theurl et al, Naqvi et al, Pasricha SR et al, Kim J et al, Cheng PP et al. However, the measured level(s) of marker differ in terms of concentration which may be due to selected method of analysis and sensitivity of kit(s) and heterogeneity of population used in the study.¹²⁻¹⁵

Table 2: Comparison of biochemical markers between normal and IDA group

Parameters (ng/mL)	Normal group	95% CI	IDA group	95% CI	P value	Remark
Serum ferritin	65.67±16.76	(59.8242,71.5257)	13.23±5.53	(11.8042,14.6657)	0.022	NS
Serum hepcidin	54.06±15.46	(48.6650,59.4572)	33.23±12.46	(30.0279,36.4680)	0.000	S
Serum IL6	10.53±2.91	(9.5171,11.5493)	7.62±3.51	(6.71852,8.53547)	0.907	NS

*Value represents (Mean±SD).

Receiver operating curve and scattered plot correlation curve

Iron deficiency anaemia is characterized by many abnormal laboratory features. Because none of these are unique, a small deviation from normal will detect most cases of iron deficiency (high sensitivity), but also falsely identify non-iron deficient subjects as being iron deficient (low specificity). On the other hand, a large deviation from normal will exclude most non-deficient patients (high specificity), but miss many iron deficient subjects (low sensitivity). The tradeoff shown graphically is so called receiver operating characteristic curve (ROC). These curves are constructed by plotting the sensitivity against the false positive rate (1-specificity) at various values of the analyte. The ROC curves for serum hepcidin and serum ferritin as predictors of IDA was constructed using MedCalc software. Further, the correlation(s) between different biomarker(s) was assessed by plotting the bio exponents on X and Y axis: the scattered correlation curve so obtained were used to compare correlation coefficient and level of significance at 95% confidence level with defined confidence interval.

In case of normal group, the scatter correlation curve revealed an excellent positive correlation between serum ferritin and hepcidin wherein for the sample size of 34 participants, correlation coefficient obtained on regression of curve was 0.9780, significance level was P<0.0001 and 95% confidence level interval was found to be 0.9561 to 0.9891. The values suggest positive

correlation between the hepcidin and ferritin indicating if ferritin level may define the cut off point for anemia then hepcidin could equally and effectively be used for defining and determining IDA. In the present study in IDA group wherein the sample size was 60; the Pearson’s scatter correlation plot shows a negative correlation between hepcidin and ferritin i.e. r = -0.06851 with a level of significance p = 0.6030 at confidence intervals of -0.3169 to -0.1887. Thus, in the sub group of IDA a negative correlation between hepcidin versus ferritin was found suggestive of functional erythropoiesis induced by anaemia in bone marrow which might suppress hepcidin expression in IDA group. The negative correlation due to enhanced erythropoiesis induced by anaemia may be yet stronger for its effect than that of iron positive regulation on expression of hepcidin. The findings are in agreement with the results of Vokurka et al. The obtained Pearson’s scatter correlation plot diagram for IL-6 and hepcidin in the case of IDA group the correlation coefficient r = -0.0431; P = 0.7436 at 95% confidence interval 0.2938 to 0.2131. Area under the ROC curve is considered as an effective measure of inherent validity of a diagnostic test. This curve is useful in (i) finding optimal cut-off point to least misclassified disease or non-diseased subjects, (ii) evaluating the discriminatory ability of a test to correctly pick diseased and non-diseased subjects; (iii) comparing the efficacy of two or more tests for assessing the same disease; and (iv) comparing two or more observers measuring the same test (inter-observer variability). ROC curve between sensitivity and 1-specificity is a useful method to evaluate the performance of a diagnostic test in

classification of subjects in to two categories positive and negative. ROC curve may be used to judge how well the test performs. If area under the curve is near 1 it has higher chance of correct classification and when it is near 0, higher chance of incorrectly classifying in opposite group. The value 0.5 shows the test is no better than just tossing a coin for classification into positive or negative. In present study, we determined the cutoff points for serum hepcidin levels to differentiate IDA from healthy individuals (Figure 1). The cutoff point had strong confidence interval and valuable predictive potential. Using Youden index (J) the Cutoff point differentiating IDA from healthy group was ≤ 34.55 ; AUC 0.845 ($P < 0.0001$), 95% Confidence interval was 0.755 to 0.911, and sensitivity was 98.33% and specificity 52.94% in case of IDA group.^{16,17}

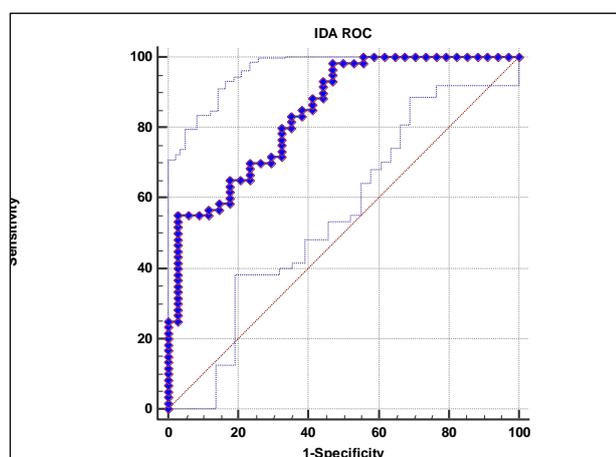


Figure 1: Receiver operating characteristic curves for serum hepcidin in IDA group.

DISCUSSION

Hepcidin is a master regulator of iron metabolism. Its production is homeostatically regulated by anemia and hypoxia; in addition to being regulated by inflammation and oxidative stress. Hepcidin is encoded as an 84-amino acid prepropeptide, which is cleaved to yield the 60-amino acid form called prohepcidin, which is further processed to yield the 25-amino acid form of Hepcidin. Hepcidin negatively regulates intestinal iron absorption, iron recycling by macrophages, iron release from hepatic stores and, during pregnancy, iron transfer in placenta. In turn, hepcidin secretion is regulated by iron stores, oxygenation, and inflammatory signals (chiefly IL-6). It is well demonstrated that bone morphogenetic proteins (BMPs) are a group of growth factors that activate the transduction signal by interacting with specific receptors. They use intracellular and extracellular iron detection mechanisms to alter hepcidin expression. BMPs increase hepcidin both in vivo and in vitro. BMP6 is the primary regulator of endogenous hepcidin. Hemojuvelin (HJV) is a BMP co-receptor specialized for iron regulation. The hepcidin adjustment process in the iron response occurs in the liver on the membranes of hepatocytes. Once

BMP6 binds the co-receptor HJV, the SMAD transcriptional system (SMAD1/5/8) is activated. The activated SMAD complexes bind directly to BMP responsive elements on the hepcidin promoter, thus inducing hepcidin transcription.^{18,19} Hepcidin synthesis is reportedly stimulated by elevated plasma iron concentration, infection and/or inflammation, and is suppressed in conditions that demand increased serum iron, such as increased or ineffective erythropoiesis, hypoxia, anemia and also iron deficiency.²⁰ In present study, we observed statistically significant lower values of RBC count, Hb gm/dl, MCV, MCH, MCHC in IDA group then the normal group. The MCH and MCV indicated that patients with IDA were microcytic and hypochromic. The biomarker serum ferritin levels were low in IDA group (13.23 ± 5.53 ng/ml) than the normal group (65.67 ± 16.76 ng/ml). Serum hepcidin levels were significantly low in IDA group (54.6 ± 15.46 ng/ml). The classic iron deficiency anemia in humans are associated with low hepcidin expression, thus hepcidin turns to be a potential marker for detection of iron deficiency anemia. Moreover, the development of diagnosis and therapy for anemia based on hepcidin may provide a more effective approach to prevent toxicity associated with iron overload.^{21,22}

CONCLUSION

This is the pioneer study of its kind, (as per reported literature) conducted in India which is a multi-parameter based study conducted comprehensively to explore the use of a biomarker i.e. hepcidin in detection and diagnosis in early stage anemia. Present findings coordinate well and are in good agreement with the observation(s) and results reported elsewhere in the literature. Moreover, there was a marked difference in the reported serum level(s) of the biomarker(s). The reported relatively low levels may be attributed to the participant population, its heterogeneity in terms of age; sex and disease history if any and also due to the method of analysis selected and sensitivity of kit(s) used for the purpose. Nevertheless, in various group(s) the pattern of biomarker(s), and their relative correlation were the same with good appreciable AUC on ROC curves; and high specificity and sensitivity revealing that hepcidin could conclusively be used to diagnose iron deficiency anaemia.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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