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Assessment of the TGF-β gene and its target *miR-1908-5p* gene expression and its role in the treatment of beta-thalassemia in Iraqi patients

Manal Lafta Abdulhassn, Hiba Muneer Abdel Hassan Al-Khafaji[1], Maryam Qasim Mohammed[2]

Abstract:

BACKGROUND: Thalassemia is potentially inherited fatal anemia and can cause health problems when left untreated. Beta-thalassemia is the most severe formula of the illness. Transforming growth factor-beta (*TGF-β*) is a cytokine that has multicellular functions. The miRNAs have been confirmed to play important roles in gene expression regulation during the development of a variety of human diseases. In a variety of hematological diseases, miRNAs influence the TGF-*β* signaling pathway. The etiology of the disease may be supported by the dysregulation of this interaction.

OBJECTIVES: This study aimed to estimate the role of *TGF-β* and its target *miR-1908-5p* expression and its effect on the prognosis and treatment of beta-thalassemia (β-thal) in Iraqi patients.

PATIENTS, MATERIALS, AND METHODS: The case–control study encompassed 100 Iraqi individuals, categorized into two groups: Fifty patients diagnosed with β-thal and fifty apparently healthy controls. Blood samples were collected from Baghdad Teaching Hospital at the Medical City in Baghdad from December 2023 to February 2024. Hematological and biochemical parameters were measured. Total RNA was extracted and converted into complementary DNA, finally, to assess the levels of *TGF-β* and *miR-1908-5p* gene expression by the reverse transcription-polymerase chain reaction.

RESULTS: The results showed that a decrease in the hemoglobin (Hb) level was significantly correlated with a significant increase in the ferritin level and the white blood cell (WBC) count in thalassemia patients in comparison to healthy controls, with a significant difference ($P < 0.01$). The expression of the *TGF-β* gene was notably lower in thalassemia patients (0.783) compared with control, with an increase in the *miR-1908-5p* gene expression level (1.431); furthermore, the correlation between them using Spearman's coefficient was negative with a significant difference of 0.04.

CONCLUSION: A low level of Hb in patients acts as a pointer to increase the level of the ferritin and the WBC count. A low level of gene expression of the *TGF-β* gene targeted by high-level *miR-1908-5p* gene expression in Iraqi patients with β-thal is considered a good biomarker according to the ROC curve test. It might be possible to regulate *TGF-β* expression by modifying *miR-1908-5p*, therefore could improve the effectiveness of gene-based therapies in hematological therapies.

Keywords:

Beta-thalassemia, *miR-1908-5p* and gene expression, transforming growth factor-beta

Department of Chemistry and Biochemistry, College of Medicine, Mustansiriyah University, [1]Department of Applied Sciences, University of Technology, [2]Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq

Address for correspondence:
Ms. Maryam Qasim Mohammed, Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq.
E-mail: maryamqasim.ms.c.mic.2020@uomustansiriyah.edu.iq

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Introduction

A hereditary disorder affecting blood cells is called thalassemia defined as low hemoglobin (Hb) level and a decrease in red blood cell (RBC) count relative to normal count. Hb, the component of RBCs that carries oxygen, is deficient in thalassemia so anemia symptoms appear, which is like stress and exhaustion.[1,2] Hemolytic anemia is induced by beta-thalassemia (β-thal) due to mutations in the β-chain globin gene locus during the development from embryos to adults and is the most prevalent kind, characterized by hypochromic anemia and microcytosis.[3,4] Al-Allawi *et al*. mention that the prevalence of β-thal carrier state varies between 3.7% and 4.6% in the various parts of Iraq.[5] Diverse origins, including Mediterranean, Asian, Indian, Turkish, Kurdish, Iranian, Egyptian, and Saudi Arabian origins, are responsible for the mutations associated with β-thal.[6] Thousands of newborns with β-thal are born each year. The most common regions for β-thal cases are the Mediterranean region, North Africa, the Middle East, India, Central Asia, and Southeast Asia.[7-9] With a predictable prevalence incidence of 37.1/100,000 in Iraq and a rate of carrier is 4.1% in north-eastern Iraq (Sulaymaniyah), β-thal is a serious public health concern in Iraq, particularly the Kurdistan Region.[10]

A pleiotropic peptide, transforming growth factor-beta (*TGF-β*) belongs to the *TGF-β* super-family of cytokines, which is a cellular protein that has an essential role in apoptosis, stem cell development, erythroid differentiation than erythropoietin, and cell growth regulation.[11]

MicroRNAs (*miRs*) are a class of noncoding RNAs through an approximate length of (22) nucleotides that standardize the expression of their target genes through posttranscriptional mechanisms. Transcription, nuclear processing, cytoplasmic processing, formation of the RNa-induced silencing complex, and translation inhibition through enzymatic messenger RNA (mRNA) degradation are the main mechanisms by which miRNAs control gene expression.[12,13] These mechanisms also include chromatin remodeling, proliferation, differentiation, and apoptosis.[14,15] It is believed that *miRs* regulate the homeostatic conditions and pathological conditions of a variety of diseases, including infections, hematologic disorders, and endometrial disorders, as well as cancer.[16,17] Since the target mRNA of the miRs binds to the 3′-untranslated region, they are thought to be negative regulators of gene expression., prevent translation and/or cause the transcript to degrade, thereby lowering the rate at which proteins are synthesized.[18] miRNAs play a role in the variability of biological processes, comprising cell division, proliferation, and apoptosis. As a result, they are involved in the pathophysiology of a widespread range of diseases.[19]

MiR-1908-5p is a comparatively recently identified *miR* that has been linked to an increasing variety of molecular processes. Many disorders involve the aberrant expression of *miR-1908-5p*.[20] Via the 3′ untranslated region binding to target genes, *miR-1908-5p* can decrease the expression of at least 27 target genes.[21] Eleven factors influence the expression of *miR-1908-5p*: Long noncoding RNA HOXA transcript at the distal tip, adipokines (tumor necrosis factor-alpha, leptin, and resistin), nuclear factor kappa B-cell, cholesterol, free fatty acid, stearoyl-CoA desaturase, and transcription factors that related to immunity (signal transducer and activator of transcription 1, the retinoblastoma protein, and interferon regulatory factor 1.[21,22] The current study was designed to measure some biochemical parameters, assess the gene expression of *TGF-β* and *MiR-1908-5p,* and evaluate their role as biomarkers in the treatment of this disease.

Patients, Materials, and Methods

A study designed as a case–control study with a total of one hundred Iraqi individuals enrolled in this study, classified into fifty Iraqi patients (22 males and 28 females; mean of age 12.03 ± 0.51) and the age range was (6–17 years) were clinically diagnosed with β-thal by consultant physicians at Baghdad Teaching Hospital at the Medical City in Baghdad from December 2023 to February 2024. Fifty apparently healthy participants (as the control group) (26 males and 24 females; mean of age 14.23 ± 0.76) and age range (8–24 years) were registered in the present study. All the subjects were informed about the goal of the present study and provided informed consent.

Patients inclusion criteria

Patients: Individuals diagnosed with β-thal.

Control: Individuals apparently healthy and without blood-associated disease.

Patients exclusion criteria

Patients: Individuals infected with any blood-associated disorder.

Control: Participants with a family history of β-thal.

The study scheme of the current investigation is illustrated in Figure 1.

Blood samples collection

About (5 mL) of venous blood samples from the patients and the control group were collected in EDTA tubes and later in Trizol (TransGen Biotech, China) to preserve

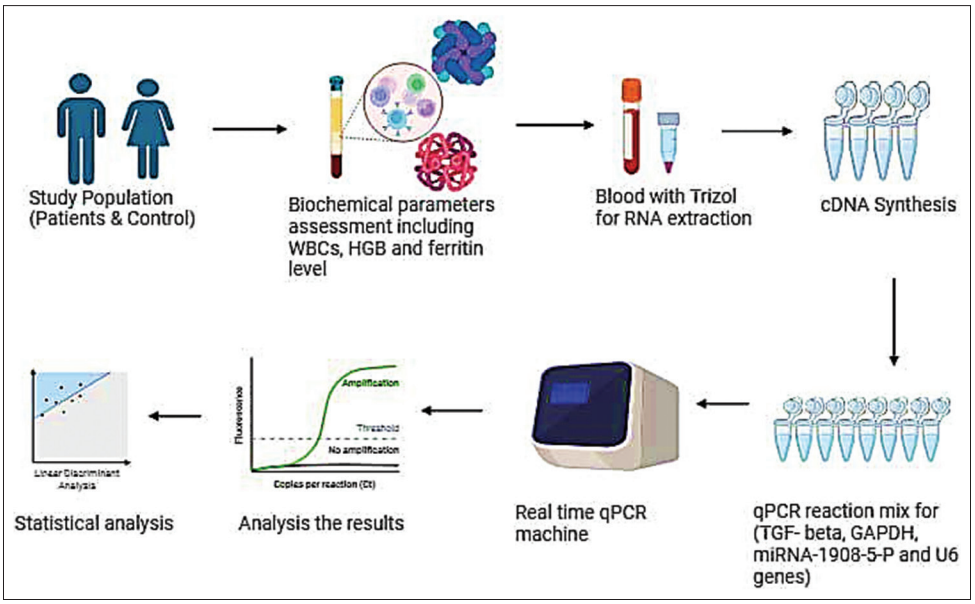

Figure 1: Study proposal of the current manuscript (using Biorender.com)

the RNA from being destroyed. The amount of 250 µl from the EDTA tube was mixed with 750 µl Trizol in an Eppendorf tube.

Biochemical parameters

Biochemical parameters in the current study including ferritin, Hb, and white blood cells (WBCs) were evaluated using a hematology analyzer (Mindary, Germany) and cobas E411/Roche (Roche, Germany).

RNA extraction

Using TransZol Up Plus RNA Kit (ER501-01) (TransGen Biotech company, China) following the manufacturer's procedure, total RNA was directly extracted from the whole blood samples. The concentration and purity of extracted RNA were estimated using the NanoDrop (Thermo Fisher Scientific, USA) to determine the concentration and purity of extracted RNA samples for further evaluation in quantitative real-time polymerase chain reaction (RT-qPCR).

The cDNA synthesis was verified using the EasyScript® One-Step gDNA Removal and cDNA Synthesis (TransGen Biotech company, China) Super Mix method. As required for cDNA synthesis, reaction mix, random primer, anchored oligo dT, genomic DNA remover, RNase-free water, reverse transcriptase, and the remaining total RNA were added as templates. In a thermal cycler, there are three stages: The first involves 10 min at 25°C, the second involves 15 min at 42°C, and the last stage involves inactivating the enzyme for 5 s at 85°C.

The primers used in the present investigation were created specifically for Alpha DNA Company in Canada. The sequences were shipped there in lyophilized form, and they were subsequently diluted with nuclease-free water until the concentration of 10 pmol/µL was achieved. Primers used in the study according to their reference sequence for *TGF-β* were (F-GTACCTGAA CCCGTGTTGCT) and (R-CAACTCCGG TGACATCAAAA), while *miR-1908-5p* (F-CGGCGGGGA CGGCGATTGGTC) and (R-CGAGGAAGAA GACGGAAGAAT).[23] While Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene according to previous investigation,[24] U6 forward and universal reverse primer.[25,26]

Quantitative real-time polymerase chain reaction runs

The QIAGEN Rotor gene Q Real-time PCR System (Qiagen, Germany) was used to accomplish the polymerase chain reactionqRT–PCR. With the (TransStart® Top Green qPCR) Super Mix kit, the expression levels and fold changes of the *TGF-β, GAPDH, U6, and miR-1908-5P* genes were evaluated by calculating the threshold cycle (Ct). The RT-PCR phases and temperature according to[27] with annealing temperature 58℃ for *U6, GAPDH, and TGF-β* while *miR-1908-5p* was 52℃.

Ethical approval

Ethical approval was obtained from the Scientific Committee of the Department of Chemistry and Biochemistry of Mustansiriyah University, College of Medicine with reference number (8363) on December 3, 2023. Before their involvement in the research, every participant signed an informed consent form.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) software version 26 was employed. Fold expression was

detected depending on the ΔCt (Ct of Target gene subtract Ct of Internal control) and ΔCt calibrator (Highest Ct of Target gene for control group subtract mean of internal control gene for control group).[28] The receiver operating characteristic (ROC) test was utilized to assess if the *TGF-β* gene and *miR-1908-5p* function as a diagnostic test, and the correlation test was employed to establish the correlation between each of the two quantitative variables. The statistical significance was evaluated using *P* values that were either equal to or less than 0.05 and considered significant.

Results

The current study showed that the mean ± standard error for age for both patients and healthy people were (12.03 ± 0.51) and (14.23 ± 0.76), respectively, and there were no significant differences with a *P* value of 0.8 (nonsignificant), as shown in Table 1.

The sex investigation for both groups of the current study showed that the males were (22) 44% and (28) 56% for patients and healthy people, respectively, while the percentage of females and males was (24) 48% and (26) 52%, respectively. The results showed that there were no significant differences between them, with a *P* value of 0.5 as in Table 2.

According to the Shapiro–Wilk and the Kolmogorov–Smirnov normality tests, the biomarker parameters of the current study such as Ferritin, Hb, and WBC do not undergo normal distribution. The median ± standard deviation (SD) deviation was measured by the Mann–Whitney *U*-test, as shown in Table 3.

Assessment of the extracted RNA purity and concentration of this study showed high purity (2.0–2.2) of RNA for both patients and control samples. Total RNA was effectively extracted from total participants, the concentration of total RNA ranged from 77 to 89 (ng/μL).

Figure 2a shows the amplification of the *TGF-β* gene, the picture was taken directly from the qPCR real-time apparatus. The Ct values varied between the (21.16–24.08) cycles. Figure 2b shows the gene dissociation curves of the *TGF-β* gene the melting temperatures varied from 78°C to 81°C. The images were captured using the Qiagen Rotor-Gene Q qPCR apparatus.

Figure 3a shows the amplification curve of the *miR-1908-5p* gene, the picture depicted is directed from a Qiagen Rotor gene qPCR real-time apparatus with the Ct value between (11.14–16.24) cycles. Figure 3b shows the gene dissociation curves of the *miR-1908-5p* gene the melting temperatures varied from 78°C to 81°C.

The gene expression level of the *TGF-β* gene and the microRNA was calculated based on the Δct and Δct Calibrator equations of the scientist Levak, where the ΔCt was extracted by subtracting the arithmetic mean of the *TGF-β* gene and its keeping gene and then calculating the fold expression, the *TGF-β* fold expression value was (0.783) in patients indicating downregulation of gene while (1.431) act as fold expression of *miR-1908-5p* in patients group that represent a little upregulation of *miR-1908-5p* expression. The results of this manuscript revealed that there is a noticeable decrease in the gene expression of the *TGF-β* gene, while the expression of *miRs* increased. This may be due to targeting the gene by *miRs*, which reduces the signals and expression of the target gene, and thus we may be able to benefit from it in preparing treatments for thalassemia patients, as shown in Tables 4 and 5. The fold changes in gene expression for both BTK and *miR_1908-5p* between the study groups (control and thalassemia patients) are presented graphically in Figure 4.

Spearman's rho coefficient was used to find the correlation value between *TGF-β* and *miR-1908-5p*, which was (0.197) and there was a significant difference value of 0.04. Finally, a simple linear regression equation was calculated to confirm the significant differences and a negative correlation between the two parameters above, as shown in Figure 5.

To determine whether it was possible to use the *TGF-β* gene or the *miR-1908-5p* as a biomarker, the ROC curve test was performed and the sensitivity and specificity for

Table 1: Mean±standard error of age according to the study group

Groups	Mean±SE	*P*
Patients	12.03±0.51	0.8
Control	14.23±0.76	

SE=Standard error

Table 2: Sex investigation among the study groups

Gender	Groups		χ^2	*P*
	Patients, *n* (%)	Control, *n* (%)		
Male	22 (44)	26 (52)	0.752	0.5 (NS)
Female	28 (56)	24 (48)		
Total	50	50		

NS=Not significant

Table 3: Biomarker parameters in patients and control

Parameters	Groups	Median±SE	*P-value*
Ferritin concentration (ng/mL)	Patients	3313.6±551.06	0.001**
	Control	46.23±2.96	
Hemoglobin (g/dl)	Patients	8.26±0.12	0.001**
	Control	15.2±0.91	
White blood cell(*10^9/L)	Patients	14.27±1.48	0.001**
	Control	6.74±0.26	

*Mann-Whitney U Test used, **Mean high significant (*P*-value ≤ 0.01).
SE=Standard error

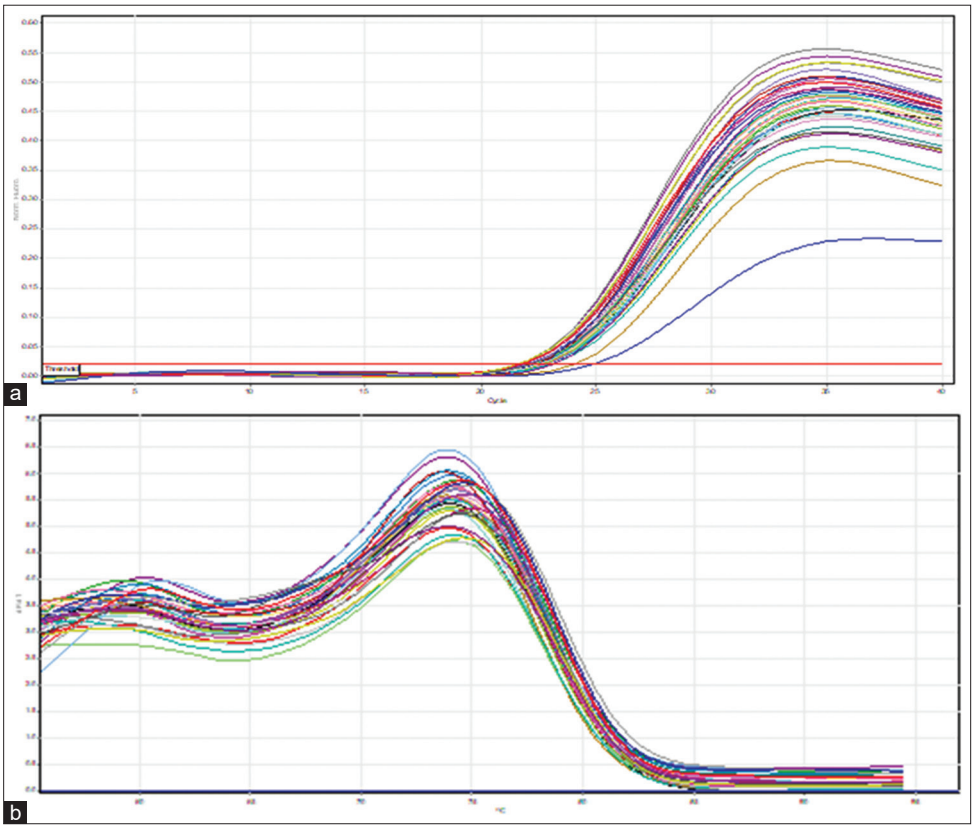

Figure 2: The *TGF-β* gene expression Results. (a) the amplification curve, (b) The dissociation curves

Table 4: Fold expression of transforming growth factor-beta gene and their target miRNA according to Δct method

Group	Mean TG	Mean HKG	Δct	2^{-}Δct	Experimental	Fold	*P*-value
TGF-β gene							
Patients	22.65	14.59	8.063	0.004	0.783	0.783	0.01**
Control	21.97	14.27	7.71	0.005	1.00	1.00	
miR-1908-5p							
Patients	12.03	13.75	0.874	0.546	1.431	1.431	0.2 N.S
Control	16.74	12.04	1.392	0.381	1.00	1.00	

**Mean high significant (*P*-value ≤ 0.01). N.S=non-significant, TG=Target gene, HKG=Housekeeping gene, TGF-β=Transforming growth factor-beta

Table 5: Fold expression of transforming growth factor-beta gene and their target miRNA according to ΔΔct method

Group	Mean TG	Mean HKG	Δct	Δct calibrator	ΔΔct	2^−ΔΔct	Fold
TGF-β gene							
Patients	22.65	14.59	8.063	8.29	−0.2275	1.170804	0.783
Control	21.97	14.27	7.71	8.29	−0.58	1.494849	1.00
miR-1908-5p							
Patients	12.03	13.75	0.874	3.85	−2.97573	7.866526	1.431
Control	16.74	12.04	1.392	3.85	−2.45848	5.496369	1.00

TG=Target gene, HKG=Housekeeping gene, TGF-β=Transforming growth factor-beta

each of them were measured.[29] The sensitivity was 88% and the specificity was 84% for the *TGF-β* gene, which is considered a good indicator for its use as a biomarker, while the sensitivity was 78% and the specificity was 48% for the *miR-1908-5p*, which is considered a failure indicator, as shown in Table 6 and Figure 6.

Discussion

A genetic abnormality in the DNA of the cells that make up Hb causes thalassemia, and this mutation is passed down genetically from parents to offspring. A fatal genetic illness known as β-thal is brought on by

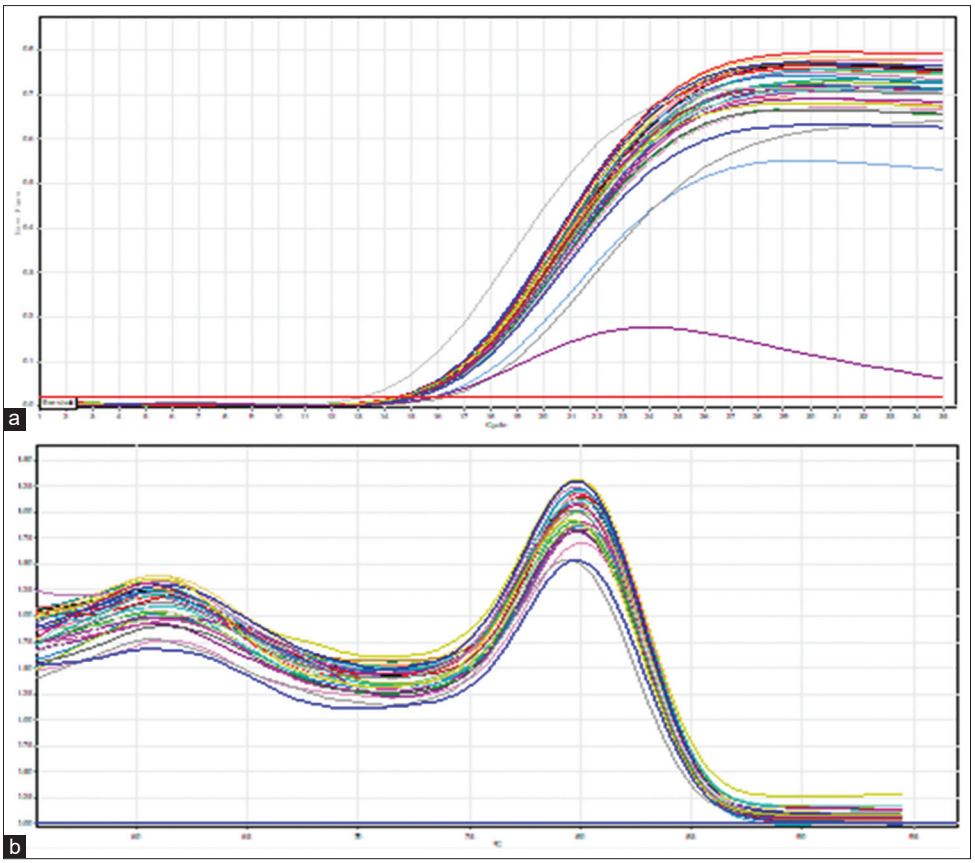

Figure 3: (a) Amplification of *miR-1908-5p* gene, (b) Melting curve of *miR-1908-5p* gene

Table 6: ROC curve of *TGF-β* and *miR-1908-5p*

Parameter	AUC	AUC explanation	Optimal cutoff	SN%	SP%	*P-value*
TGF-β	0.878	Excellent discrimination	22.36	88%	84%	0.0001***
miR-1908-5p	0.534	No discrimination	14.5	78%	48%	0.5 N.S

***Mean a high significant *P*-value (*P*-value ≤0.001), N.S mean non-significant. AUC=Area under the curve, TGF-β=Transforming growth factor-beta

mutations in the β-globin gene.[30] *TGF-β* is a negative regulatory factor in erythrocyte differentiation and maturation, just like erythropoietin.[31] The present study found an elevation in biochemical parameter ferritin and WBCs in patients compared to a control group, this can be explained by hyperactivities of the immune system in thalassemia patients receiving blood frequently from different donors. Alternatively, it may result from a high percentage of broken RBCs both inside and outside the bone marrow, which stimulates the kidney's production of erythropoietin hormone, which in turn stimulates the bone marrow to produce more red and WBCs. In addition, thalassemia disease increases monocyte count, which breaks down the defective RBCs.[32,33] A previous investigation by Abdullah *et al*., 2020 consistent with the current study on WBC and Hb levels which include the WBC count of patients (18.77 ± 13.74), the control group (7.77 ± 1.55) indicates a significant increase of WBC count ($P < 0.01$), the concentration of Hb in patients was significantly lower ($P < 0.01$) in thalassemia patients (8.95 ± 2.88 g/dL) when compared with the control group (13.58 ± 0.86 g/dL). Low Hb levels in thalassemia patients due to genetic mutations in the globin gene disrupt normal Hb production and this study confirmed that, additionally as a complication effect of degradation of RBCs, Hb level is decreased. In patients with transfusion-dependent thalassemia (TDT) and non-TDT, high ferritin levels and low Hb levels were correlated with complications and survival.[34] According to a previous study, there was a significant decrease in Hb ($P < 0.001$) values with mean ± SD (7.2 ± 1.5, 13 ± 1.4) in β-thal patients compared to controls found by Karim *et al*., 2016.[35] Clinical studies have looked into the *TGF-β* gene as a possible target for β-thal; however, the *TGF-β* gene expression level was lowered due to the influence of *miR-1908-5p*. *TGF-β* is a tumor-suppressor gene that functions by causing a variety of cell types to mediate its anti-proliferative effects.[36,37] Beehler *et al*., 2021 showed that *miR-1908-5p* reduces *TGF-β* signaling revealed according to their analysis that the *TGF-β* pathway activity was reduced by 22% in response to *miR-1908-5p*

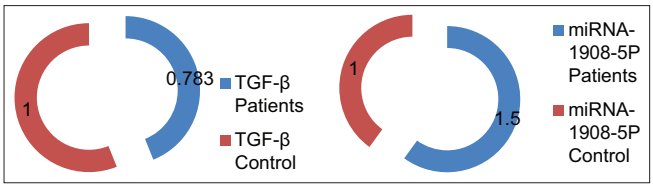

Figure 4: Fold of gene expression of transforming growth factor-beta and *miR-1908-5p* genes

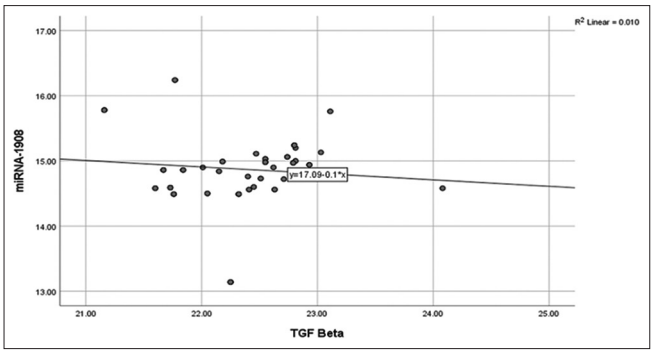

Figure 5: Regression test between transforming growth factor-beta (*TGF-β*) gene and *miR-1908-5p* dependent on equation (Y*miR-1908-5p* = 17.09-0.1X *TGF-β*)

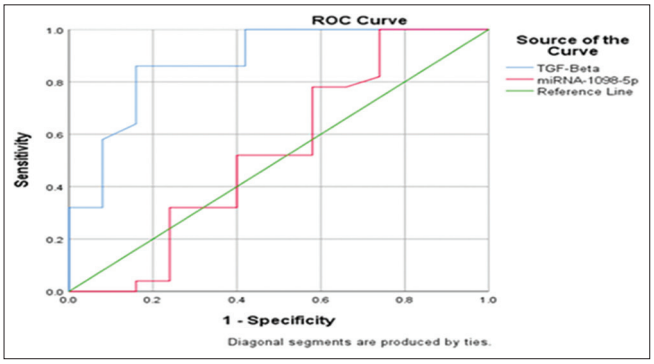

Figure 6: Receiver operating characteristics curve of transforming growth factor-beta and *miRNA-1908*

treatment. They further show that *miR-1908-5p* mimic treatment reduced *TGF-β* expression by 60% specifically. *MiR-1908-5p* reduces *TGF-β* mRNA abundance, thereby reducing the level of BMP1 (bone morphogenetic protein-1) and LDLR (low-density lipoprotein receptor) cleavage this can be done by *TGF-β* treatment.[38]

As a posttranscriptional regulator, *miR-1908-5p* affects the expression of many genes related to iron metabolism, erythropoiesis, and hematopoiesis, and the aberrant expression of *miR-1908-5p* in thalassemia patients suggests its potential as a noninvasive biomarker for disease diagnosis and monitoring. The correlation between *TGF-β* and *miR-1908-5p* was negligible but significant and was confirmed using the regression equation, which also showed a significant difference. Because miR-1908-5p targets the *TGF-β* gene, thalassemia patients' low *TGF-β* gene expression is a biomarker. The regression equation, which also revealed a substantial difference, supported the minor but significant association between *TGF-β* and *miR-1908-5p*. Verification of the ROC curve test results showing *TGF-β* is a useful biomarker for Iraqi thalassemia patients. *TGF-β* gene and *miR-1908-5p* may serve as the indicators for β-thal prognosis, and a coordinated therapy strategy based on gene expression and miRNAs may be employed to effectively treat β-thal. Finally, the complicated interplay between *TGF-β* and miR-1908-5p in thalassemia emphasizes the complicated nature of the condition and the possibility of creating more specialized treatments. Through an examination of the molecular mechanisms that underlie their interaction, investigators may develop a significant understanding of the etiology of thalassemia and discover innovative treatment approaches.

Conclusion

One prospective molecular marker for the initial diagnosis of β-thal might correspond to the *TGF-β* gene. Prior studies highlighted how *miR-1908-5p* is a target of *TGF-β*, which negatively regulates its activity. This demonstrated that the gene may be a promising target for thalassemia management. The *TGF-β* gene may have less expression in thalassemia patients because *miR-1908-5p* targets it, causing patients' expression of the *miR-1908-5p* gene to be higher than that of a healthy control.

Acknowledgment

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Nil.

Conflicts of interest

There are no conflicts of interest.

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