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The Role of *Hemoglobin-Alpha2* and *miR-4459* Gene Expression in the Progression of Major and Intermediate Beta Thalassaemia in Iraqi Patients

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Abstract

Background. Beta-thalassemia, a genetic hemoglobin production condition, has three clinical types: β-Thalassemia Major (βTM), β-Thalassemia Intermedia (βTI), and β-Thalassemia Minor. **Aim.** The study explores the impact of *hemoglobin-alpha2* (*HBA2*) and *miR-4459* gene expression on βTM and βTI illness severity, potentially influencing thalassemia diagnosis and treatment through novel diagnostic techniques. **Methods.** The research was conducted with care, dividing patients into three groups: 73 with βTM, 27 with βTI, and 50 healthy volunteers. Data was collected through patient interviews and clinical examinations at Al-Krama Teaching Hospital. The investigation focused on *miR-4459* and *HBA2* genes using qRT-PCR. **Results.** Research showed β-thalassemia major and intermedia patients had decreased *HBA2* gene expression, particularly in βTM, and higher *miR-4459* gene expression, indicating high confidence in the findings. **Conclusion.** The *HBA2* and *miR-4459* genes have the potential to act as biomarkers for the early detection of βTM and βTI.

Keywords: Beta-thalassemia major, beta-thalassemia intermedia, hemoglobin-alpha2

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Introduction

Thalassaemia is largely viewed as an autosomal recessive disease in many parts of Iraq (1). Three normal forms result from the four hemoglobin chains (α, β, γ, and δ): A (2α with 2β, 93% to 97%), A2 (2α with 2δ, 1.5% to 3.5%), and F (2α with 2γ, 21.5% to 3.5%). The percentages of the latter two hemoglobin types rise in beta-thalassemia because they lack β chains. The alpha-globin gene on chromosome 16 is primarily the site of mutations that cause β-thalassemia (2). Differences in *HBA2* function or levels might potentially account for part of the documented clinical variability across patients with βTM and βTI. Blood transfusions are the standard treatment for thalassemia. Hemopoietic growth factor production is frequently found to be a controlled cascade reaction (3).

Dysregulated miRNA may have a role in the genesis of some hematological diseases (4). Consequently, to fill in some of the gaps related to β-thalassemia, our study had to focus on a *miR-4459* component. Moreover, a thorough understanding of the roles played by gene polymorphism might advance our understanding of the genetics behind the

heterogeneity of human phenotypes (5). This study aimed to analyze the effect of *HBA2* and *miR-4459* gene expression with βTM and βTI as predictive indicators of illness progression. Previous research in this area had not been properly examined.

Materials and methods

Sample Collection

Three groups were randomly selected to participate in this study. The patients' ages varied from one to forty-six. The first group consisted of 73 patients with βTM, the second group of 27 patients with βTI, and the third group consisted of 50 healthy persons in the same age range for both sexes who were also included in the study. Data was collected through clinical examinations, patient interviews, and history collection, with thalassemia patients diagnosed using hemoglobin electrophoresis, full blood counts, and serum ferritin levels. The patients with β-thalassemia minor were excluded from the investigation due to the simplicity of the results. In addition, patients with hepatitis B and C and those who had splenic surgery were excluded since they can negatively impact the results.

Blood Sample Collection

Peripheral venous blood samples weighing five milliliters were taken from individuals diagnosed with βTM and βTI, in addition to healthy controls. An Eppendorf tube containing TRIzol was filled to 750 µL with 250 µL of blood from "Ethylene Diamine Tetra-Acetic Acid" (EDTA) tubes.

The Eppendorf tube was then frozen at -20 ºC until it was needed for genetic research.

RNA Extraction

1. In accordance with the manufacturer's instructions, all blood samples were extracted to produce total RNA using TransGen Biotech's ER501 RNA Kit TransZol Up Plus reagent.

2. Total miRNA was extracted from all blood samples using the EasyPure® miRNA Kit (TransGen, Biotech, ER601-01) reagent in accordance with the manufacturer's instructions.

The concentration and purity of the extracted RNA were measured using the OneC Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) in order to evaluate the quality of samples for RT-qPCR analysis (6). The RNA samples' concentrations ranged from 62 to 96 ng/µl, and they were examined at two distinct wavelengths (260 and 280 nm) to determine the purity of the RNA. An A260 to A280 ratio of about 2.0 showed the quality of the RNA sample.

The process of creating cDNA from mRNA.

The EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (Catalogue No. AE311) method was used to perform the cDNA synthesis; this procedure is quicker and lowers the possibility of contamination. The manufacturer required that a 20µl reaction volume be used for the reverse transcription of 4µl of total RNA. [Table 1] displays the steps.

Primer preparation

Using Primer 3 Plus V4 to create the primers, the reference sequence databases of the University Code of Student Conduct (UCSC) and the National Centre for Biotechnology Information (NCBI) programs were double-checked. They were

synthesized and lyophilized by Alpha DNA Ltd. (Canada). The primer sequences utilized in every test conducted for this study are listed in (Table 2). Primer preparation was employed in this inquiry for each test run. Following the manufacturer's instructions, 10µL of each primer stock solution was diluted in 90µL of water without nuclease to produce 200µL of functional cure. The working solution was kept at -20°C until it was needed.

Table 1: Reverse transcription of cDNA in the presence of a thermal cycler

	Step 1	Step 2	Step 3
Temperature	25ºC	42ºC	85ºC
Time	10 min.	15min.	5Sec.

Table 2: The investigation-specific primer

Primers	Direction of sequence 5'→3'		Size of the product, bp	Ta. °C
HBA2				
Forward	TTAAGCTGGAGCCTCGGTAG	20	144	58
Reverse	TGCTGCCCACTCAGACTTTA	20		
GAPDH				
Forward	GAAATCCCATCACCATCTTCCAGG	24	160	58
Reverse	GAGCCCCAGCCTTCTCCATG	20		
miRNA				
miR-4459	GAGGCGGAGGAGGTGGAGAAA	21	72	58
miRNA-U6 F.P.	A GAGAAGATTAGCATGGCCCC T	22	73	58
miR-universe R.P.	G CGAGCACAGAATTAATACGA C	22		
miRNA universal R. transcription P.	CAGGTCCAGTTTTTTTTTTTTTTTVN			

HBA2 = Hemoglobin-Alpha2, GAPDH = Glyceraldehyde-3-Phosphate Dehydrogenase, Ta = Annealing Temperature.

Quantitative Polymerase Chain Reaction (qRT-PCR) Analysis

The study used qRT-PCR to determine the expression levels of *HBA2* and *miRNA-4459* genes. The target gene's existence was confirmed using a SYBR Green assay. Alpha DNA Ltd. provided the primers, and endogenous control GAPDH and U6 mRNA levels were amplified. The fold changes and expression levels were assessed using the *TransStart*® Top Green qPCR Super Mix kit. Every response was reviewed twice. The volume needed for each component was determined using (Table 3). Based on the thermal profile, the cycling process was configured for the ensuing optimum cycles, as indicated in (Table 4).

Calculating gene expression

The availability of the calibrator value influences the ratio known as the degree of gene expression fold, the mean ΔCt of the patients, and the mean ΔCt of the control. It is impossible to complete any computation without the housekeeping gene values (7).

Statistical analysis

An ANOVA conducted a one-way analysis. Suppose the $P < 0.05$, statistical significance was taken into account. ROC Using Pearson correlation, the "receiver operating characteristic" is a measure of the correlation between two quantitative variables.

Table 3: qRT-PCR components used in the *HBA2*, GAPDH, U6, and *miRNA4459* gene expression

Components	20 µl rxn
2xTransStart® Top Green Qpcr Super Mix	10
nuclease-free water	6
Forward Primer, 10µM	1
Reverse Primer, 10µM	1
cDNA	2

Table 4: Expression levels of the gene *miRNA-4459*, *HBA2*, *U6*, and GAPDH at different temperatures

Steps	Temperature ºC	Time sec.	Cycles
enzyme activation	94	30	1
Denaturation	94	5	40
Annealing	58	15	
Extension	72	20	
Dissociation	55–95		1

Results

Gene expression of *miR-4459* and *HBA2*

The involvement of homo sapiens (*has-miR-4459*) and *HBA2* gene expression in the incidence of beta-thalassemia major and intermedia is identified in the current study, which has never been reported before. Real-time PCR quantification was utilized using SYBR green, a fluorescent dye that can identify any double-stranded DNA, including cDNA.

Quantification of GAPDH expression using real-time PCR

In this study, the housekeeping gene was utilized. Table 5 displays the Ct value of GAPDH. Each research group's GAPDH Ct levels ranged from 13 to 13.95; the mean Ct values for the βTM, βTI, and control groups were 13.82, 13.95, and 13.90, respectively. There was no appreciable variation in the GAPDH Ct value between these groups. Furthermore, as shown in Table 5, each research group's 2-ΔCt value was computed and compared to the control to investigate the variance of the overall change in GAPDH expression [Figures 1, A, and B].

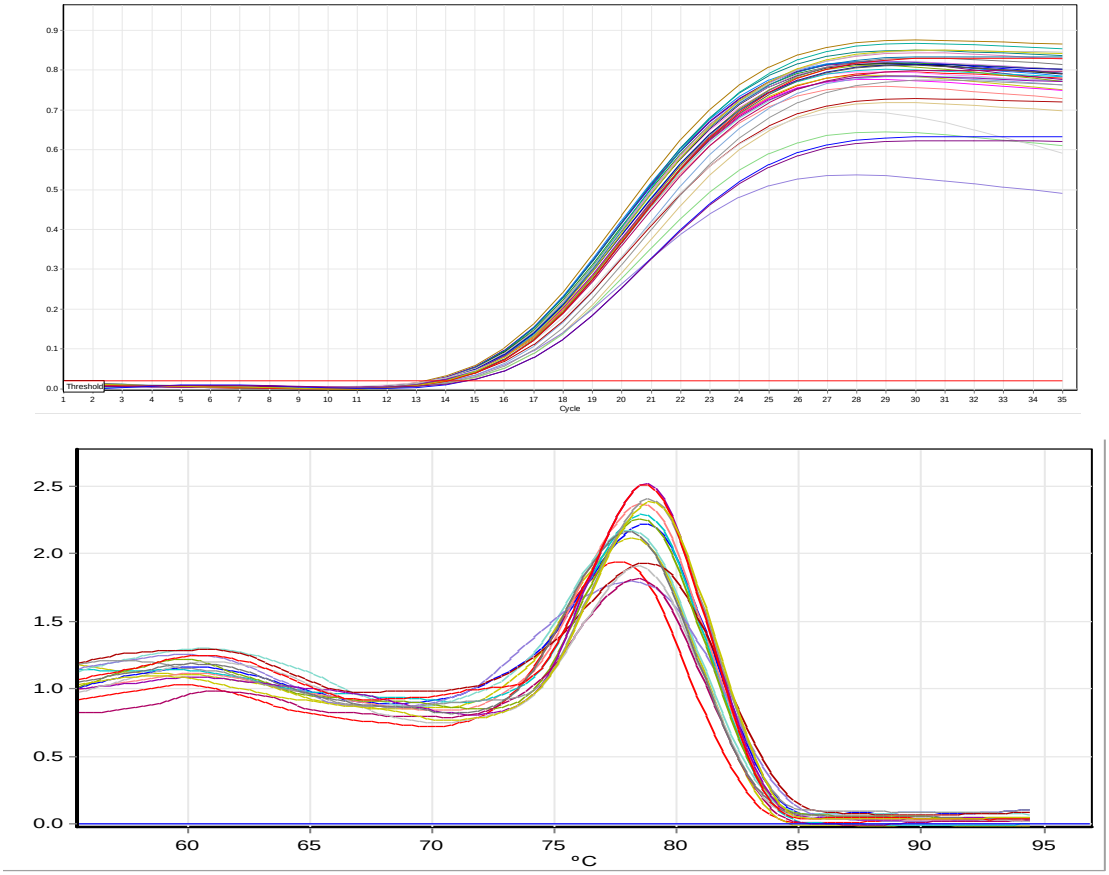

Figure 1: A. The GAPDH amplification plot; B. The GAPDH expression melting curves.

Real-time PCR quantification of HBA2 expression

Real-time PCR was performed to quantify HBA2 mRNA expression in blood samples from β-thalassemia major (βTM), β-thalassemia intermedia (βTI), and healthy control groups. The mean Ct values for HBA2 amplification were 16.26 and 15.57 in the βTM and βTI groups, respectively, compared with 9.26 in the control group (Table 5). Normalization of HBA2 expression was carried out using GAPDH as an internal reference gene. The mean ΔCt

values were 2.43 for βTM, 1.62 for βTI, and −4.63 for the control group (Table 5). Relative gene expression was calculated using the $2^{-}\Delta\Delta Ct$ method. The mean $2^{-}\Delta\Delta Ct$ values were 0.18 for the βTM group and 0.32 for the βTI group, whereas the control group showed a mean value of 24.81 (Table 5). Each qPCR reaction was performed in duplicate. Amplification plots and melting curve analyses for HBA2 are presented in (Figures 2, A, and B).

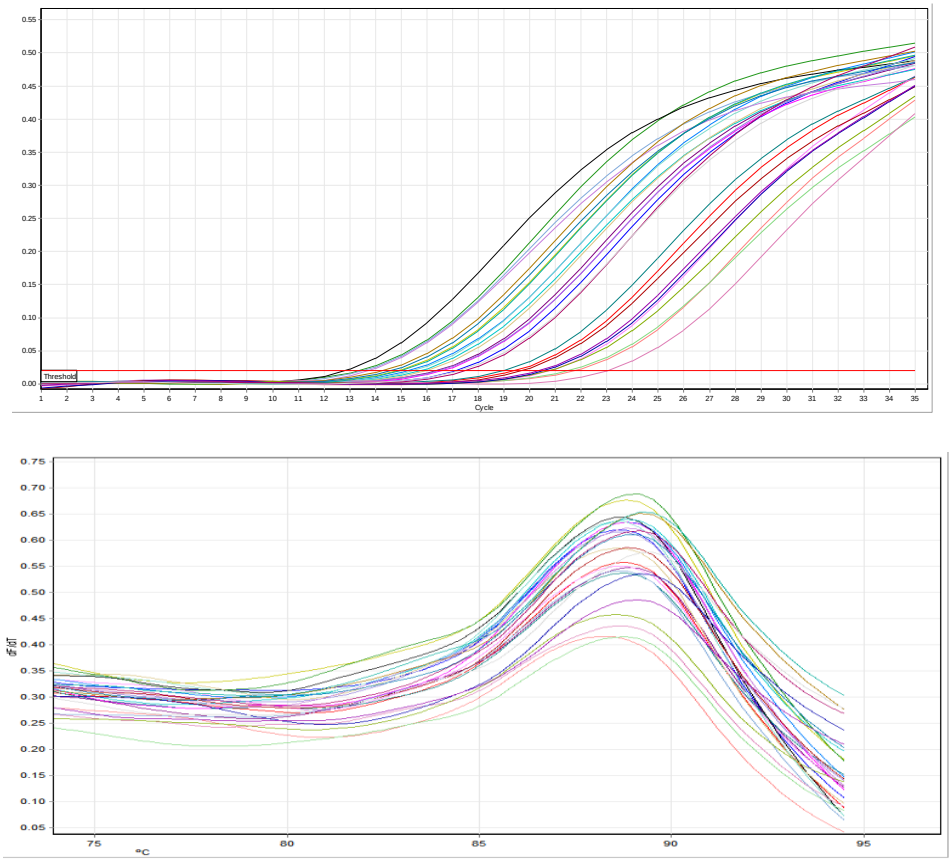

Figure 2: A. *HBA2* gene amplification plot; B. Expression of *HBA2* gene melting curves

Table 5: The $2^{-\Delta Ct}$ method was used to quantify the *HBA2* gene expression level in the control, β-thalassemia major, and β-thalassemia intermedia.

Groups	means Ct of *HBA2*	means Ct of GAPDH	ΔCt means Ct of *HBA2*	$2^{-\Delta Ct}$	Experimental /control groups	fold of expression of a gene
βTM	16.26	13.82	2.43	0.18	0.18/24.81	0.007
βTI	15.57	13.95	1.62	0.32	0.32/24.81	0.013
Control	9.26	13.90	-4.63	24.81	24.81/24.81	1.00

βTM = β-thalassemia major, βTI = β-thalassemia intermedia, *HBA2* = Hemoglobin-Alpha2, *GAPDH* = Glyceraldehyde-3- Phosphate Dehydrogenase, Ct = threshold cycle.

Real-time PCR-based quantification of miRNAU6 expression

The Ct value of the housekeeping gene used in this study, miRNAU6, is shown in Figures 3, A, and B, as well as in Table 6. The Ct values for miRNAU6 varied throughout study organizations, ranging from 16 to 16.4. The mean Ct values for the βTM, βTI, and control groups were (16.29), (16.40), and (16.29), in that order. The findings indicate that there was no significant difference in the means of the miRNAU6 Ct levels between these groups. As also shown in (Table 6), additional analysis of the variance of the overall change in miRNAU6 expression in each research group was conducted utilizing the $2^{-\Delta Ct}$ value and the ratio of each study group's $2^{-\Delta Ct}$ relative to the control.

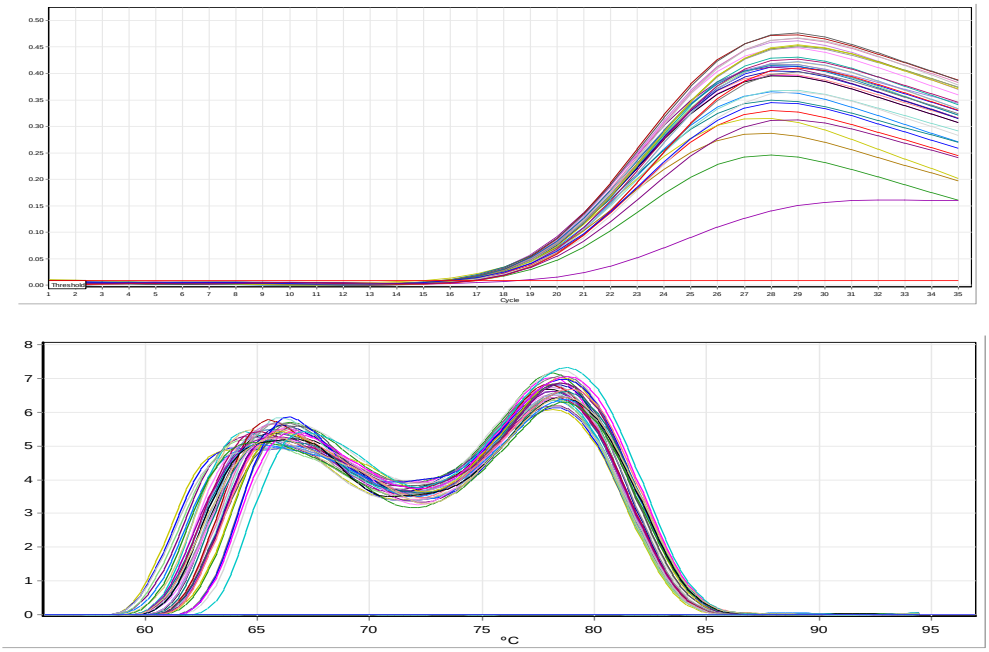

Figure 3: A. *miRNA U6* gene amplification plot; B. *miRNA U6* gene expression melting curves

Table 6: The *miR-4459* gene mRNA expression level was determined in the control, β-thalassemia major, and β-thalassemia intermedia using the $2^{-\Delta Ct}$ method.

Groups	Means Ct of *miR4459*	Means Ct of U6	ΔCt means Ct of *miR4459*	$2^{-\Delta Ct}$	experimental / control groups	fold of gene expression
βTM	14.05	16.29	-2.23	4.70	4.70/2.11	2.22
βTI	14.35	16.40	-2.05	4.14	4.14/2.11	1.96
Control	15.21	16.29	-1.08	2.11	2.11/2.11	1.00

βTM = β-thalassemia major, βTI = β-thalassemia intermedia, Ct = threshold cycle.

Real-time PCR quantification of *miR-4459* expression

Table 6 presents the miR-4459 cDNA amplification average Ct values (14.05 and 14.35, respectively) in blood samples from βTM and βTI patients in relation to the corresponding Ct values (15.21) for the control group. Significant differences were between the research groups' mean CT findings. The initial mRNAs discovered in the samples were consistent with this result. It is also possible that the βTM and βTI study groups express these genes at higher levels than the control group because there was a connection between the patient group and more copies of mRNAs. Findings indicated that elevated expression of the *miRNA-4459* gene was present in all patient groups, uggesting a potential role for the *miR-4459* gene as a diagnostic tool for β-thalassemia early identification. Dissociation curves and amplification plots for the *miR-4459* gene are displayed in [Figures 4, A, and B].

Mean ΔCt values were -2.23, -2.05, and -1.08 for the βTM, βTI, and control groups, respectively. Significant differences existed between the research groups. The matching expression of the *miR-4459* gene has been identified by each study group utilizing the $2^{-\Delta\Delta Ct}$ values. Table 6 displays the outcomes. We have compared the $2^{-\Delta\Delta Ct}$ outcomes of each group to those of the control group's members. $2^{-\Delta\Delta Ct}$ mean values for the βTM and βTI groups were (4.70) and (4.14), respectively. However, the $2^{-\Delta\Delta Ct}$ mean for the control group was (2.11).

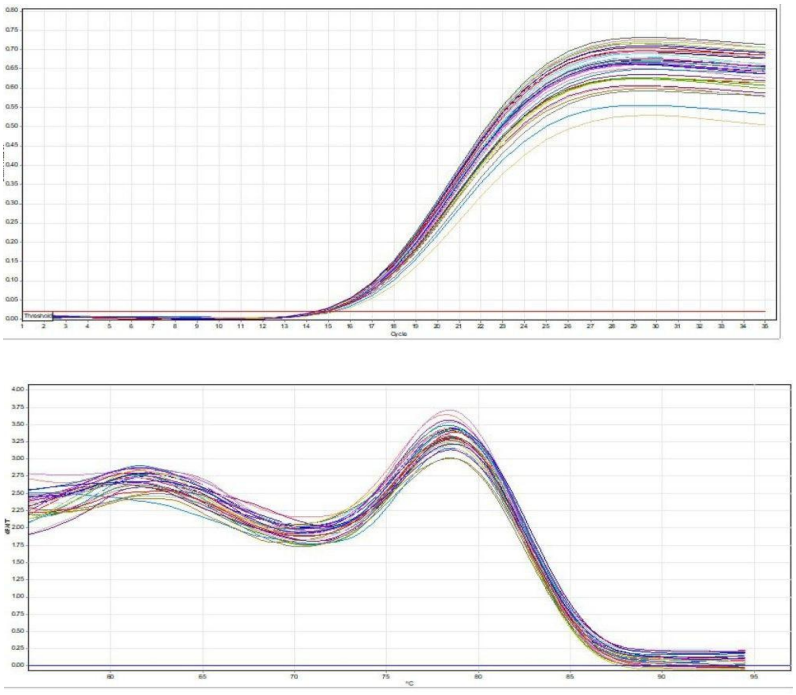

Figure 4: A. the *miR-4459* gene amplification plot; B. the *miR-4459* gene expression melting curves.

Hemoglobin-alpha2's receiver perating characteristic curve (ROC)

According to the results shown in [Table 7] and [Figure 5], there was an *HBA2* threshold of 0.2847 between sensitivity (98) and specificity (92).

The outcome showed that the *HBA2* area under the curve was 0.98. The area under the curve is ideal for predicting the prognosis of an illness when its value is 0.001 or greater.

Table 7: The *HBA2* and *miR-4459* receiver operating characteristic curve data

Parameters	AUC	Explanation	*P*-value	The best cut-off	Sensitivity %	Specificity %
miR-4459	0.86	**Very Good**	*0.001*	1.4794	82	84
HBA2	0.98	**Excellent**	*0.001*	0.2847	98	92

AUC = Area Under the Curve, *HBA2* = Hemoglobin-Alpha2.

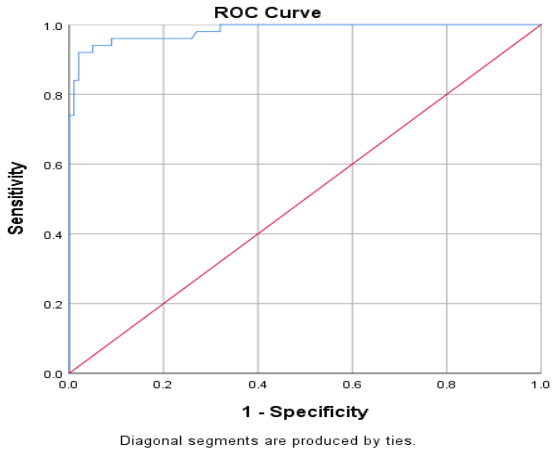

Figure 5: The receiver operating characteristic of the HBA2

The *miR4459* receiver operating characteristic curve

The results in [Table 7] and [Figure 6] demonstrate that the threshold for *miR4459* between sensitivity (82) and specificity (84) was 1.4794. The area

under the curve for *miR-4459* was 0.86 based on the results. When the area under the curve has a value of 0.001 or above, it is ideal for predicting the prognosis of an illness.

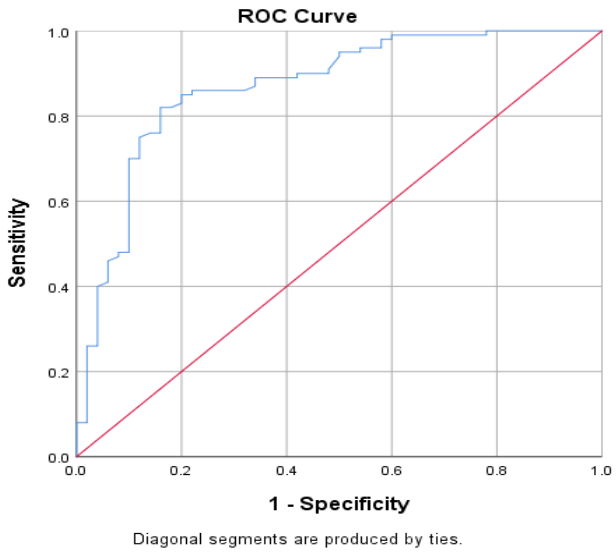

Figure 6: Receiver operatg characteristic curve *miR-4459*

Discussion

The present study was designed to elucidate the role of hemoglobin alpha-2 (HBA2) and miR-4459 gene expression in the progression of major and intermediate beta thalassaemia among Iraqi patients. The observed variations in gene expression levels among the βTM, βTI, and control groups highlight the molecular differences associated with disease severity. In the current analysis, the gene fold ratios normalized to GAPDH demonstrated distinct expression patterns between patient groups and healthy individuals, supporting the reliability of GAPDH as an internal control gene due to its stable expression across samples. Quantitative real-time PCR analysis relies on cycle threshold (Ct) values to assess gene expression levels, where lower Ct values reflect higher transcript abundance and higher Ct values indicate reduced gene expression (8). In this context, the differences in Ct values observed among the study groups suggest altered regulation of target genes in β-thalassaemia patients. Such molecular alterations are consistent with the pathophysiological mechanisms of the disease, in which imbalanced globin chain synthesis and disrupted gene regulation contribute to disease progression.

These findings provide molecular evidence that differential gene expression may play a role in distinguishing between major and intermediate forms of beta thalassaemia (8). The rationale for using reference genes in molecular research is that they show constant expression in the cells or tissues under investigation (9). In gene expression studies, the GAPDH gene is one of the reference genes most often utilized (10). Their utility in molecular research is predicated on the idea that housekeeping genes are expressed consistently in the cells under investigation (11). One of the housekeeping genes most frequently utilized in partners for gene expression data is GAPDH. The present findings demonstrate a marked reduction in HBA2 gene expression in both βTM and βTI

patients compared with healthy controls. This reduction may reflect the impaired globin chain synthesis associated with β-thalassemia, where imbalance between α- and β-globin chains leads to ineffective erythropoiesis.

The more pronounced decrease observed in βTM compared to βTI patients could be attributed to the greater clinical severity of βTM and its association with more extensive genetic and hematological alterations. Similar reductions in HBA2 expression have been reported in previous studies, supporting the role of altered globin gene regulation in the pathophysiology of β-thalassemia. Current findings imply that the βTM and βTI research groups express the *HBA2* gene at lower levels than the control group and that there may be a relationship between the patients' group and fewer mRNA copies. These results showed that HBA2 gene expression is decreased in all patient categories, especially in βTM. The control group expresses the *miR-4459* gene at lower levels than the βTM and βTI study groups, and there was a link between the patient group and more mRNA copies. The *miR-4459* gene was expressed at higher levels in all patient groups, indicating that the *HBA2* and *miR-4459* genes might serve as indicators for the early identification of β-thalassemia. HBA2 is crucial for diagnosing β-thalassemia, but factors influencing HbA2 levels in certain illnesses, including beta and alpha-thalassemia, have been extensively studied (12). It was commonly recognized that carriers of β-thalassemia have elevated levels of HBA2. β-thalassemia major carriers run the danger of being undiagnosed and passing the illness on to their progeny (13).

In a separate study, researchers discovered polymorphisms in the genes. Compared to the control group, individuals with beta-thalassemia major exhibited more homogeneity and diversity (14). A second study (15), however, discovered that four mutations were selected for molecular diagnosis in Iraq. Research has linked the polymorphism of the AHSP gene to the development of β-thalassemia disease (16). MicroRNAs, which are small, non-coding, single-stranded RNA molecules of 20–23 nucleotides, are frequently needed for base pair binding to the 3ρ-UTRs of numerous target genes in order to silence those genes (17) post-transcriptionally.

Important regulatory molecules called microRNAs are found in cells and are necessary for several vital biological functions (18). Due to the significant function that miRNAs play in cells, several diseases have been found to be associated with the dysregulation of miRNAs (19). By focusing on miRNAs, β-thalassemia and other hemoglobinopathies linked to an excess of free α-globin chains may be reduced (20). Given its ability to differentiate between two patient states, the ROC analysis may be used in clinical epidemiology to assess the precision of medical diagnostic techniques (21). The predictive test's false-positive rate (X-axis) is the independent variable. The area under the curve provides a useful means of evaluating the diagnostic test's sensitivity and specificity, which together define its inherent validity. The real positive rate for the predictive test, or Y-axis, is the dependent variable (22). To avoid missing or inaccurate diagnoses, more study on uncommon genes is necessary (23).

Conclusion

According to findings, patients with β-thalassemia major and intermedia have reduced levels of *HBA2* gene expression in comparison to the control group. These results showed that *HBA2* gene expression is decreased in all patient categories, especially in βTM. Furthermore, *miR-4459* gene expression was greater than normal in all patient groups. That β-thalassemia major and intermedia might be identified early on using the *HBA2* and *miR-4459* genes. The *HBA2* and *miR-4459* genes are being proposed as potential biomarkers for the early detection of β-thalassemia major and intermedia.

Conflicts of Interest

There are no competing interests between them, as the author attests.

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