Clinical and molecular correlation of hepcidin RNA expression in sickle cell patients with iron overload

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Background/aim
Iron overload is the main concern in treatment of hemolytic diseases with repeated blood transfusion, especially sickle cell disease (SCD). Hepcidin has appeared as the key iron metabolism regulator. Erythroferrone (ERFE) is postulated to function as the chief erythroid regulator. Transferrin receptor 2 (TfR2) acts as an iron sensor on erythroid cells. Our aim is to evaluate serum levels of hepcidin, ferritin, ERFE, and TfR2 and its correlation with molecular genetic study of hepcidin gene expression for SCD patients
Patients and methods
Patients: 103 children aged 6–18 years with SCD were recruited from the Pediatric Hematology Clinic at the National Research Center and Abo-Elrish Hospital (Cairo University), and 55 healthy children with matched age and sex served as the control group. Methods: laboratory analysis and enzyme-linked immunosorbent assay tests on patient samples were performed for serum hepcidin, ERFE, ferritin and TfR2, and hepcidin gene expression was performed by quantitative real-time PCR.
Results
Hepcidin RNA expression level showed significant correlation with the duration of the disease and blood transfusion frequency (r=–0.33, P<0.001) (r=–0.270, P 0.006), respectively.
Conclusion
Combination of enzyme-linked immunosorbent assay and molecular studies of hepcidin RNA expression could be a diagnostic marker to be used in conjunction with analytical techniques to detect iron overload in pediatric sickle cell disease.

Keywords:
erythroferrone, ferritin, hepcidin, iron overload, sickle cell disease

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Introduction

Iron storage mainly occurs in the liver (hepatocyte) in the form of ferritin. Iron is a major analyte in which its metabolism is adjusted mainly by its storage and erythropoietic capacities [1]. Iron overload is one of the causes or morbidity and mortality in patients with hemolytic anemia. Iron overload is a main concern in the treatment of hemolytic diseases with repeated blood transfusion especially sickle cell disease (SCD). However, in untreated SCD iron storage (ferritin) rarely exceeds 2000 ng/ml in the absence of repeated blood transfusion which may lead to iron deficiency presentation in nontransfused SCD population [2,3].

Mechanisms underlying the distribution of iron in SCD are mediated by various factors including blood transfusion regimens and primary site of red blood cell destruction. The distribution of iron largely reflects iron storage with a lower proportion of transfused iron distributes extrahepatically in SCD with less common complications of iron overload to the heart and the endocrine system [4–7].

In SCD, hemolysis is mainly intravascular which provides a potential mechanism for iron elimination via urinary and biliary excretion as hemoglobin and hemosiderin [8].

Hepcidin has appeared as the key iron metabolism regulator [9]. Many factors contribute to the level of hepcidin including iron erythropoiesis and inflammation. Moreover, hepcidin expression is regulated by transferrin through transferrin receptor 2 (TfR2). TfR2 was shown to act as an iron sensor on erythroid cells [10].

Furthermore, bone marrow-derived factors may play a role in iron regulation through growth differentiation factor 15, twisted gastrulation protein homolog, and recently identified erythroferrone (ERFE) [2]. ERFE is postulated to function as the chief erythroid regulator. ERFE is an erythropoiesis-driven regulator of iron metabolism [11]. ERFE helps the suppression of iron regulator hepcidin to increase iron absorption and mobilization of iron from cellular iron stores (liver, spleen) [9]. Although ERFE emerges to be an important iron regulator for erythropoiesis, TfR2 appear to be a crucial factor in this regulation [12,13], in which Trf2 is regulated by erythropoietin through an accompanied erythropoietin receptor on erythroblasts and inversely affects its sensitivity to erythropoietin [10]. The increased erythropoietin secretion in response to anemia stimulates erythroblasts to produce ERFE [1].

Nonetheless, the main erythroid regulator of iron remains intangible. Our aim is the evaluation of serum levels of hepcidin, ferritin, ERFE and TfR2, and its correlation with the molecular genetic study of hepcidin gene expression.

Patients and methods

Patients

One hundred and three children aged from 6 to18 years with SCD were recruited from the Pediatric Hematology Clinic at the National Research Centre and from the Pediatric Hematology Departments at Abo-Elrish Hospital, Cairo University.

Ethical consideration

The study was approved by the National Research Centre Ethics Committee, Egypt with approval no. 161/28 and blood samples from patients and control were collected upon written informed consent in accordance with the Declaration of Helsinki.

Study design

In our cross-sectional study, 103 SCD patients were recruited and 55 healthy age-matched and sex-matched children were selected as the control group. The studied patients with SCD were divided into five groups according to disease duration: less than 3 years duration (n=24), 3–10 years duration (n=15), 11–13 years duration (n=22), 14–15 years duration (n=27), and more than 15 years duration (n=15).

Methods

All patients and controls were subjected for the following:

(1) Clinical assessment: detailed medical history was taken, regarding previous blood transfusion (type, frequency, and amount/year), chelation, and encountered complications (attacks of painful hemolytic crises, thrombotic manifestations, or chest problems). Clinical examination was performed (organomegaly, pallor, jaundice, bone aches).

(2) Laboratory investigations: 5 ml venous blood was withdrawn for laboratory assessment. Routine laboratory tests: complete blood count, serum aspartate, and alanine aminotransferases. Serum iron was measured using the automated analyzer Olympus AU-400 (Shinjuku City, Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol was done for serum hepcidin [14], ferritin [15], TfR2 [16], and serum ERFE [17], using ELISA kits of Bioassay Technology Laboratory (Shanghai, China).

(3) RNA extraction and complementary DNA (cDNA) synthesis
Total RNA was isolated from peripheral mononuclear cells, including lymphocytes and monocytes using a QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instruction [18].
Reverse transcription reactions were performed using a high-capacity cDNA archive kit according to the manufacturer's instruction (Applied Biosystems, Foster City, California, USA).

(4) Real-time quantitative PCR
Real-time PCR was performed with Stratagene Mx3000P (Agilent Technologies Inc., Santa Clara, California, USA). The PCR reaction was carried out in a final volume of 25 µl containing 2 µl cDNA, 12.5 µl 2× SYBR Green Master Mix (Applied Biosystems), 0.5 µl of 25 nmol/l sense and antisense primers, and deionized water up to 25 µl. The PCR conditions consisted of 40 cycles at 95°C for 15 s and 60°C for 60 s. The sequences of the primers were as follows: GAPDH: sense primer 5′-CCACCCAGAAGACTGTGGAT-3′, antisense 5′-TTCAGCTCAGGGATGACCTT-3′, and hepcidin: sense primer 5′-CACAACAGACG GGACAACTT-3′, antisense5′-CGCAGCAG AAAATGCAGATG-3′. We used the comparative (ΔΔCT) method where we compared the CT values of the target gene (hepcidin) with the reference gene (GAPDH).

Statistical analysis

All data were statistically described in terms of mean ±SD, median and range, or frequencies. Comparison of

numerical variables between the study groups was done using Student's *t* test for independent samples in comparing normally distributed data and Mann–Whitney *U* test for independent samples when not normally distributed. Correlation between various variables was done using the Spearman rank correlation equation for nonlinear relationship. *P* values less than 0.05 were considered statistically significant. All statistical calculations were done using the computer program SPSS (Statistical Package for the Social Sciences; SPSS Inc., Chicago, Illinois, USA), version 21 for Microsoft Windows.

Results

A total of 103 of SCD patients were recruited and 55 healthy children were selected for comparison. demographic and laboratory data of the studied groups obtained are shown in Table 1.

Table 1 Laboratory routine investigations of sickle cell disease patients

	Mean±SD
Age (years)	13.77±3.27
RBCs (10^{12}/l)	3.86±0.92
HB (g/dl)	8.91±3.14
HCT%	32.42±5.33
MCV (fl)	81.95±7.42
MCH (pg)	28.62±3.1
MCHC (g/dl)	32.17±2.93
RDW%	13.57±0.91

HB, hemoglobin; HCT, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cell; RDW, red cell distribution width.

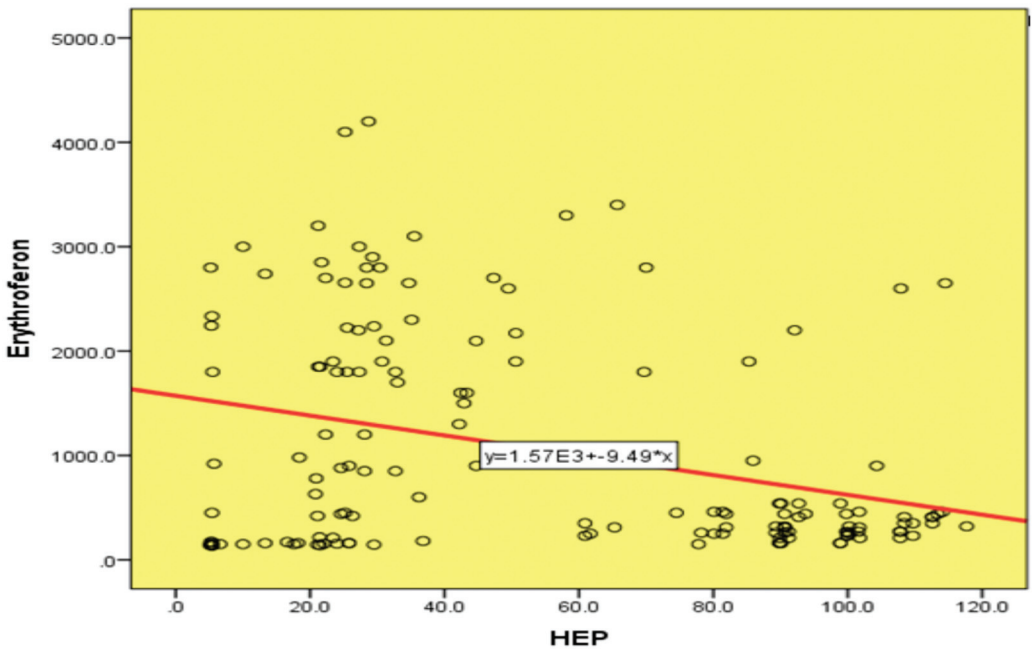
The present results indicated that serum levels of ERFE, TfR2, and ferritin showed statistically significant increase ($P<0.001$) in SCD patients compared with the control group. Meanwhile, as expected hepcidin serum levels showed a statistically significant decrease ($P<0.001$) in the patient group compared with the control group, as shown in Table 2. Moreover, our data indicated significant correlation between serum levels of hepcidin and ERFE in SCD patients as demonstrated in Fig. 1.

In our study, hepcidin RNA expression levels displayed a significantly increased level that ranged between 0.001 and 0.791, with a mean value of 0.118 in SCD patients in comparison with our control group. Furthermore, a negative correlation was found between

Table 2 Serum levels of erythroferrone, hepcidin, transferrin receptor 2, and ferritin in sickle cell disease patients and control

	Cases (*N*=95) (mean±SD)	Controls (*N*=55) (mean±SD)	*P* value
Erythroferrone (pg/ml)	1468.6±1114.0	319.6±107.3	<0.001
Hepcidin (ng/ml)	32.5±24.5	94.5±13.6	<0.001
Transferrin receptor 2 (pg/ml)	771.52±432.68	260.25±85.13	<0.001
Ferritin (ng/ml)	562±302	82±31	<0.001

Figure 1



Graph demonstrating the correlation between hepcidin and erythroferrone serum levels in SCD patients. SCD, sickle cell disease.

Table 3 Hepcidin RNA expression in sickle cell disease patients and controls

Hepcidin RNA expression (ΔΔCT)	SCD patients (*N*=95)	Controls (*N*=55)	*P* value
Median	0.022	1.014	<0.001
Mean±SD	0.118±0.188	1.028±0.113	

SCD, sickle cell disease.

Table 4 Spearman rank correlation between serum levels of ferritin, hepcidin, hepcidin RNA expression and erythroferrone with disease duration and blood transfusion frequency among the sickle cell disease patients

	Ferritin (*N*=95)	Hepcidin (*N*=95)	Hepcidin expression (*N*=95)	Erythroferrone (*N*=95)
Disease duration	*r*=0.232	*r*=−0.007	*r*=−0.659	*r*=0.227
	P=0.019	*P*=0.948	*P*=0.000*	*P*=0.021
Blood transfusion frequency per year	*r*=0.288	*r*=−0.081	*r*=−0.270	*r*=−0.095
	P=0.003*	*P*=0.419	*P*=0.004*	*P*=0.339

*Statistically significant correlation at *P* value less than 0.005.

serum ERFE and serum hepcidin among the SCD patient group at *P* value less than 0.001 (Table 3).

Hepcidin RNA expression levels showed high significant correlation in the SCD patient in relation to the duration of the disease and the blood transfusion frequency per year ($P<0.001$) ($P=0.004$), respectively, which highlights its importance as a predictor in the detection of iron overload in SCD patients.

Moreover, ferritin showed high significant correlation with the blood transfusion frequency per year ($r=0.288$, $P=0.003$).

On the contrary, serum ERFE, TfR2 showed no significant correlation in relation to the duration of diseases and blood transfusion (Table 4).

Discussion

SCD patients are characterized by chronic hemolytic anemia with ineffective erythropoiesis. In untreated SCD, iron storage(ferritin) rarely exceeds 2000 ng/ml in the absence of repeated blood transfusion, which may lead to iron deficiency in nontransfused SCD population [5,6]. However, iron overload is a main concern in the treatment of hemolytic diseases with repeated blood transfusion especially SCD [19].

Our study revealed statistically significant increase of serum levels of ERFE, TfR2, and ferritin in SCD patients than that detected in the control group. However, hepcidin serum levels showed a statistically significant decrease in children with SCD. Hepcidin gene expression showed a significant low expression in these patients.

Hepcidin plays a key role in iron homeostasis and regulation. This mechanism is mediated by the iron exporter ferroprotein placed enterocytes and macrophages, whereas hepcidin overexpression causes iron-deficiency anemia. However, inactivation of hepcidin causes severe iron overload [20].

In our study, significant low serum hepcidin levels have been found in SCD patients. These findings agree with previously published studies [21–23] that reported lower hepcidin expression levels in thalassemia and other disorders with abnormal erythropoiesis. In the aforementioned disorders, increased iron release from recycling macrophages and absorptive enterocytes stimulated by reduced hepcidin leads to release ferroprotein leading to increasing availability of iron for erythropoiesis.

Hepcidin mRNA expression was significantly downregulated among SCD patients compared with controls. Comparable study results showed hepcidin RNA expression was downregulated in hepatic tissues obtained from hemolytic anemia patients [24]. In the Handa *et al.* [24] study hepatic hepcidin expression was elevated with concomitantly iron hepatic deposition in nonalcoholic fatty liver disease patients and in nonalcoholic steatohepatitis patients.

Moreover, our findings of hepcidin mRNA expression is significantly correlated with the duration of disease and frequency of blood transfusion per year. Our study may reflect its importance as a predictor of iron overload in children with SCD [25].

Concerning the finding of significantly higher levels of ERFE with lower serum hepcidin in children with SCD than controls may be explained by the fact ERFE stimulates hepcidin suppression during erythropoiesis stimulation. ERFE is produced by erythroblasts in response to erythropoietin.Moreover, a highly significant negative correlation between serum

ERFE and hepcidin variables were found in our patients with SCD.

ERFE regulates the accessibility of iron according to erythropoiesis stimulation. It acts on hepatocytes to decrease hepcidin through a bone morphogenetic protein pathway. Through hepcidin suppression and persistent stimulation for iron delivery, it could contribute to iron overload in hemolytic anemias [26,27].

In the Grotto *et al*. [27] study, SCD patients showed higher sTfR levels than in normal population, which matches our results regarding the increase in serum levels of TfR2 and ferritin in the SCD patient group [28]. TfR2 acts as an iron sensor on erythroid cells. Overproduction of sTfR may be a common finding in conditions when erythropoiesis is impaired by deficiency of iron. Therefore, increasing sTfR and decreasing hepcidin will lead to increase iron uptake [29].

To increase reliable diagnostic achievements, further genetic-based studies are required to open up new horizons for future research in order to approach appropriate monitoring and treatment of iron overload in SCD patients.

Conclusion

Our study concluded that a combination of noninvasive and cost-effective (ELISA) and molecular studies of hepcidin RNA expression may be a diagnostic marker to be used in conjunction with current analytical techniques to detect iron overload in pediatric SCD.

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Conflicts of interest

There are no conflicts of interest.

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