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Assessment of alpha hemoglobin stabilizing protein gene expression in relation to hematological parameters in Iraqi patients with beta-thalassemia syndrome

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Abstract:

BACKGROUND: Beta-thalassemia syndromes are inherited disorders marked by reduced beta-globin production, leading to excess free α-globin chains that cause ineffective red blood cell production, anemia, and oxidative stress. The heterozygous (trait) form causes mild anemia, while the homozygous form leads to severe, transfusion-dependent anemia. Genetic modifiers such as α-globin mutations and hereditary persistence of fetal hemoglobin (HPFH) can reduce disease severity by balancing globin chains. Alpha hemoglobin (Hb) stabilizing protein (*AHSP*) plays a protective role by binding free α-globin, preventing its toxic effects. This chaperone function helps mitigate damage in conditions like β-thalassemia.

OBJECTIVES: The aims of this study were to evaluate AHSP gene expression in patients with beta-thalassemia syndrome and correlate its expression with hematological parameters.

MATERIALS AND METHODS: Thirty-five beta-thalassemic pediatric patients' leftover samples were collected from thalassemia center in Baghdad/Iraq. The patients randomly selected regarding sex. Twenty control leftover samples were collected from Al-Kadhimiya pediatric hospital, who were age and sex matched with the patient's group. *AHSP* mRNA of 35 patients, patients with beta-thalassemia major (β-TM) included 18 patients and 17 patients with beta-thalassemia intermedia, and 20 normal controls were measured using real-time reverse transcription-polymerase chain reaction (qRT-PCR). *AHSP* RNA of each sample was calculated by relative quantification using the equation of folding = $2^{-\Delta\Delta^{CT}}$. ΔΔCT = ΔCT Treated – ΔCT Control.

RESULTS: There was a statistical difference found between the control and patients with beta-thalassemia syndrome regarding hematological parameters and *AHSP* gene expression and a highly significant difference between β-TM and intermedia regarding complete blood count finding, duration of blood transfusion, and high-performance liquid chromatography finding, while there was no statistical difference regarding *AHSP* gene expression that was seen between those two groups.

CONCLUSION: The expression level of the *AHSP* gene was elevated in thalassemic individuals compared to the healthy control, and it was higher in patients with the BI group than thalassemia major group.

Keywords:

Alpha hemoglobin stabilizing protein gene expression, beta-thalassemia syndrome, reverse transcription-polymerase chain reaction

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Introduction

Thalassemia is an inherited autosomal recessive blood disorder characterized by abnormalities in the synthesis of the beta-globin chains of hemoglobin (Hb). This condition is particularly prevalent in the Eastern Mediterranean region, including Iraq, where it presents with a broad spectrum of clinical manifestations. The milder form, known as thalassemia minor, typically occurs in individuals with a heterozygous mutation, resulting in mild anemia. In contrast, the more severe form, beta-thalassemia major (β-TM), usually results from homozygous or compound heterozygous mutations. Individuals with β-TM experience severe, life-threatening anemia that necessitates lifelong, regular blood transfusions.[1] These patients often suffer from multiple transfusion-related and disease-related complications, such as cardiac abnormalities, endocrine dysfunctions (including hypoparathyroidism and hypogonadism), diabetes mellitus, and liver diseases, which significantly impact their quality of life and long-term prognosis.[2]

Alpha Hb stabilizing protein (*AHSP*) is an erythroid -specific molecular chaperone protein that is highly expressed during red blood cell (RBC) development. *AHSP* plays a critical role in maintaining the stability of free alpha-globin chains, which are produced in excess in beta-thalassemia due to the impaired synthesis of beta chains. Unbound alpha-globin is highly unstable and prone to oxidation, aggregation, and precipitation, all of which contribute to ineffective erythropoiesis and the pathogenesis of anemia in β-thalassemia.[3] *AHSP* binds specifically and reversibly to free alpha-globin, stabilizing its structure and preventing its aggregation and incorporation into RBC membranes. This action significantly reduces the oxidative damage and cellular toxicity associated with free alpha-globin chains.[4]

Mutations or altered expression of the *AHSP* gene may influence the clinical severity of β-thalassemia. Co-occurrence of *AHSP* mutations in individuals with β-thalassemia has been associated with a worsened phenotype, suggesting that *AHSP* plays a modulatory role in the disease's pathophysiology. Given its protective function, *AHSP* has been proposed as a potential therapeutic target or biomarker for disease severity in beta-thalassemia. Understanding the expression patterns and regulatory mechanisms of *AHSP* could pave the way for novel strategies aimed at alleviating disease burden.[5]

Therefore, this study aims to investigate the expression levels of the *AHSP* gene in patients with beta-thalassemia syndromes and to correlate these levels with hematological parameters and the duration between required blood transfusions. This correlation may help clarify the role of *AHSP* in modulating disease severity and transfusion dependency, potentially contributing to more personalized and effective management approaches for patients suffering from β-thalassemia.[6]

Materials and Methods

This is an observational analytical study aimed at detecting *AHSP* gene expression and correlating it with clinical data.

Study design

This was a case–control study, involving beta-thalassemia patients as cases and age- and sex-matched healthy individuals as controls. Leftover samples collected from thalassemia center in Baghdad/Iraq and Al-Kadhimiya Pediatric Hospital for the control from June 2023 to June 2024/Baghdad.

Patients

A 35 beta-thalassemia pediatric patients' leftover samples (18 with β-TM and 17 with beta-thalassemia intermedia) and 20 control leftover samples were age- and sex-matched with the patient's group.

Inclusion

- Pediatric patients diagnosed with β-TM or intermedia
- Availability of leftover blood samples
- Age- and sex-matched healthy pediatric controls.

Exclusion

Patients with other hematological disorders or those lacking parental consent were excluded from the study.

Ethical approval

This study was approved by the (Institute Review Board) Al-Nahrain University's College of Medicine, Baghdad, Iraq (Approval No. 20250387) 13/4/2025, in accordance with the ethical guidelines of the Declaration of Helsinki.

Statistical analysis

In this study, appropriate statistical tests were employed to analyze the data based on the type and distribution of variables. The unpaired *t*-test was used to compare the means of normally distributed continuous variables between two independent groups. For nonnormally distributed data, the Mann–Whitney *U*-test was applied as a nonparametric alternative. Categorical variables were analyzed using the Fisher's exact test and Yates' corrected Chi-square test, depending on the expected frequency distribution, to assess associations between the groups. These statistical methods ensured accurate and reliable interpretation of the relationship between *AHSP* gene expression and clinical parameters in beta-thalassemia patients.

Reverse Transcription quantitative polymerase chain reaction (RT-qPCR) was used to evaluate the gene expression as shown in figure 1. For each tube, 0.25 mL of the blood was added to 0.75 mL of TRIzolTM Reagent, the lysate was homogenized by pipetting up and down several times, Quantus Fluorometer was used to detect the concentration of extracted RNA or cDNA to detect the quality of samples for downstream applications, and RNA concentration values were detected.

These primers were used for AHSP gene amplification supplied by Macrogen Company in a lyophilized form: [7]

- AHSP Forward: 5'-CCTGTTAGAC CTGAAGGCAGATGGC-3'
- AHSP Reverse: 5'-AGTCCTCCACC ACAGTCACCATGT -3'

GoTaq® 1-Step reverse transcription-quantitative polymerase chain reaction System, Promega, USA. Were used (GoTaq® 1-Step RT-qPCR System), is a reagent system for quantitative analysis of RNA using a one-step RT-qPCR protocol.

BRYT Green® dye, a unique fluorescent DNA-binding dye included in the GoTaq® 1-Step RT-qPCR System, has a higher fluorescence increase than SYBR® Green I when it binds to double-stranded DNA.

1. Using the TRIzol™ Reagent technique, RNA was extracted from the sample
2. Estimate the yield of RNA and cDNA: The fluorescence method
3. Primer preparation

These primers were supplied by the Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease-free water to give a final concentration of 100 pmol/µl as a stock solution.

A working solution of these primers of 10 pmol/µl was prepared from 10 µl of primer stock solution, which was stored at – 20◦C and added to 90 µl of nuclease-free water.

4. Reaction setup and thermal cycling protocol: One-Step RT-PCR
5. Gene expression levels were analyzed using the relative quantification method, with fold change calculated by the 2–ΔΔCT formula as follows:

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

$$\Delta\Delta CT = \Delta CT \text{ Treated} - \Delta CT \text{ Control}$$

$$\Delta CT = CT \text{ gene} - CT \text{ House Keeping gene}$$

Complete blood count (CBC) parameters and high-performance liquid chromatography finding were recorded from the patient's records.

Results

This case–control study involved 35 pediatric thalassemic patients with 20 healthy matched controls; male was 22 (62.9%), female constitutes 37.1%, and age range was from 3 years to 17 years.

About hematological parameters and ASHP gene expression, there was a significant difference between the beta-thalassemia syndrome group and the control, as shown in Table 1.

Organomegaly was seen in 25 patients, as a splenomegaly in 28%, hepatosplenomegaly in 42.9%, and splenectomy detected in 2.8% (one patient), while no organomegaly was found in 25.7% of the patients, as shown in Table 2.

As we compare between the thalassemia major (TM) and intermedia group, we found a highly significant difference regarding hematological parameters and organomegaly, while the *AHSP* gene expression, there was no statistical differences between the thalassemic groups, as shown in Tables 3 and 4.

About the duration in months for the blood transfusion, it was significantly lower duration interval between the transfusions in patients with β-TM than those with thalassemia intermedia with a *P* value of < 0.001, as shown in Table 3.

There was a statistically significant difference regarding organomegaly between β-TM and intermediate, so splenomegaly and hepatosplenomegaly were seen in higher frequency in TM than thalassemia intermedia (TI) with *P* value of 0.021 [Table 4].

There was no significant correlation found apart from the RBC count between the level of the expression of the *AHSP* gene and any of the other hematological parameters or the duration intervals between the transfusions, as shown in Table 5.

The receiver operating characteristic curve for *AHSP* between thalassemic cases and control shows a cutoff value of 1.497 with a sensitivity of 71.45% and specificity of 85%.

Discussion

Beta-thalassemia syndrome is still a wide field for researches since the complication of the disease and the treatment still considered a major challenge to

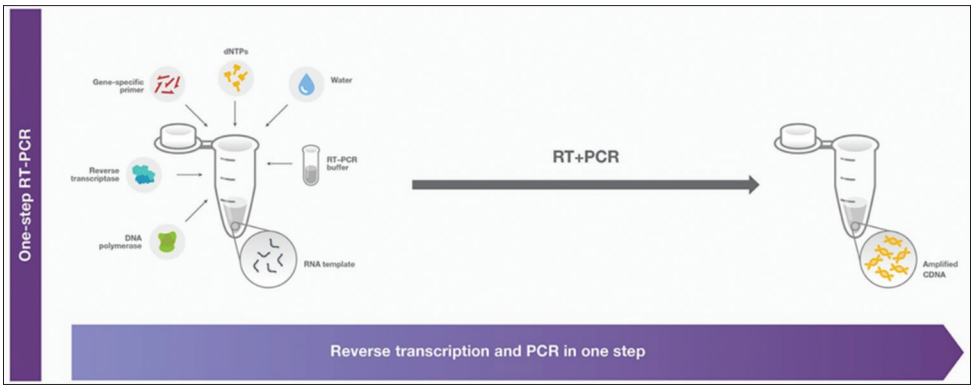

Figure 1: Experimental methods and workflow experiment overview. RT = Reverse transcription, PCR = Polymerase chain reaction

Table 1: Comparison of hematological parameters and alpha hemoglobin stabilizing protein gene expression between thalassemia syndrome patients and control

Parameter	Thalassemia (*n*=35)	Control (*n*=20)	*P*	Test used
Age (years)				
Mean±SD	10.37±4.41	8.15±3.22	0.075**	Mann–Whitney test
Median (range)	10.0 (3–17)	7.5 (4–16)		
Hb (g/dL)				
Mean±SD	7.3±1.35	12.27±0.51	<0.001*	Unpaired t-test
Median (range)	7.1 (5.0–9.7)	12.25 (11.6–13.3)		
PCV (%)				
Mean±SD	21.7±4.64	38.2±0.82	<0.001*	Unpaired t-test
Median (range)	21.9 (14.0–29.8)	38.0 (37.0–39.9)		
MCV (fL)				
Mean±SD	65.84±8.34	82.27±1.69	<0.001**	Mann–Whitney test
Median (range)	68.5 (50.3–76.0)	82.35 (78.8–85.5)		
MCH (pg)				
Mean±SD	20.67±1.82	27.5±1.35	<0.001**	Mann–Whitney test
Median (range)	20.7 (17.8–25.3)	27.05 (26.0–30.9)		
RBC ($\times10^3$/mm^3)				
Mean±SD	2.68±0.43	4.53±0.12	<0.001**	Mann–Whitney test
Median (range)	2.6 (2.2–3.9)	4.5 (4.3–4.7)		
AHSP expression				
Mean±SD	51.59±144.0	1.03±0.53	0.002**	Mann–Whitney test
Median (range)	5.24 (0.13–700.03)	0.89 (0.19–2.34)		

**P* value by unpaired *t*-test, ***P* value by Mann–Whitney test. *AHSP*=Alpha hemoglobin stabilizing protein, Hb=Hemoglobin, MCV=Mean corpuscular volume, MCH=Mean corpuscular Hb, RBC=Red blood cell, PCV=Packed cell volume, SD=Standard deviation

Table 2: The frequency and percentage of organomegaly in thalassemia cases

Organomegaly	Thalassemia (*n*=35), *n* (%)
Splenomegaly	10 (28.6)
Hepatosplenomegaly	15 (42.9)
Splenectomy	1 (2.8)
No organomegaly	9 (25.7)

the thalassemic centers and the families of those patients, finding a possible target to ameliorate those complications. Since *AHSP* is recognized as a genetic modifier of β-thalassemia, and given that variations in its gene expression can influence disease severity, evaluating *AHSP* expression levels and their correlation with hematological parameters, organomegaly, and blood transfusion frequency may provide valuable insights. Such findings could support recommendations for therapeutic strategies aimed at upregulating *AHSP* expression, thereby improving alpha-globin chain stabilization and reducing the alpha/beta chain imbalance characteristic of the disease. As the *AHSP* activity could relieve some of the toxic effects of alpha-globin excess, while lower *AHSP* expression might worsen the clinical outcome.[8]

Regarding demographic characteristics, there were no significant differences observed between the studied groups and the control.[9-11]

In regard to hematological parameters measured by the CBC, there were a highly significant difference observed between the patients with thalassemia syndrome and the

Table 3: Comparison of parameters between thalassemia major and thalassemia intermediate

Parameter	Thalassemia major (*n*=18)	Thalassemia intermediate (*n*=17)	*P*
Age (years)			
Mean±SD	10.72±3.85	10±5.02	0.708*
Median (range)	10.5 (3–16)	9 (4–17)	
Hb (g/dL)			
Mean±SD	6.22±0.73	8.44±0.8	<0.001*
Median (range)	6.25 (5–8.1)	8.5 (7.1–9.7)	
PCV (%)			
Mean±SD	17.93±2.45	25.7±2.53	<0.001**
Median (range)	18.2 (14–23.7)	25.5 (21.9–29.8)	
MCV (fL)			
Mean±SD	59.99±7.08	72.04±3.93	<0.001**
Median (range)	61.25 (50.3–75)	73.4 (60.5–76)	
MCH (pg)			
Mean±SD	19.56±1.66	21.85±0.95	<0.001*
Median (range)	19.2 (17.8–25.3)	22 (19.5–23.8)	
RBC (×10^3/mm^3)			
Mean±SD	2.39±0.12	2.99±0.44	<0.001**
Median (range)	2.4 (2.2–2.7)	2.9 (2.6–3.9)	
HbA (%)			
Mean±SD	0.36±0.65	34.48±19.39	<0.001**
Median (range)	0.2 (0–3)	28.5 (13–80)	
HbA2 (%)			
Mean±SD	2.17±0.91	3.95±1.44	<0.001*
Median (range)	2.05 (0.4–3.8)	4.5 (1–6.1)	
HbF (%)			
Mean±SD	97.47±1.88	61.57±19.62	<0.001*
Median (range)	97.8 (94–99.5)	67 (15.5–86.2)	
Duration intervals of blood transfusion (months)			
Mean±SD	0.72±0.12	3.35±0.93	<0.001**
Median (range)	0.75 (0.5–1)	3 (2–5)	
AHSP gene expression			
Mean±SD	49.87±128.5	53.4±167.19	0.369**
Median (range)	2.62 (0.13–531.13)	7.95 (0.53–700.03)	

P* value by Mann–Whitney test,*P* value by unpaired *t*-test. *AHSP*=Alpha hemoglobin stabilizing protein, Hb=Hemoglobin, MCV=Mean corpuscular volume, MCH=Mean corpuscular Hb, RBC=Red blood cell, PCV=Packed cell volume, SD=Standard deviation

Table 4: Comparison of sex and organomegaly between thalassemia major and thalassemia intermediate cases

Parameter	Thalassemia major (*n*=18), *n* (%)	Thalassemia intermediate (*n*=17), *n* (%)	*P*
Sex			
Male	14 (77.8)	8 (47.1)	0.086*
Female	4 (22.2)	9 (52.9)	
Organomegaly			
Splenomegaly	6 (33.3)	4 (23.5)	0.021**
Hepatosplenomegaly	11 (61.1)	4 (23.5)	
Splenectomy	1 (5.6)	0	
No organomegaly	0	9 (52.9)	

**P* value by Fisher's exact test, ***P* value by Yates' Chi-square test

Table 5: Comparison of parameters between thalassemia major and thalassemia intermediate cases

Parameter	*AHSP* gene expression			
	Thalassemia (*n*=35)		Control (*n*=20)	
	r	*P*	*r*	*P*
Age (years)	0.071	0.684	−0.051	0.830
Hb (g/dL)	−0.063	0.721	0.106	0.657
PCV (%)	−0.046	0.792	0.182	0.442
MCV (fL)	−0.100	0.568	−0.025	0.915
MCH (pg)	−0.046	0.794	−0.178	0.453
RBC (×10^3/mm^3)	−0.112	0.520	0.550	0.012
HbA (%)	−0.071	0.687		
HbA2 (%)	0.236	0.172		
HbF (%)	0.053	0.764		
Duration intervals of blood transfusion (months)	−0.135	0.438		

Hb=Hemoglobin, *AHSP*=Alpha hemoglobin stabilizing protein, MCV=Mean corpuscular volume, MCH=Mean corpuscular Hb, RBC=Red blood cell, PCV=Packed cell volume

control regarding the median of RBC count, Hb level, hematocrit, mean corpuscular volume, mean corpuscular Hb (MCH) and MCH concentration which were lower in the patients compared to the control with a *P* value of < 0.001**,** and this finding expected since patients with thalassemia had a defective hematopoiesis and deformity in the red cell membrane resulting in lysis, anemia, and

microcytosis. Similar finding by Karim MF in a study performed in Bangladesh 2016.[11]

Clinically severe anemia were observed more in β-TM than thalassemia intermedia; organomegaly were observed in the majority of the cases in the form of splenomegaly and hepatosplenomegaly, and in comparison, between beta thalassemia major (BM) and beta thalassemia intermedia (BI), the organomegaly observed more in BM with all patients had organomegaly while half of the patients with BI had no organomegaly.[12-14]

A highly significant difference was observed between BM and BI regarding the levels of HbA percent, HbA2 percent, HbF percent, and duration between blood transfusions; these differences related to the severity of anemia and the ineffective erythropoiesis and the type of beta-globin mutation that relate to the production of HbA0, similar findings.[15-17]

The frequency of blood transfusions was found to be more in BM than the BI aiming to managing anemia,[18,19] controlling of the ineffective erythropoiesis,[20] and improving well-being and oxygen transport.

Regarding *AHSP* gene expression, it was noted that there was a higher level of expression in both BM and BI compared to the control. *AHSP* expression was higher in BI patients compared to BM, consistent with findings by Kong Y *et al*., who suggested that a low β/α globin chain ratio may lead to increased *AHSP* expression. This upregulation likely serves to stabilize and solubilize excess free alpha chains in patients with β-thalassemia, thereby mitigating their toxic effects by preventing oxidative-induced alpha-globin precipitation. However, this increase in *AHSP* expression is not sufficient to fully counteract the harmful impact of unpaired alpha chains.[21-23]

In this study, *AHSP* gene expression was not associated with severity of symptoms in beta-thalassemia patients. Similar findings were seen in other studies.[24,25]

This study demonstrates that at a cutoff > 1.497, *AHSP* gene expression can discriminate thalassemia cases from healthy control with specificity of 85% and sensitivity of 71.4%.

Conclusion

The expression level of the *AHSP* gene was found to be elevated in individuals with beta-thalassemia, with significantly higher levels observed in patients with thalassemia intermedia compared to those with TM. These findings suggest that *AHSP* may act as a genetic modifier, potentially influencing the clinical severity of beta-thalassemia.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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