The Correlation of β/α mRNA Ratio with Clinical and Hematological Parameters in Patients with β-thalassemia Syndrome

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Abstract

Background	Thalassemias are a group of genetically transmitted blood diseases characterized by defects in the production of α- or β-chains of hemoglobin called α-thalassemia and β-thalassemia, respectively. One of the common features of β-thalassemia is ineffective erythropoiesis because of the imbalance of globin chain production, which result in increased apoptosis during erythroblast maturation.
Objective	To evaluate the ß/α globin mRNA ratio in patients with ß-thalassemia syndrome and to correlate the ß/α ratio with hematological and clinical condition of the patients.
Methods	Thirty-five patient samples were collected from Thalassemia Centre of Ibn Al-Balady Hospital; 18 patients with β-thalassemia major and 17 patients with β-thalassemia intermedia. The patients were randomly selected regarding sex, whereas their age ranged from 3 to 17 years. Along with those samples, twenty control leftover samples that were the remaining of samples collected for laboratory investigation, were taken from Al-Kadhimiya Pediatric Hospital, and were age and sex matched with the patients group. The α and β globin chain ration were calculated by the real-time reverse transcription-polymerase chain reaction (qRT-PCR). The β/α -globin mRNA ratio of the samples was measured by the $2-\Delta\Delta CT$ method.
Results	Analysis of the β-globin/α-globin mRNA ratio showed that disease severity increased with a reduction of the ratio. There was a highly significant difference in α level, ß level and ß/α ratio among studied groups with a p value of ($p < 0.001$). The highest level was in control group, followed by that in β-thalassemia intermedia and the lowest was in β-thalassemia major.
Conclusion	The severity of ß/α globin chain imbalance showed a significant and negative correlation with the mean corpuscular volume and there was no significant correlation between the ß/α ratio and markers of erythropoiesis in ß-thalassemia major and ß-thalassemia intermedia patients. The ß/α ratio was a good tool for diagnosis in ß-thalassemia major and ß-thalassemia intermedia as compared to control group with high sensitivity and specificity. A ß-chain gene expression was the lowest in ß-thalassemia major followed by ß-thalassemia intermedia as compared to control group.
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List of abbreviations: Hb = Hemoglobin, HPFH = Hereditary persistence of fetal hemoglobin, MCH = Mean corpuscular hemoglobin, MCHC = Mean corpuscular hemoglobin concentration, MCV = Mean corpuscular volume, PCV = Packed cell volume, RBC = Red blood cells, WBC = White blood cells,

Introduction

Thalassemias are genetic diseases that are transmitted as autosomal recessive. It is one of the most common single

gene diseases in the world. It results from an imbalance in amount of hemoglobin chains production due to mutations in globin chains [1].

The α- and β-thalassemia syndromes are the most important clinical thalassemia. The α-thalassemia is due to deletion or less commonly to mutation that affect one or more of the duplicated α-globin genes, which is situated on chromosome 16, whereas β-thalassemia is due to point mutations or less commonly to deletion in β-globin gene, which is situated on chromosome 11. These leads to absence or reduction in globin chain synthesis [2].

An imbalanced quantity of β-globin chain production leads to relative increase of α-chain, which is precipitate in erythrocytes, therefore, the clinical phenotype of β-thalassemia related to the excess amount of α-chain, ranging from asymptomatic phenotype the thalassemia minor to severe anemia (thalassemia major). Major thalassemia is transfusion-dependent. It appears in infancy or childhood and it characterized by absence or reduce of normal hemoglobin and severe anemia, enlargement of the heart, liver, spleen, and skeletal deformation [3].

Although the reduction of β-chain production leads to β-thalassemia but the main problem is due to the free α-globin chains, which produce oxidative stress in red blood cells, which leads to hemolysis in red blood cells [4].

The same gene mutation in patients with β-thalassemia have remarkable differences in hematological and clinical symptoms. Several factors like environmental or genetic modifiers are involved in the disease severity of β-thalassemia [5].

Erythropoiesis in β-thalassemia patients is due to proportional increment of free α-globin [6]. The major cause of disease severity is the level of imbalance in the α-globin versus β + γ-globin production ratio rather than the reduction of ß-globin chain production [7].

There is a twofold increment in β-thalassemia trait in the production of α-globin, which have nearly normal hematopoiesis with only mild microcytosis and hypochromia of the red blood cells [8].

The α/β ratio in patients with thalassemia intermedia is typically 3-4/1 because of the presence of reduced amount of β-globin synthesis with γ-globin synthesis to qualify the consequences of excess α-globin production [8].

Patients with β0-thalassemia have marked chain imbalance and it is the underlying basis for their severe phenotype [8].

The unpaired α-globin forms molecular aggregates, will precipitate, and form inclusions, which cause damage to the cell membrane and the membranes of intracellular organelles [7].

One of the most toxic products of unpaired α-chains is hemichromes, which attach to the cell membrane and leads to clustering of band 3, one of the major constituents of cell membrane [7].

The formation of α-chain inclusions occurs early during erythropoiesis and peaks in the polychromatophilic erythroblasts, leading to cellular apoptosis [9].

The severity of β-thalassemia decreased by increased production of fetal hemoglobin (HbF) [10]. The increase in HbF (α and γ chain) is due mainly to selective survival of the cells containing HbF and not to increase synthesis of γ-globin, therefore, there is still excess α chain and ineffective erythropoiesis. Only if there is another mutation such as hereditary persistence of fetal hemoglobin (HPFH) that result in increased synthesis of γ-globin, in this case it will attach to α chain and result in less inclusion and ameliorate the signs and symptoms of thalassemia. The increment in γ-globin synthesis reduces the α/β-chain ratio. As a result, there is improvement in the ineffective erythropoiesis, which cause the disease. That leads to decreased hemolysis, and increased of hemoglobin levels because of improved survival of red cells that contain high levels of HbF [10].

Severe anemia and erythroid hyperplasia, bone marrow expansion and extramedullary hematopoiesis are the causes of ineffective erythropoiesis [11].

β-thalassemia minor causes microcytosis and mild anemia due to decreased HbA synthesis. Patients with minor β-thalassemia have one unaffected β-globin gene and they can produce sufficient amount of hemoglobin without causing significant erythroid hyperplasia. Also, the reduction in hemoglobin level is overcome by an increase in other hemoglobin forms mostly HbA2 [11].

The objectives of this study was to evaluate the ß/α globin mRNA ratio in patients with ß-thalassemia syndrome and to correlate the ß/α ratio with hematological and clinical condition of the patients.

Methods

For all patients and control samples, 0.25 ml of EDTA blood sample was added to 0.75 ml TRIzol for RNA extraction for polymerase chain reaction (PCR).

PCR

Using GoTaq® 1-Step RT-qPCR System, Promega, USA. GoTaq® 1-Step RT-qPCR System used for quantitative analysis of RNA by a one-step reverse transcription-quantitative PCR (RT-qPCR) protocol.

The GoTaq® 1-Step RT-qPCR System act as Reverse Transcriptase and qPCR Master Mix for one-step RT-qPCR quantification.

The GoTaq® 1-Step RT-qPCR System based on the fluorescent DNA-binding dye called BRYT Green® dye, which gives greater fluorescence enhancement when bind to double-stranded DNA (dsDNA) than does SYBR® Green I.

1. RNA was isolated from sample by using TRIzol Reagent.
2. Determine RNA, cDNA yield: Fluorescence Method. To know the quality of samples, a fluorometer was used to know the concentration of extracted RNA or cDNA. For 1 µl of RNA or cDNA, 199 µl of diluted Quantifluor Dye was added after 5 min

incubation at room temperature in dark place.
3. Primer preparation: These primers were in a lyophilized form. It then dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution.

By adding 10 µl of primer stock solution (stored at freezer -20°C) to 90 µl of nuclease free water a working solution of 10 pmol/µl was prepared.
4. Reaction setup and thermal cycling protocol: One Step RT-PCR.
5. Analysis gene expression using pfaffi method.

Relative quantification

Folding $= 2^{-\Delta\Delta CT}$

$\Delta\Delta CT = \Delta CT$ Treated - ΔCT Control

$\Delta CT = CT$ gene - CT House Keeping gene.

Statistical analysis

Data were analyzed with the statistical package for social sciences software (SPSS); median (range) and frequency and percentages were used to describe continuous and categorical variables, respectively. Independent samples t-test (or Mann–Whitney U-test) and chi-squared test were used to compare continuous and categorical variables, respectively, between two groups. One-way ANOVA was employed for three groups. Correlation analysis was performed with Pearson or Spearman correlation. $P<0.05$ was considered statistically significant.

Results

In this study, the median of α globin gene expression was (0.60) in thalassemia major, (0.87) in thalassemia intermedia and (1.48) in control group. The median of β globin gene expression was (0.12) in thalassemia major, (0.44) in thalassemia intermedia and (1.57) in control group. The median of ß/ α globin genes ratio was (0.24) in thalassemia major, (0.50) in thalassemia intermedia and (1.05) in control group. There was a highly significant difference in α mRNA level, ß mRNA level and ß/α ratio

among studied groups. The p value was (p <0.001); the highest level was in the control group, after that in ß-thalassemia intermedia and then in ß-thalassemia major as shown in table 1.

The ß/α ratio was not significantly correlated to hepatosplenomegaly and frequency of blood transfusion duration in patients with ß-thalassemia major, and patients with ß-thalassemia intermedia (Table 2).

The correlations of ß/α ratio to hematological parameters in patients with ß-thalassemia major revealed a negative correlation with MCV, whereas, in patients with ß thalassemia intermedia, the ß/α ratio significantly correlated to white blood cells (WBC) count, lymphocyte and neutrophil count as shown in table 3.

Table 1. Comparison of alpha gene expression, beta gene expression and ß/α ratio among patients with thalassemia major, thalassemia intermedia and control subjects

Characteristic		Thalassemia major $n = 18$	Thalassemia intermedia $n = 17$	Control $n = 20$	p
α gene expression	Median (IQR)	0.60 (0.21) C	0.87 (1.29) B	1.48 (1.04) A	<0.001 K
	Range	0.11-1.20	0.29-3.95	0.68-4.86	HS
ß gene expression	Median (IQR)	0.12 (0.10) C	0.44 (0.67) B	1.57 (0.83) A	<0.001 K
	Range	0.00-0.21	0.13-3.02	0.74-6.11	HS
ß/α ratio	Median (IQR)	0.24 (0.18) C	0.50 (0.20) B	1.05 (0.27) A	<0.001 K
	Range	0.00-0.54	0.32-0.80	0.90-1.20	HS

n: number of cases, IQR: inter-quartile range, K: Kruskal Wallis test, HS: highly significant at p ≤0.01, Capital letters (A, B and C) were used to indicate the level of significance following post hoc Dunn's test so that similar letters indicate no significant difference whereas, different letters indicate significant difference

Table 2. Correlations of ß/α ratio to splenomegaly, hepatosplenomegaly and frequency of blood transfusion duration in patients with ß-thalassemia

Characteristic	Thalassemia major		Thalassemia intermedia	
	r	p	r	p
Splenomegaly	-0.055	0.828 NS	-0.241	0.351 NS
Hepatosplenomegaly	0.055	0.828 NS	0.057	0.829 NS
Frequency of blood transfusion	-0.242	0.333 NS	0.329	0.198 NS

r: correlation coefficient, NS: not significant at p >0.05

Table 3. Correlations of ß/α ratio to hematological parameters in patients with ß-thalassemia

Characteristic	Thalassemia major		Thalassemia intermedia	
	r	p	r	p
Hb	-0.460	0.055 NS	-0.135	0.605 NS
PCV	-0.388	0.111 NS	-0.021	0.936 NS
MCV	-0.478	0.045 S	0.091	0.729 NS
MCH	-0.170	0.499 NS	-0.024	0.927 NS
MCHC	-0.201	0.424 NS	-0.297	0.246 NS
RBC	-0.318	0.199 NS	-0.059	0.823 NS
Ferritin	0.238	0.342 NS	-0.042	0.873 NS
Platelet	-0.058	0.820 NS	-0.088	0.737 NS
WBC	-0.363	0.139 NS	0.486	0.048 S
Lymphocyte	-0.248	0.321 NS	-0.494	0.044 S
Neutrophil	0.291	0.241 NS	0.570	0.017 S
Monocytes	-0.243	0.331 NS	-0.329	0.197 NS
Eosinophils	0.048	0.849 NS	0.229	0.378 NS
Basophils	*	*	0.358	0.158 NS

r: correlation coefficient; NS: not significant at $p > 0.05$; S: significant at $p \leq 0.05$; *: basophil is constant in patients with beta thalassemia major, Hb: hemoglobin, PCV: Packed cell volume, MCV: Mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, RBC: Red blood cells, WBC: White blood cells.

Discussion

The ß/α globin chain imbalance is central to the ineffective erythropoiesis in β-thalassemia, as it triggers a sequence of events that lead to premature cell death [12].

There was a highly significant difference in α gene expression, ß gene expression difference and ß/α ratio level among studied groups, with p value of (p<0.001); the highest level was in control group, followed by β-thalassemia intermedia and then by major β-thalassemia.

This result was comparable with Ranjbaran et al. [3], Watanapokasin et al. [13] and Ahmedy et al. [14] studies, which found that α-globin gene expression was higher in thalassemia intermedia group versus thalassemia major, while β-globin gene expression was lower in thalassemia major group compared with thalassemia intermedia (p<0.001) and β/α-globin genes ratio was higher in control group compared with both thalassemia groups (p<0.001).

The severity of β-thalassemia depends on the degree of imbalance between ß and α-chains and the amount of the unpaired α-chain. So, the factors that reduce the extent of chain imbalance and the extent of α-chain excess in the red cell precursors will affect the phenotype [15]. The severity of β-thalassemia increased when the ß/α ratio decreased. As this result in precipitation of extra α-chain in erythrocyte membrane, which eventually result in ineffective erythropoiesis and hemolysis. Moreover β/α ratio was significantly higher in control group compared to both thalassemia groups, the decline in this ratio is mainly because of α-chains rather than decrease β-chains formation and the major pathophysiological basis of β-thalassemia is a free α-globin chains which produce oxidative stress in red blood cells, which leads to ineffective erythropoiesis and erythrocytes hemolysis [16].

In this study, the median of α, ß-globin gene expression and the median of ß/α globin genes ratio in thalassemia major, thalassemia intermedia and control group were comparable to other studies [3,14,16].

The ß/α ratio was not significantly correlated to splenomegaly, hepatosplenomegaly and

frequency of blood transfusion duration in patients have β-thalassemia major and β-thalassemia intermedia. No similar studies were found. This may be due to small size sample.

The ß/α ratio was negatively correlated to MCV in patients have β-thalassemia major. This was comparable to other study [2], the ß/α ratio correlates with the severity of anemia. whereas, in patients with β-thalassemia intermedia, the ß/α ratio were significantly correlated to WBC count, lymphocyte count and neutrophil count. No similar result was found. This may be due to infection because thalassemia patients more susceptible to infection

In conclusions, the severity of ß/α globin chain imbalance showed a significant and negative correlation with MCV and there was no significant correlation between the ß/α ratio and markers of erythropoiesis in ß-thalassemia major and ß-thalassemia intermedia patients; including clinical findings, frequency of blood transfusion and most of the hematological parameters in the studied samples. The ß/α ratio was a good tool for diagnosis in ß-thalassemia major and ß-thalassemia intermedia as compared to control group with high sensitivity and specificity. A ß-chain gene expression was the lowest in ß-thalassemia major followed by ß-thalassemia intermedia as compared to control group.

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Author contribution

Dr. Yousif: Samples collection, laboratory work and writing the draft of the article. Dr. Al-Mamoori: helped in data analysis and revising the article manuscript.

Conflict of interest

Authors declare that there is no conflict of interest.

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