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Evaluation of Caspase-8 and Caspase -9 Genes Expression and their effecting in Erythropoiesis in Patients with β -Thalassemia Major

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Abstract

One of the most prevalent hereditary hemoglobinopathies worldwide is β -thalassemia. Finding out how certain apoptosis genes (Casp-8 and Casp-9) affect erythropoiesis in patients with β -thalassemia major was the aim of this investigation. This study involved 60 patients with β -thalassemia major, according to the age the highest percentage of incidence in group (21-30) years with percentage (60%), depending on the sex; the results recorded there were (53.35%) of male patients and (46.65%) female patients. The analysis of complete blood count parameters revealed a significantly decrease in red blood cell count was observed in BT patients ($4.091 \pm 0.04 \times 10^6/\mu\text{l}$) compared to controls ($5.951 \pm 0.08 \times 10^6/\mu\text{l}$), the hemoglobin levels (HGB) were significantly lower in BT patients ($10.99 \pm 0.11 \text{ g/dL}$) compared to controls ($15.88 \pm 0.366 \text{ g/dL}$), the mean corpuscular volume was significantly lower in BT patients ($61.28 \pm 5.20 \text{ fL}$) compared to controls ($90.86 \pm 4.20 \text{ pg}$). White blood cell (WBC) showed significantly increase in patients with β -thalassemia ($12.03 \pm 1.06 \times 10^3/\mu\text{l}$) compared to control ($8.28 \pm 0.22 \times 10^3/\mu\text{l}$) and the Platelets levels are significantly higher in thalassemia patients ($373.64 \pm 21.01 \times 10^3/\mu\text{l}$) compared to healthy control group ($279.18 \pm 9.02 \times 10^3/\mu\text{l}$). The quantitative analysis of RT-PCR of *Casp-8* and *Casp-9* genes showed significantly increase in patients (4.38) and (3.58) respectively compared to healthy control (1.00). In conclusion; the overexpression of the *Casp-8* and *Casp-9* genes are significantly affected erythropoiesis, which will reflect in a better knowledge of the pathophysiology of β -thalassemia major disease. This disease's inefficient erythropoiesis is caused by the overexpression of *Casp-8* and *Casp-9* genes that linked to apoptosis.

Keywords: Beta Thalassemia Major, Erythropoiesis, *Casp-8* Gene, *Casp-9* Gene, Real Time PCR.

1. Introduction

Thalassemia was a common monogenic genetic disorder that caused anemia all throughout the world. The two main classifications for it are α -thalassemia and β -thalassemia. Anemia in β -thalassemia results from both a reduction in the lifespan of circulating red blood cells and inefficient erythropoiesis. This results in the buildup of toxic, mismatched α -globin chains, which are thought to cause the bone marrow's erythroid precursor cells to undergo apoptosis (Cao and Galanello, 2012)^[14]. A complex network of oxygen sensors and regulating cytokines controls the physiological process known as erythropoiesis, which produces red blood cells (RBC) at a steady rate and maintains the Hb content at a surprisingly stable level throughout life. The development of multipotent hematopoietic progenitors in a single lineage is the first step in the highly controlled process that produces adult RBC (Tusi *et al.*, 2018)^[22]. Erythroid precursors go through an expansion phase of proliferation after the stem cell progenitors commit, which is followed by a stage of gradual maturation. This process, which is frequently explained in a sequential manner, is actually continuous and marked by gradual changes in gene expression profiles leading to the development of adult erythrocytes (Chen *et al.*, 2009; Dulmovits *et al.*, 2017; An *et al.*, 2014)^[6, 8, 4]. The relative excess of α -globin chains causes erythroid precursors to undergo apoptosis, which results in intravascular and medullary hemolysis (Oikonomidou *et al.*, 2018)^[19].

Together with enhanced proliferation and apoptosis, thalassaemia appears to be made worse by the altered differentiation of erythroid progenitors (Liban *et al.*, 2008). ultimately resulting in systemic iron excess, splenomegaly, anemia, and extramedullary hematopoiesis (Talawy *et al.*, 2020) [21]. A family of proteases known as caspases, which are aspartate-specific and cysteine-dependent, are necessary for apoptotic programming. There is a description of two groups of caspases: effectors (caspase-3 and caspase-7) and initiators (caspase-8 and caspase-9) (Hengartner, 2000; Yi and Yuan, 2009) [13, 23]. Following a cell surface receptor ligand contact, the death receptor pathway triggers the activation of caspase-8 (Locksley *et al.*, 2001) [17]. On the other hand, intracellular damage and changes in the mitochondrial membrane potential (mitochondrial route) trigger the activation of caspase-9 (Orrenius, 2004; Green and Kroemer, 2004) [20, 11]. The cell death program is made possible by activated caspase-8 and caspase-9, which in turn trigger effectors such as caspase-3 that cleaves GATA-1, Tal1 (Zeuner *et al.*, 2003) [24], and proteins involved in the cytoplasm, nucleus, and DNA integrity. This study was aimed to detect the impact of some apoptosis genes (*Casp-8* and *Casp-9*) in Erythropoiesis in Patients with β TM.

2. Materials and Method

2.1. Subjects

In this study, two groups have been examined: Patients Group include (60) β TM patients were collected between May and July of 2024. Al-Zahra Teaching Hospital in Wasit Province, Iraq, provided all of the cases. Participants in this study ranged in age from 20 to 50 years. Healthy Control Group consists of (20) individual in different ages. All individuals were chosen dependent on several criteria, including: Healthy participants should have no medical history of heart disorders, renal disorders, or diabetes. The control group might be in the approximate age range with the patient's group.

2.2. Blood Sampling

Five milliliters of venous blood were drawn from each patient and a healthy control. For the CBC and Hb electrophoresis analysis, which were carried out directly by the Hematology Analyzer (Sysmex, Japan) and the Sebia Hb electrophoresis technique, each blood sample was promptly placed in dipotassium-EDTA Vacutainer® tubes.

2.3. Ethics Approval

Prior to their inclusion in the study tests, the Iraqi Ministry of Health and the Ethics Committee of the Department of Biology, College of Science, University of Wasit, gave their approval. Every person taking part in the study had their father's signed written approval.

2.4. Gene Expression

Following the manufacturer's instructions, the TRIzol® LS Reagent was used to extract the total RNA from each sample. The WizScript™ RT FDMix Kit was used to reverse-transcribe total RNA to complementary DNA (cDNA). A 20 μ l reaction volume was used for the procedure. The following program was used to complete the reverse transcription phase in a single cycle: 25°C for 10 minutes, 42°C for 10 minutes, 85°C for 5 minutes, then 4°C until the run was finished. Using quantitative real-time PCR (qRT-PCR), the expression levels of the *Casp-8* and *Casp-9* genes were estimated. EVA Green was utilized to verify the target gene's expression. The *Casp-8* and *Casp-9* genes' mRNA levels were normalized using the reference β -actin gene's amplified mRNA levels. Lyophilized primers were dissolved in free DNase/RNase water to produce a stock solution with a final concentration of 100 pmol/ μ l. To create a work solution with a final concentration of 10 μ M, the primers were reconstituted with 10 pmol/ μ l in 90 μ l of demonized water, table (1).

Table 1: Primers Sequence that used in the study

Genes	Forward Primer	Reverse primer	Reference
<i>Casp-8</i>	AGAAGGGTCATCCTGGGAGA	TCAGGCTTCCTTCAAGGCTGC	Mohamed and Altaee, 2023 [18]
<i>Casp-9</i>	AGGCCCATATGATCGAGGA	TCGACAACTTTGCTGCTTGC	Abbas and Al- Saffar, 2025 [2]
β -actin	TCGACAACTTTGCTGCTTGC	GGGGTACTTCAGGGTGAGGA	

2.5. Thermal Profile

The program of the qPCR reactions of *Casp-8*, *Casp-9* and β -

actin genes were illustrated in table (2).

Table 2: Thermal profile of targeted genes

Steps	Temperature (°C)	Duration	Cycles
Initial Denaturation	95	5 min	1
Denaturation	95	40 sec	40
Annealing	<i>Casp-8 gene</i>	40 sec	
	<i>Casp-9 gene</i>	40 sec	
	β -actin gene	40 sec	
Extension	72	40 sec	1
Final Extension	72	5 min	
Hold	4	10 min	

2.6. Statistical Analysis

According to the Livak and Schmittgen equation (2001) [30], Ct and Ct were computed. To assess the impact of various elements in the parameter research, the means were calculated in accordance with the Statistical Analysis System-SAS (2018) [31]. Means were compared using the

Least Significant Difference (LSD) test. For every sample variable, including Ct values and gene expression levels, the means and standard deviations were noted. This includes the test and housekeeping genes' values. For all tests, a P value of less than 0.01 was deemed highly significant.

3. Results and Discussion

3.1. Distribution of patients depending to age

The 60 β TM patients in this study ranged in age from 10 to 50 years. The following is the greatest incidence percentage:

The age group of 10–20 years old reported 23%, followed by the age group of 21–30 years old with 60%, and the age group of >30 years old with 17%. The age and incidence proportion in β TM were displayed in Table (3).

Table 3: Distribution of patients according to the age

Age group	Patients	Control	P-value
10-20	14 (23%)	2 (10%)	0.001 **
21-30	36 (60%)	12 (60%)	
>30	10 (17%)	6 (30%)	
Total	60	20	

* ($P \leq 0.05$), ** ($P \leq 0.01$).

3.2. Distribution of patents according to sex

Gender distribution data for both patients and control group are shown in table (4). The results recorded there were

(53.35%) of male patients and (46.65%) female patients with significant differences $P \leq 0.05$ between patients and healthy control group.

Table 4: Distribution of patents according to sex

Gender	Patients No (%)	Control No (%)	P-value
Male	32 (53.35%)	10 (50.0%)	0.0417 *
Female	28 (46.65%)	10 (50.0%)	
Total	60	20	

* ($P \leq 0.05$), ** ($P \leq 0.01$).

The results of the present study showed that 14(58.33%) of the positive diagnosed cases were males and 10 (41.67%) were females for all the studied type of mutations (table 4-4). Statistical analysis showed that there are non-statistically significant differences at ($p > 0.05$) between males and females in both patients and control groups. This is due to the fact that β TM is an autosomal (non sex- linked), recessively inherited disease, also the genetic disorder is caused by abnormalities in the beta-globin gene not on a sex

chromosome. For that both sexes are equally affected with β TM (Taher *et al.*, 2021) [10].

3.3. Complete Blood Count Parameters

The analysis of complete blood count (CBC) parameters revealed statistically significant differences between β TM patients and healthy controls across several hematological indices, as summarized in Table (4).

Table 5: Comparison between patients and control in CBC parameters

Group	Mean $\pm$ SE				
	RBC ($\times 10^6 / \mu\text{L}$)	WBC ($\times 10^3 / \mu\text{L}$)	PLT ($\times 10^3 \mu\text{L}$)	HGB (g/ dL)	MCV (pg)
Patients	4.091 $\pm$ 0.04	12.03 $\pm$ 1.06	373.64 $\pm$ 21.01	10.99 $\pm$ 0.11	61.28 $\pm$ 5.20
Control	5.951 $\pm$ 0.08	8.28 $\pm$ 0.22	279.18 $\pm$ 9.02	15.88 $\pm$ 0.36	90.86 $\pm$ 4.20
T-test	0.178 **	3.197 *	64.602 **	0.605 **	1.738 **
P-value	0.0001	0.0219	0.0046	0.0001	0.0001

* ($P \leq 0.05$), ** ($P \leq 0.01$).

RBC: red blood cell, HGB: hemoglobin, MCV: mean corpuscular volume, WBC: White blood cell, PLT: Platelets

A significantly decrease in red blood cell (RBC) count was observed in β TM patients (4.091 $\pm$ 0.04 $\times 10^6 / \mu\text{L}$) compared to controls (5.951 $\pm$ 0.08 $\times 10^6 / \mu\text{L}$). This paradoxical erythrocytosis, despite underlying anemia, is attributed to chronic ineffective erythropoiesis and compensatory bone marrow hyperplasia in response to hypoxia caused by impaired hemoglobin synthesis. The bone marrow increases RBC production in an attempt to enhance oxygen-carrying capacity, although the produced cells are typically microcytic and hypochromic (Cappellini *et al.*, 2021) [5]. The results showed the hemoglobin levels (HGB) were significantly lower in BT patients (10.99 $\pm$ 0.11 g/dL) compared to controls (15.88 $\pm$ 0.366 g/dL, $p < 0.0001$). This reduction reflects the defective synthesis of β -globin chains, resulting in ineffective hemoglobin formation and chronic anemia (Kurtz *et al.*, 2022) [15]. The mean corpuscular volume (MCV) and was significantly lower in BT patients (MCV: 61.28 $\pm$ 5.20 fL)

compared to controls (MCV: 90.86 $\pm$ 4.20 pg, $p < 0.0001$). These values are indicative of microcytic, hypochromic anemia, a hallmark of thalassemia due to insufficient hemoglobin content per red cell (Galanello and Origa, 2021) [9]. However, compared to the healthy control group (8.28 $\pm$ 0.22 $\times 10^3 / \mu\text{L}$), patients with β TM had a considerably higher number of WBC (12.03 $\pm$ 1.06 $\times 10^3 / \mu\text{L}$). This finding may be explained by the following factors: First, the kidney's release of "erythropoietin hormone," which stimulates the WBC, and general medical problems and immune system hyperactivation in patients who regularly receive blood from a variety of donors, as evidenced by the development of fever shortly after transfusion (Cousens *et al.*, 2021) [7]. Platelets (PLT) levels are significantly higher in β TM patients (373.64 $\pm$ 21.01 $\times 10^3 \mu\text{L}$) compared to healthy control group (279.18 $\pm$ 9.02 $\times 10^3 \mu\text{L}$) this is could be associated to the proliferation and development of marrow mononuclear cells to produce colony-forming unit megakaryocytes (Al-Fartosy *et al.*, 2011) [3].

3.4. Genes Expression

In this study, a quantitative analysis of RT-PCR of *Casp-8* and *Casp-9* genes showed significantly increase in β TM patients compared to control group as shown in table (5). The change in gene expression was calculated using a relative quantitative measurement (Livak and Schmittgen, 2008). This is based on the normalization of the Ct values for calculating Δ Ct and represents the difference between the average Ct values of *Casp-8* and *Casp-9* cDNA amplification replica for each case and case of *GAPDH* gene.

Table 6: Fold of *Casp-8* and *Casp-9* genes expression. Depending on $2^{-\Delta\Delta C_t}$ method.

Genes folding	Group	n	M	SD	p
Cas-8 gene	Patients	60	4.38	3.11	<0.001
	Control	20	1.00	0.00	
Cas-8 gene	Patients	60	3.58	2.18	<0.001
	Control	20	1.00	0.00	
* (P<0.05), ** (P<0.01).					

n = sample size; M = mean; SD = standard deviation; p = probability value.

Cas-9 protein is an apoptotic initiator enzyme, is an aspartate-specific cysteine protease that plays a central role in the intrinsic (mitochondrial) pathway of apoptosis. Upon activation, Cas-9 forms a complex with Apaf-1 and cytochrome C, leading to the activation of downstream effector caspases, such as caspase3 and caspase7, which execute the apoptotic process (Shoshan-Barmatz *et al.*, 2013)^[11]. By depolarizing the mitochondria and sequentially activating caspase-9 and caspase-3, a decrease in erythropoietin (EPO) levels causes cell cycle arrest and progenitor apoptosis (Asano *et al.*, 2012)^[29]. Some studies claim that interactions between Fas-expressing erythroblasts and FasL result in caspase-8 activation and upregulation, differentiation arrest, and apoptosis. These events, in turn, lead to the negative regulation of erythropoiesis within the bone marrow's erythroid blood islands on a different level (De Maria *et al.*, 1999; Centis *et al.*, 2000)^[28, 1].

There is a clear evolutionary link between the absence of mitochondria and the mature erythrocyte's lack of an inherent apoptotic pathway. Research on the apoptotic machinery in erythrocytes has demonstrated that RBCs contain significant levels of caspase-3 and caspase-8, but it has also made it evident that caspase-9, apoptotic protease activating factor 1 (Apaf-1), and CytC are absent (Berg *et al.*, 2001)^[26]. In addition to the caspase already stated, erythrocytes also contain Fas, FasL, and Fas-associated protein with death domain (FADD), all of which are essential components of the extrinsic apoptotic pathway. According to Manda *et al.* (2005), The detergent-resistant membrane (DRM) fraction of oxidatively stressed and aged erythrocytes contains all of these components. Notably, activated caspases have the ability to break down erythrocyte membrane proteins that are essential for preserving cell shape and the mechanical properties of the erythrocyte membrane. Caspases 8 mediate the degradation of β -spectrin. Tests on erythrocyte ghosts have shown that spectrin undergoes proteolytic cleavage at residue 470, among other sites (Toporkiewicz *et al.*, 2013)^[25].

Conclusions

The overexpression of *Casp-8* and *Casp-9* genes are expected to have a significant impact in the Erythropoiesis and this reflecting in an understanding of the pathophysiology of β TM disease. The upregulation of this genes related to apoptosis

and contributing to the disease's ineffective erythropoiesis. The high level of expression probably acts as a potential indicator of apoptotic activity and disease progression. The reduction of *Casp-8* and *Casp-9* genes could be a potential therapeutic strategy to improve erythropoiesis in β TM.

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