Original Article

Circulating MicroRNA-146a as a Biomarker Related to Oxidative Stress in Thalassemia Patients

Nawal Khinteel Jabbar and Heba Hani Hamzah

ABSTRACT

Objective: To examine the miRNA-146a expression in thalassemia patients with chronic anemia and its relationship to oxidative stress in these individuals.

Study Design: Case-control study

Place and Duration of Study: This study was conducted at the Diwaniyah Women and Children Teaching Hospital and the University of Al-Diwaniyah Al- Qadisiyah, College of Sciences, Iraq from October 2022 to February 2023.

Methods: A total of 80 thalassemia patients and 40 healthy controls, measuring serum ferritin levels with the Cobas e411 analyzer and interleukin-6 using sandwich immunodetection. It also assessed advanced oxidation protein products (AOPPs) colorimetrically, along with catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA) levels. Furthermore, serum miRNA-146a expression was analyzed through quantitative polymerase chain reaction.

Results: When comparing patients to controls, serum ferritin levels increased considerably ($P<0.05$), and patient groups had significantly higher activity levels of SOD, CAT, AOPP, and MAD. Patients also had higher levels of the gene miRNA-146a ($p< 0.05$).

Conclusion: There is a reciprocal relationship between increasing oxidative stress and the expression of the miRNA-146a gene, contributing to disease onset and enhancing antioxidant enzyme effectiveness. In thalassemia, the pathophysiology of oxidative stress drives the expression of miRNA-146a.

Key Words: Oxidative Stress, Thalassemia, MicroRNA

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INTRODUCTION

Thalassemia is a hereditary form of chronic anemia characterized by a reduction or absence of α- or β-globin chain synthesis, leading to hemolytic, hypochromic, microcytic anemia. Beta thalassemia is specifically caused by various point mutations on chromosome 11 affecting the β-globin gene. It is categorized into three primary categories depending upon their clinical presentation: major, intermedia, and minor.[1]

Oxidative stress occurs when the production of reactive oxygen species (ROS), like free radicals and ions, exceeds the ability of natural antioxidants to neutralize them, resulting in an excess of oxidants.[2]

[1.] Department of Chemistry, College of Sciences, Al Qadisiyah University, Iraq.

Correspondence: Heba Hani Hemzah, Department of Chemistry, College of Sciences, Al Qadisiyah University, Iraq.
Contact No: 0785 531 5583
Email: university/Iraqhibahani984@gmail.com

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Overproduction of ROS can be detrimental since it readily reacts with biological systems' lipids, proteins, and DNA.[3] Reactive oxygen species (ROS) are crucial for initiating signaling pathways that lead to cell damage and death, either through direct harm to biomolecules or by altering proteins and genes.[4] Antioxidant enzymes are crucial for detoxifying free radicals and reducing oxidative stress by facilitating the removal of reactive oxygen species (ROS).Superoxide Dismutase (SOD) is an antioxidant protein that converts superoxide anions into hydrogen peroxide (H_2O_2), which is subsequently detoxified into oxygen and water by catalase or glutathione peroxidise.[5]

AOPPs are oxidative indicators that can trigger inflammation by activating monocytes. MDA is a ketoaldehyde produced from the peroxidative breakdown of unsaturated lipids during arachidonate metabolism.[6]

MicroRNA (miRNA) is an endogenous gene that produces small RNA molecules that bind to specific target RNAs, leading to their decay or repression of translation.[7] significant roles in controlling gene expression as a number of human disorders develop. MiRNAs play a crucial role in maintaining erythroid homeostasis and regulate the expression of α-, β-, and γ-globin genes. It is important to examine how miRNAs influence globin expression in β-thalassemia.[8]

METHODS

The study involved 120 respondents divided into two groups: healthy controls and TM patients. Data collected included age, sex, splenectomy status, and other health issues, with the control group carefully selected to ensure no prior history of diabetes, hypertension, or other conditions. The study documented patients' age, gender, BMI, smoking status, family history, and any medications. It included laboratory test analyses from Nabu Scientific Foundation in Baghdad, the Thalassemia Center's Women and Children Teaching Hospital in Diwaniyah, and the Biochemistry Lab at Al-Qadisiyah University's College of Sciences. Patients with splenectomy, infections, chronic bone inflammation, liver failure, cardiac disease, and other blood conditions were excluded from this study.

Five milliliters of blood were collected from each subject, divided into two tubes, with four milliliters placed in a gel tube. The serum was extracted by centrifugation at 3600 rpm for 10-15 minutes and divided into four parts: one stored at -40°C for miRNA-146a analysis and the others at -20°C. Additionally, 1 mL of blood was collected in a K2EDTA tube for a complete blood count (CBC).

Spectrophotometry was used to determine the serum's activity (SOD). The calculation of (CAT) was done using UV spectrometry. Spectrophotometry was used to calculate the concentrations of (MDA).[9] AOPP's concentration was measured using UV spectroscopy.[10] Serum levels of miRNA-146a were measured using quantitative polymerase chain reaction (qPCR). RNA was extracted from 0.3 mL of serum using TRIzole™ reagent, and cDNA was generated using the Protoscript® first-strand cDNA synthesis kit from NEB, UK. The Polymerase Chain Reaction was conducted using Luna Universal qPCR Master Mix from NEB, UK. The complementary DNA produced was combined with forward and reverse universal primers for miR-146a and the cDNA Bright Green master mix. As an internal control, gene U6 was used. In order to determine the proportional levels of miR-146a and ($2^{-\Delta\Delta Ct}$), (2(-Ct)), A threshold cycle (Ct) by comparison was utilized, and A fold change in expression was observed in the results (Table 1).

SPSS version 26 was used for all analyses. A t-test assessed continuous data with a normal distribution. The chi-square test compared categorical variables, reporting data as frequency and percentage. The Pearson correlation test linked normally distributed quantitative data, examining the relationship between two continuous variables with a correlation coefficient (r) and a significance level of $P < 0.05$.

RESULTS

Patients with TM had slightly lower body mass index (BMI) values compared to controls. White blood cell (WBC) levels were significantly higher in the TM group, while hemoglobin (Hb) and red blood cell (RBC) levels were considerably lower. Additionally, the TM group exhibited higher random blood sugar (RBS) and packed cell volume (PCV) levels, but lower mean corpuscular volume (MCV) compared to controls. The mean corpuscular hemoglobin (MCH) was significantly higher in the control group, with a p-value greater than 0.05 (Table 2).

SOD activity was elevated in the TM group as compared to the control group (Fig. 1). CAT activity was elevated in TM as compared to the control group (Fig. 2). AOPP levels were significantly higher in the TM group compared to the control group, which also had elevated MDA levels (Figs. 3-4). Figure 5 presents a study comparing serum ferritin levels between patients and healthy controls. Additionally, qPCR miRNA analysis revealed that the TM group had significantly higher serum levels of miRNA-146a expression than the control group.

Tables 3 show the relationships between miRNA-146a and indicators of oxidative stress in patients with thalassemia (SOD, Catalase, MDA, and AOPP). The current findings indicate no significant relationship between miRNA-146a and any of the factors (Table 4).

Table No.1: Quantitative PCR analysis primers

Primers	Sequence	Product Size (bp)
miR-146_RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACT GGATACGACAACCCA	
miR-146 For miR-146 Rev	GGGTGAGAACTGAATTCCA CAGTGCGTGTCGTGGAGT	
U6 For U6 Rev	CTCGTTCGGCAGCACA AACGCTTCACGAATTTGCGT	94

Table No.2: The demographics of thalassemia patients and healthy control participants

Characteristic	**Patients (*n* = 80)**	**Healthy control (*n* = 40)**	***P***
Age (years)			
Mean ±SD	12.47±6.42	14.60±6.49	0.092† (NS)
Range	2 – 33 years	4 – 30 years	

<12, *n* (%)	37 (46.3%)	14 (35.0%)	0. 498¥ (NS)
12-17, *n* (%)	27 (33.7%)	16 (40.0%)	
≥ 18, *n* (%)	16 (20.0%)	10 (25.0%)	
Sex (M/F)			
Male, *n* (%)	46 (57.5%)	18 (45%)	0.196¥ (NS)
Female, *n* (%)	34 (42.5%)	22 (55%)	
Body mass index (BMI) (Kg/m^2)			
Mean±SD	12.41±4.07	15.73±4.87	0.001† (S)
Range	4.72–22.70	6.7–25.8	
White blood cells count µL			
Mean±SD	14.74 ± 19.03	9.04±11.33	0.101† (NS)
Range	2.57 – 109.20	4.10- 78.00	
Red Blood Cells count µL			
Mean ±SD	3.12±0.49	4.70±0.49	< 0.001† (HS)
Range	1.68 – 4.94	3.89- 5.74	
Haemoglobin (Hb) g/dl			
Mean± SD	7.92±1.22	12.92±1.72	< 0.001† (HS)
Range	3.70 -11.10	10.01-16.70	
Packed cell volume (PCV) %			
Mean± SD	24.01±3.31	38.80±4.82	< 0.001† (HS)
Range	13.60 -30.60	30.10-49.10	
Mean Corpuscular Volume (MCV) fl			
Mean± SD	76.18±6.53	82.11±5.51	< 0.001† (HS)
Range	51.30 -88.50	67.10-91.30	
Mean corpuscular hemoglobin (MCH) pg			
Mean± SD	26.42 ± 5.99	27.57± 2.37	0.247†(NS)
Range	18.90 -75.50	20.80-31.10	

n: number of cases; SD: standard deviation; †: independent samples t-test; HS: Highly significant at P ≤ 0.001. NS: not significant at P > 0.05

Table No.3: Correlation between miRNA-146a and oxidative stress parameters (SOD, CAT, MDA and AOPP) in patients with thalassemia

Oxidative stress parameters	miRNA-146a	
	r	P
SOD	0.172	0.314
Catalase	0.161	0.407
MDA	0.017	0.917
AOPP	0.208	0.279

Table No.4: Correlation between serum ferritin, antioxidant enzyme and oxidative stress parameters (SOD, CAT, Catalase, MDA and AOPP) in patients with thalassemia

Oxidative stress parameters	Serum ferritin	
	R	P
SOD	0.198	0.078
Catalase	0.092	0.416
MDA	0.020	0.857
AOPP	0.297*	0.039

r: correlation coefficient.

Figure No. 1: SOD levels in healthy controls and patients

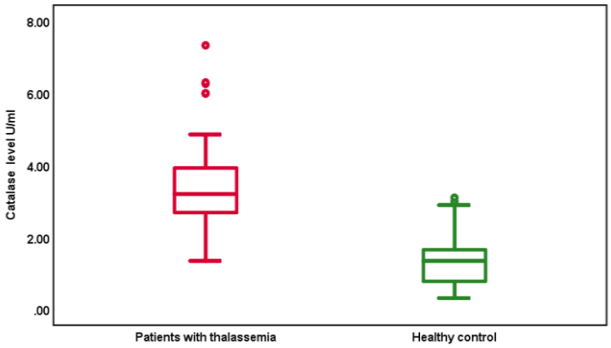

Figure No. 2: Catalase levels in healthy controls and patients

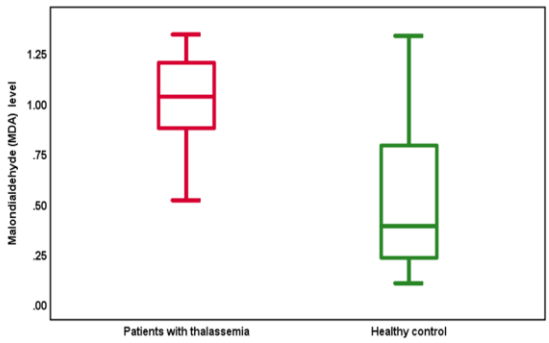

Figure No. 3: The levels of malondialdehyde (MDA) in patients and healthy controls

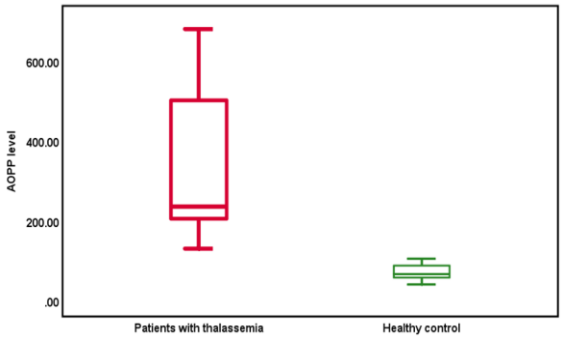

Figure No. 4: AOPP levels for both healthy controls and patients

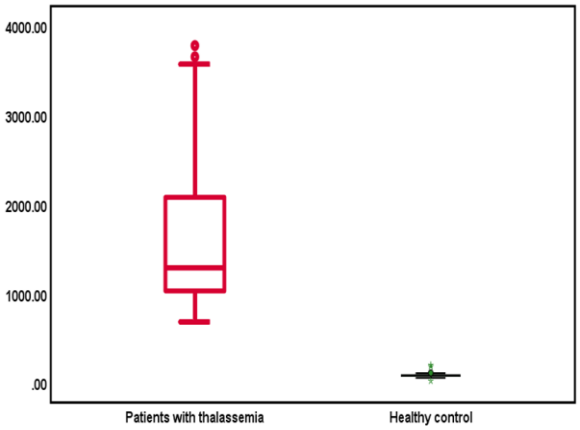

Figure No. 5: Serum ferritin levels in healthy controls and patients

DISCUSSION

In thalassemia disorders, oxidative stress is primarily caused by redox reactions of hemoglobin involving hydrogen peroxide (H_2O_2) and superoxide anion radicals (O2•). The Haber-Weiss process leads to the formation of molecular oxygen and hydroxyl radicals (•OH), with Fe3+ catalyzing this reaction and potentially generating •OH. Additionally, the absence of the β-globin chain causes unpaired α-chains to self-aggregate, further damaging oxidized membranes and affecting immature erythroblasts in the bone marrow. Thalassemia red cells exhibit in vivo oxidative damage and heightened susceptibility to external oxidant stress.[11] Thalassemic red blood cells (RBC) may experience biochemical and metabolic changes due to chronic oxidative stress, which is linked to the accumulation of excess alpha-globin chains, iron decompartmentalization, and the release of free iron.[12]

Patients with thalassemia were found to have elevated plasma malonyldialdehyde (MDA) levels, as determined by the spectrophotometry method. MDA indicates significant oxidative damage. A previous study found that thalassemia major patients receiving regular transfusions had higher levels of free and total MDA compared to thalassemia intermedia patients.[13] Another study showed lipid peroxidation products (such MDA) were more prevalent in thalassemia patients[14]. The research found elevated plasma MDA levels, suggesting that ongoing blood transfusions may lead to peroxidative tissue damage due to secondary iron overload in patients.

SOD is a protective antioxidant, Researchers previously discovered increased SOD activity in thalassemia patients.[15] In vivo lipid peroxidation is caused by thalassemia and the concomitant iron overload, and SOD and glutathione peroxidise (GPx) levels rise as a result of the compensatory rise in lipid peroxidation.[16] Compared to both healthy individuals and beta-thalassemic carriers, the catalytic activities of SOD and GPx were significantly greater in beta-thalassemic erythrocytes. Increased AOPP levels have been linked to monocyte activation, according to reports.[17] Previous studies have shown that elevated red cell SOD levels in thalassemic individuals are a response to or a compensatory mechanism for the elevated generation of superoxide radicals.[18] While the catalase result in β-thalassemia patients was much higher than in controls, this result was consistent.[19] A plausible explanation for the higher red cell catalase levels seen in the more severe genotype of β-thalassemia. Higher concentrations of hydrogen peroxide can directly damage catalase.

Ferritin 4-4 h and ferritin 4-24 h groups had significantly higher serum AOPP levels than the control group, supporting previous findings of elevated AOPP levels in thalassemia patients. It highlighted a correlation between serum ferritin and AOPP levels, as well as between blood transfusions and AOPP levels. Furthermore, ferritin and MDA levels were significantly higher in transfusion-dependent thalassemia (TDT) compared to non-transfusion-dependent thalassemia (NTDT). Furthermore, it was discovered that the concentrations of miRNAs in serum were constant, repeatable, and consistent between members of the same species showed that serum miR-146a levels were considerably higher in thalassemia patients.[20]

CONCLUSION

The globin gene expression could change the classification of thalassemia and alleviate its severe symptoms. Identifying miRNAs associated with the

disease's development is valuable for developing new diagnostic markers and treatment strategies for thalassemia.

Author's Contribution:

Concept & Design or acquisition of analysis or interpretation of data:	Nawal Khinteel Jabbar, Heba Hani Hamzah
Drafting or Revising Critically:	Nawal Khinteel Jabbar, Heba Hani 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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