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The Harmony of Iron Regulating Genes and Genes of Antioxidant Enzymes in Thalassaemia Patients

Nuha S. Thanoon[1], Mohammed K. J. Alnori[2*], Ali S. Alchalabi[3]
[1]*Ninevah Health, Ninevah, Iraq*
[2]*College of Pharmacy, University of Mosul, Mosul, Iraq*
[3]*College of Veterinary Medicine, University of Mosul, Mosul, Iraq*

Abstract

The purpose of this work was to investigate the expression of iron-regulating genes and their relationship to enzymatic antioxidant genes in thalassaemic patients, as well as to detect distinct forms of thalassaemia using an RT-PCR technique using a presence/absence protocol. This study included 50 patients who were admitted to Mosul's Al-Hadba'a Hospital. Ten healthy subjects and forty thalassaemic patients were aged eight to seventeen years. Freshly blood samples were collected using EDTA for molecular processes, as gene expression and genomic identification of thalassaemia types. The outcomes indicated that the foxO1 gene was significantly upregulated in thalassaemic patients compared to healthy participants, but hepcidin expression was non-significantly downregulated. Similarly, the enzymatic antioxidant gene GSH-Px1 demonstrated significant downstream regulation expression in thalassaemic participants compared to healthy ones, even though the expression of SOD1 and CAT antioxidant enzyme genes did not differ across investigated patients. Catalase expression is associated inversely with foxO1 expression. The presence/absence approach showed that 8.4% and 5.1% of individuals had positive α-thalassaemia based on α1 and α2 mutations in their gDNA samples, respectively, compared to healthy patients. Furthermore, 33.6%, 36.5%, and 16.4% of blood samples with an unknown type of thalassaemia tested positive for β-thalassaemia because their gDNA samples had codon8/9, codon 41/42, and IVS-I-5 mutations, respectively. Our findings suggest that the RT-PCR approach is the most effective for studying gene expression and molecular identification of thalassaemia types.

Keywords: *Antioxidant Enzymes, Foxo1, Hepcidin, Thalassemia.*

Introduction

Many biological functions require iron, a mineral that can be hazardous in excess. The body has to control how much iron it gets from food since it cannot expel excess iron [1]. Hepcidin is a hormone produced in the liver that mediates this control [2]. Additionally, hepcidin regulates the release of iron from hepatocytes, which store iron, and macrophages, which recycle iron. Hepcidin inhibits the release of iron into the plasma by binding to ferroportin, the sole known iron export protein, and causing its internalization and destruction [3]. The following factors significantly influence hepcidin and, by extension, systemic iron homeostasis: body iron storage, infection and inflammation, hypoxia and erythropoiesis, and, to a lesser extent, testosterone [4].

Overproduction of hepcidin leads to iron-restricted anaemias in chronic inflammatory diseases and inherited iron-refractory anaemia, while deficiency causes iron overload in hereditary hemochromatosis and non-transfused β-thalassemia [5]. Strong radicals can be created from relatively stable oxidants

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****Corresponding Author: alnorimkj@uomosul.edu.iq***

by the majority of transition metals, including iron. The Fenton and Haber-Weiss reactions are the mechanisms by which excess iron catalyzes the production of hydroxyl radicals from activated oxygen species [6]. Thalassaemia patients are highly vulnerable to free radical damage. Thalassaemia causes oxidative damage due to free radical generation, lipid peroxidation, and iron toxicity [7]. In vitro, unstable haemoglobins have been shown to produce free oxygen radicals. The more methemoglobins these free radicals produce, the more reversible and irreversible hemichromes are formed [8]. Non-hemoglobin iron levels increase in thalassemic RBCs [9]. The Fenton reaction catalyzes lipid and protein peroxidation using free irons, ferritin aggregates, and hemosiderin deposits.

The World Health Organization (WHO) lists thalassaemia as one of the most common hereditary disorders worldwide, with an estimated 7% global carrier prevalence. It is highly common in the Middle East and Mediterranean region, with estimated carrier rates as high as 30%. The result of thalassaemia syndromes, which are inherited as autosomal recessive disorders, is defective globin synthesis [10]. There are several ways they might manifest themselves, ranging from carriers with no symptoms to severe thalassaemia requiring regular blood transfusions. One allele mutation is the cause of the carrier status known as the thalassaemia trait [11]. The molecular characterization of two types of thalassemic abnormalities in the target population is a necessary precursor for the construction of an effective prenatal diagnosis service. Thus, the study's goal is to explore the link between iron-controlling genes and certain enzymatic antioxidant genes alongside the molecular identification of two forms of thalassaemia based on the presence or absence of specific mutations in hemopathy patients using an RT-PCR approach.

Materials and Methods

Ethical Approval

The Mosul City Health Department issued an ethical authorization under No. (48386). This study involved 50 individuals who were presented to the Al-Hadba'a Hospital in Mosul. Ten healthy participants and seventy thalassaemic patients ranged in age from eight to seventeen years. The hospital provided blood samples meticulously and cautiously. Iron and enzymatic antioxidant genes were expressed using RT-PCR on a portion of the blood samples after extracting total RNA; the remaining portion was utilized for gDNA extraction and then preserved in a deep freeze at -80°C for molecular study.

RT-PCR Analysis for Gene Expression

The quantitative endpoint for real-time PCR is the threshold cycle (Ct), which is defined as the PCR cycle at which the fluorescent signal of the reporter is detected. The data obtained from reverse transcription-PCR (RT-qPCR) were compared to the expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as an endogenous control (relative quantification). The initial stage in the PCR process was to isolate total RNA using an Addbio RNA extraction kit and following the manufacturer's instructions. The isolated RNA was then converted to cDNA using AddScript Rt Master (Addbio, Korea) reverse transcriptase. Priming took place at 25°C for 10 minutes, followed by reverse transcription at 50°C for 60 minutes, RT inactivation at 80°C for 5 minutes, and holding at 12°C. The cDNA samples of the iron-controlling genes (hepcidin and foxO1 genes) and enzymatic antioxidant genes (catalase, GSH-Px1, SOD1) were used for the last stage of RT-PCR (table 1), which was done using the Step-One Applied Biosystems tool system (USA). A final amount of 25 µl was used for each reaction, which included 5 µl of cDNA, 0.5 µmol of sense and antisense specific

primers (2 µl per primer), 5.5 µl of PCR water, and 12.5 µl of Add SYBER Master (Addbio, Korea). The template cDNA was denaturized with a 3-minute pre-incubation at 95 °C. This was followed by 40 cycles of amplification: denaturation (15 s at 95 °C), annealing (15 s at 60 °C), and extension (30 s at 72 °C). At 72 °C, fluorescence was measured after each cycle. Each experiment had a negative control run that did not employ any cDNA templates. Plotting the Ct values (cycle threshold) against the log cDNA dilution resulted in the creation of standard curves for each target and monitoring gene. This made it possible to perform relative quantification after PCR. The data was retrieved as Ct values, and the fold change was calculated with the 2^-(ΔΔCt) method. The gene expression was calculated using the following formula: Target gene = 2−1Ct, i.e. [2− (Ct target gene−Ct GAPDH)].

Table 1. Sequences of Studied Genes for Gene Expression

Hepcidin	CAC AACAGACGGGACAACTT	F
	CGCAGCAGAAAATGCAGATG	R
FoxO1	AACTTTCGCTTAGTGGAACGT	F
	ACCCTCATACCTTTGGAACAG	R
Catalase	GTTACTCAGGTGCGGGCATTCTAT	F
	GAAGTTCTTGACCGCTTTCTTCTG	R
GSH-Px1	AACCAGTTTGGGCATCAGGAGA	F
	TCTCGAAGAGCATGAAGTTGGG	R
SOD1	GTGGAGAACCCAAAGGGGAGTT	F
	TTTCATGGACCACCAGTGTGC	R
GAPDH	ATGACATCAAGAAGGTGGTG	F
	CATACCAGGAAAATGAGCTTG	R

Presence/Absence Approach for Molecular Detection of 2-Types of Thalassaemia

Genomic DNA was extracted from freshly provided blood samples with EDTA by using of gDNA kit, Addbio gDNA kit, Korea according to the kit protocol for blood samples. The typical DNA yielded from 200µl of whole blood was about 5-15 µg which depends on the quantity of White blood cells. The final reaction volume was 20µl, with 10µl Tag master mix, 2µl negative control block internal positive control IPC, 1µl negative control IPC, 1µl primer, 2µl isolated gDNA, and 4µl PCR-water. Samples from both the patient and control groups are analyzed for each mutation using disease phenotypes. Each sample is subjected to two PCR reactions (two tubes), one for determining the presence of the normal allele (using the normal primer) and the other for detecting the presence of the mutant allele. The Presence/Absence protocol conditions are as follows: pre-PCR read at 60°C for 30 seconds, holding stage step 1 at 50°C for 2 minutes, step 2 at 95°C for 10 minutes, followed by cycling stage for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, and finally post-PCR read at 60°C for 30 seconds (Table 2).

Table 2. Sequences of Studied Mutations for Detection of Thalassemia Types

Mutation	**Sequences for α-thalassaemia**
α1	5′- CCAAGCATAAACCCTGGCGCGCT-3′
	3′- CCATGCTGGCACGTTTCTGAG-5′
α2	5′-CCAAGCATAAACCCTGGCGCGCT-3′

	3′-AACACCTCCATTGTTGGCACATTCC-5′
Sequences	
Internal primers	Primer A (Forward): 5 ′-CAA TGT ATC ATG CCT CTT TGC ACC -3 ′
	Primer B (Reverse): 5 ′-GAG TCA AGG CTG AGA GAT GCA GGA- 3 ′
Common primer	Common primer C: 5 ′-ACC TCA CCC TGT GGA GCC AC3'
	Common primer D: 5 ′-CCC CTT CCT ATG ACA TGA ACT TAA-3 ′
Sequences for ß-thalassaemia	
IVS- I- 5	5 ′-CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG-3 ′
Codon 8 /9	5 ′-CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC-3 ′
Codon 41/42	5 ′-GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT-3 ′

Statistical Analysis

Data were reported as mean ± SE using IBM's version 26 of SPSS software from the USA. A t-independent student t-test was then used to compare healthy and thalassaemic subjects. Then, a *Pearson correlation test* was performed among the analyzed parameters, and $P > 0.05$ was used to indicate a significant difference. While using percentages to explore the percentage of each type of thalassaemia.

Results

Gene Expression of Iron Regulating Genes and Antioxidant Enzymes Genes

The gene expression analysis found notable upregulation of the foxO1 gene in thalassaemic patients compared to healthy participants, while hepcidin expression shows non-significant downregulation in thalassaemic compared to healthy subjects. Likewise, the enzymatic antioxidant gene GSH-Px1 showed important downstream regulation expression in thalassaemic participants compared to healthy ones, despite SOD1 and CAT antioxidant enzyme genes showing no variation in expression between examined subjects (Figure 1).

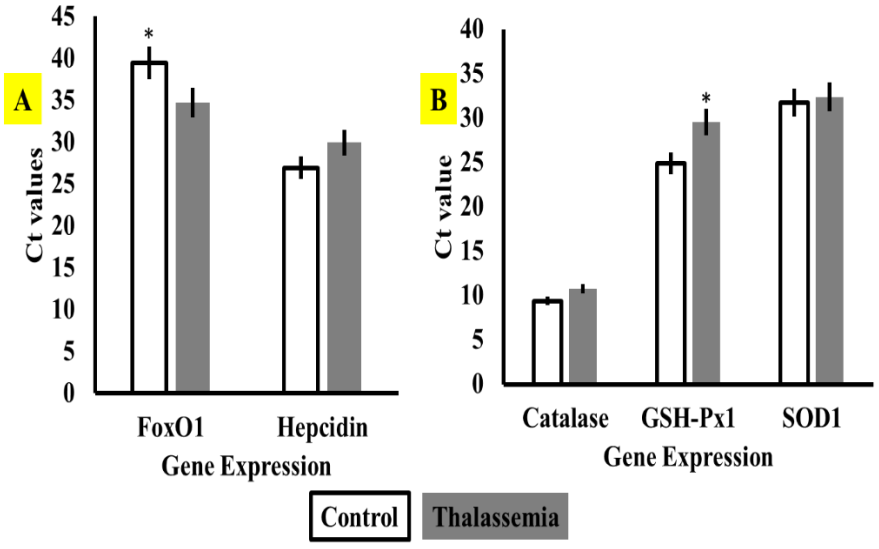

Figure 1. Gene Expression Profile of Iron Regulating Genes (A) and Genes of Antioxidant Enzymes (B). Data Expressed as Mean±SD of the Ct Values. * Indicatres Significant Difference at $p<0.05$ Using Independent Student t-test. SOD1=Superoxide Dismutase, GSH-Px=Glutathione Peroxidase

Correlation Between Iron Regulating and Antioxidant Enzymes Genes

To determine the association between these genes within the participants, a correlation test was run between antioxidant enzymes and iron-regulating genes. The findings showed a significant vice-versa connection between the antioxidant enzymes SOD1 and CAT at *P* 0.000 with an *r-value* of -.481 and a strongly direct link between GSH-Px1 and catalase at *P* value 0.004 with an *r-value* of 0.323. Additionally, there was a negative association between the foxO1 gene and the antioxidant enzyme CAT at *P* 0.001 and *r* value -0.355 (Table 3).

Table 3. Correlation Between Iron Regulating Genes and Enzymatic Antioxidant Genes

		FoxO1	Hepcidin	Catalase	GSH-Px1	SOD1
FoxO1	Pearson Correlation	1	-0.087	**-0.355****	-0.089	0.148
	Sig. (2-tailed)		0.448	0.001	0.438	0.197
Hepcidin	Pearson Correlation		1	0.035	0.033	-0.020
	Sig. (2-tailed)			0.761	0.772	0.863
Catalase	Pearson Correlation			1	**0.323****	**-0.481****
	Sig. (2-tailed)				0.004	0.0001
GSH-Px1	Pearson Correlation				1	**-0.262***
	Sig. (2-tailed)					0.021
SOD1	Pearson Correlation					1
**Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).						

Molecular Detection of α Thalassaemia

Using the RT-PCR approach for detecting the presence and absence of certain mutations in the participant blood samples with unknown types of thalassaemia in 50 subjects (healthy and non-healthy) revealed that 8.4%, 5.1% had positive *α*-thalassaemia according to the presence of α1 and α2 mutations in their gDNA samples concerning healthy subjects that revealed 2% and 0% presence of the same mutations in their gDNA samples (Table 4).

Table 4. The Percentage of Absence and Presence of Primer in *α*-Thalassaemic Subjects

Mutation	Healthy subjects (n=10)		Thalassaemic subjects (n=70)	
	Presence%	Absence%	Presence%	Absence%
α1	2	98	8.4	91.6
α2	0	100	5.1	94.9
Total α-thalassaemic subjects	2%		13.5%	
Note: positive results in healthy subjects it mean careers but without symptoms				

Molecular Detection of ß Thalassemia

The 50 individuals (healthy and non-healthy) whose blood samples had an unknown type of thalassaemia were found to have 33.6%, 36.5%, and 16.4% positive *ß*-thalassaemia based on the presence of codon 8/9, codon 41/42, and IVS-I-5 mutations in their gDNA samples, despite the healthy subjects illustrated 0%, 0%, and 0.5%,

respectively, of the same mutations in their gDNA samples (Table 5).

Table 5. The Percentage of Absence and Presence of Primer in *ß*-Thalassemia Subjects

Mutation	**Healthy subjects (n=10)**		**Thalassemia subjects (n=40)**	
	Presence%	**Absence%**	**Presence%**	**Absence%**
Codon 8/9	0	100	33.6	66.4
Codon 41/42	0	100	36.5	63.5
IVS-I-5	0.5	99.5	16.4	83.6
Total *ß*-thalassaemic subjects	0.5%		86.5%	
Note: positive results in healthy subjects it mean carriers but without symptoms				

Discussion

In contrast to healthy individuals, thalassaemic patients exhibited strong upstream regulation of the foxO1 gene and no alteration in hepcidin regulation to iron. Furthermore, a comparison of thalassaemic individuals' GSH-Px1 enzymatic antioxidant gene expression to that of healthy participants demonstrated significant downstream regulatory expression; in contrast, the expression of the SOD1 and CAT antioxidant enzyme genes did not differ among the people under investigation similar to these findings, [12] discovered that the median serum hepcidin levels were not substantially different among the three groups of thalassaemia patients, supporting the idea that hepcidin down-regulation caused by thalassaemia can lead to iron overload. As a result of the down-regulation of hepcidin, liver production of hepcidin will be reduced, resulting in a fall in serum levels due to excessive erythroid signals [13-15]. This iron overload situation could have occurred from regular blood transfusions in ß-thalassaemia minor subjects as opposed to ß-thalassaemia major patients [16].

Haeme and iron are powerful oxidising agents. Surprisingly, iron, whether free or coupled to haeme and Hb, can operate as a Fenton reagent in the Haber-Weiss cycle, which produces the extremely reactive hydroxyl radical. Unlike ROS, hydroxyl radicals cannot be removed enzymatically and can cause significant oxidative damage. Iron chelators have been found to inhibit lipid peroxidation in RBC membranes, implying that iron plays a role in ROS production and oxidative damage [17]. The fluctuation in enzymatic antioxidants gene expression in thalassaemic patients as downregulation could be interpreted due to that the home is a hydrophobic molecule that links with membrane lipids and proteins, boosting oxidation processes. Heme triggers NF-κB, redox-sensitive genes that raise inflammation by coupling to receptors, enzymes, and genes. This impacts cell function, metabolism, and gene expression. Haemoglobin may deteriorate DNA, promote caspases and cathepsins, drop mitochondrial activity, and suppress antioxidant enzymes like glutathione reductase. This opinion is more reliable and constant with [18].

Interestingly, because RBCs lack mitochondria, energy production is dependent on the anaerobic breakdown of glucose via the glycolytic route, followed by lactate formation. The main reducing agent in metHb is NADH, which is created during glycolysis and retains Hb ferrous. Higher metHb generation in β-thalassaemia may improve glycolysis by increasing NADH demand.NADPH is the reducing agent used by glutathione reductase to convert oxidised glutathione (GSSG) to two molecules of glutathione (GSH). GSH is an important antioxidant molecule in the glutathione cycle,

and many enzymes use it as a reducing agent. Glutathione peroxidase and peroxiredoxin generate GSSG while reducing peroxides and organic hydroperoxides. This opinion supports our outcomes in this research and is also similar to the findings of previous works by [19-21]. Another option that supports our findings is what [18] found in mice lack of superoxide dismutase 1 (SOD1) or peroxiredoxin (Prx) 2 results in anaemia due to heightened oxidative stress. Thus, some Prxs' redox status is systemically altered to a more oxidised state as a result of SOD1 deficiency, which has been linked to anaemia and numerous ailments.

Molecular analysis of thalassaemias' types revealed that 8.4% and 5.1% had positive *α*-thalassaemia according to the presence of α1 and α2 mutations in their gDNA samples concerning healthy subjects. Whereas, 33.6%, 36.5%, and 16.4% positive *β*-thalassaemia based on the presence of codon8/9, codon 41/42, and IVS-I-5 mutations in their gDNA samples. Based on previous works by different researchers [22-35]. Our study found two mutations in α-thalassaemia haemopathy. The −α1 and −α 2 deletions made up the majority of the detected alterations. The prevalence of the −α3.7 deletion is comparable with research conducted in the eastern Mediterranean and globally [36-38]. Furthermore, other studies from the Arabian Peninsula show that this mutation is fairly widespread, with 45% of neonates carrying it in the United Arab Emirates. Interestingly, our findings are in constant with other studies in Kuwait and Saudi Arabia. This mutation is the most frequent α mutation reported in Kuwait and eastern Saudi Arabia, with 45% of the population heterozygous. [39, 40]. The current research registers only 6 cases of α1 and 3 cases of α2 haemopathy despite the prevalence of both variants in the population. The low prevalence of α-thal (α+ and α0 abnormalities) in our population may explain the sporadic character of Hb H illness and Hb Bart's hydrops fetalis in Mosul. It's possible that we underestimated the prevalence of α+ problems. Although α-thal is not a big clinical problem in Mosul, it can have a considerable impact on haematological and clinical phenotypes in populations with a high β-thal rate.

The present investigation documents just 19 cases of codon 8/9, 21 cases of codon 41/42, and 10 cases of IVS-I-5 haemopathy disease in blood samples of thalassaemic children. This result is supported by other studies done in Iraq [22, 24, 27, 29]. The RT-PCR technique utilized in this study produced good results in diagnosing the many varieties of thalassaemia that had recently entered our city of Mosul for diagnosing thalassaemia species. This approach has already been utilized in other research in various countries, and it has been proven that the PCR reactions are multiplex, identifying several genotypes at the same time, decreasing the time and expense of screening and prenatal testing. These technologies should make it easier to test for carriers and identify couples who are at risk for α-thalassemia [30, 31, 33]. Other works by other researchers used other new approaches that support our approach in the diagnosis of thalassaemia, who found that the real-time gap-PCR and HRM analysis have additional advantages, including less manpower, faster turnaround time, and a lower risk of PCR carryover contamination [25, 30].

Conclusion

Per the current study, hepcidin exhibited little downregulation in thalassaemic patients, although foxO1 showed overexpression. Likewise, the enzymatic antioxidant gene was notably downregulated in thalassaemia patients as opposed to healthy children, and catalase expression was inversely linked with foxO1 expression. Subsequently, the most effective, rapid, and precise way for diagnosing the two forms of thalassaemia is the RT-PCR technique.

Conflict of Interest

The authors had no conflict of interest

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