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Biochemical and Genetic Study in Blood of β– Thalassaemia Children in Mosul City, Iraq

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Abstract

The present study aimed to demonstrate the extent to which the activity of a number of enzymes and genetic variation of β-globin genes were affected in the blood of 65 children with β - thalassemia major of both sexes. The patients, with an age range of 2 – 15 years, were registered in the Thalassemia Center at Ibn Al-Atheer Teaching Hospital for Children in the city of Mosul / Iraq. They were under continuous treatment after being diagnosed by specialist doctors. The study also involved 30 healthy children of both sexes with the same age range who were considered as a control group.

The results showed significant increases ($p \leq 0.05$) in the activities of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), glucose 6- phosphate dehydrogenase (G6PD), and adenosine deaminase (ADA) in the serum of treated patients by 73% , 53%, 8%, 9%, and 54%, respectively, compared with the healthy children group. Also, the results showed significant increases in the activities of G6PD and ADA in the red blood cells (RBCs) of the patients by 7% and 43%, respectively, compared with the control group.

When determining the genetic variation of the β-globin gene depending on the PCR technique, the results did not show any genetic variation in the size of PCR band. However, the results of the sequencing showed variations in the nucleotides that included the conversion of the nucleotides (A) to (C) in position (250), (T) to (C) in position (426), (C) to (A) in position (623), (G) to (A) in position (630), and (T) to (A) in position (724). Also, the results demonstrated the detection of three transversion mutations and two transition mutations in β-globin gene in the children with β-thalassemia.

Keywords: β - Thalassaemia major, Glucose 6- phosphate dehydrogenase (G6PD), Adenosine deaminase (ADA), genetic variation, β-globin gene.

دراسة كيموحيوية ووراثية في دم الاطفال المرضى المصابين بالـ β– Thalassaemia في مدينة الموصل، العراق

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الخلاصة

تهدف هذه الدراسة الى توضيح مدى تأثير فعالية عدد من الانزيمات والتباين الوراثي لجين الـ β–globin في دم 65 طفل مريض بالـ β–thalassemia من كلا الجنسين وبفئة عمرية تراوحت بين (2–15)

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سنة من المراجعين الى مكز الثلاسيميا في مستشفى ابن الاثير في مدينة الموصل \ العراق، والذين يواصلون العلاج تحت اشراف اطباء مختصين، كما شملت الدراسة 30 طفل لا يعانون من الاصابة بالثلاسيميا من كلا الجنسين وبنفس الفئة العمرية اعتبرت كمجموعة سيطرة.

كذلك اظهرت نتائج الدراسة ارتفاعا معنويا ($P\leq0.05$) بمستوى فعالية الانزيمات Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline phosphatase (ALP), Glucose 6-phosphate dehydrogenase (G6PD) and Adenosine deaminase (ADA) في مصل دم الاطفال المصابين بمرض الـ β-thalassemia بنسب مئوية 73%, 53%, 8%, 9% and 54% على التوالي مقارنة مع مستواها في الاطفال الاصحاء (مجموعة السيطرة)، كذلك اظهرت نتائج الدراسة انخفاضا معنويا في مستوى فعالية الانزيمات G6PD و ADA في كريات الدم الحمراء للاطفال المصابين بالـ β-thalassemia وبنسب مئوية 7% و 43% على التوالي عند مقارنتها مع مجموعة السيطرة.

وعندما تم تحديد التباين الوراثي للجين β-globin بالاعتماد على تفاعل الـ PCR فان نتائج الدراسة لم تظهر اي اختلاف في حجم الحزمة الناتجة من تفاعل الـ PCR بالنسبة لعينات الاطفال الاصحاء والمرضى الذين شملتهم الدراسة، بينما نتائج اختبار تحديد تتابع النيوكليوتيدات Sequencing اظهرت تغاير في عدد من النيوكليوتيدات اذ تحول النيوكليوتيد A الى C في الموقع 250 كما تغير النيوكليوتيد T الى C في الموقع 426 كما تغير النيوكليوتيد C الى A في الموقع 623 كما استبدل النيوكليوتيد G الى A في الموقع 630 كذلل تحول النيوكليوتيد T الى A في الموقع 724، وقد تم في هذه الدراسة تحديد ثلاث طفرات تحول وطفرتين انتقال في جين الـ β-globin لدى الاطفال المصابين بمرض الـ β-thalassemia.

Introduction

β-thalassemia is one of the most widely distributed epidemic single-gene diseases around the world. It has a number of defects in hemoglobin synthesis, including that resulting from the decreased yield of β-globin protein. Clinically, thalassemia can be divided into three types, namely thalassemia major, thalassemia minor, and thalassemia intermediate [1, 2].

β-thalassemia is common in all communities in the world. The majority of β- thalassemia cases are caused by different types of mutations in β-globin gene, including deletion, insertion, and conversion of one or more nucleotides within the gene sequence[3, 4]. Higher than 200 mutations associated with β-thalassemia disease have been determined around the world [2, 4]. Because of the high variety of mutations in β-globin gene, they are different from one population to another ; in every affected population, there are a lot of changes in β-globin nucleotides that lead to uncommon genetic mutations [5, 6].

More than 90 % of β-globin mutations have been determined by the PCR-technique, which is used extensively in the laboratories due to the ease of detection of such mutations [7,8].

However, PCR sequencing normally detects one mutation for each reaction and can be exhausting and expensive [9, 10].

The biological processes that occur within all living organisms are chemical reactions, mostly regulated by enzymes which are biological catalysts. Without enzymes, many of these reactions would not take place. Enzymes catalyze all processes of cell metabolism. Enzymes also have valuable medical applications, including those of the diagnosis of specific diseases. It is known that all tissues and organs of the body of a living organism contain thousands of different types of enzymes, each being specific to a particular chemical reaction. The content of a specific enzyme varies from one tissue to another, but it is generally higher than that in the blood serum. Therefore, any damage that occurs in these tissues and organs leads to leakage of their components into the circulatory system, which increases their concentrations in the blood serum. The amount of this increase indicates the extent of damage to these tissues and related organs. This shows the importance of assessing the activity of enzymes in different disease states [11, 12].

Damages to the organs are commonly diagnosed by measuring the activities of certain enzymes in the blood. Hence, the current study focused on detecting the levels of several enzymes in the blood of children with β-thalassemia. These included ALT, which is located mostly in the cytoplasm, and AST, which is located mostly in the mitochondria, because they are indicators of liver function. The study also involved measurements of ALP, which is located in the osteoblasts and the cells of the liver

and kidneys, because it is an indicator of bone and liver functions [11, 13]. G6PD levels were also tested since it is one of the main enzymes involved in the metabolic pathway of phosphodiesterase that supplies energy to cells such as erythrocytes by maintaining enzyme activity and NADP level. In turn, it maintains the level of glutathione in these cells, which aids in protecting red blood cells from oxidative damage [12, 13, 14]. In addition, the study included the analysis of ADA level, which is an enzyme involved in purine metabolism. The determination of ADA may show the reflection of purine accumulation or the affected gene that is responsible of ADA production. This shows that ADA plays an important role in the rapid growth of tissues by reutilization of nucleotides which are required for RNA and DNA synthesis. The physiological role of ADA is related to lymphocytic proliferation and somatic cells differentiation [15, 16].

Materials and methods

Samples Collection

This study included 65 children patients with β - thalassaemia major of both sexes (33 males and 32 females) with ages that ranged between 2 and 15 years, who registered in the Thalassemia Center at Ibn Al-Atheer Teaching Hospital for Children in the city of Mosul. The patients were under continuous treatment after being diagnosed by specialist doctors depending on the laboratory tests of blood film, Hb – electrophoresis, and iron levels. In addition, 30 healthy children of both sexes with the same age range were included as a control group. Venous blood was collected and separated in two types of tubes; EDTA tubes for DNA extraction and anticoagulant-free jell tubes. The blood samples with hemolysis were neglected. Serum was separated and kept at -20 °C until analysis was performed.

RBC Separation and Hemolysis

A volume of 4-5 ml of non-clotting blood was collected in a tube containing sodium citrate (an anticoagulant) to separate the RBCs using the method of Beulter *et al.* [17]. Then, the haemolysis of RBC was achieved using the method of Price and Stevens [18] for the purpose of detecting the presence of the enzymes of G6PD and ADA.

Determination of Biochemical Parameters

The blood serum and the haemolysis product of RBCs were used to estimate the activities of a number of enzymes. Test kits from Randox (England) and BioMerieux (France) were used to estimate the activities of ALT, AST, and ALP. They were determined in the serum based on the well-established spectrophotometric methods, according to the manuals supplied.

Statistical analysis of the results was carried out with Duncan's test [19].

Genotyping

The DNA was extracted from the peripheral blood using a modified method [20]. The purity of the genomic DNA was determined by using BioDrop specttrophotometer. The samples were stored at -20 °C.

Detection of β-thalassemia mutation by DNA sequencing

In this test, 100 ng of template were added to 10 pmole of each primer for every PCR reaction. The sequences of primers used are shown in Table- 1 [2]:

Table 1-Show the sequence of primers that used in genotyping test

Primer	Sequence of primer	Size of PCR product
forward primer	5-TCCAACTCCTAAGCCAGTGC-3	804 bp
Revers primer	5-CGATCCTGAGACTTCCACACTG-3'	

A total volume of 20 µl of the master mix (BioLaps) was utilized in PCR reaction and the conditions of the reaction are described in Table-2.

Table 2-Show the PCR program steps that used in Genotyping test

No.	Stage	Temperature	Time	Cycle number
1	Initial denaturation	95	5 min.	1
2	Denaturation	95	45 sec.	35
3	Annealing	58	1 min.	
4	Extension	72	1 min.	
5	Final extension	72	7 min	1

Results and Discussion

Enzyme Activity

1. Activities of Transaminases in Patients' Serum

The results in Table-3 show significant increases ($p \leq 0.05$) in the activities of ALT and AST in the serum of patients with β - thalassaemia major by 73% and 53%, respectively, compared with the healthy children group. The results of this study are consistent with results of many previous studies, as they indicated increased activities of ALT and AST in the serum of patients with β - thalassaemia major [21- 25].

The increased ALT and AST activities in the serum of children with β - thalassaemia major may be attributed to damage to liver cells, and not necessarily cell death, which leads to an increase in their cellular permeability due to a change in the chemical composition of the cell membranes, leading to the release of these enzymes into the blood circulation and thus an increase in their activities in the blood stream [26,27]. Liver damage is especially associated with frequent blood transfusions [28-30], which points out that liver fibrosis is one of the complications of thalassemia that leads to increased activities of thses enzymes due to iron overload.

2. Activity of Alkaline Phosphatase in Patients' Serum

Table-3 shows a slightly significant increase ($p \leq 0.05$) in the activity of ALP in the serum of children with β - thalassaemia major by 8% compared with the healthy children group. The results of this study are in agreement with the findings of previous reports [21- 25, 31, 32].

ALP is an important enzyme in clinical diagnosis in terms of its different activity in different body tissues. This enzyme is found in the liver and bone marrow and considered as a diagnostic marker for several diseases, such as liver and bone diseases, thalassaemia, hemolytic anemia and cancer [24, 29, 33, 34].

3. Activity of Glucose 6- Phosphate Dehydrogenase in Patients' Serum and Hemolysis Product of RBCs

The results in Table-3 show a slightly significant increase ($p \leq 0.05$) in the activity of G6PD in the serum of children with β - thalassaemia major by 9% compared with the healthy children group. Also, Table-3 show a slightly significant increase ($p \leq 0.05$)
in the activity of G6PD in the hemolysis product of RBCs of children with β - thalassaemia major by 7% compared with the healthy children group. The results of this study are in agreement with the findings of earlier studies [14, 21, 35, 36, 37].

Table 3-A comparison of several enzymes activities in the serum and hemolysis product of RBCs between children with β - thalassaemia major and healthy control group

Studied groups / **Enzymes Activity**	**Healthy control (n = 30)**			**Patients (n = 65)**		
	Mean ± SD*	**% activity**	**% change**	**Mean ± SD**	**% activity**	**% Change**
ALT (U/l)	9.324±0.21 b	100	-	16.131±2.42 a	173	+73
AST (U/l)	8.280±0.16 b	100	-	12.683±1.5 a	153	+53
ALP (Kau/dl)	15.599±0.53 b	100	-	16.839±1.03 a	108	+8
G6PD (mU/dl)	12.05±2.14 b	100	-	13.17±0.7 a	109	+9
G6PD in hemolyzed RBCs ($mU/10^9$ RBC)	135.8±2.14 b	100	-	145.4±12.4 a	107	+7
ADA (nmol/min/mg)	16.95±1.3 b	100	-	26.04±1.76 a	154	+54
ADA in hemolysis of	64.41±5.04 b	100	-	92.25±14.56 a	143	+43

RBC (nmol/min/mg)						

*The numbers followed by different letters horizontally indicate a significant difference at $p \leq 0.05$ according to Duncan's test.

Ponnazhagan and Sarker [36] reported that the increase the activity of this enzyme occurs in order to overcome the deformation of RBC and the auto-oxidation reactions . They also indicated that the increase in the activity of G6PD in the case of β – thalassaemia is due to the increase in the number of reticulocytes.

4. Activity of Adenosine Deaminase in Patients' Serum and the Hemolysis Product of RBC

Table-1 shows a significant increase ($p \leq 0.05$) in the activity of ADA enzyme in the serum of patients with β - thalassaemia major by 54% compared with the healthy children group. Also, Table-1 shows a significant increase ($p \leq 0.05$) in the activity of ADA in the hemolysis product of RBCs of patients with β - thalassaemia major by 43% compared with the healthy children group. The results of this study are in agreement with previous findings [25,29,38,39,40] in various patients with major and intermedia thalassaemia, acute and chronic leukemia, hepatitis, and toxoplasmosis.

Our results might be in agreement with previous interpretations. The increased activity of ADA in the serum is attributed to disturbances in the body's organs, especially the liver and spleen, which leads to enlargement and necrosis of their cells or an increase in the rate of degradation of immature cells, resulting in leakage of their enzymes into the serum. Previous studies [40, 41, 42] reported elevated red cell's ADA activity in patients with Diamond-Blackfan anemia (DBA) and other blood diseases. It has been suggested that this disorder is caused by a disturbance in the formation of normal erythrocytes, which may be the result of a defect in the differentiation of erythrocyte stem cells.

Genotyping

Figure-1 shows the PCR product for amplification of β-globin gene in one case of the present study, with a band size of 804 bp and without any variation between patients.

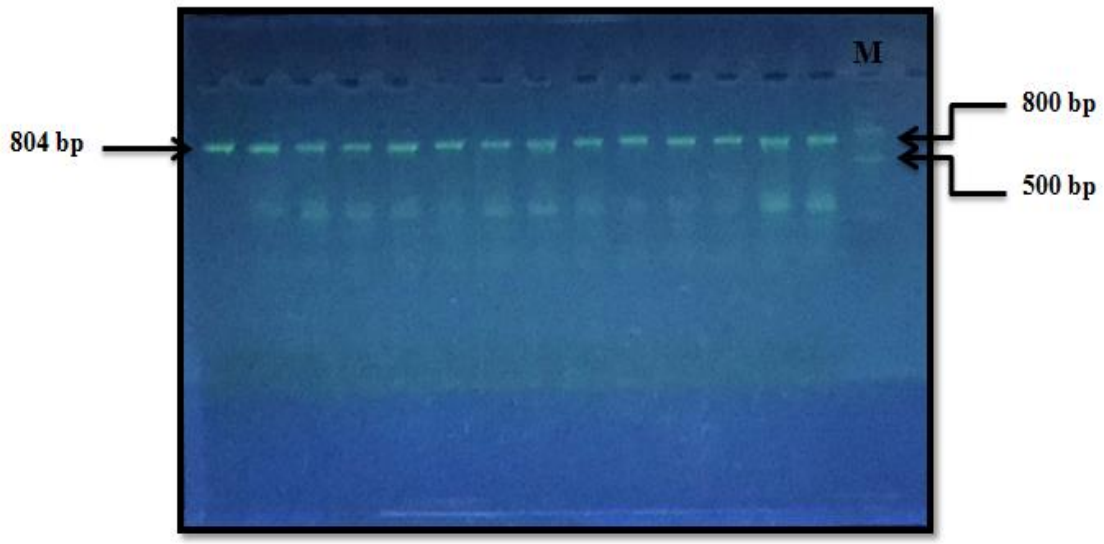

Figure 1-The PCR product for β-Globin gene in children with β-thalassemia. The M ladder has a size of 100bp and the PCR product has a size of 804 bp, being separated in 2% concentration of agarose gel.

In this study, the result of DNA sequencing for the amplified PCR product of β-Globin gene showed the presence of changes in the sequence for some nucleotides in the β-Globin gene for a selected case as compared to the healthy β-globin gene sequence found in the database of the National Centre of Biotechnology Information (NCBIsequence ID: LC507563.1).

Table 2-Positions and types of the various mutations in the β-Globin gene in children with β-thalassemia

T	Location	Nucleotide	Sequence ID	Mutation type	Gaps	Identity
1	MK476502.1	A ⟶ C	250	Transversion	1 %	98 %
2	MK476503.1	T ⟶ C	426	Transition	4 %	95 %
3	LC507563.1	C ⟶ A	623	Transversion	1%	98 %
4	MK476496.1	G ⟶ A	630	Transition	4 %	91 %
5	MK476503.1	T ⟶ A	724	Transversion	2 %	97 %

The results of DNA sequencing showed the presence of five mutations corresponding to β-globin gene, two of which were transition mutations and three were transversion mutations. In details, replacements were recorded of the A nucleotide to C nucleotide in position 250, T nucleotide to C nucleotide in position 426, C nucleotide to A nucleotide in position 623, G nucleotide to A nucleotide in position 630, and finally T nucleotide to A nucleotide in position 724. These variations in Nucleotide sequences may cause defects in the protein that is encoded by β-globin gene. Also, this mutation can lead to a disturbed gene expression of β-globin gene, thus causing a damage in the synthesis of haemoglobin proteins in children with β-thalassemia [3, 4, 43].

Conclusion

The results of this study demonstrate increases in the enzyme activities of ALT, AST, ALP, G6PD, and ADA in the serum and hemolysis product of RBCs of children with β-thalassaemia. We conclude that it is possible to consider the activities of these enzymes as one of the diagnostic markers for β-thalassaemia major, in addition to the possibility of predicting the extent of damage caused by injury. Also, we found five different mutations in β-globin gene, divided into two transition mutations and three transversion mutations.

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