Original Article

Evaluation of gal-9 Gene Expression and its Impact in some Apoptotic Proteins (Cas3,7) Levels in Patients with Beta Thalassemia Major

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ABSTRACT

Objective: To investigate the genetic relationship of affected samples with healthy controls.
Study Design: Case-control study
Place and Duration of Study: This study was conducted at the Al Kut Hospital for Women and Children, Iraq from 1st November 2023 to 29th February 2024.
Methods: There were 74 children in patient's group and 40 children in control group. 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. Molecular and serological analyses were carried out using quantitative real-time PCR and ELISA techniques, respectively.
Results: There were 38 (51.35%) males and the 36(48.65%) females in patients group while in control group, there were 25 males and 15 females. The largest group of patients between 10-20 years, 38 (51.35%), followed by those between 21-30 years, 15 (20.27%). The ELISA assay revealed there is no significant rise in cas-3 (976.93±55.58 pg/ml) and control group (945.87 ±92.57pg/ml). Whereas there was a significant decrease in levels of Cas-7(2267.90±315.56pg/ml) compared to healthy control group (2267.90±315.56pg/ml). The gal-9 gene expression was assessed by quantitative real-time PCR, which revealed a substantial ($P \leq 0.05$) drop in patient expression (0.0054 ±0.0004) as compared to the control, which recorded 1.00±0.00 fold.
Conclusion: The decreased level of galectin-9 production suggested there are many mutations in gal-9 gene this reflects in increased levels of Cas3 and Cas7 in patients with beta thalassemia major.

Key Words: Beta thalassemia major,Gal-9gene,Caspase,Quantitiverealtime PCR

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INTRODUCTION

Beta thalassemia major, a hereditary blood disorder, is characterized by reduced or nonexistent β-globin chain synthesis. This lowers RBC hemoglobin, RBC production, and anemia.[1] Old red blood cells' hemoglobin is broken down mostly by the spleen. In β-TM, hemopoiesis is accelerated to address anemia, leading to increased synthesis and removal of aberrant red blood cells. Additional changes include extramedullary hematopoiesis and splenomegaly. An increase in blood transfusions, red blood cell hemolysis, and iron deposition and buildup may cause splenomegaly.[2] Chronic hypoxia causes anemia and growth hormone deficiency (GHD) in thalassemia patients due to the liver's defective somatomedin production and rapid red blood cell destruction.[3] (Some gene expression is affected by promoter methylation.[4] Low Cas-8 levels and methylation may be diagnostic markers for certain malignancies.[5] owing to their intracellular and extracellular immune control mechanisms, galectin-mediated immune regulation is complicated owing to their extensive organ expression. Galectins regulate adaptive and innate immune responses via cellular and extracellular pathways. Galectins activate macrophages, attract immune cells, and facilitate phagocytosis to govern adaptive immunity via T cell activities and innate immunity through macrophage activation.[6,7] Nucleotide substitutions and frame shift mutations might impact β-globin gene transcription, splicing, and mRNA translation, resulting in β-globin chain absence or reduced synthesis.[8] Gal-9

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gene expression and caspase (3,7) levels in beta thalassemia major patients were the main focus of this study.

METHODS

The Iraqi Ministry of Health and the Ethics Committee of the Department of Biology, College of Education, University of Al Qadisiyah approved their participation in the research tests. All research participants obtained their fathers' written approval. A control group of 40 healthy individuals and 74 βTM patients hospitalized to Al Kut Hospital for Women and Children each had three milliliters of blood taken. The healthy control group and patients were 21–30. The hospital's consulting physician diagnosed the ailment. Three milliliters of blood were placed in EDTA tubes for molecular analysis and three in plain tubes for ELISA testing. Following manufacturer directions, the Enzyme Linked Immunosorbent Assay measured IL-37 and IL-35 levels in patients and controls. Human Caspase 7 and 3 ELISA Kits (SL3104Hu, SL2079Hu).This research uses TransGen Biotech-China.

The manufacturer's instructions were followed to extract total RNA from each sample using TRIzol®LS Reagent. Transcription of whole RNA to complementary DNA was done using the Easyscript® Kit. A 20µl reaction volume was employed for the technique. The reveres transcription phase was completed in one cycle using the following program: 25°C for 10 minutes, 42°C for 10 minutes, 85°C for 5 minutes, then 4°C until the run was ended. Using quantitative real-time PCR, gal-9 gene expression levels were determined. This expression was validated using TransStart® Top Green qPCR Super Mix (SYBR Green).The reference gene, glyceraldehyde 3-phosphaee dehydrogenase gene (gapdh), was amplified to standardize gal-9 mRNA levels.

Quantitative real-time PCR was done using specified primers. The reaction procedure was: Lyophilized primers were reconstituted in DNase/RNase-free water to a stock solution concentration of 100 pmol/µl. Reconstituted 10 pmol/µl primer in 90 µl deionized water provided a final concentration of 10µM in the working solution. Initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 40 seconds, annealing at 58°C for gapdh and gal3 for 40 seconds, extension at 72°C for 1 minute, 35 cycles, and a hold at 4°C for 1 cycle. Gapdh and gal-9 gene primers were
F: 5′-AACTTTGGCATTGTGGAAGG-3′,
R: 5′-ACACATTGGGGGTAGAACA-3′ and
F: 5′-TCAGAGGTTCCACATCAA-3′,
R: 5′-CCACAGCATTCTCATCAA-3′, respectively.

Using the Livak and Schmittgen[9] equation, ΔCT and ΔΔCT were calculated. This research used SAS to analyze the effects of many characteristics. The least significant difference (LSD) test determined mean differences.

RESULTS

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 (Table 1).

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 juxtaposed with the healthy control group (Table 2).

Caspase-3 and Caspase-7 were included in this study as proteins contribute in apoptotic processes. There were no significant changes in Caspase-3 protein in patients (976.93±55.58pg/ml) and healthy control group (945.87±92.57pg/ml) while there were a significant increase in Caspase-7 protein (2267.90 ±315.56pg/ml) compared to healthy control group (917.50± 162.07pg/ml) [Table 3].

Table No.1: Gender distribution among patients with β-thalassemia major and the control group

Gender	Patients	Control	P-value
Male	38 (51.35%)	25 (62.50%)	0.0419*
Female	36 (48.65%)	15 (37.50%)	
*P≤0.05			

Table No.2: Age distribution of β-thalassemia major patients and control group

Age (years)	Patients	Control	P-value
<10	12 (16.22%)	-	0.000**
10-20	38 (51.35%)	10 (25.00%)	
21-30	15 (20.27%)	24 (60.00%)	
>30	9 (12.16%)	6 (15.00%)	
**P≤0.01			

Table No.3: Comparison between patient and control groups in Cas-3 and Cas-7

Group	Caspase-3pg/ml	Caspase-7pg/ml
Patients	976.93 ±55.58	2267.90 ±315.56
Control	945.87 ±92.57	917.50 ±162.07
T-test	106.49 NS	878.72**
P-value	0.7661	0.0497
*P≤0.05,NS: Non-Significant		

No significant variations were seen in the Ct values of gapdh between individuals and the healthy control group (1±0.00). The average fold change of GAPDH

gene expression in the patient groups was (0.98 ± 0.08) [Table 4, Fig. 1]. Gene expression of gal-9was recorded significant decrease (P≤0.05) in patients (0.0054±0.0004) fold compared to control that recorded (1.00 ±0.00) fold (Table 5, Fig. 2).

Table No.4: Comparison of gapdh fold between study groups depending on 2- ΔCt 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				

Table No.5: Comparison of gal-9 gene fold between study groups depending on $2^{-\Delta Ct}$Method

Group	Ct gal-9	Ct GAPDH	ΔCt	ΔΔ Ct	Fold change
Patients	20.15	20.94	-0.2121	-8.9973	0.0054 ±0.0004
Control	20.72	18.16	2.558	2.558	1.00 ±0.00
T-test	--	--	--	--	0.571*
P-value	--	--	--	--	0.03673
*P≤0.05					

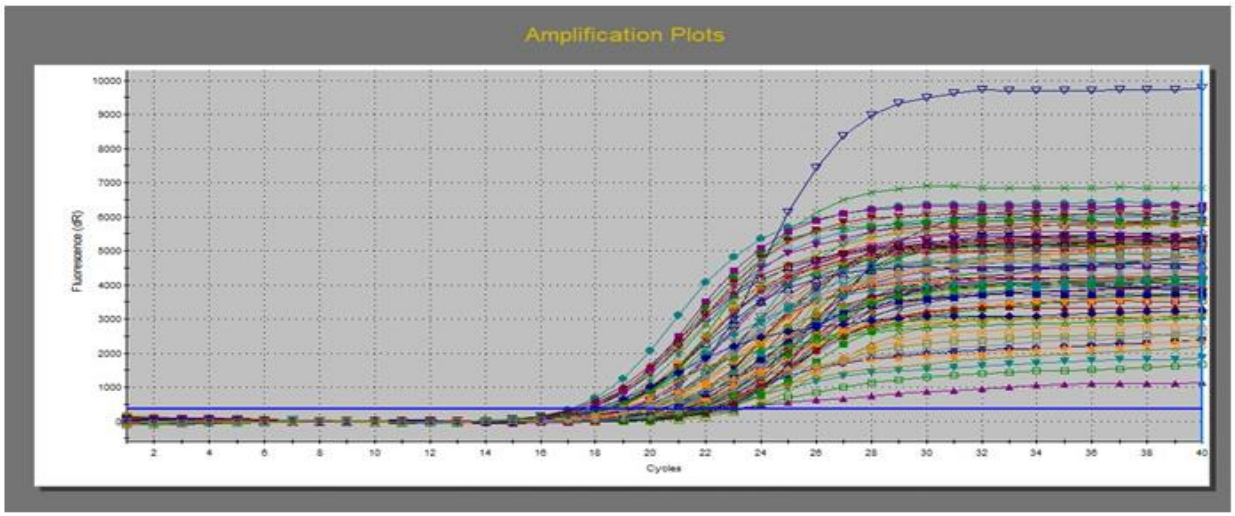

Figure No. 1: Gapdh genes amplification plots by qPCR. Ct values were ranged from 23.32 to 25.4. The photograph was taken directly from Qtower2.0/2.2

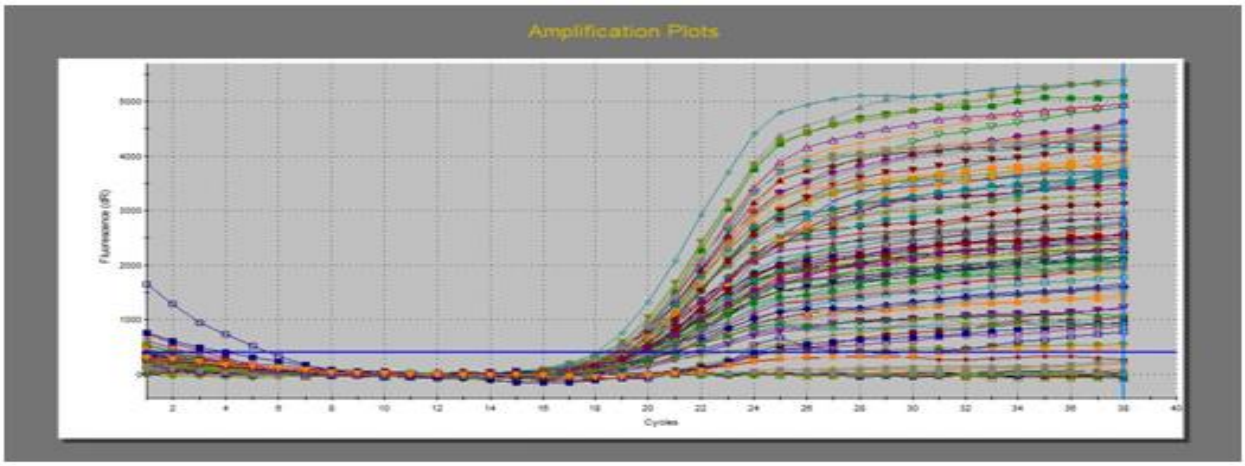

Figure No. 2: gal-9geneamplification plots by qPCR .Ct values was ranged from 27.11 to 31.18.The photograph was taken directly from Qtower2.0/2.2

DISCUSSION

Beta-thalassemia is an autosomal recessive illness that affects both sexes equally and is caused by defects in the β-globin gene on chromosome 11.[10,11] Researcher found similar results in his research of 1800 Iraqi βTM patients in Baghdad. In Saudi Arabia and Al-Bahrain, Al-Awamy[12] found 40% female cases and 60% male cases.

Regular blood transfusions may lengthen life, says one study. However, the strength varied, so some persons had just modest affects. In untreated βTM, hemolytic and severe anemia leads to marrow expansion and hyperplasia. In severe anemia, death might occur within five years. Long-term erythrocyte breakdown causes chronic bilirubin overproduction, which may lead to pigmentary gallstones and hemosiderosis from excess iron in the reticuloendothelial system, notably the liver, pancreas, and heart. Deaths from hemostasis may occur before 25.[13] Takeshita[14] also noted that high transfusion and chelating therapy enhanced survival beyond 30.

Apoptotic caspases kill many old or changed blood cells, which bone marrow, spleen, and liver macrophages remove. The last step of neutrophil and eosinophil lifespan is apoptosis.[15] The reticuloendothelial system eliminates erythrocytes after 100–120 days of senescence.[16] Even without mitochondria and the Apaf-1 adaptor protein, these cells may die via eryptosis, which may include caspases.[17] Platelets, ten-day-old anucleate cytoplasmic fragments, die by caspase-dependent apoptosis when Bcl-XL expression decreases.[18] After genetically modifying Caspase-3, which inhibited cell death in response to oxidative stress (OS), mitochondrial dysfunctions included a swollen morphology, disrupted cristae, decreased membrane potential, increased ROS concentrations, and deficits in mitochondrial oxidative phosphorylation (OXPHOS) enzymes. The fact that caspase-3 gene knockdown (KD) significantly lowered the expression of mitochondrial biogenesis transcriptional activators such Tfam and Nrf-1 suggests that procaspase-3 has a non-apoptotic role in mitochondrial biogenesis.

The idea behind the use of housekeeping genes in molecular research is that these genes are consistently expressed in cells.[19] Gapdh is one of the most often employed housekeeping genes, based on the gene expression data.[20] Robert et al[21] examined the expression of 1718 genes using qRT-PCR. They employed seventy-two distinct kinds of healthy human tissues, using the gapdh gene as a reference gene. They found that gapdh is a very reliable technique for qRT-PCR normalization when applied in clinical studies.

Galectins are the oldest mammalian glycan binding protein (GBP) family and the first to regulate the immune system. Vertebrates contain over 16 prototype galectins (galectin-1 to galectin-16) based on their structural properties.[22] Adipocytes express galectin-12, whereas gastrointestinal epithelial cells, the thymus, and endothelial cells express galectin-9.[23] Galectins in the extracellular environment may bind several glycoconjugates, including those on the cell surface and matrix.

Galectins may influence intracellular activities via glycan-dependent or glycan-independent interactions, unlike other GBPs.[24] Galectins are expressed in different organs and cross-act via several cellular and extracellular mechanisms to modulate immunology. Galectins regulate adaptive and innate immune responses in many ways. Explain galectins' basic properties to understand their regulatory roles in innate immunity, including immune cell migration, phagocytosis, and macrophage activation, and adaptive immunity control through T cell biology mechanisms.

CONCLUSION

The decreased level of galectin-9 production suggested there are many mutations in gal-9 gene this reflects in increased levels of Cas3 and Cas7 in patients with beta thalassemia major**.**

Author's Contribution:

Concept & Design or acquisition of analysis or interpretation of data:	Abbas Abed Radam, Sundus Kareem Hamzah
Drafting or Revising Critically:	Abbas Abed Radam, Sundus Kareem Hamzah
Final Approval of version:	All the above authors
Agreement to accountable for all aspects of work:	All the above authors

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