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Cytokine Dependent Hematopoietic Cell Linker (CLNK) is Highly Elevated in Blood Transfusion Dependent Beta-Thalassemia Major Patients

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Abstract

Beta-thalassemia major (β-TM) is a severe form of thalassemia caused by mutations in the β-globin gene, resulting in partial or complete deficiency of β-globin chains. This deficiency results in oxidative stress, dyserythropoiesis, and chronic anemia. Cytokine dependent hematopoietic cell linker (CLNK) belongs to the adaptor protein family and has the capacity to interact with multiple signaling proteins thereby modulating signal transduction.

The aim of the present study was to examine CLNK in sera of β-TM patients and examine its association with iron overload biomarkers.

Sixty β-TM patients, aged 3-12 years old and undergoing blood transfusions, and 30 healthy control children were recruited and CLNK, ferritin and iron status parameters were measured.

The results showed a significant increase ($p<0.001$) in serum CLNK levels in β-TM patients as compared with normal controls. The increased levels of CLNK were significantly associated with increased ferritin levels.

Increased CLNK levels in β-TM may be explained by reciprocal effects between immune signaling and immature erythrocytes, which, release soluble receptors and signaling molecules, including CLNK, in the blood.

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Keywords: CLNK, thalassemia, ferritin, iron overload.

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Introduction

Beta-thalassemia major (β-TM), a hereditary anemia characterized by absent or decreased production of β-globin chains, is associated with considerable morbidity and mortality.[1] These patients are treated with continuous administration of blood transfusion to raise the depletion of red blood cells (RBCs).[2] The treatment regimen causes iron overload whereby excess iron accumulates in the body organs tissues such as heart, liver and endocrine glands causing damage to the organs.[3,4] Eryptosis (suicidal erythrocyte death) contributes to or even accounts for the anemia in β-TM, and involves Ca^{2+} entry and ceramide, caspase, and tyrosine kinase signaling. Inhibitors of eryptosis may prevent anemia in clinical conditions associated with enhanced eryptosis.[5] Fetal hemoglobin (HbF), which consists of two γ-globin chains and two α-chains, is normally replaced by adult hemoglobin (HbA) at birth, whilst in β-TM, γ-chain production continues into adulthood.[6] In patients with β-TM, cAMP levels are elevated in both red blood cells and nucleated erythroblasts and the transcription factor cAMP response element binding protein is phosphorylated in nucleated erythroblasts, while its phosphorylation levels correlate with HBG mRNA levels of the patients.[7] Many signaling molecules, such as mitogen-activated protein kinases and signal transducers and activators of transcription proteins, are phosphorylated at variable levels.[7,8] Plasma levels of cytokines, such as erythropoietin, stem cell factor and transforming growth factor-β are increased in β-TM patients, and these cytokines induce both γ-globin gene (HBG) mRNA expression and cAMP response element binding protein phosphorylation.[7]

The Src family of protein tyrosine kinases (SFKs) is a family of non-receptor tyrosine kinases which play a central role in the regulation of hematopoietic cell functions and is are involved in regulation of membrane transport.[9] SFKs play a key role in regulating signal

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transduction by a diverse set of cell surface receptors. SFKs exhibit many molecular strategies to couple receptors with the cytoplasmic signaling machinery.[10] Src-family kinases are implicated in regulation of erythrocyte membrane cation transport including tyrosine phosphorylation regulating K/Cl cotransport.[9]

Cao et al., (1999)[11] have identified a Src homology 2 domain-containing leukocyte protein of 76 kD (SLP-76)-related molecule which they have termed CLNK (cytokine-dependent hemopoietic cell linker). Hematopoietic precursors secrete numerous regulatory molecules that form the basis of the intercellular cross-talk networks regulating in an autocrine and/or a paracrine manner various stages of normal human hematopoiesis.[12] CLNK, the third member of the SLP-76 family, is a cytokine inducible (IL-2 and IL-3) adaptor protein.[13] CLNK is expressed exclusively in cytokine-stimulated hemopoietic cells, including IL-2-induced T cells and NK cells, and IL-3-induced myeloid cells and mast cells.[14] CLNK may undergo tyrosine phosphorylation in response to immunoreceptor, but not cytokine, stimulation.[11] Inhibition of tyrosine dephosphorylation may alter the shape of RBCs into echinocytes, indicating loss of interaction between the cytoskeleton and lipid bilayers, and stimulates microvesicle production *in vitro*.[15,16,17] Nevertheless, no studies have examined CLNK in β-TM patients.

Hence, the present study examines serum levels of CLNK in β-TM patients as compared with healthy controls and the associations between CLNK levels and serum iron status. The specific hypothesis is that serum CLNK is significantly increased in β-TM children.

Subjects and Methods

Subjects

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Sixty β-TM patients aged 3-12 years participated in the present study. All of them were on blood transfusion regimen in the Thalassemia Unit at "Al-Zahra'a Teaching Hospital" in Najaf city, Iraq. The diagnosis was made by hematologists according to ICD-10-CM Diagnosis Code D56.1 (2019). Moreover all patients showed haematological tests and haemoglobin HPLC analysis indicative of β-TM. Haemoglobin HPLC were done by using (VARIANTTM β-T Short Program) HPLC instrument. Normal range for HbA2 is typically between 1.75 and 3.25% of total haemoglobin while heterozygous β-T conditions yield HbA2 levels between 4.0 and 9.0%. The normal range for HbF is typically less than 1% of the total haemoglobin. Heterozygous and homozygous condition of β-T yield HbF ranges of 1-5% and 80-100%, respectively.[18]

All patients were treated with blood transfusions and received approximately 15 ml of packed red blood cells/kg of body weight at each transfusion (2-6 week intervals) to maintain Hb levels above 9.5 g/dl. Patients were treated with chelation therapy with desferrioxamine B (Desferal) at least four times a week (subcutaneous infusion). The range of dose was 30 to 60 mg/kg body weight/day. The median duration of β-TM was 6.8 years with a range of 2.1 to 9.1 years. The median of the duration of treatment was 4.2 with a range from 1.2-8.3 years. Serological serum CRP was negative in all samples i.e. less than 6mg/L. CRP test was used to exclude inflammation that causes changes in the acute phase reactant proteins.[19] None of the patients had undergone splenectomy. Endocrinologic, hepatologic and cardiac evaluations were regularly performed and showed normal results at the time of sample collection. Blood samples from patients were collected 7-10 days after the last transfusion and just before the next transfusion. Only patients who are free from any other systemic disease were participated in the study. Thirty apparently healthy children were carefully chosen as the control group. Their age range and mean were comparable to that of patients. None of the controls was anaemic or had

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any obvious systemic or hematological diseases. The study was approved by the local IRB (institutional review board) of the University of Kufa (REC number: 1321). All participants included in this study and their first degree relatives (or guardians) gave written informed consent after appropriate explanation according to the Declaration of Helsinki.

Methods

Five milliliters of blood was collected from all selected children and separated into two aliquots. One aliquot was transferred into an EDTA tube for hematological analysis and the rest was transferred into a plain tube in order to separate serum by centrifugation at 3000rpm for 10 minutes. The Human CLNK quantitative test (Bioassay Technology Laboratory®, China) is based on a solid-phase enzyme-linked immunosorbent assay (ELISA). Serum ferritin concentration was determined by using Vidas Ferritin; an automated quantitative test using enzyme linked fluorescent assay (ELFA) technique. The fluorescence intensity was measured by automated analyzer (miniVidas, Biomeraux-France). Serum iron and TIBC were estimated by kits supplied by Biolabo, France. The other iron status-related parameters were computed using formulas as explained previously:[19,20] unsaturated iron-binding capacity (UIBC), the amount of protein (apotransferrin) still available to bind iron, can be estimated from the formula: UIBC=TIBC - Serum iron. Transferrin saturation percentage (TS%) was calculated as: TS%=(Serum Iron/TIBC)*100%. Transferrin concentration can be calculated using the following formula: Transferrin Conc. (g/L) =S.Iron (µmol/L)/ (TS%*3.98). Estimated total iron body stores (ETIBS) were calculated using the following equation: ETIBS (in µmol) = (Serum ferritin in µg/L) ×143.

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Statistical Analysis

Chi square test (χ^2 test) was used to check associations between categorical variables and analysis of variance (ANOVAs) to check differences in continuous variables between groups. Ln transformations were used to normalize the distribution of variables were needed. Multivariate general linear model (GLM) analysis was used to examine the effects of diagnosis (β-TM versus controls) on the different biomarkers, which were entered as dependent variables. Power analysis, using a 2-tailed test at α=0.05 and assuming an effect size of 0.3 with power of 0.80, shows that the required sample size is 90 participants. We computed two z unit weighted composite scores containing Ln(CLNK) namely, zLn(CLNK)+zLn(Ferritin) and zLn(CLNK)+zLn(Ferritin)+zLn(Iron)). Receiver operating characteristic (ROC) curves were utilized to determine the sensitivity and specificity of the outcome variables to differentiate between patients and controls. The bootstrapped (n-=2000) Area Under the Curve (AUC) was calculated to determine the diagnostic accuracy for β-TM. An optimal diagnostic tool has an AUC of 1, which indicates 100% sensitivity and specificity.[21] Tests were 2-tailed and a p-value of 0.05 was used for statistical significance. All statistical analyses were performed using IBM SPSS windows version 25, 2017.

Results

Table 1 presents the socio-demographic, clinical data, iron status parameters, as well as CLNK and the composite scores in β-TM patients and controls. There were no significant differences in the sex ratio between the both categories, while age was somewhat higher in controls than in patients (without p-correction). Hb and PCV were significantly lower in β-TM as compared with controls. Serum iron, TS%, ferritin, and EIBC were significantly higher in β-TM

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than in controls. CLNK and the CLNK-containing composite scores were significantly higher in patients as compatred with healthy controls. In the total study group, we found significant correlations between CLNK and Hb (p=-0.341, p=0.001), PCV% (p=-0.338, p=0.001), ferritin (p=0.452, p<0.001), but not iron or transferrin. In β-TM there was a significant association between CLNK and number of transfusions (p=0.305, p=0.018).

Table 2 displays the results of a multivariate GLM analysis with CLNK and the zCLNK+zFerritin+zIron composite score as dependent variables and with diagnosis as primary explanatory variable, while we adjusted for possible age and sex effects. There was a highly significant effect of diagnosis with an effect size of 0.635, while age and sex had no significant effect on the biomarkers. Tests for betwee-subject analyses showed that there are strong associations between diagnosis and both CLNK and the composite score. We also performed an univariate analysis with CLNK as dependent variable and found that the significant effects of diagnosis were no longer significant after inducing ferritin in the analysis. The last univariate analysis in Table shows that 20.4% of the variance in CLNK was explained by ferritin.

The results of ROC analysis including AUC are presented in **Table 3**. The bootstrapped area under the ROC curve was 0.738 for CLNK while that of ferritin was 0.978. Combining CLNK with ferritin and / or iron did not improve the diagnostic performance of ferritin.

Discussion

The major finding of this study is that CLNK and the CLNK-containing composite scores showed a highly significant increase in patients as compatred with healthy controls. Moreover, the increased levels of CLNK were explained at least in part by increased levels of ferritin. The increased CLNK levels in β-TM patients may be explained by reciprocal effects of aberrant

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immune signaling coupled with the immaturity of RBCs. In β-TM, many RBCs in the blood stream contain nucleic acids (reticulocytes), while RBCs have short lives and, therefore, may become hemolysed thereby activating release of soluble receptors and signal transducing molecules, including CLNK. The latter is the third member of the SLP-76 adaptor protein family and is expressed in cytokine-activated hematopoietic cells including mast cells and NK cells.[11,22] As other family members, CLNK contains 3 protein interaction domains, including amino terminus tyrosine residues, which are phosphorylated by Src family PTKs and also Syk.[23] The SLP-76-related adaptor molecule CLNK physically and functionally interacts with HPK-1 in immunoreceptor-activated hemopoietic cells. CLNK has the capacity to substitute at least partially for SLP-76 during immunoreceptor signaling, even though it binds to a distinct set of partners.[14]

The pathophysiological mechanisms of ineffective erythropoiesis in β-TM could be the result of several mechanisms of which the final consequence is the arrest of maturation and increased apoptosis of erythroblasts during their terminal differentiation stage.[25] CLNK can function as a signaling scaffold downstream of multiple immune receptors.[14,22,23] Other findings on the transcriptional regulation of the CLNK gene helps to understand how lineage-specific immunoreceptor signaling pathways are established and regulated during activation and differentiation of various hematopoietic cells.[24] Increased level of several inflammatory cytokines has been reported in β-TM and may contribute to ineffective erythropoiesis, through anemia associated with chronic disease.[25]

Our results also showed the typical picture of iron overload state and anemia in β-TM, including higher serum iron, TS%, ferritin, and EIBC and lower Hb and PCV in β-TM as compared with controls. Our findings are in accordance with Kuldeep *et al*., (2011)[26] who found

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a significant increase in serum iron in patients with β-TM as compared with healthy controls. These results are also in accordance with those of previous studies.[3,27] The predominant mechanism driving the iron loading process includes transfusion therapy and enhanced intestinal absorption secondary to ineffective erythropoiesis.[2] Serum ferritin measurement and noninvasive imaging techniques are available to diagnose iron overload, quantify its extent in different organs, and monitor clinical response to therapy.[2] Higher levels of serum ferritin (especially over 2500 µg/l) are associated with iron-related organ failure particularly in the liver and heart.[27] This organ damage is not only related to oxidative stress end products,[18] but also to the direct effect of iron overload on the tissues. In the current study, serum CLNK was significantly associated with number of transfusions, ferritin and iron. The overall positive correlation between CLNK and iron status parameters such as ferritin indicates the dependence of the CLNK level on iron overload status. In patients with transfusion-dependent β-TM, red blood cell transfusion is the mainstream management although regular blood transfusions may cause iron overload leading to organ damage.[28] A positive association between duration of blood transfusion and degree of body iron was observed previously.[29] Furthermore, some studies reported positive correlations between serum ferritin and duration of transfusion and age of the patients.[29]

Moreover, the interaction between CLNK and iron overload biomarkers may be explained by the formation of microvesicles in RBCs. Bailey et al. (2007)[7] found that the cyclic adenosine monophosphate (cAMP)-dependent pathway plays a role in γ-globin gene (HBG) expression in adult erythroblasts in β-thalassaemia. Their results also demonstrate that the cAMP-dependent pathway is augmented by multiple cytokines, which play a role in regulating Hb expression in β-TM.[7] Hemoglobinopathies such as sickle cell disease and thalassemia are accompanied by a substantial increase in microvesicle levels.[16,30] Phosphorylation of band 3 is

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associated with clustering and correlates with microvesicle formation during storage and in the RBCs of patients with thalassemia intermedia.[16,31] The involvement of various signaling pathways in RBC vesiculation is supported by the relative large numbers of signaling proteins in microvesicles obtained from the plasma of a healthy donors.[32] RBCs generate microvesicles to remove damaged cell constituents such as oxidized Hb and damaged membrane constituents, thereby prolonging the lifespan of RBCs. Damage to Hb, in combination with altered phosphorylation of membrane proteins such as band 3, lead to a weakening of the binding between the lipid bilayer and the cytoskeleton, thereby increasing membrane budding and microparticle shedding. The contribution of RBC-derived microvesicles to inflammation, thrombosis and autoimmune reactions emphasizes the need for a better understanding of the mechanisms and consequences of microvesicle generation.[33] These factors may underpin the associations among CLNK and RBCs, which undergo hemolysis in β-TM patients.

Limitations of the study

The limitation of the study was the smaller sample size. Therefore, it is considered a pilot study and further studies on a larger cohorts are warranted.

Conclusion

In the present study we introduced a new biomarker (CLNK) to diagnose β-TM and iron overload in β-TM. The serum CLNK concentrations are higher in thalassemic patients as compared with controls. CLNK level depends on number of transfusion, Hb, ferritin, and iron.

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Ethical approval: The ethics committee of Kufa University approved this study (REC number: 1321).

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Table 1. Socio-demographic, clinical and biomarker data in TM patients as compared with healthy controls.

Parameter	Control	Patients	F or χ^2	df	p-value
Sex (M/F)	15/15	27/33	0.20	1	0.654
Age yrs.	8.8±2.6	7.2±3.0	6.76	1/88	0.011
Hb g/dl	13.47±0.99	7.65±2.03	219.35	1/88	<0.001
PCV %	41.03±2.77	22.43±6.55	220.82	1/88	<0.001
Iron µM	28.6±12.8	39.8±13.2	14.54	1/88	<0.001
TIBC µM	63.87±10.39	71.22±19.00	3.89	1/88	0.052
UIBS µM	35.27±11.65	31.46±17.45	1.17	1/88	0.283
TS%	44.49±17.28	57.31±17.231	11.03	1/88	0.001
Transferrin mg/L	160.47±26.12	178.94±47.73	3.89	1/88	0.052
Ferritin * mg/L	59.0-227.0	51.8-9598.0	256.61	1/88	<0.001
EIBS* mmol	8.44-32.46	7.41-1372.51	256.61	1/88	<0.001
CLNK* ng/ml	1.95±3.06	6.24±4.78	20.89	1/88	<0.001
zCLNK+zFerritin (z scores)*	-1.07±0.48	0.54±0.72	122.92	1/88	<0.001
zCLNK+zFerritin+zIron (z scores)*	-1.06±0.52	0.53±0.71	120.71	1/88	<0.001

Results expressed as mean±standard deviation or as minimum and maximum

(*): Processed in Ln transformation when compared.

zCLNK+zFerritin: computed as z transformation of Ln transformation of CLNK (zCLNK) plus zLnFerritin.
zLnCLNK+zLnFerritin+zLnIron: computed as zLnCLNK plus zLnFerritin plus zLnIron.

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Table 2. Results of multivariate GLM analysis with the biomarkers as dependent variables and diagnosis as explanatory variable while adjusting for extraneous variables.

Tests	Dependent Variables	Explanatory variables	F	df	p-value	Partial η^2
Multivariate	CLNK and zCLNK+zFerritin+zLnIron	Diagnosis	73.85	5/85	<0.001	0.635
		Sex	0.49	5/85	0.612	0.011
		age	2.95	5/85	0.058	0.065
Tests for between-subject effects	CLNK	Diagnosis	20.65	1/86	<0.001	0.194
	zCLNK+zFerritin+zIron	Diagnosis	128.04	1/86	<0.001	0.598
Univariate	CLNK	Model	11.79	2/87	<0.001	0.213
		Diagnosis	0.99	1/87	0.321	0.011
		Ferritin	2.38	1/87	0.127	0.027
Univariate	CLNK	Model	22.59	1/88	<0.001	0.204
		Ferritin				

zCLNK+zFerritin+zIron: computed as z transformation of Ln transformation of CLNK (zCLNK) plus zLnFerritin plus zLnIron.

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Table 3. Results of the ROC curve and bootstrapped AUC analysis

Parameter	Bootstrapped AUC (n=2000)	Bootstrap 95% CI
CLNK	0.738	0.622-0.859
Ferritin	0.978	0.940-1.000
zCLNK+zFerritin	0.952	0.907-0.987
zCLNK+zFerritin+zIron	0.948	0.896-0.986

zCLNK+zFerritin: computed as z transformation of Ln transformation of CLNK (zCLNK) plus zLnFerritin.
zLnCLNK+zLnFerritin+zLnIron: computed as zLnCLNK plus zLnFerritin plus zLnIron.