Original Article

Serum hepcidin level evaluation in children with acute lymphoblastic leukemia during different treatment phases; the influence of erythroid activity and iron stores

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ABSTRACT

Background: Hepcidin is the master regulator of iron homeostasis but until now, data about its expression in acute lymphoblastic leukemia (ALL) is scarce. **Objectives:** To evaluate hepcidin level in a group of ALL children in different treatment phases, investigating its relation to serum ferritin and erythroid activity. **Materials and Methods:** Forty ALL children were included and categorized into; Group I: Included 20 newly diagnosed ALL children who were evaluated at diagnosis and after remission. Group II: Included 20 ALL children in the maintenance phase of therapy. Twenty age and gender matched healthy children were enrolled as a control group. Complete blood count including reticulocytes %, liver functions, renal functions, and C-reactive protein were assayed. Serum hepcidin and ferritin were measured by enzyme-linked immunosorbent assay. **Results:** Serum hepcidin and ferritin levels were significantly higher among both ALL groups compared to the controls. These values were higher before therapy than after remission in the newly diagnosed group as well as than the maintenance group. Before therapy, both serum hepcidin and ferritin levels had significant negative correlation with hemoglobin and reticulocytes % while directly correlated with each other. **Conclusion:** Hepcidin level increased in ALL children at diagnosis and in different treatment phases. The highest rise was at diagnosis. These results indicate that hepcidin level among ALL patients is under the opposing effects of the iron stores and erythroid activity with the net level is determined by the strength of each stimulus.

Key words: Acute lymphoblastic leukemia, hepcidin, iron stores

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a malignant clonal proliferation of lymphoid progenitor cells most commonly of the B-cell lineage (B-ALL) that accounts for 81% of childhood leukemias.[1] It is a heterogeneous disease in which many genetic lesions result in the development of multiple biological subtypes.[2]

Anemia remains one of the most common complications of childhood cancer and plays a critical role in the quality of life of these children.[3] Approximately, 75% of children who present with ALL will have significant anemia with hemoglobin (Hb) level <10 g/dL.[4]

A number of contributors have been proposed to account for this dysfunction including the effects of a chronic disease,

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neoplastic infiltration of the bone marrow (BM), specific nutritional deficits, chronic bleeding, frequent infections, and autoimmune hemolytic processes.[5]

Hepcidin, a 25-amino acid antimicrobial peptide synthesized in the liver, is the central iron-regulatory hormone that mediates the homeostasis of extracellular iron concentrations.[6] It inhibits the intestinal absorption of iron by enterocytes in the duodenum and iron release by macrophages and hepatocytes.[7] This action is mediated through binding to and inducing the internalization and degradation of ferroportin, the only known exporter of iron.[8]

Hepcidin expression is stimulated when iron stores increase[9] as well as during inflammation. Conversely, it is inhibited by anemia/hypoxia and heightened erythropoiesis drive.[10] Damage of the regulating mechanisms of hepcidin plays a role in the pathogenesis of some diseases including anemia from neoplastic diseases.[11] In these pathologic situations, antagonistic signals for hepcidin regulation can occur simultaneously.[12]

Currently, there is scant information about hepcidin expression in acute leukemia.

Hence, the aim of this study was to evaluate hepcidin level in a group of ALL children in different treatment phases, and its relation to iron stores and the erythroid marrow activity.

MATERIALS AND METHODS

Subjects

This case-control study included 40 children with precursor B-ALL attending Hematology and Oncology Unit, Menoufia University Hospital, Egypt. Diagnosis of ALL was performed according to standard clinical, morphological, cytochemical, and immunophenotypic criteria.

Patients were treated according to St. Jude ALL total therapy study XV treatment protocol.[13] According to this protocol, the patients were categorized into risk groups depending on pretherapeutic factors and the response to the induction therapy. The inclusion criteria were precursor B-ALL children, on total XV treatment protocol, and of low-risk category. The exclusion criteria included patients <1 year and >18 years old, those with trisomy 21, T-cell ALL, risk categories other than low risk, patients on other therapy protocols, patients who failed to achieve remission after induction or who were relapsed, those with serum creatinine >0.7 mg/dl or serum total bilirubin >1 mg/dl, and those on recombinant human erythropoietin therapy.

Twenty age and sex matched healthy children were enrolled as a control group.

The included children were categorized into 3 groups:

Group I: Included 20 newly diagnosed ALL children (13 males and 7 females) with their ages ranged from 3 to 10 years (mean age of 5.15 ± 2.06 years, median of 5). This group was re-evaluated after achieving complete remission as per protocol (<5% blast cells in BM aspirate and minimal residual disease <0.01%).

Group II: Included 20 ALL children who were in the maintenance phase of therapy (after recovery of re-induction 2) during the study time (14 males and 6 females) with their ages ranged from 3 to 9.5 years (mean age of 5.3 ± 1.53 years, median of 5).

Group III: Included 20 ages and gender matched healthy children (14 males and 6 females) with their ages ranged from 3 to 10 years (mean age of 6.25 ± 1.75, median of 6).

The study was approved by the Ethics Committee, Faculty of Medicine, Menoufia University, Egypt, and the informed consent was obtained from the legal guardians of the included children before participation.

In brief, St. Jude ALL Total Therapy Study XV treatment protocol consists of the following phases:

A. Remission induction therapy (6 weeks). It begins with prednisone, vincristine, daunorubicin, asparaginase, and triple intrathecal treatment, followed by cyclophosphamide plus cytarabine plus 6-mercaptopurine
B. Consolidation treatment (8 weeks) consisting of high-dose methotrexate at 2.5 g/m^2 (every other week for 4 doses) and daily 6-mercaptopurine
C. Maintenance treatment (120 weeks for girls and 146 weeks for boys). Its backbone contains daily oral 6-mercaptopurine and weekly intravenous or intramuscular methotrexate. Re-induction I and II are given at weeks 7–9 and 17–19, respectively. Dexamethasone with vincristine and triple intrathecal treatment are added at certain weeks.

Methods

The study design

The study investigated serum hepcidin level in 2 different patient groups. The first comparison was of newly diagnosed ALL children to the same group after getting remission (paired comparison) and to healthy controls (nonpaired comparison). The other comparison was between another groups of ALL children who were in the maintenance phase of therapy at the study time with the healthy controls. Moreover, both groups were compared to each other.

The clinical evaluation included full history taking and thorough clinical examination.

Laboratory investigations included complete blood count by coulter counter with reticulocytes counting by manual method, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (LTEC Kit, England), blood urea nitrogen (BUN) by urease-colorimetric method, and serum creatinine by fixed rate kinetic chemical method (Diamond diagnostics international kits, England). C-reactive protein (CRP) was determined (Kraemer Blvd. Brea, CA, USA). Serum levels of ferritin and hepcidin were determined by enzyme-linked immunosorbent assay (ELISA), using Monobind Inc., Human Ferritin ELISA kit, USA, and EIAab® Human Hepcidin ELISA kit, China, respectively.

For all evaluations, blood samples were taken before blood transfusion or after at least 72 h if blood transfusion was indicated at the due time of the evaluation.

Statistical analysis

Sample size calculation in the present case-control study was calculated using OpenEpi, Version 3, open source calculator SSCC (www.openepi.com/SampleSize/SSCC.htm). With incidence of ALL of 4:100,000, two-sided confidence level (1-alpha) of 95%, power 80%, ratio of controls to cases of 1:1, and the least extreme odds ratio to be detected 0.00, the total sample size (Fleiss with CC) was 40 children (20 for each patient group and 20 for the controls).

Statistical analysis was performed using SPSS-20 (SPSS Inc., Chicago, IL, USA). Continuous variables were presented as mean ± standard deviation while for categorical variables (gender, pallor, lymphadenopathy, hepatosplenomegaly, and bleeding) numbers (%) were used. Chi-square test was used for qualitative variables (gender). Compatibility with normal distribution was evaluated using Shapiro–Wilk normality test. The difference between 2 groups was performed by student's *t*-test for parametric continuous variables (age, Hb, ALT, AST, creatinine and hepcidin) and by Mann–Whitney U-test for nonparametric variables (red blood cells [RBCs] count, reticulocytes %, white blood cells [WBCs] count, platelet count, BUN, CRP, and ferritin). Paired *t*-test and Wilcoxon Signed Rank test were used for paired analysis of parametric (Hb, ALT, AST, creatinine, and hepcidin) and nonparametric variables (RBCs count, reticulocytes %, WBCs, platelet count, BUN, CRP, and ferritin), respectively. Pearson correlation (r) was the test used to measure the association between two quantitative parametric variables (for hepcidin with Hb), and Spearman correlation coefficient was applied for nonparametric data (for hepcidin with reticulocytes %, CRP, and ferritin; and for ferritin with Hb, reticulocytes %, and CRP). All tests were two-tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

The clinical characteristics of the patient groups were represented in Table 1. B-ALL children at diagnosis had significant lower Hb, RBCs, and reticulocytes % with significant higher CRP, serum ferritin, and hepcidin levels as compared to their levels after remission and to the controls. After remission, ALL children had significant lower Hb and significant higher CRP, serum ferritin, and hepcidin levels compared to the controls. Compared to the maintenance group, Group I ALL children at diagnosis had significant lower Hb, RBCs counts, and reticulocytes % and significant higher CRP. Both at diagnosis and after getting remission, this group had significant higher ferritin and hepcidin levels in relation to the maintenance group.

Regarding the maintenance group children, they had significant higher CRP, serum ferritin, and hepcidin levels and significant lower Hb compared to control group [Table 2 and Figure 1].

The correlation analysis revealed that both serum hepcidin and ferritin had significant negative correlation with Hb level and reticulocytes % while they had a positive correlation with each other among ALL children at diagnosis. In the same group, serum ferritin correlated positively with the CRP [Table 3 and Figure 2]. No significant correlation was found between serum hepcidin or ferritin, and the other tested parameters among Group I after remission or in the maintenance group [Table 3].

DISCUSSION

In this study, anemia was more prevalent at diagnosis than other phases. The lowest Hb level and reticulocytes % were at diagnosis. Anemia, at this time, is mainly related to defective erythropoiesis caused by blast cells infiltration

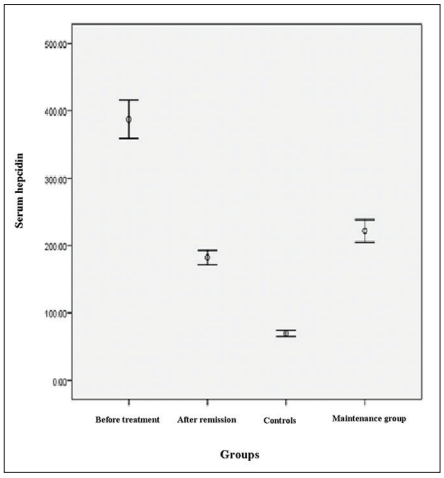

Figure 1: Serum hepcidin level (ng/ml) in the studied groups

Table 1: The characteristics of the studied groups

	Group I (at diagnosis) (*n*=20)	Group I (after remission) (*n*=20)	Group II (maintenance group) (*n*=20)	Group III (control) (*n*=20)	*P*
Age (years)	5.15±2.06	-	5.3±1.53	6.25±1.75	>0.05
Gender, male, *n* (%)	13 (65)	-	14 (70)	14 (70)	>0.05
Pallor, *n* (%)	20 (100)	6 (30)	7 (35)	0 (0)	
Lymphadenopathy, *n* (%)	7 (35)	0 (0)	0 (0)	0 (0)	
Hepatosplenomegaly, *n* (%)	15 (75)	0 (0)	0 (0)	0 (0)	
Bleeding, *n* (%)	9 (45)	0 (0)	0 (0)	0 (0)	

n: Number

Table 2: Comparisons between the studied groups regarding the laboratory parameters

	Group I (at diagnosis) (*n*=20)	Group I (after remission) (*n*=20)	Group II (maintenance group) (*n*=20)	Group III (control) (*n*=20)
Hb (g/dL)	6.1±2.1$^{\#,\&}$	10.8±1.1$^{*,\#}$	11.0±0.9$^{\#}$	12.6±0.4
RBCs count (10^{12}/L)	2.6±0.9$^{\#,\&}$	3.8±0.7*	6.2±8.4	4.9±0.3
Reticulocytes (%)	1.4±0.7$^{\#,\&}$	2.08±0.2*	2.05±0.2	2.11±0.1
WBCs count (10^3/mm^3)	33.5±23.4$^{\#,\&}$	2.8±1.4$^{*,\#,\&}$	4.4±0.5$^{\#}$	5.4±0.9
Platelets count (10^3/mm^3)	61.2±47.1$^{\#,\&}$	214.4±82.6$^{*,\#}$	250.3±60.7$^{\#}$	307.50±33.06
Serum ALT (IU/L)	13.9±3.2$^{\#}$	13.2±1.9	13.1±1.9	11.9±0.9
Serum AST (IU/L)	29.8±6.1$^{\#}$	28.2±3.5$^{\#}$	28.6±4.1$^{\#}$	24.1±3.3
Serum creatinine (mg/dL)	0.35±0.05$^{\#}$	0.30±0.06*	0.34±0.06$^{\#}$	0.28±0.07
Blood urea nitrogen (mg/dL)	12.25±2.44$^{\#}$	9.75±1.74$^{*,\#}$	10.10±2.1$^{\#}$	7.60±1.0
CRP (mg/L)	50.25±12.28$^{\#,\&}$	14.8±4.54$^{*,\#}$	12.65±4.46$^{\#}$	3.43±1.13
Serum ferritin (ng/mL)	1265.3±252.9$^{\#,\&}$	793.5±206.5$^{*,\#,\&}$	604.3±184.7$^{\#}$	166.6±44.3
Serum hepcidin (ng/mL)	387.6±60.9$^{\#,\&}$	221.5±36.4$^{*,\#,\&}$	181.9±22.7$^{\#}$	69.8±9.7

*Significant compared to before treatment, $^{\#}$Significant compared to the control group, $^{\&}$Significant compared to the maintenance group. *n*: Number, Hb: Hemoglobin, RBCs: Red blood cells, WBCs: White blood cells, ALT: Alanine transaminase, AST: Aspartate transaminase, CRP: C-reactive protein

Table 3: Correlations of serum hepcidin and ferritin with Hb, reticulocytes percentage, and ferritin among patient groups

	Group I (at diagnosis)		Group I (after remission)		Group II (maintenance group)	
	r	*P*	*r*	*P*	*r*	*P*
Serum hepcidin						
Hb (g/dL)	−0.54	**0.01**	0.06	0.82	−0.19	0.41
Reticulocytes (%)	−0.62	**0.003**	−0.11	0.67	−0.005	0.98
CRP (mg/L)	0.6	0.8	0.1	0.66	−0.09	0.7
Serum ferritin (ng/dL)	0.81	**<0.0001**	0.04	0.86	−0.29	0.2
Serum ferritin						
Hb (g/dL)	−0.46	**0.04**	0.5	0.18	0.04	0.86
Reticulocytes (%)	−0.52	**0.02**	−0.02	0.92	−0.39	0.08
CRP (mg/L)	0.46	**0.04**	−0.05	0.83	−0.09	0.7

Bold numerical values indicate significance. Hb: Hemoglobin, CRP: C-reactive protein

of the BM,[14] while with BM recovery from blast cells infiltration, chemotherapy BM depressive effect was responsible for anemia after remission and during the maintenance phase.

Recovery of the erythropoietic activity in this study was reflected by the significant rise in reticulocytes % in ALL children after remission meaning that the major recovery of BM erythroid activity occurred in the stage of remission induction.

Hepcidin contribution in anemia of malignancy was previously suggested in few reports.[11] In this study, serum hepcidin was significantly higher in ALL children at diagnosis in comparison to its level after remission and in both situations it was significantly higher than the control values. This is in agreement with what was reported by Cheng *et al*.[15] In this group also serum hepcidin had a negative correlation with Hb level which is consistent with what was reported by others,[15,16] denoting that the degree of anemia is another hepcidin regulator.

The maintenance group also exhibited higher hepcidin level compared to the controls (although being significantly lower than the newly diagnosed cases both at diagnosis and after remission).

Serum hepcidin rises among our studied ALL children despite the presence of anemia and could denote the presence of other confounding factors that could override

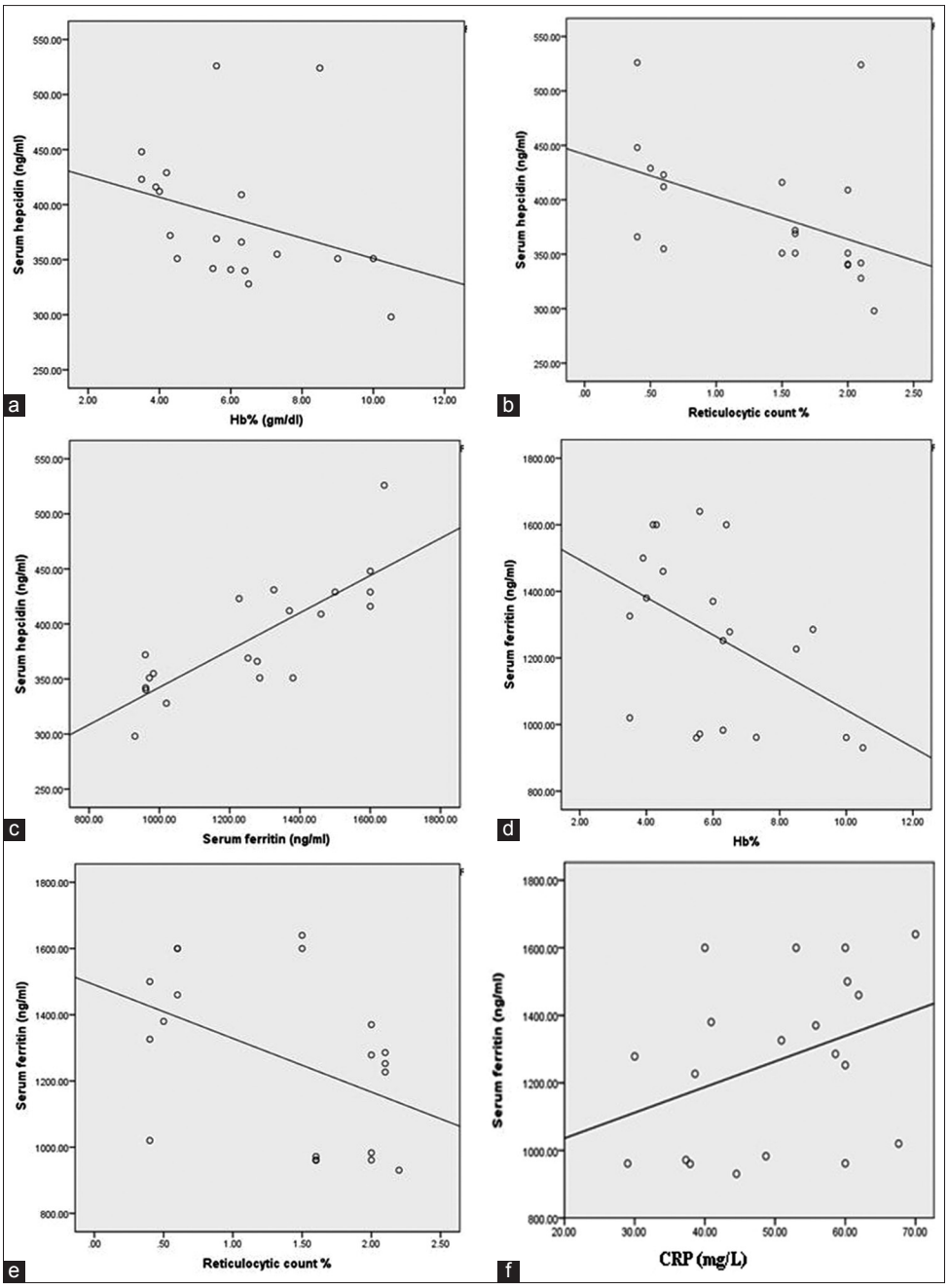

Figure 2: Significant negative correlations between serum hepcidin and ferritin with hemoglobin, reticulocytes %; (a, b, d, and e) significant positive correlation between hepcidin and ferritin, (c) significant positive correlation between ferritin and C-reactive protein, (f) among the newly diagnosed acute lymphoblastic leukemia children before therapy

the expected depressive effect of anemia upon hepcidin production.

Hepcidin production is regulated by at least 3 factors: Iron load,[9] inflammatory stimuli such as interleukin-6,[17] and unknown erythropoietic signals.[18] All of which can be present in ALL.

Iron metabolism and erythropoiesis are closely linked.[19] Imbalance of iron metabolism has been associated with several cancers including leukemia.[20]

Serum ferritin is supposed to represent a reliable marker of body iron stores[21] in most physiological and pathological conditions.[22] However, ferritin is an acute phase reactant that increases in the setting of inflammation.[23]

Patients with acute leukemia in different stages are a candidate for iron overload due to multiple blood transfusion.[24] In this study, serum ferritin was significantly higher in all patient groups compared to the controls and took the same pattern of serum hepcidin level. The higher ferritin before treatment could be related

mainly to the inflammatory status - where this group had the highest CRP level that had a positive correlation with serum ferritin - and to decreased iron utilization caused by suppressed erythropoiesis.[22] After remission and in the maintenance group, both inflammation and blood transfusion-induced iron overload could be the contributors of this elevation.

The parallel increase in serum hepcidin and ferritin in the studied groups supports the role of increased iron load in enhancing hepcidin expression. In addition, the significant positive correlation between hepcidin and ferritin in the newly diagnosed cases before treatment augments this role.

Usually, hepcidin and serum ferritin respond similarly to inflammation and changes in iron stores, and this is reflected in the strong correlation between hepcidin and ferritin.[25,26] Moreover, hepcidin mRNA correlated with iron stores in human liver biopsies.[16]

The different influences of erythroid activity and iron stores on hepcidin expression during different disease phases were apparent in this study. Before treatment, ALL children had the highest hepcidin and ferritin (related mainly to inflammation) levels with inverse relation between serum hepcidin and the reticulocytes % (reflecting the erythroid marrow activity). This indicates that, at this phase, hepcidin was regulated mainly by the erythroid marrow activity and inflammation.

An elevated hepcidin expression as a proposed cause of anemia of inflammation[27] might explain the inverse correlation between hepcidin and Hb observed in this group at diagnosis and could highlight the complexity of hepcidin regulation.[28]

The significant decline of serum hepcidin levels in ALL children after remission and in the maintenance phase (who had more or less recovered erythroid activity) despite the presence of iron load with its enhancing effect could propose that increased erythropoietic activity may play the main role in suppressing hepcidin production during these phases as previously suggested.[29] However, to override hepcidin regulation by iron, very high erythropoietic activity was required,[19] which is not our case where there was still lower although nonsignificant reticulocytes % in comparison to the controls. This indicated that increased iron stores among these patients were the main drive for high serum hepcidin.

In accordance with our result, elevated hepcidin level was reported by Eisfeld *et al*.[28] among acute myeloid leukemia patients before stem cell transplantation suggesting that hepcidin synthesis and up-regulation remain intact despite intensive chemotherapy.

To summarize, the results of this study confirmed the enhanced hepcidin expression among ALL children at different disease stages being highest in the newly diagnosed group before therapy.

CONCLUSIONS

Serum hepcidin was found to be high among ALL children at diagnosis and was associated with decreased erythroid activity. This level significantly declined upon complete remission and was lower in the maintenance group due to marrow recovery. These results indicate that hepcidin level among ALL patients is under the opposing effects of the inflammatory stimuli, iron stores, and erythroid activity with the net level is determined by the strength of each stimulus as previously reported.[12]

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Conflicts of interest
There are no conflicts of interest.

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