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Original Article

Deciphering the Impact of miR-92a-3p on Controlling BCL11A Gene Expression and Its Implication in Easing Thalassemia Symptoms

Mohammed M. Salih Al-Khafaji, Anas Y. Al-Hayawi

Department of Biology, College of Education for Pure Sciences, Tikrit University, Tikrit, Iraq

Abstract

Background: Thalassemia, a prevalent genetic disorder, is characterized by a point mutation affecting the globin gene expression. One manifestation of this disorder, β-thalassemia, arises from compromised β-globin chain production. **Objectives:** The objective of this research was to explore the presence of miR-92a expression in the bloodstream of individuals affected by severe thalassemia. Moreover, the study aimed to uncover the functional role of miR-92a and its influence on the expression of γ-globin (HGB gene) and oxidative stress within blood cells. **Materials and Methods:** As an initial step, the real-time quantitative polymerase chain reaction (RT-qPCR) was employed to assess gene expression levels subsequent to RNA extraction. The process involved the addition of the polyadenosine monophosphate enzyme Poly-A to facilitate the production of mature messenger RNA (mRNA) from DNA. **Results:** The study revealed significantly elevated miR-92a expression in blood cells. Furthermore, higher levels of gamma-globin, glutathione (GSH), and superoxide dismutase (SOD), along with a decrease in malondialdehyde (MDA) and BCL11A expression, were identified as targets of miR-92a-3p and were downregulated by this microRNA. Additionally, individual knockdown of BCL11A led to increased expression of β-globin (HBB gene), SOD, and GSH, accompanied by a reduction in MDA levels. However, the patterns observed were altered upon subsequent inhibition of miR-92a. **Conclusion:** The findings of this study propose that through the suppression of BCL11A, miR-92a may elevate γ-globin levels while concurrently mitigating oxidative stress in blood cells.

Keywords: BCL11A, gene expression, miR-92a-3p, symptoms, thalassemia

Introduction

Thalassemia, a prevalent genetic disorder, is characterized by a point mutation affecting the globin gene expression. One manifestation of this disorder, β-thalassemia, arises from compromised β-globin chain production. The condition presents in three primary forms: major thalassemia, intermediate thalassemia, and minor thalassemia.[1] Clinical symptoms, like mild microcytic and hypochromic anemia, are closely linked to the severity of the disease. Mediterranean countries often experience the prevalence of thalassemia syndromes and iron overload.[2] In notable cases of β-thalassemia, patients require frequent blood transfusions to manage severe anemia.[3] This inherited blood disorder is widespread across the Mediterranean region, the Middle East, Africa, and Southeast Asia.[4] It results from genetic defects, including mutations or deletions, leading to varying degrees of anemia. β-thalassemia is categorized into three main types: thalassemia minor (also known as β-thalassemia carrier or BTT), β-thalassemia intermedia (BTI), and mediterranean anemia (BTM).[5] Iron overload is a prominent concern in thalassemia syndromes in Mediterranean countries, primarily due to blood transfusions.[6] Patients with β-thalassemia major

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Address for correspondence: Dr. Mohammed M. Salih Al-Khafaji, Department of Biology, College of Education for Pure Sciences, Tikrit University, Tikrit, Iraq.
E-mail: Mohammad.alkhafaji78@tu.edu.iq

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experience severe anemia necessitating frequent blood transfusions, whereas those with BTI have a milder form of the disease,[7] which can lead to various associated conditions, including hepatitis.

MicroRNAs, small endogenous noncoding RNAs typically composed of about 22 nucleotides, play a significant role in regulating gene expression post-transcriptionally.[8] They achieve this by specifically binding to target messenger RNA (mRNA) sequences in the 3'-untranslated region (UTR), coding region, or 5'-UTR.[9] Given that most miRNAs recognize imperfect matches with target mRNAs, each miRNA can have numerous mRNA targets, and conversely, a single mRNA can be regulated by multiple miRNAs.[10] Due to their essential function in biological processes, abnormal miRNA expression has been linked to pathological mechanisms in various liver diseases. Specific miRNA dysregulation, coupled with their roles, has been observed in liver conditions such as HIV/HCV infection, cirrhosis, and hepatocellular carcinoma (HCC).[11]

Materials and Methods

Samples collection

A total of 90 blood samples were collected, comprising 60 samples from individuals diagnosed with β-thalassemia and an additional 30 samples from healthy individuals serving as the control group. The study focused on subjects within the age range of 10–25 years. The blood samples were procured from patients visiting the Hematology and Thalassemia Hospital (currently Al-Hadbaa Specialized Hospital) situated in the Nineveh governorate. This hospital, which caters to over 1500 patients on a regular basis, provided the context for sample collection. Ethical protocols were diligently adhered to, with patient and parental consent secured prior to the sample collection process. Moreover, the study design received formal approval from the hospital's Ethics Committee.

As a basic first step to perform the RT-PCR instantaneous polymerase chain reaction to estimate the level of gene expression, the TransZol Up Plus RNA Kit equipped by TRANS was used.

Poly-A enzyme

Following the RNA extraction procedure, the polyadenosine monophosphate enzyme, Poly-A, is introduced to facilitate the completion of the mature messenger RNA (mRNA) production process. Additionally, this step contributes to the subsequent reactivation of miRNA in the cytosol, adhering to the instructions provided in the pre-designed kit. The Poly-A enzyme was isolated and purified from *Escherichia coli* (*E. coli*) using a recombinant vector that contains the Poly-A polymerase gene. The enzyme's function involves catalyzing the addition of adenosine monophosphate (AMP), derived from adenosine triphosphate (ATP), to the 3' end of RNA, essentially appending a Poly-A tail to the RNA molecule. The prepared mixture is then placed in an incubator at 37°C for 10–20 min. The reaction is subsequently terminated by subjecting the mixture to a temperature of 65°C for 20 min within a water bath.

Preparation of cDNA for RNA samples

Gene expression measurement was carried out using the qRT-PCR technique, employing the two-step thermal cycling method. This approach involves the conversion of single RNA strands into complementary DNA (cDNA) strands. To achieve this, the Easy Script First-Strand cDNA Synthesis kit, provided by TRANS, was utilized, following the instructions provided in the kit. Upon completing the necessary additions, the samples were then transferred to a thermocycler device to initiate the reaction. The thermal cycling program encompassed distinct conditions: an initial denaturation step at 25°C for 10 min, followed by an annealing step at 42°C for 20 min, and finally, an extension step at 85°C for 5 s. Each of these steps constituted a single cycle of the process [Table 1].

U6 is the reference gene for miR-92a, while β-actin was the reference gene for the BCL11A gene, and the prefixes were lyophilized by Macrogene which was diluted as per the instructions of the processing company.

qRT-PCR procedure

The qRT-PCR reaction was conducted at the University of Tikrit's Department of Central Laboratories, utilizing a Germany-origin Biomolecular System device. Prior to this, the cDNA samples were meticulously prepared as per the established procedure. These prepared cDNA samples were placed within a frozen rack tube holder. Subsequently, the reaction mixture was meticulously prepared, drawing from the resources provided by the perfect star green quantitative polymerase chain reaction (qPCR) Super Mix extraction kit, supplied by the TRANS company. The composition of the reaction mixture was determined based on the additives outlined in Table 2. This comprehensive approach facilitated the accurate execution of the qRT-PCR reaction.

Upon completing the required additions, the sample plate was transferred to the designated device, and the

Table 1: RT-PCR primers

Primers	Sequences
BCL11A	F5' – AATTCATCTTCCCTGCGCCA-3' R5' – GCTTAAGTGCTGGGGTTTGC-3'
miR-92a	F5' – TATTGCACTTGTCCCG-3 R5' – GCAGGGTCCGAGGTATTC-3'
U6	F5' – CTCTCGCTTCGGCAGCACA-3 R5' – ACGCTTCACGAATTTGCGT-3'
β-actin	F5' – GGATTCCTATGTGGGCGACGA-'3 R5' – GCGTACAGGGATAGCACAGC-3

interaction was initiated following a specific program. The program's sequence included an initial denaturation step at 94°C for 10 s, succeeded by 45 cycles of a second denaturation at 94°C for 5 s, followed by an annealing step at 60°C for 10 s, and an extension step at 72°C for 10 s. The final extension was carried out at 72°C for 5 min. This comprehensive process was performed in triplicate, and subsequent readings were averaged to ensure accuracy.

Statistical analysis was conducted utilizing the Livak method of $2^{-\Delta\Delta Ct}$. In this context, ΔΔCt represented the difference between the threshold cycle (Ct) value of the target gene and the Ct value of the reference gene for the patient group, subtracted from the same calculation for the control group.

For the determination of human B cell lymphoma/leukemia 11A (BCL11A) levels, an assay kit manufactured by the Chinese company Sunlong was used. Calculations were performed following the steps outlined in the attached instructions.

To measure malondialdehyde (MDA) levels, the researchers used the thiobarbituric acid (TBA) method. This method relies on the reaction of lipid peroxides, especially MDA and TBA, in an acidic medium. MDA levels, which are indicators of lipid oxidation, were measured with this procedure. The methodology for calculating this figure follows the guidelines established by researcher Shah-Guidct.

Estimation of the efficiency of the superoxide dismutase (SOD) enzyme

BCL11A levels were determined using an apparatus provided by (Sunlong Company, China). The filling steps included in the kit were followed, and the experiment was performed. This particular kit used an ELISA sandwich system, which included a pre-coated microplate loaded with specific antibodies targeting the HB-*β* protein.

SOD enzyme activity was evaluated by the modified petro blue tetrazolium (NBT) method. Sodium cyanide was used as an enzyme peroxidase inhibitor in this method. This method relies on indirectly assessing the efficacy of the SOD enzyme by monitoring changes in the optical density of the formazan product formed as a result of O2 depletion. This decrease leads to the formation of nitroblue tetrazolium (NBT) dye. The measurement of this compound is affected by ventilation, according to the protocol established by Brown & Goldstein in 1983. Notably, the decrease in formazan optical density indicates the ability of the SOD enzyme which is increased.

Table 2: qRT-PCR reaction mixture

Primers	Sequences
2 µL	Template cDNA.
10 pmol, 0.5 µL	Forward primer.
10 pmol, 0.5 µL	Reverse primer.
10 µL	*Perfectstart* Green qPCR SuperMix.
7 µL	RNase-free water.

Results

The analysis of the 60 samples comprising the patient group and the 30 samples forming the control group yielded statistically significant differences in relation to age, thalassemia severity (classified as major or intermediate), and Ferritin levels. However, no significant disparity was observed concerning gender. Figure 1 visually represents the relationships between these two sample groups.

The utilization of quantitative real-time quantitative polymerase chain reaction (RT-qPCR) facilitated the evaluation of miRNA-92a within the thalassemia-infected group. The results exhibited positive miRNA-92a expressions, as evidenced by Ct values. These values were compared against a housekeeping gene and a control group. The observed miRNA expressions were characterized by varying levels of fluorescence intensity, spanning from high to moderate. This outcome is depicted through [Figures 1 and 2].

The results of miR-92a melting and amplification curves [Figures 3 and 4] were sharp curves with a narrow top, indicating that pure and homogeneous PCR products were produced. One peak is observed for the amplicon. These results show that the melting curve represents one pure amplicon per sample.

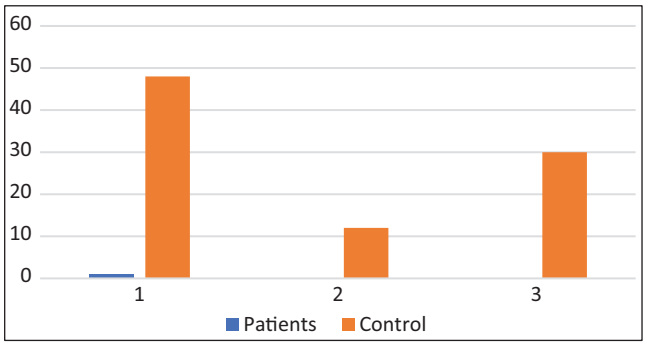

Figure 1: Ct values of miRNA and H.K. genes

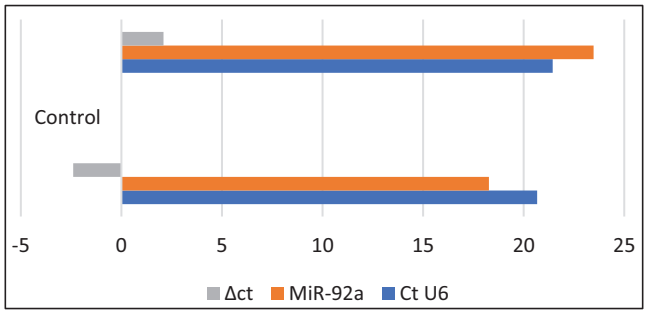

Figure 2: Samples collection

Figure 3: Melting curves

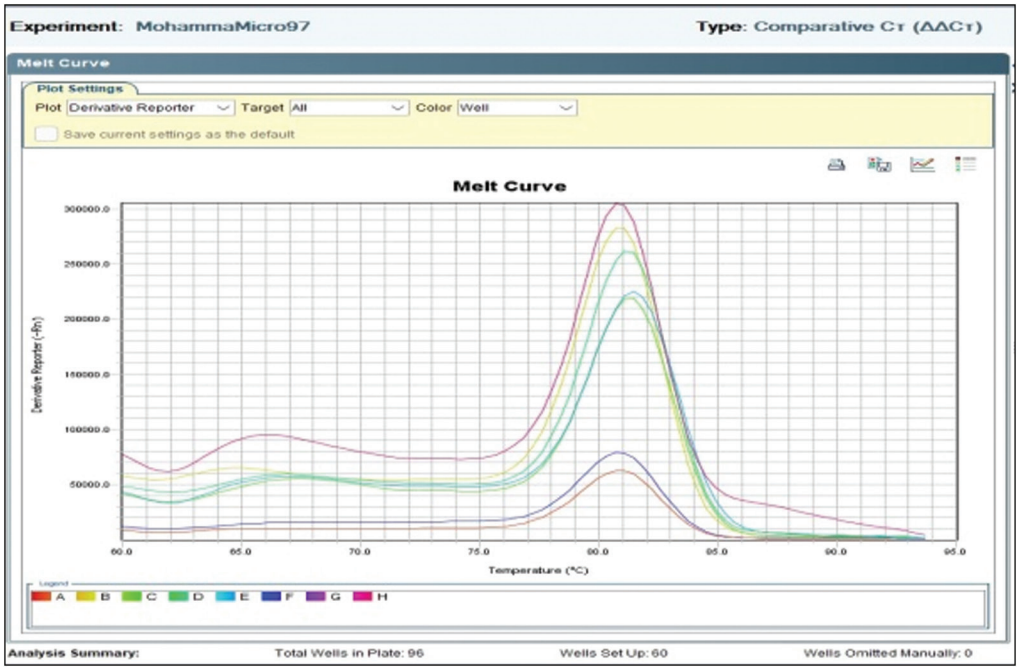

Figure 4: Amplification curve

The results obtained from miRNA92a expression analysis, based on the Livak equation,[12] indicate a substantial increase in expression. The fold expression value of 13.58 reflects a level that is 12 times greater than that observed in healthy individuals. This finding is reinforced by the *t* test results for the mean ± standard deviation (STD) of ΔCt values, which demonstrates a significant disparity between patients and controls. The ΔCt values for patients showed a mean of −1.8116 ± 3.0991, whereas the control group displayed a mean of 1.3693 ± 3.1778. The *P* value, which stands at < 0.00001, further underscores the high significance of these results, with a threshold set at $P < 0.05$.

Table 3 presents a comprehensive overview of miRNA-93a levels compared to the housekeeping (H.K.) gene. Notably, a lower Ct value of 18.27 for miRNA-93a indicates a heightened gene expression level in patients. On the other hand, BLC11A exhibited an increased Ct value of 26.0461, accompanied by a fold expression value of 0.12339, which signifies a lower level of expression. This relationship between Ct values and target gene levels is indicative of

Table 3: The miRNA-92a expression according to the Livak equation

Genes	Fold expression	ΔCt P. Mean ± SD	ΔCt C.	*P* value	Results
miRNA-92	10.78509	−3.431 ± 3.0991	1.3693 ± 3.177	0.0001	The result is significant at $P < 0.05$
BLC11A	0.12339	2.2294 ± 4.6457	4.9148 ± 5.9259	0.020837	The result is significant at $P < 0.05$
γ-globin	9.042677423	−0.253 ± 3.6943	2.5404 ± 4.2779	0.001875	The result is significant at $P < 0.05$

P: Patients (60), C: Control (30)

the inverse correlation observed. Furthermore, the H.K. gene's mean Ct value of 20.33 serves as a reliable reference for detecting miRNA, ideally falling within the range of 20–30 ΔCt as a constant benchmark.

The research highlights the detectability of miRNAs in diverse biofluids, including blood, which has opened up novel avenues for biomarker discovery in thalassemia. Elevated blood miRNA levels are associated with acute thalassemia infections. Taking miR-92a as an example, the study investigated miRNA92a expression in whole blood samples from both patients and healthy volunteers with thalassemia. The results unveiled an upregulation in miRNA-92 gene expression, coupled with a downregulation in BLC11A expression among patients, as compared to the H.K. gene. This genetic control marker for miRNA detection exhibited an inversely proportional concentration to the studied genes. These findings hold potential for the prediction of thalassemia risk.

To investigate the potential impact of miR-92a-3p on γ-globin and oxidative stress within blood cells through the regulation of BCL11A and the γ-globin gene, a comprehensive approach was employed. Using the qRT-PCR and ELISA techniques, the experimental setup aimed to reduce the expression of BCL11A via miRNA action. Consequently, this reduction resulted in a decreased concentration of the BCL11A protein. This downregulation increased the expression of the γ-globin gene, giving a fold expression value of 0.12 for BCL11A and 9.04 for γ-globin.

To determine the extent of oxidative stress, MDA, SOD, and GSH levels were measured in each experimental group. Statistical analysis was performed with a significance of $P < 0.05$, as shown in Figure 5. The results of this study reinforced the aforementioned findings, indicating that miR-92a-3p plays a role in inhibiting BCL11A, increasing HGB gene expression, and subsequently reducing oxidative stress in red blood cells.

To quantify the BCL11A protein, a standard curve was plotted using the absorbance values on the vertical axis and the concentration of the standard solution on the horizontal axis [Figure 6] An Excel program was used to obtain the curve equation, which was then used in determining the absorbance of the samples. The resulting concentrations were measured in units of pg/mL. Analysis of the data from the standard curve

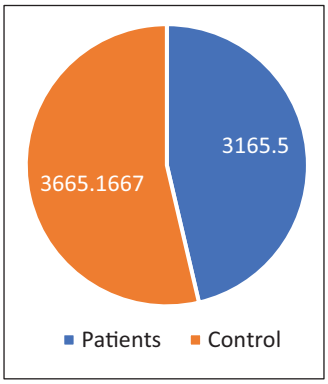

Figure 5: GSH, SOD, and MDA mean concentration

equation provided insight into the concentrations of the samples, revealing an average concentration of 3665.16 in patient samples and 3165.5 in healthy individuals. Statistical assessment indicated that there was no significant difference between patients and healthy subjects (P value of 0.302291 at $P < 0.05$). This slight increase in patient sample concentrations compared to healthy samples aligns with the notion of reduced gene expression activity of the BLC11A protein due to miRNA-mediated silencing. These findings are consistent with the gene expression results discussed earlier [Figure 7].

Impaired biosynthesis of the β-globin chain in thalassemia β leads to the accumulation of alpha-globin chains that are incompatible with β-globin. This results in the failure of hemoglobin formation, as well as iron overload due to frequent blood transfusions and oxidative stress-induced tissue injuries. The findings from the present study demonstrated a slight increase in the oxidizing factor test (SOD) for thalassemia patients compared to healthy individuals. The average value of the serum samples for patients was 2.8514 ± 1.8107, whereas the results for healthy samples were 2.4105 ± 1.6228. However, this difference was not statistically significant at $P < 0.05$ ($P = 0.263161$). Figure 5 illustrates the levels of glutathione (GSH), where the mean value of serum samples for patients was 36.832 ± 1.8107, while the results for healthy samples were 23.7071 ± 1.7499. There was a significant difference at $P < 0.05$ (P value = 0.027561) between these groups. Furthermore, the study revealed a notable increase in the oxidizing factor test (MDA) for thalassemia patients

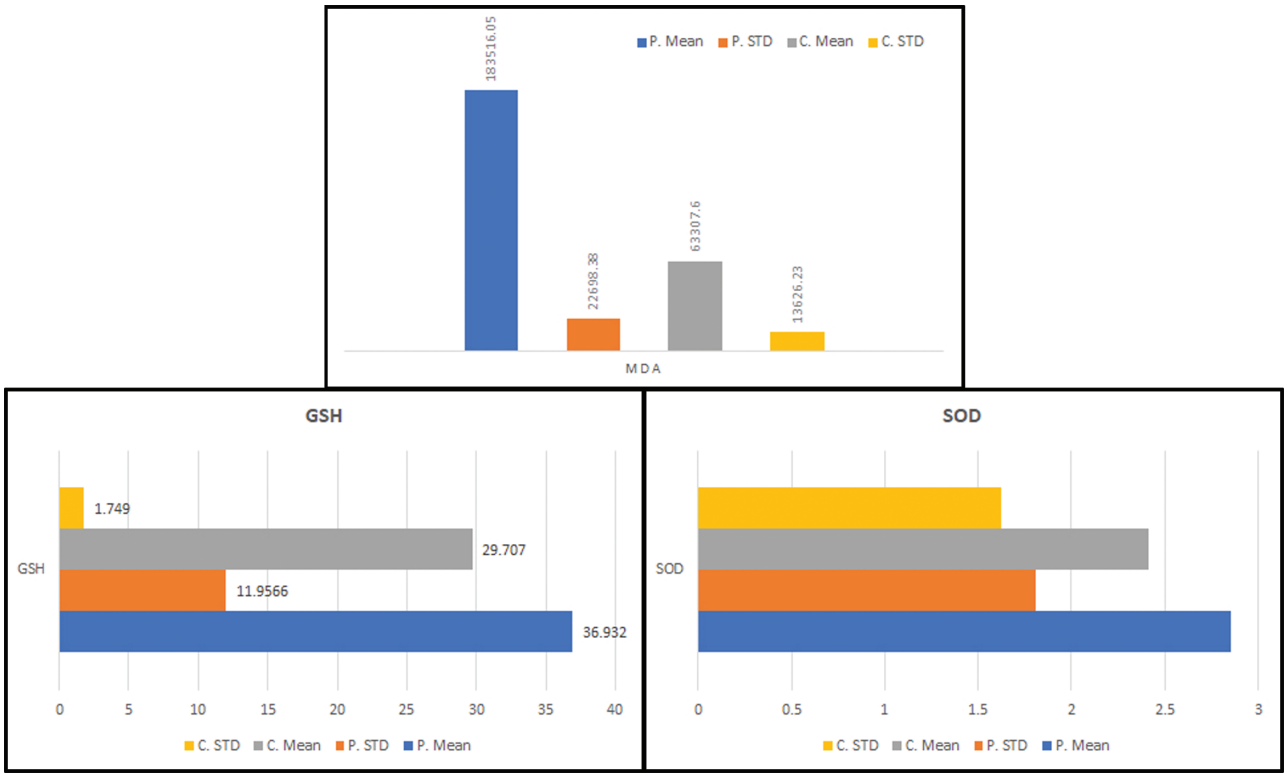

Figure 6: Standard curve for BLC11A gene

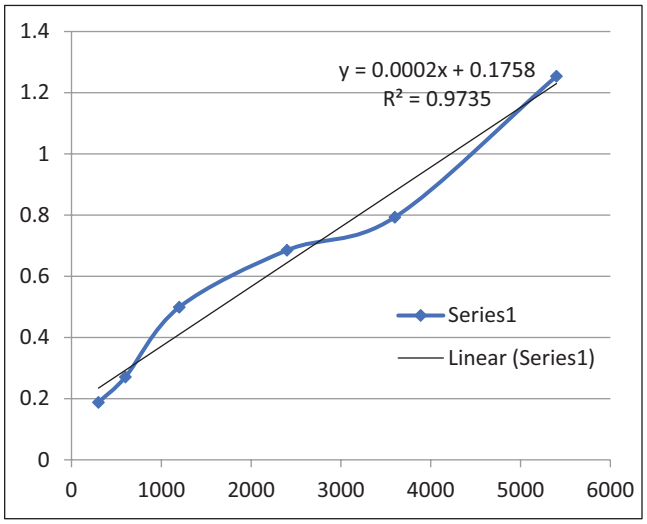

Figure 7: Concentration of BLC11A gene

compared to healthy individuals. The mean value of serum samples for patients was 22.698 ± 183516.05, whereas for healthy samples, it was 63307.6 ± 13.626. This difference was highly significant at a probability level of <0.05 ($P < 0.00001$).

Discussion

The imbalance of globin chains resulting in an excess of iron can exacerbate oxidative damage in red blood cells (erythrocytes). It is established that increasing levels of HbF can alleviate symptoms of β-thalassemia.[13,14] Moreover, it is widely recognized that BCL11A suppresses the production of γ-globin,[15] as well as γ-globin.[16] This study aimed to elucidate the role of miR-92a-3p in γ-globin production and the significance of both BCL11A and miR-92a. The study findings demonstrated that miR-92a-3p enhances γ-globin production and reduces oxidative stress in blood cells. Specifically, our data revealed that miR-92a regulates the expression of HGB which encodes γ-globin and oxidative stress in β-thalassemia blood cells.

Expression of miR-92a-3p was found to be higher in blood cells of β-thalassemia patients compared to healthy controls. Notably, miR-92a-3p expression was positively correlated with HbF levels in these patients. Growing evidence suggests that microRNAs such as miR-144, miR-326, and miR-2355-5p play a physiological role in controlling γ-globin (HGB) expression.[17,18] Similarly, our study unveiled that overexpressing miR-92a-3p could elevate the expression of the γ-globin (HGB) gene in β-thalassemia blood cells, while downregulating miR-92a-3p consistently inhibited β-globin expression. This investigation contributes to the understanding of miR-92a-3p's influence on γ-globin.

In individuals with thalassemia, abnormal accumulation of reactive oxygen species (ROS) signifies oxidative stress in erythrocytes and is associated with morphological abnormalities in these cells.[19] One metabolic mechanism

triggered by ROS is apoptosis, which contributes to thalassemia pathophysiology.[20] Previously, it was posited that miR-92a-3p could intensify oxidative stress and apoptosis caused by low-density lipoprotein in human umbilical vein endothelial cells.[21] In rats with catheter-related thrombosis, resistance training led to the downregulation of miR-92a-3p along with a reduction in MDA activity and ROS generation.[22]

These findings establish a connection between oxidative stress damage and miR-92a. The study initially demonstrates that overexpression of miR-92a-3p reduces oxidative stress in blood cells. It is known that inhibiting BCL11A binding can enhance gamma-globin gene expression in mice, as BCL11A functions as a transcriptional regulator of the gene.[23] In this study, the knockdown of BCL11A led to an accumulation of gamma-globin. Furthermore, functional experiments illustrated that suppressing BCL11A prevented oxidative stress and cell death in erythroid progenitor cells. This connection between BCL11A and oxidative stress is supported by the vulnerability of midbrain dopaminergic neurons expressing BCL11A to neurodegeneration under oxidative stress conditions.[24] Notably, this study is the first to demonstrate BCL11A's impact on oxidative stress in β-thalassemia.

Numerous investigations have suggested that microRNAs (miRs) can interfere with BCL11A expression to influence gamma-globin production. For instance, elevated levels of miR-30a enhance gamma-globin expression by inhibiting BCL11A in the blood cells of individuals with β-thalassemia.[25] MiRNA-210, by suppressing BCL11A mRNA, is involved in blood cell differentiation and the activation of the gamma-globin gene.[25] Through bioinformatics prediction, luciferase reporter assay, and AGO2-RIP test, BCL11A was identified as a target gene of miR-92a-3p. The negative correlation between miR-92a-3p and BCL11A levels was confirmed through Pearson correlation analysis, as well as gain- and loss-of-function experiments. Importantly, compared to solely knocking down BCL11A, simultaneous suppression of miR-92a-3p and BCL11A resulted in increased MDA levels, decreased SOD and glutathione (GSH) levels, and accelerated erythrocyte apoptosis in β-thalassemia blood cells. By inhibiting BCL11A mRNA, it was demonstrated that miR-92a enhances gamma-globin expression and reduces oxidative stress in blood cells. These results support the association between miR-92a-3p and BCL11A, highlighting a potential role for miR-92a in regulating gamma-globin expression. This suggests a novel approach for treating β-thalassemia by modulating miR-92a function. To advance the clinical management of β-thalassemia, further comprehensive animal studies are urgently needed to elucidate the roles of the miR-92a/BCL11A axis *in vivo*.

Conclusion

This study focused on the role of miR-92a-3p in severe thalassemia, with particular emphasis on the effects of HGB γ-globin expression and oxidative stress in red cells. Increased globin levels and reduced oxidative stress in red cells were observed. The study also showed that miR-92a-3p targets BCL11A, which is known to inhibit γ-globin synthesis, and its inhibition resulted in γ-globin secretion expressed was further increased influencing oxidative stress levels. These findings confirm the potential of miR-92a-3p as a therapeutic target for the management of thalassemia by modulating γ-globin expression and reducing oxidative stress. By shedding light on the complex relationship between miR-92a-3p, BCL11A, γ-globin, and oxidative stress, this review addresses the challenges posed by thalassemia. Using a novel approach for the development of alternative strategies, the validation of these findings requires further investigation through *in vivo* studies to pave the way for potential future therapeutic applications.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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