

Article

Relation between interferon-gamma gene expression and its serum level with thyroid hormone status and Ferritin level in Iraqi sickle cell anemia patients

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Abstract

The hereditary hemoglobinopathy known as sickle cell disease is characterized by abnormal hemoglobin synthesis, hemolytic anemia, and intermittent obstruction of tiny blood arteries. The current investigation aims to study the relationship between interferon-gamma gene expression and serum levels with thyroid hormone status and Ferritin levels in sickle cell anemia patients. Blood samples were collected from 50 patients suffering from SCA and 50 healthy volunteers as a control group. The results of the relation of IFN- γ serum level with T3, T4 and TSH levels showed a positive relationship between IFN- γ serum and TSH levels. At the same time, an inverse association to T3 and T4 levels with high significance also showed high IFN- γ gene expression (10.78 ± 5.06 Fold) in the patient group as compared with control (2.079 ± 0.52 Fold) at a significant difference, as well as the results found acquire strong positive association between IFN- γ serum level and IFN- γ mRNA expression in the patient group compared with the control group. The current study concluded that there was an inverse association among T3, T4 and IFN- γ serum with high significance, as high gene expression of IFN- γ , and a strong positive association between IFN- γ serum level and IFN- γ mRNA expression.

Keywords: IFN- γ , SCA, Ferritin, TSH, T3, T4.

Introduction

Sickle cell anemia (SCA) is a hereditary disorder in which the body produces red blood cells with a sickle or crescent shape. It affects many people all over the world. In sickle cell anemia, the red blood cells harden, stick together, and become a crescent or sickle. They only live for 10 to 20 days, while normal red blood cells are soft, round, and move quickly through blood vessels and live for 120 days². A lack of red blood cells can be caused by abnormal cells being trapped in small blood vessels and delaying or obstructing blood and oxygen flow to various parts of the body (anemia)³. Sickle cell anemia is characterized by fatigue and weariness, intense pain, visual issues, painful swelling in the feet and hands, persistent pain episodes, growth retardation, recurrent bacterial infections, and other symptoms that vary from person to person and over time⁴. Even though there is no treatment for sickle cell anemia, some drugs can alleviate discomfort and assist in preventing issues connected with the condition⁵. In-depth genetic and immunological studies and

The link between genetic and immunological parameters may provide a better understanding of this disease and thus provide strategies for diagnosis and treatment⁶. One of the critical parameters may be interferon-gamma (IFN- γ) because this parameter has a role in infection and inflammation, as well as IFN- γ has been shown to affect globin gene expression in cord blood and erythroblasts derived from normal adult progenitors⁷. In SCA, cellular expression of IFN- γ influences pivotal pathways that contribute to disease pathogenesis⁸. Patients with vaso-occlusive crises (VOC), a hallmark of SCA associated with severe pain due to occlusion of blood vessels, have a higher level of circulating IFN- γ compared to those who are not in crisis condition⁹. Moreover, increased IFN- γ production was associated with higher hospital admissions for VOCs¹⁰. Ferritin is the cell's primary iron-storage molecule. When a person with SCD is experiencing a crisis due to chronic inflammation, infection, or liver disease, their blood levels of this acute phase reactant are greater. One of the most excellent techniques to detect iron homeostasis in SCA is by serum ferritin levels. People with SCD who have chronic hemolysis and get many blood transfusions may absorb more iron from their gut¹¹. In SCD, erythrocytes result in microvascular occlusion, leading to acute complications in the endocrine system and chronic organ damage. People with sickle cell anemia have several indications for a red blood cell transfusion. One complication of a red blood cell transfusion is iron overload¹². Iron overload has been linked to several endocrine disorders¹³. Previous research has shown that the thyroid hormone controls how fast erythropoiesis happens in people with SCD. This is because erythropoietin speeds up the process. Thyroid function hormones such as thyroxine (FT4), triiodothyronin (FT3), and thyroid stimulating hormone (TSH) should thus be tested for in persons with SCD¹⁴. There is limited data on IFN production in SCD. Furthermore, there is a relation with hormonal status; therefore, this study was conducted to investigate the relation between IFN- γ gene expression and its serum level with thyroid hormone status and Ferritin level in Iraqi SCA patients.

Materials and Methods

Six ml of venous blood was obtained from 50 patients suffering from SCA aged between 20 and 60 who attended the genetic hematology center located in Ibn Al-Baladi hospital in Baghdad, and 50 healthy individuals as control at age were matched with patients. Each sample was divided into two parts: the first (2ml) was put into an EDTA tube and subjected to Trizol preservation (Invitrogen/ Thermo Fisher/USA) for RNA extraction and subsequent IFN- γ gene expression, While the second part (4 ml) put in gel tube for two hours and centrifuged at 5000 rpm for 6 min, the serum was gathered and stored at -20 C for serological analysis.

Serological analysis

Serum levels of Ferritin, T3, T4 and TSH were determined by using an automated immunocheiluminometric analyzer (DiaSorin Ltd, Wokingham, Berkshire, UK). Ferritin's average value range between 12-300 ng/ml for male and 12-150 ng/ml for female, the average value of T3 range from 80-220ng/dL, the average value of T4 5.0-12.0 ng/dL and TSH average value range 0.5-5.0 ml/L.0.4-4.5, while the serum level of IFN- γ was measured using the ELISA technique and IFN- γ Elisa kit (Abcam/USA).

Molecular analysis

According to Mohammad's instructions, total RNA was isolated from blood using an RNA purification kit from Pioneer Korea, and Nanodrop (Pioneer Korea) measured the RNA's concentration and purity¹⁶. Volume 20 μ L of total RNA was reverse transcribed into complementary DNA (cDNA) employing WizScript™ RT FDmix Kit (Wizbiosolutions/ South Korea). As the Table illustrates, further heat cycler stages condition cDNA reverse transcription (1).

Step	Step 1	Step 2	Step 3	Step 4
Temperature	25 c	42°C	85 C	4 C
Time	10 min	30 min	5min	∞

Table 1. Thermal cycler steps of conditions cDNA Reverse Transcription.

The reaction mixture was performed using Beta active, and IFN- γ gene primers were provided by Macrogene /Koria as listed with their sequences in Table (2).

Gene		Sequence (5'-3')	size (bp)	Reference
IFN- γ	Foreword	GAGTGTGGAGACCATCAAGGAAG	124	(Wang et al. ¹⁷)
	Reverse	TGCTTTGCGTTGGACATTCAAGTC		
Beta-actin	Foreword	CCTGGCACCCAGCACAAT	144	Hua et al. ¹⁸
	Reverse	GGGCCGGACTCGTCATAC		

Table 2. Primers of IFN- γ and Beta-actin genes.

The RT-PCR test was performed with an RT-PCR instrument (Thermo Fisher/USA), and the cycling software procedure was set for optimal cycles and temperature profiles, as stated in Table (3). The Livak formula estimated gene expression levels and fold inductions by monitoring the cycle threshold (Ct).

Step	Temperature	Time	Cycles
Enzyme activation	50°C	2min	1
Initial denaturation	95	10 min	1
Denaturation	95°C	15 sec	40
Annealing/Extending	60°C	1 min	
Melting curve	60-95	2-5 sec/step	1

Table 3. Program of qPCR.

Statistical Analysis

Utilizing the Statistical Analysis System- SAS19 software, the influence of various variables on research parameters was determined. To compare means substantially, the Least Significant Difference –LSD test (Analysis of Variation-ANOVA) was

applied. The chi-square test was used to compare percentages (0.05 and 0.01 probability) substantially.

Results

Regarding gender, the distribution of SCA patients was 23 males 46% and 27 females 54%, with no significant difference as shown in table (4).

Gender	Patients No. (%)
Male	23(46 %)
Female	27 (54%)
Total	50
P-value	0.061

Table 4. Distribution of SCA patients according to gender.

The results of estimation of ferritin, T3, T4 and TSH levels in the serum of SCA patients and control groups revealed that the levels of ferritin and TSH were elevated in patients groups which ferritin levels reached (503.11 ±97.9 ng/ml) in patients and (34.33 ±9.06 ng/ml) in controls as well as TSH level was (9.01±1.03 mIU/L) in patients and (2.6±0.77 mIU/L) in controls with high significant differences (P<0.01). In contrast, the level of T3 and T4 were lowered, where the level of T3 was (78±6.4 ng/dL) in patients and (140±9.9 ng/dL) in controls, while level of T4 was (4.5±0.5 ng/dL) when compared with control group (7.7±0.7 ng/dL), with high significant differences (P<0.01) as explained in table (5).

Group	Mean ± SE			
	Ferritin (ng/ml)	TSH (mIU/L)	T3 (ng/dL)	T4 (ng/dL)
SCA Patients	503.11 ±97.9	9.01±1.03	78±6.4	4.5±0.5
control	34.33 ±9.06	2.6±0.77	140±9.9	7.7±0.7
LSD value	149.08 **	0.096**	62.988**	2.589 **
P-value	0.0001	0.0001	0.0001	0.0001
** (P≤0.01).				

Table 5. Comparison between patients and control groups according to Ferritin, TSH, and T4 levels

The results of the effect of gender on Ferritin, TSH, T3 and T4 levels in SCA patients found that there was a highly significant difference (P≤0.01) in Ferritin levels between male (722.18 ±31.17 ng/ml) and female (350.08 ±53.52 ng/ml), and significant difference (P≤0.05) according to TSH levels in males (10.07±14.34 mIU/L) when compared with females (8.66±20.21 mIU/L). At the same time, there was no significant difference regarding T3 and T4, whereas the T3 level was (79.01±0.11 ng/dL) in males and (78.1±0.28 ng/dL) in females, as the value of T4 was (4.17±0.68 ng/dL) in male and (4.81±0.85 ng/dL) in female as clarified in table (6).

Gender	Mean ± SE			
	Ferritin (ng/ml)	TSH (mIU/L)	T3(ng/dL)	T4 (ng/dL)

Male	722.18 ±31.17	10.0 7±14.34	79.01±0.11	4.17±0.68
Female	350.08 ±53.52	8.66±20.21	78.1±0.28	4.81±0.85
P-value	0.0001**	0.051*	0.481NS	0.561NS
** (P≤0.01). * (P≤0.05), NS: Non-Significantly.				

Table 6. Effect of gender on Ferritin, TSH, T3 and T4in SCA patients.

The result of the correlation of IFN- γ serum level with SCA found that the serum level of IFN- γ was increased in the SCA patient group (51.75 ±4.92 pg /ml) as compared with the control group (15.73±1.3 4 pg /ml) with highly significant difference (P<0.01), as displayed in Table (7).

Group	Mean ± S.E. of serum IFN- γ (pg/ml)
Patients	51.75 ±4.92
Control	15.73 ±1.34
LSD value	9.969 **
P-value	0.0001
** (P≤0.01).	

Table 7. Comparison between serum IFN- γ levels in patients and control groups.

According to the relation of IFN- γ serum level with thyroid hormone levels asT3, T4 and TSH in patients and control groups, the results showed a positive relationship between IFN- γ serum and TSH levels. At the same time, an inverse association to T3 T4 with high significance (p< 0.001) is illustrated in Table (8).

Group	Mean ± SE			
	T3 (ng/dL)	T4 (ng/dL)	TSH (mIU/L)	IFN- γ (pg/ml)
Patients	78±6.4	4.17±0.68	9.01±1.03	51.75 ±4.92
Control	140±9.9	7.7±0.7	2.6±0.77	15.37 ±1.34
LSD value	62.988**	2.589 **	0.096**	9.969 **
P-value	0.0001	0.0001	0.0001	0.0001
** (P≤0.01).				

Table 8. Relationship between IFN- γ serum level with thyroid hormone levels in patients and control groups.

The result of the current study showed high gene expression of IFN- γ expression (fold expression) in the patient group as compared with control at a significant difference (P<0.05). In contrast, the gene expression of IFN- γ reached (10.78 ±5.06 Fold) in the patient group, while in the control group was (2.079 ±0.52 Fold) as illustrated in Table (9).

Group	Mean ± SE		
	Delta CT	Delta Delta CT	Folding
Patients	0.624 ±0.42	-1.868 ±0.42	11.78 ±6.06
Control	2.29 ±0.33	4.33 ±0.32	2.080 ±0.53
LSD value	---	---	8.489 *
P-value	---	---	0.0467
* (P≤0.05).			

Table 9. Comparison between IFN- γ gene expression in patient and control groups.

As shown in Table (10), the current investigation revealed a considerable positive correlation between IFN- γ serum level and IFN- γ mRNA expression in the patient group compared to the control group.

Group	Mean \pm SE	
	IFN- γ serum (pg/ml)	IFN- γ gene expression (Fold)
Group: A	51.75 \pm 4.92	11.78 \pm 6.06
Group: C	15.73 \pm 1.34	2.080 \pm 0.53
LSD value	9.969 **	8.489 *
P-value	0.0001	0.0467
** (P \leq 0.01).* (P \leq 0.05),		

Table 10. Correlation between sera IFN- γ and IFN- γ gene expression

Discussion

Among Iraqis, sickle cell disease is the most deadly and widespread inherited disease 20. The spread of the disease may be caused by the habit of inbreeding, which contributes significantly to the spread and prevalence of most diseases of genetic origin 21. According to the results of dividing patients according to gender groupings, it was determined that gender had no significant influence on the spread of the disease, with 23 (46%) in males and 27 (54%) in females. As regards the results of evaluating the levels of ferritin and the function of thyroid hormones, the results showed that ferritin (503.11 \pm 97.9 ng/ml) and TSH (9.01 \pm 1.03 mIU/L) were high in the group of patients compared to the control group where were (34.33 \pm 9.06 ng/ml) for ferritin and (2.6 \pm 0.77 mIU/L) for TSH, at the same time the results showed a decrease in T3 (78 \pm 6.4 ng/dL) and T4 (4.5 \pm 0.5 ng/dL) levels in patients compared to the control (140 \pm 9.9 ng/dL) and (7.7 \pm 0.7 ng/dL) respectively, with highly significant differences (P \leq 0.01). However, variations in the severity of the endocrine abnormalities may be connected, at least in part, to the severity of SCA. The inverse relationship between the level of TSH and T3 T4 is evident and proved, as a drop in T3 T4 leads to a rise in TSH. So, the significant drop in the serum level of fT3 could be because T3 secretion has been slowed down 22. In the case of SCA, to relieve symptoms and prevent disease complications, taking into account the high rate of chronic hemolysis, patients resort to blood transfusions to increase the blood's ability to carry oxygen and replace abnormal red cells with normal cells. In the current study, most patients had previously been subjected to frequent blood transfusions, which, as a consequence of this therapeutic process, led to increased iron absorption in the gastrointestinal tract, which, as a result, increased iron levels and higher than normal ferritin values. Repeated blood transfusions lead to the accumulation of exogenous iron, and due to the human body not having an effective physiological mechanism to excrete excess iron, exogenous iron can accumulate, circulate as non-transfer bound iron, and types reactive oxygen, which enters tissues and leads to ultimate organ damage 23. The response to oxidative stress may be responsible for the pathophysiological increase of secondary imbalances in SCA patients, as the metabolic effects of thyroid hormones are directly related to active oxygen species, production and oxidative stress, and these conditions are also associated with SCA disease. Also, patients with SCA have been reported to have endocrine dysfunction that often affects growth, development, and metabolism 24. Regarding the effect of gender on Ferritin, TSH, T3 and T4 levels in SCA patients, the results found that there was a high significant difference (P \leq 0.05) in Ferritin levels between male (722.18 \pm 31.17 ng/ml) and female (350.08 \pm 53.52 ng/ml), and significant difference (P \leq 0.05) in TSH level between male (10.07 \pm 14.34 mIU/L) and female (8.66 \pm 20.21 mIU/L). In contrast, the results

showed no significant difference according to T3 (79.01 ± 0.11 ng/dL) and T4 (4.17 ± 0.68 ng/dL) in males when compared with females (78.1 ± 0.28) and (4.81 ± 0.85) respectively, In women, during menstruation, pregnancy or after menopause, TSH levels may drop slightly outside the normal range, due to the fluctuation of estrogen levels²⁵. Concerning the result of the correlation of IFN- γ serum level with SCA, it found that the serum level of IFN- γ was elevated in the patient's group (51.75 ± 4.92 pg/ml) as compared with the control group (15.73 ± 1.34 pg/ml) with highly significant difference ($P < 0.01$). Researchers think that even though IFN- γ production is linked to vaso-occlusive crises, people with SCA also have a more inflammatory profile than healthy people. Chronic inflammation in SCA is associated with endothelial damage, an increase in the formation of reactive oxygen species, and red blood cell destruction. Leukocytes, erythrocytes, and platelets are making more adhesion molecules, and more pro-inflammatory cytokines are being made²⁶. Regarding the relation of IFN- γ serum level with thyroid hormone levels, the results showed a positive relationship between IFN- γ serum level and TSH level. At the same time, we found an inverse association among T3 and T4 with high significance ($p < 0.001$). Researchers indicated that pro-inflammatory cytokines such as IFN- γ play a role in lowering peripheral thyroid hormone levels during illness. On the other hand, it has been suggested that thyroid hormones make more inflammatory cytokines²⁷. According to the relation between IFN- γ gene expression and SCA disease, the result showed high gene expression of IFN- γ expression (10.78 ± 5.06 Fold) in the patient group as compared with the control (2.079 ± 0.52 Fold) at a significant difference ($P < 0.05$). Patients with sickle cell disease experience frequent vaso-occlusive events that are marked by ischemia-reperfusion injury and inflammation; in addition, these patients also experience vascular occlusive crises, a defining feature of SCA that is accompanied by excruciating pain from vascular obstruction, and they have a higher level of circulating IFN- γ ²⁸⁻²⁹. Moreover, increased IFN- γ production was associated with more hospital admissions for SCA. Furthermore, the IFN- γ level is associated with an increase in ferritin level related to iron overload, a distinguishing feature of the disease³⁰. On the subject of the correlation between sera IFN- γ and IFN- γ gene expression, the result showed a strong positive association between IFN- γ serum level and mRNA IFN- γ expression in the patient group compared with the control group. Normally, there is a positive relationship between the cytokine level in the serum and its level of messenger RNA, as the translation of its code gives the product of cytokine in the blood as an immune defense response against infections associated with the disease.

Conclusion

Current study concluded that the distribution of SCA patients according to gender showed no significant difference between male and female. as well as revealed that the levels of ferritin and TSH were elevated in patients group when compared with control, while, the level of T3 and T4 were lowered in patients as compared with control at high significant differences, also the effect of gender on Ferritin, TSH, T3 and T4 levels in SCA patients found that there was high significant difference in Ferritin level and significant difference according to TSH level in males when compared with females, while the results showed no significant difference in males when compared with females regarding to T3 and T4, furthermore IFN- γ serum level found elevated in SCA patient group as compared with control group with highly significant difference, moreover there was a positive relationship between IFN- γ serum and TSH levels, while found inverse association among T3, T4 and IFN- γ serum with high significance, likewise showed high gene expression of IFN- γ in patient group, also results showed acquire strong positive association between

IFN- γ serum level and mRNA IFN- γ expression in patient group compared with the control group.

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Conflicts of interest

The author declares no conflicts of interest.

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