International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 2s, 2025
https://www.theaspd.com/ijes.php

Evaluation of *gal-3* Gene Expression and its related to some Immunological Markers in patients with Beta Thalassemia Major

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Abstract: One of the most prevalent hereditary autosomal single-gene illnesses in the world, beta-thalassemia major is also present in the Iraqi population. In order to perform this study, 75 blood samples were taken from pediatric hospital patients with β-thalassemia major in Wasit Province, and 25 people who appeared to be in good condition served as controls. The study period was extended from November 2023 to February 2024.The findings displayed the gender-specific distribution of β-thalassemic major (βTM) patients, with no discernible differences between the 38 males (51.35%) and the 36 females (48.65%). The age distribution of the βTM patients showed that the largest group of patients were between the ages of 10 and 20, represented by 38 (51.35%), followed by those between the ages of 21 and 30, represented by 15 (20.27).The When compared to the healthy control group, which had a record of 172.45 ±37.27 ng/ml and 24.55 ±6.35 pg/ml, respectively, the ELISA assay revealed a significant rise in IL-35 and IL-37 (371.64 ±21.01 pg/ml) and 69.56 ±5.76 pg/ml). The gal-3 gene expression was assessed by quantitative real-time PCR, which revealed a substantial (P≤0.05) drop in patient expression (0.374±0.18) as compared to the control, which recorded 1.00 ±0.00 fold.

Keywords: Beta thalassemia major, gal-3 gene, Quantitive Real Time PCR

1. INTRODUCTION:

The basic characteristic of beta thalassemia major, a category of hereditary blood disorders, is the reduction or lack of β-globin chain synthesis. This leads to a decrease in hemoglobin in red blood cells (RBCs), a drop in RBC production, and ultimately anemia (Cossio et al., 2016). The primary organ for hemoglobin degradation of aged red blood cells is the spleen. In β-TM, hemopoiesis is accelerated to make up for anemia, which leads to a rise in the creation of aberrant red blood cells and their subsequent removal. Other alterations include extramedullary hemopoiesis, which causes splenomegaly. Other causes of splenomegaly include an increase in blood transfusions and the breakdown of red blood cells, in addition to iron deposition and buildup (Kolnagou et al., 2013). In patients with thalassemia, growth retardation (GR), changes in appearance, bone deformity, and failure of pubertal development are caused by chronic hypoxia that results in anemia and growth hormone deficiency (GHD), which is caused by the liver's defective production of somatomedin and the rapid destruction of red blood cells (Badfar et al., 2016). Promoter methylation effecting in some of genes expression (Jabir and Hamzah, 2018 a,b) Mutations such as nucleotide substitutions and/or frameshifts of the insertion/deletion type have been reported to interfere with the transcription of the β–globin gene, splicing procedures and translation of β -globin gene mRNA, resulting in either absence or reduction of synthesis of β -globin chains (Kayisli *et al.,* 2005).The purpose of this study was to ascertain the expression of the gal-3 gene and how it relates to certain immunological variables in individuals with beta thalassemia major.

2. 2. Materials and Methods:

2.1. Ethical 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 Education, University of Al Qadisiyah, gave their approval. Every participant in the study had their father's signed written consent secured.

2.2. Blood Sample Collection:

Twenty-five people who appeared to be in good health served as a control group, and fifty βTM patients who were admitted to the Al Kut Hospital for Women and Children each had three milliliters of blood drawn. Patients and the healthy control group were between the ages of 21 and 30. The hospital's consulting physician has made a clinical diagnosis of the illness. Five milliliters of blood were separated into two tubes: three milliliters in a plain tube for the ELISA test and three milliliters in EDTA tubes for molecular analysis.

2.3. Enzyme Linked Immunosorbent Assay:

The evaluate the levels of IL-37 and IL-35 in patients and control group the Enzyme Linked Immunosorbent Assay was done according to the manufacturer's instructions. The Human Interleukin-37 ELISA Kit Cat. No: SL2231Hu and Human Interleukin-34 ELISA Kit Cat. No: SL2231Hu / TransGen Biotech- China were used in this study.

2.4. Gene Expression:

2.4.1. RNA Extraction and cDNA. Synthesis:

Following the manufacturer's instructions, the TRIzol®LS Reagent was used to extract the total RNA from each sample. Using the Easyscript® Kit, total RNA was reverse-transcribed to complementary DNA (cDNA). A 20µl reaction volume was used for the procedure. The following program was used to complete the reveres 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.

2.4.2.Quantitative Real Time-PCR:

The *gal-3* gene expression levels were calculated using quantitative real-time PCR (qRT-PCR). The TransStart® Top Green qPCR Super Mix (SYBR Green) was utilized to validate this expression. The mRNA levels of the gal-3 gene were normalized using the amplified mRNA levels of the reference gene, glyceraldehyde 3-phosphaee dehydrogenase gene (*gapdh*).

2.4.3.PCR Reaction and Program:

Using particular primers, a quantitative real-time PCR reaction was conducted. The reaction program was as follows: Lyophilized primers were dissolved in free DNase/RNase water to yield a final concentration of 100 pmol/µl as stock solution. To create a 10µM concentration as work primer, 10 pmol/µl was resuspended in 90 µl of demonized water to reach a final concentration of 10µM as work solution. Initial denaturation: 95°C for 5 minutes (on cycle), Denaturation: 95°C for 40 seconds, Annealing (*gapdh* = 58°C gal3=55°C) for 40 seconds, Extension: 72°C for 1 minute, and the run was completed with 35 cycles, followed by a 1-cycle holding at 4°C.The sequences of *gapdh* gene primers was F:5′-AACTTTGGCATTG TGGAAGG-3′, R:5′-ACACATTGGGGGTAGAACA-3′ [9] and *gal-3* gene was (F:5′- TGATGTTGC CTTCCACTT -3,R:5′- ACTGTCTTTCTTCCCTTCC -3′**.**

2.5. Statistical Analysis:

According to the Livak and Schmittgen equation [2001], ΔCT and ΔΔCT were computed. The impact of several elements in features was examined in this study using the statistical analysis system, or SAS program [2004]. To find the significant difference between means, the Least Significant Difference (LSD) test was employed.

3. Results and Discussion:

3.1.Gender Distribution:

Gender-specific distribution results of β-thalassemic major (βTM) patients showed no significant differences, with 38 samples (51.35%) being male and 36 samples (48.65%) being female. In contrast, the control group had 25 samples (62.50%) males and 15 samples (37.50%) females, as indicated in table (1).

Table (1): Gender distribution of β-thalassemia major patients and control.

Gender	Patients No (%)	Control No (%)	P-value
Male	38 (51.35%)	25 (62.50%)	0.0419 *
Female	36 (48.65%)	15 (37.50%)	

*** (P≤0.05), ** (P≤0.01).**

The current finding was supported by observations made by Lucarelli et al. (1996) and Galanello R, Origa R ,(2010), who claimed that β-thalassemia is an autosomal recessive disease that affects both sexes equally and is brought on by abnormalities in the β-globin gene on chromosome 11. This result was comparable to that of Shamsi Al-Dinn (2005), who conducted his study on 1800 Iraqi βTM patients in Baghdad. However, Al-Mosaway's (2004) study indicated that 40% of the affected individuals were female, and 60% of the cases were male in their studies conducted in Saudi Arabia and Al-Bahrain, respectively, Jassim et al. (1998) and Al-Awamy (2000) also noted a similar conclusion. Additionally, our findings were mostly consistent with those of Hickman's (1999) study conducted in England.

3.2. Age Distribution:

When the βTM patient samples were distributed by age, the results showed that the largest group of patients were between the ages of 10 and 20 (38, or 51.35%), followed by those between the ages of 21 and 30 (15, or 20.27%). These two groups form the higher attendance group of age when compared to the healthy control group, as shown in table (2).

Table (2): Age distribution of β-thalassemia major patients and control group.

Age (Year)	Patients (%)	Control (%)	P-value
<10 yr.	12 (16.22%)	0 (0.00%)	
10-20 yr.	38 (51.35%)	10 (25.00%)	0.0001 **

21-30 yr.	15 (20.27%)	24 (60.00%)
>30 yr.	9 (12.16%)	6 (15.00%)

*** (P≤0.05), ** (P≤0.01).**

According to this research, receiving blood transfusions on a regular basis can extend life. There was a range of intensity, though, so some people might only have minor effects. However, marrow enlargement and hyperplasia result from hemolytic and severe anemia in the untreated βTM. Death could happen within the first five years if the anemia is that bad. Chronic bilirubin overproduction brought on by long-term erythrocyte breakdown puts the patient at risk for pigmentary gallstones and hemosiderosis from the buildup of excess iron in the reticuloendothelial system, especially the liver, pancreas, and myocardium. Hemostasis-related deaths can happen before the age of 25 (Jackson, 2004). Additionally, Takeshita (2002) and Cao and Gallanello (2003) explained that although survival after the age of 30 was rare, the prognosis has improved with the use of a hyper transfusion regimen combined with chelating treatment.

3.3. Immunological Assay:

Interleukin-35 and interleukin-37 were included as significant immunological indicators in this study's detection of immunological disturbances. ELISA kits were used to estimate the levels of IL-35 and IL-37. In comparison to the healthy control group, which had records of 172.45±37.27 ng/ml and 24.55±6.35 pg/ml, respectively, Table 3 demonstrated a considerable rise in IL-35 and IL-37 (371.64 ±21.01 pg/ml) and 69.56 ±5.76 pg/ml).
respectively.

Table(3): Comparison between patient and control in IL-37 and IL-35

Group	**Mean ±SE**	
	IL-37 (pg/ml)	**IL-35 (pg/ml)**
Patients	69.56 ±5.76	325.27 ±27.94
Control	24.55 ±6.35	172.45 ±37.27
T-test	19.259 **	97.317 **
P-value	0.0001	0.0024

**** (P≤0.01).**

The anti-inflammatory and immune-modulating properties of the discovered cytokines, interleukin (IL-35) and interleukin (IL-37), have been reported in relation to a variety of inflammatory diseases, auto-immune disorders, cancers, infectious diseases, and sepsis. According to Yoshimoto and Yoshimoto (2013), there have been reports of either cytokine being decreased or, in certain situations, raised, which has led to a contribution to the pathogenesis of disease. The study's findings suggested that Khalaf et al. (2022) were greedy. They gave their approval for the substantial rise in IL-37 levels in β-thalassemia patients' serum as compared to the healthy control group. It has been demonstrated that interleukin-37, a member of the interleukin family, can lower the activity of both

innate and specific immune responses. It is an anti-inflammatory cytokine that immune cells produce and inhibits the production of inflammatory cytokines in a variety of diseases (Ding et al., 2016). Immunoregulatory cell populations and immunosuppressive cytokines help to maintain the equilibrium between inflammatory and anti-inflammatory immune responses. In order to restrict inflammatory reactions, the inhibitory cytokine interleukin-35 (IL-35), which is a member of the IL-12 family, can effectively reduce T cell proliferation and induce regulatory T cells that produce IL-35. A rising body of research over the last ten years has shown that IL-35 is crucial in regulating immune-related conditions such as cancer, infectious diseases, and autoimmune diseases (Ya et al., 2021).

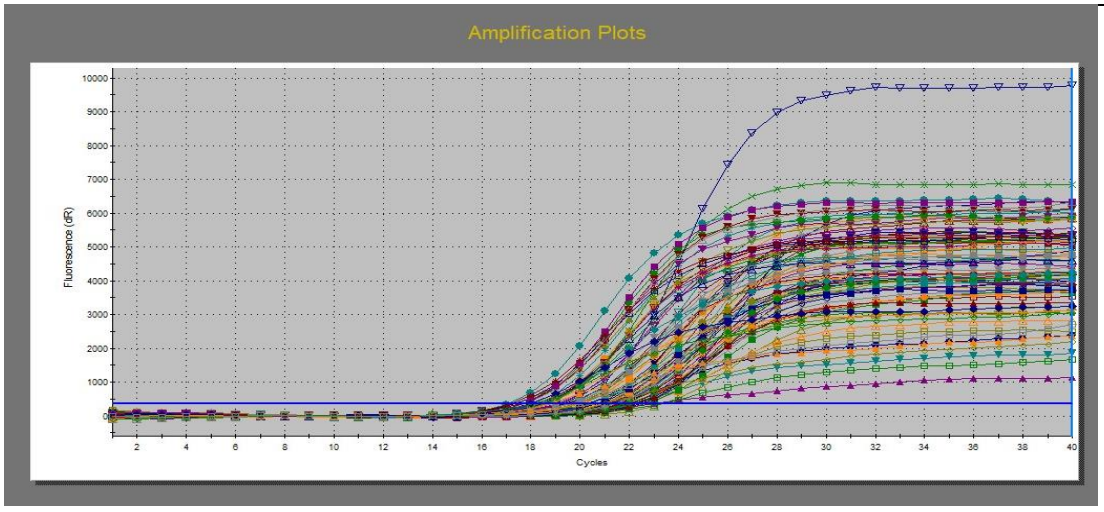
3.4. *gapdh* gene Expression:

There were no significant differences of Ct value of *gapdh* in subjects and healthy control group (1±0.00)**.** The mean fold of *gapdh* gene expression in the patients groups was (0.98 ± 0.08), Table (2), Figure(1).

Table (2): Comparison of *gapdh* fold between study groups depending on $2^{-\Delta Ct}$ Method.

Group	Mean Ct of *gapdh*	2^-ct	Expression group/ control group	mean fold of *gapdh* expression
Patients	29.977	9.5 E10	9.5 E10/9.7 E10	0.98 ± 0.08
Control	29.946	9.7 E10	9.7 E10/9.7 E10	1 ± 0.00
LSD value	~	---	---	0.217 NS

NS: Non-Significant.



Figure(1): *gapdh* genes amplification plots by qPCR .Ct values was ranged from 23.32 to 25.4.The photograph was taken directly from Qtower2.0/2.2

The usage of housekeeping genes in molecular research is predicated on the underlying premise that the cells' expression of these genes is constant (Reboucas, 2013). According to the gene expression data, gapdh is one of the most often used housekeeping genes (Barber et al., 2005). Using qRT-PCR, Robert et al. (2005) investigated the expression of 1718 genes. They used the gapdh gene as a reference gene in seventy-two different types of healthy human tissues. When used in clinical investigations, they discovered that gapdh is a very dependable method for qRT-PCR normalization.

3.5. *gal-3* Gene Expression:

Expression of the ***gal-3*** gene was significant decreased (P≤0.05) in patients (0.374 ±0.18)fold compared to control that recorded (1.00 ±0.00)fold, table (4) and figure (1).

Table (4): Comparison between control and patient groups in Folding of *gal-3* gene expression

Group	Ct *gal-3*	Ct *GAPDH*	ΔCt	ΔΔ Ct	Fold change
Patients	20.01	20.94	-0.9379	-2.3461	0.374 ±0.18
Control	19.58	18.16	1.5256	1.5256	1.00 ±0.00
T-test	~	~	~	~	0.517 *
P-value	~	~	~	~	0.0469

*** (P≤0.05).**

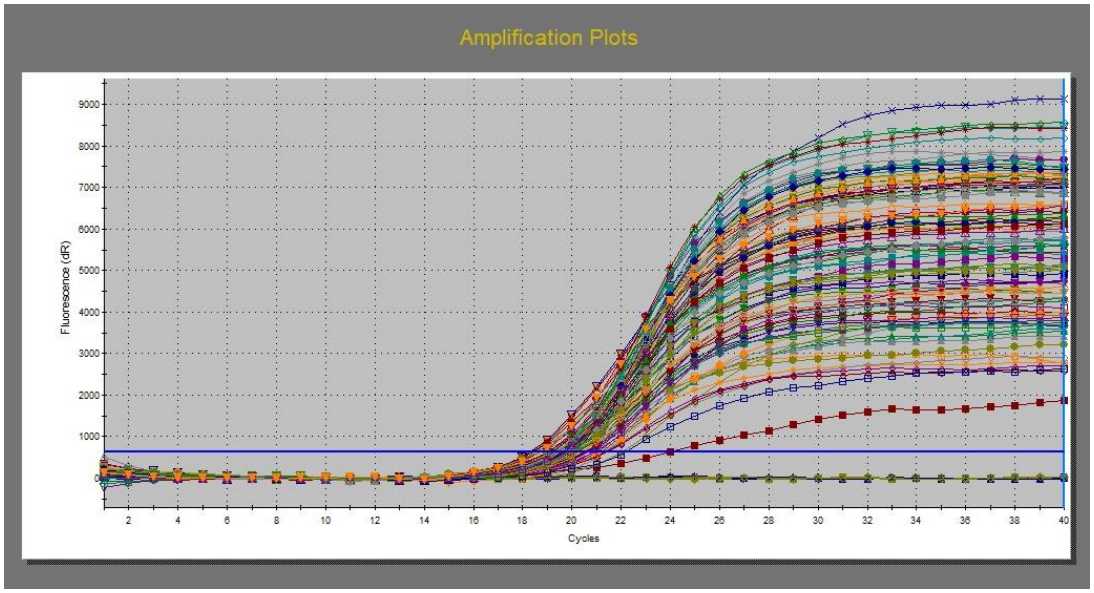


Figure (2): *gal-3* gene amplification plots by qPCR .Ct values was ranged from 28.34 to 32.13.The photograph was taken directly from Qtower2.0/2.2,

This study was focused on galectin-3 among several potential genes. Galectin-3 belongs to a broad family of animal lectins that bind galactosides. During phagocytosis, macrophages exhibit elevated expression of galectin-3 at phagocytic cups and phagosomes (Sano et al 2003). Laminin, collagen, synexin, and integrins are among the ligands found in the extracellular matrix that Galectin-3 interacts with (Ochieng et al., 2004). 1-integrin has been demonstrated to mediate the pathway of galectin-3

import from the extracellular milieu to the cytoplasm (Furtak et al., 2001). While intracellular galectin-3 controls the cell cycle and apoptosis, extracellular galectin-3 mediates cell motility and cell-cell contacts (Kim et al., 1999). The growth-promoting effect of galectin-3 is mostly dependent on the promotor activity of cyclin D1, and overexpression of galectin-3 alters the expression levels of cell cycle regulators, including cyclin D1 (Lin et al., 2002). Galectins are expressed in many tissues, coupled with their ability to regulate immune activities through diverse intracellular and extracellular processes, galectin-mediated immune regulation shows a high level of complexity. The diverse mechanisms by which galectins can regulate adaptive and innate immune responses. general characteristics of galectins and explore their roles in regulating adaptive immunity, in particular T cell biology, and in regulating innate immunity, including macrophage activation, immune cell recruitment and phagocytosis (Mendez-Huergo *et al.*, 2017; Girotti *et al.*,2020)

CONCLUSION:

The present study, showed the decreased level of galectin-3 production suggested there are many mutations in gal-3 gene this reflects in increased levels of IL-35 and IL-37 in patients with Beta Thalassemia Major

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