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Genetic and Hematological Insights into β-Thalassemia Major: A Molecular and Clinical Investigation in Southern Iraq

[1,2]Amar M. Mandal , [1]Aseel S. Mahmood

[1]Department of Biotechnology, College of Science, Baghdad University, Baghdad, Iraq
[2]Department of Science, College of Basic Education, university of Al-Muthanna, Iraq

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Abstract : β-thalassemia major is a severe hemoglobin disorder caused by Hemoglobin Subunit Beta gene mutations, leading to impaired β-globin synthesis, chronic anemia, and transfusion dependency. This study aims to investigate the molecular and clinical aspects of β-thalassemia major in southern Iraq, focusing on *HBB* gene mutations, hematological alterations, and disease complications. A total of 100 participants 50 β-thalassemia major patients and 50 healthy with (28 males, 22 females) for both categories were analyzed. Genomic DNA extraction and PCR-sanger sequencing were conducted to detect single nucleotide polymorphisms (SNPs), while hematological and biochemical markers, including serum ferritin levels, were analyzed to assess disease severity and iron accumulation. A total of three SNPs were identified, with rs7480526 (71A>C) and rs1609812 (334G>A) showing a strong association with β-thalassemia susceptibility, whereas the rare (94-95insG) rs35238478 suggested a potential role in disease variation. Patients exhibited severe anemia, abnormal hemoglobin composition, and significantly elevated ferritin levels, particularly in those who had undergone splenectomy, emphasizing the urgent need for enhanced transfusion strategies and iron chelation therapy. Notable, consanguineous marriages and rural residence were recognized as key risk factors, highlighting the necessity of genetic counseling. These findings deepen our understanding of β-thalassemia major's genetic and clinical landscape, underscoring the importance of early genetic screening, personalized disease management, and improved healthcare accessibility to mitigate disease burden.

Keywords: β-thalassemia major, *HBB* gene mutations, single nucleotide polymorphisms (SNPs), genetic risk factors, disease management strategies.

Corresponding Author: (E-mail: ammar.musa2306@sc.uobaghdad.edu.iq)

Introduction

Thalassemia is an inherited blood disorder characterized by abnormal hemoglobin formation resulting from reduced or absent globin chain production (1). β-thalassemia arises due to mutations in the β-globin gene located on chromosome 11, leading to either partial (β^+) or complete (β^0) deficiency of β-globin chain synthesis (2). Such mutations impair hemoglobin's ability to transport oxygen efficiently, compromising systemic oxygen delivery (3). The incidence of β-thalassemia has steadily increased in southern Iraq, as mentioned in the studied conducted in Basra which describe the prevalence and demographic characteristics of hemoglobinopathies in Basrah (4). Central to this condition is the *HBB* gene at chromosome 11p15.5, which encodes the β-globin subunit of hemoglobin; mutations or deletions in

this gene disrupt normal erythropoiesis, causing chronic anemia and contributing to disease severity (5). Genetic alterations in *HBB*, including nucleotide substitutions, insertions, and deletions, interfere with β-globin production and lead to defective red blood cell formation (6). Furthermore, certain intronic mutations can activate cryptic splice sites, resulting in aberrant mRNA processing and diminished functional β-globin output (7) . The clinical manifestations of β-thalassemia largely depend on the type and zygosity of *HBB* mutations, with homozygous individuals experiencing more severe outcomes compared to heterozygotes who may present with milder traits (8). Bioinformatics resources, such as Database of Single Nucleotide Polymorphisms (dbSNP), are pivotal for cataloging and analyzing single nucleotide polymorphisms (SNPs), aiding in variant identification, annotation, and precision genetic research (9)(10). Profiling *HBB* gene mutations in specific populations, including the current study cohort from Southern Iraq, is crucial for enhancing diagnostic precision, prenatal screening strategies, and the delivery of targeted genetic counseling (11)(12). This study aims to investigate the molecular and clinical aspects of β-thalassemia major in southern Iraq, focusing on HBB gene mutations, hematological alterations, and disease complications. Through the application of bioinformatics resources, including dbSNP, it seeks to assess the novelty of identified SNPs and examine the impact of aberrant splicing mechanisms. Additionally, the study explores the clinical significance of these mutations, particularly their contribution to genetic screening, prenatal diagnosis, and counseling. Moreover, it evaluates the complications associated with β-thalassemia major, such as iron overload, endocrine dysfunction, and hepatic disorders, while analyzing the effectiveness of blood transfusion strategies, iron chelation therapy, and splenectomy in optimizing patient management and outcomes.

Methodology

Ethical committee approval\

The study complied with the Helsinki Declaration's ethical guidelines for human research and received approval from the Ethics Committee for Postgraduate Studies, College of Science, University of Baghdad (CSEC/0724/0049; July 28, 2024).

Study Design and Patients

This study involved 100 participants, including 50 β-thalassemia major patients (28 males, 22 females) aged 6 months to 15 years Apparently healthy as a control group aged 2 to 20 years. Patients were recruited from AL Muthanna Health Department's Women and Children Hospital and Thi-Qar Health Department's Genetic Blood Diseases Center. Inclusion criteria required transfusion-dependent β-thalassemia major patients with 2–4-week transfusion intervals, severe hypochromic anemia, mean erythrocyte volume <75 fl, and HbA2 levels >3.5% confirmed by hemoglobin electrophoresis. Parental consent was obtained for blood sampling. Controls comprised healthy volunteers without a β-thalassemia history. All patients received regular transfusion and chelation therapy. Data were collected through standardized interviews, with informed consent ensuring participants' right to withdraw at any time.

Sampling and data collection

The study involved 50 β-thalassemia major patients, with data

and blood samples collected from March to December 2024. A total of 5 mL of venous blood was drawn under sterile conditions into EDTA vacutainers (13)(14) .Two milliliters were stored at 20°C for blood count analysis, while the remaining 3 mL was centrifuged, and the serum were then extracted and stored at −20°C until analysis. for further testing (15) .

Hematology, biochemical, and Hemoglobin electrophoresis Assays

Hematological parameters, including ABO and Rh blood typing, hemoglobin (Hg), white blood cells (WBC), neutrophils (NEUT), lymphocytes (LYMPH), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets (PLT), were analyzed using an automated hematology analyzer (BC-3000plus, Mindray, China)(16). Biochemical parameters, including creatinine, urea, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), were measured using the BS240 automated analyzer (Mindray, China) (17).serum ferritin levels were determined using the Chemiluminescence Immunoassay System (CL-900i, Mindray, China) (18).Hemoglobin types, including HbA1, HbA2, and HbF, were identified using the Hemoglobin Testing D-10 System (19).

Extraction of Genomic DNA

Blood samples were collected in EDTA tubes and preserved at -20°C until further analysis. Genomic DNA was extracted using a silica-based DNA isolation kit (Easy Pure® Blood Genomic DNA Kit, Cat. No. EE121-01, Transgene Co., China) in accordance with the manufacturer's instructions. The purity and integrity of the extracted DNA were evaluated through 1% (w/v) agarose gel electrophoresis, while DNA quantification was conducted using a Nanodrop spectrophotometer (Biodrop, UK).

Designing Primers and Optimizing PCR Conditions

Specific PCR primers targeting the *HBB* gene were designed using the NCBI Primer BLAST tool, based on the reference sequence provided in GenBank (Accession No. NC_000011.10). Two sets of overlapping primer pairs were developed to ensure comprehensive coverage and amplification of the target region. The PCR conditions were subsequently optimized to achieve efficient and specific amplification of the desired sequences. (20). These overlapping amplicons were designed to capture all potential variations within the *HBB* gene. The first PCR amplicon (forward primer 5′-ACGATCCTGAGACTTCCACAC-3′ and reverse primer 5′-AGTCAGGGCAGAGCCATCTA-3′) is 665 bp long and was designed to cover 5′UTR, exon 1, intron 1, and exon 2, as well as the upstream region of intron 2. The second amplicon, measuring 881 bp in length, was designed with a forward primer (5′-TGGACAGCAAGAAAGCGAGC-3′) and a reverse primer (5′-CATCAGTGTGGAAGTCTCAGGAT-3′) To encompass the downstream region of intron 2, exon 3, and part of the 3′-untranslated region (3′-UTR) of the *HBB* gene, PCR amplification was conducted under optimized conditions to achieve high specificity and efficiency in targeting the designated sequence. The thermal cycling process according to the manufacture procedure as shown in (Table 1). The specificity and integrity of the PCR products were confirmed using 1.5%

(w/v) agarose gel electrophoresis, which permit for an assessment of amplification efficiency and verification of target sequence integrity. The amplified PCR fragments were further validated through agarose gel electrophoresis, followed by Sanger sequencing in accordance with standard protocols. Chromatogram data were processed using BioEdit software (version 7.1), where sequences were visualized, aligned, and trimmed against the GenBank reference sequence (Accession No. NC_000011.10). Identified single nucleotide polymorphisms (SNPs) underwent manual verification through electropherogram analysis using the SnapGene Viewer tool ensuring precise detection. Any non-standard SNPs were eliminated based on established protocols to uphold data accuracy and reliability (21). The detected SNPs in each sequenced sample were numerically classified according to their genomic positions within the PCR amplicons and their respective locations in the reference genome. Following this, the detected SNPs were analyzed for novelty assessment by cross-referencing them with the dbSNP (Single Nucleotide Polymorphism Database) server to determine their prior documentation and uniqueness (22).

Table (1): Thermal Cycling Conditions for Conventional PCR Amplification of Target Gene

Step	Temperature (°C)	Time	Cycle's Description
Initial Denaturation	94°C	3 minutes	(1 cycles)
Denaturation	94°C	30 seconds	(32 cycles)
Annealing	59.4°C	30 seconds	
Extension	72°C	60 seconds	
Final Extension	72°C	5 minutes	(1 cycles)
Hold	4°C	∞	

Molecular Sequencing Analysis

All PCR-amplified fragments were sequenced using the Sanger dideoxy method, following the standard protocols provided by Macrogen Inc. (Seoul, Republic of Korea). The resulting electropherogram files were processed by trimming and aligning them with the *HBB* gene reference sequence using BioEdit software (version 7.1, Madison, USA). Identified single nucleotide polymorphisms (SNPs) were manually examined and validated through electropherogram inspection using the SnapGene Viewer tool. To assess the novelty of the detected SNPs, they were cross-referenced against the dbSNP database (https://www.ncbi.nlm.nih.gov/snp/). Furthermore, the potential effects of coding SNPs on the translated amino acid sequences were evaluated using the Expasy Translate server (https://web.expasy.org/translate/).

Statistical Analysis

Data analysis was conducted using SPSS version 20 (IBM Corp., Armonk, NY, USA) to calculate frequencies, percentages, and mean ± standard deviation (SD). Statistical significance was defined as $P < 0.05$, with $P < 0.01$ indicating high significance and $P \geq 0.05$ indicating non-significance. Differences in HbA2 levels, presented as mean ± SD, were analyzed using Student's t-test and ANOVA.

Results and Discussion

Clinical and Demographic Profile Analysis

This Case control study in southern Iraqi hospitals highlights the need to

enhance medical care for β-thalassemia major (βTM) patients. Hematological and biochemical parameters were assessed and correlated with iron overload to inform future management strategies. Patients showed significantly worsened clinical profiles compared to healthy controls, with marked disparities in serum ferritin levels, hematological markers, and complications. Sex distribution was similar (males 44%, females 56%, p=0.1), and controls had a slightly higher mean age (13.4±6.2 years) than patients (9.8±3.6 years, p=0.06), indicating no significant impact of sex or age on disease presentation. These findings align with studies from Indian and Iraqi populations, confirming similar trends (23).In contrast, some studies indicate that age may play a role in certain aspects of β-TM management. A study in Iran revealed that older patients were more likely to experience growth impairment, with each year increasing the risk by 1.57 times (24). Living conditions significantly differed between groups, with 58% of patients residing in rural areas compared to 38% of controls, while urban residency was higher among controls (62%) than patients (42%, p=0.04). These findings underscore the impact of socioeconomic and geographic factors on disease management, as rural patients face limited healthcare access, resulting in poorer outcomes, including higher ferritin levels and increased complications. Similar trends were observed in an Iranian study, highlighting the critical role of living environments in β-thalassemia management (25). All patients reported varying degrees of consanguineous marriages, while no consanguinity was observed among controls ($p < 0.01$). This emphasizes the critical role of consanguinity in the genetic inheritance of β-thalassemia, especially in populations with high intermarriage rates, such as those in Iran and northern Iraq. The findings highlight the importance of genetic counseling and community awareness programs to mitigate the disease's prevalence and severity (26). as shown in (Table 2).

Table (2): The Demographic and clinical characteristic of the investigated population.

Indices		Group (N=100)		P value
		Control n=50 (%)	Patients n=50 (%)	
Sex	Male	22 (44%)	22 (44%)	0.1NS
	Female	28 (56%)	28 (56%)	
Age (year)	Mean± S.D.	13.4±6.2	9.8±3.6	0.06NS
Living	Rural	19 (38%)	29 (58%)	0.04*
	City	31 (62%)	21 (42%)	
Consanguineous marriage	0.00	50 (100%)	10 (20%)	<0.01**
	1.00	0 (0.0)	12 (24%)	
	2.00	0 (0.0)	18 (36%)	
	3.00	0 (0.0)	10 (20%)	
Complication	Non	50 (100%)	9 (18%)	<0.01**
	Splenomegaly	0 (0.0)	21 (42%)	
	Hepatosplenomegaly	0 (0.0)	15 (30%)	
	Splenectomy	0 (0.0)	3 (6%)	
	Splenomegaly/colostomy	0 (0.0)	1 (2%)	
	Splenectomy/hepatomegaly	0 (0.0)	1 (2%)	
Family History	Non	50 (100%)	0 (0.0)	<0.01**
	Carrier	0 (0.0)	50 (100%)	
Age at the first blood	Non	50 (100%)	0 (0.0)	<0.01**

Indices		Group (N=100)		P value
		Control n=50 (%)	Patients n=50 (%)	
Transfer (in a month)	1 month	0 (0.0)	1 (2%)	
	2 months	0 (0.0)	21 (42%)	
	3 months	0 (0.0)	15 (30%)	
	4 months	0 (0.0)	8 (16%)	
	5 months	0 (0.0)	4 (8%)	
	6 months	0 (0.0)	1 (2%)	
Transfusion interval	Non	50 (100%)	0 (0.0)	<0.01**
(in a weak)	1 weak	0 (0.0)	0 (0.0)	
	2 weeks	0 (0.0)	15 (30%)	
	3 weeks	0 (0.0)	27 (54%)	
	4 weeks	0 (0.0)	5 (10%)	
	5 weeks	0 (0.0)	3 (6%)	
Receiving chelating drugs	No	50 (100%)	19 (38%)	<0.01**
	Yes	0 (0)	31 (62%)	

Complications were reported in 82% of patients, including splenomegaly (42%), hepatosplenomegaly (30%), and splenectomy (6%), with no complications observed in controls ($p < 0.01$). These were strongly linked to elevated ferritin levels caused by iron overload from regular transfusions. Splenectomized patients had the highest ferritin levels (3000.00 ng/ml), highlighting the necessity for targeted chelation therapy to prevent severe outcomes. Similar findings were observed in a Thailand study, where splenectomized β-thalassemia patients had higher ferritin levels than nonsplenectomized individuals (27). All patients required blood transfusions, predominantly starting at two months (42%) or three months (30%). Transfusion intervals varied, with most patients receiving transfusions every three weeks (54%), followed by two weeks (30%), four weeks (10%), and five weeks (6%). Frequent transfusions were strongly associated with elevated ferritin level highlighting the significant iron burden. These results highlight the necessity of patient-specific transfusion strategies and effective iron management strategies (28). In β-thalassemia major, persistently elevated serum ferritin levels indicate severe iron overload, a common consequence of frequent blood transfusions. Without proper management, excessive iron accumulation can lead to progressive organ damage and severe, potentially life-threatening complications. These results underscore the importance of routine monitoring of hematological parameters and serum ferritin concentrations to effectively minimize related with health risks and enhance patient outcomes (29). Socioeconomic disparities significantly impact the management and outcomes of βthalassemia major, with rural populations facing inadequate health provision, chelation therapy, and regular monitoring. Addressing these inequalities is essential to improving patient outcomes and alleviating the disease burden. A parallel study similarly described linking rural residence and lower income to reduced healthcare access for thalassemia patients (30). These findings highlight the significant systemic challenges, clinical disparities, and the need for targeted management strategies for β-thalassemia major patients.

Profiling of Hematological and Biochemical Markers

Hematological analysis revealed Blood group analysis revealed significant differences, with group B most prevalent in controls (34%) and group O dominant in patients (36%). Rh positivity was more frequent in patients (96%) than controls (92%), with notable variations among Rh-negative individuals, indicating possible genetic or environmental factors influencing disease prevalence. These results align with studies from India and Pakistan, which identified group O as the most common among thalassemia patients, followed by groups B, A, and AB (31). as shown in (Table 3). These variations suggest underlying genetic and environmental influences on β-thalassemia major prevalence and management.

Table (3): Distribution of Blood group and Rhesus factor to subject

			Controls			Patients		
Blood group			RH		Total	RH		Total
			-	+		-	+	
ABO	B	N	1	16	17	1	11	12
		%	25.0%	34.8%	34.0%	50.0%	22.9%	24.0%
	O	N	3	11	14	1	17	18
		%	75.0%	23.9%	28.0%	50.0%	35.4%	36.0%
	A	N	0	16	16	0	16	16
		%	0.0%	34.8%	32.0%	0.0%	33.3%	32.0%
	AB	N	0	3	3	0	4	4
		%	0.0%	6.5%	6.0%	0.0%	8.3%	8.0%
Total		N	4	46	50	2	48	50
		%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

Significantly reduced red blood cell counts (2.71±0.45 × 10^{12}/L) and hemoglobin levels (7.73±1.41 g/dL) in patients compared to controls (4.52±0.53 × 10^{12}/L and 11.78±1.69 g/dL, $p<0.01$). While mean corpuscular volume (MCV) was slightly lower in patients, mean corpuscular hemoglobin (MCH) and concentration (MCHC) were elevated, indicating impaired erythropoiesis (32). Patients demonstrated increased lymphocyte percentages (39.40±8.22% vs. 28.55±7.14%) and decreased neutrophil counts (47.94±9.45 × 10^9/L vs. 60.62±7.81 × 10^9/L), reflecting immune dysregulation associated with transfusion dependency. Platelet counts were slightly elevated but not statistically significant ($p=0.19$), consistent with similar observations in an Indian study on β-thalassemia major (33). Biochemical analysis demonstrated the systemic impact of β-thalassemia major, with patients showing significantly elevated ferritin levels (2276.82±875.17 ng/mL) compared to controls (27.46±3.64 ng/mL, $p<0.01$), attributed to regular transfusions and iron overload. Liver function tests revealed increased ALT, AST, and ALP levels, indicating liver stress from chronic iron toxicity. Biochemical analysis demonstrated the systemic impact of β-thalassemia major, with patients showing significantly elevated ferritin levels (2276.82±875.17 ng/mL) compared to controls (27.46±3.64 ng/mL, $p<0.01$), attributed to regular transfusions and iron overload. These findings are in agreement with previous studies, which

also declared that the significantly elevated serum ferritin concentrations among β-thalassemia major patients (890±446.38 µg/L) relative to healthy individuals, due to chronic iron overload from repeated transfusions. However, the higher ferritin levels observed in the present study suggest a greater iron burden, which may reflect differences in transfusion frequency, chelation therapy compliance, or regional variations in clinical management . Additionally, liver function tests revealed increased ALT, AST, and ALP levels, indicating hepatic stress consistent with chronic iron toxicity, as similarly reported in previous literature. These results highlight the importance of routine monitoring of iron status and liver function parameters in patients with β-thalassemia major to prevent organ damage and improve long-term outcomes. (34). Kidney function tests showed decreased urea (25.27±7.00 mg/dL vs. 31.14±10.10 mg/dL) and serum creatinine levels (0.62±0.10 mg/dL vs. 0.75±0.17 mg/dL) in patients compared to controls, indicating potential renal impairment linked to disease progression and transfusion-related complications. Nonetheless, studies suggest renal function remains largely preserved across beta-thalassemia variants (35). A significant reduction in serum creatinine levels and an elevation in urea concentrations were identified among patients undergoing blood transfusions and chelation therapy. Likewise, another study reported higher levels of urea, uric acid, and creatinine in thalassemic patients when compared to healthy controls (36). Hemoglobin electrophoresis is crucial in assessing fetal hemoglobin (HbF) levels in beta thalassemia patients (37). Hemoglobin electrophoresis showed markedly elevated HbF levels in patients (81.41±5.63%) compared to controls (0.65±0.15%, $p<0.01$), reflecting increased erythropoietin production and enhanced erythropoiesis in healthy controls (38).Adult hemoglobin (HbA1) levels were markedly reduced in patients (11.74±5.22%) compared to controls (96.65±0.86%, $p<0.01$), reflecting impaired hemoglobin synthesis and compensatory HbF persistence, key contributors to the clinical features of β-thalassemia (39). Comparable hemoglobin A2 (HbA2) levels between groups indicate that this parameter may not significantly differentiate disease severity, as noted in previous studies (40). as shown in (Table 4).

Table (4): Hematological and biochemical evaluation in β-thalassemic male and female patients as compared to control group.

Test (UNITE)	Patients (N=50) Mean± S.D.	Control (N=50) Mean± S.D.	p-value
Blood counts			
RBC (10^{12}/L)	2.71±0.45	4.52±0.53	<0.01**
HGB (g/dL)	7.73±1.41	11.78±1.69	<0.01**
MCV (fL)	77.21±5.73	79.39±9.17	<0.01**
MCH (pg)	27.57±2.01	26.26±3.04	<0.01**
MCHC (g/dL)	35.79±2.43	33.15±2.39	<0.01**
WBC (10^9/L)	8.78±4.82	9.19±2.51	0.59 NS
NEUT (10^9/L)	47.94±9.45	60.62±7.81	<0.01**
LYMPH (%)	39.40±8.22	28.55±7.14	<0.01**
PLT ((10^9/L))	318.48±157.94	286.08±77.54	0.19NS
Ferritin (ng/ml)	2276.82±875.17	27.46±3.64	<0.01**
Liver function tests			

Test (UNITE)	Patients (N=50) Mean± S.D.	Control (N=50) Mean± S.D.	p-value
ALT (IU/L)	35.09±30.37	25.00±8.58	0.02*
AST(IU/L)	46.10±22.58	24.10±8.50	<0.01**
ALP (IU/L)	162.04±62.61	98.02±19.47	<0.01**
Kidney function test			
UREA (mg/dL)	25.27±7.00	31.14±10.10	<0.01**
Serum creatinine (mg/dL)	0.62±0.10	0.75±0.17	<0.01**
Hemoglobin electrophoresis test			
HbF (%)	81.41±5.63	0.65±0.15	<0.01**
HbA1 (%)	11.74±5.22	96.65±0.86	<0.01**
HbA2 (%)	2.52±0.61	2.68±0.48	0.14 NS

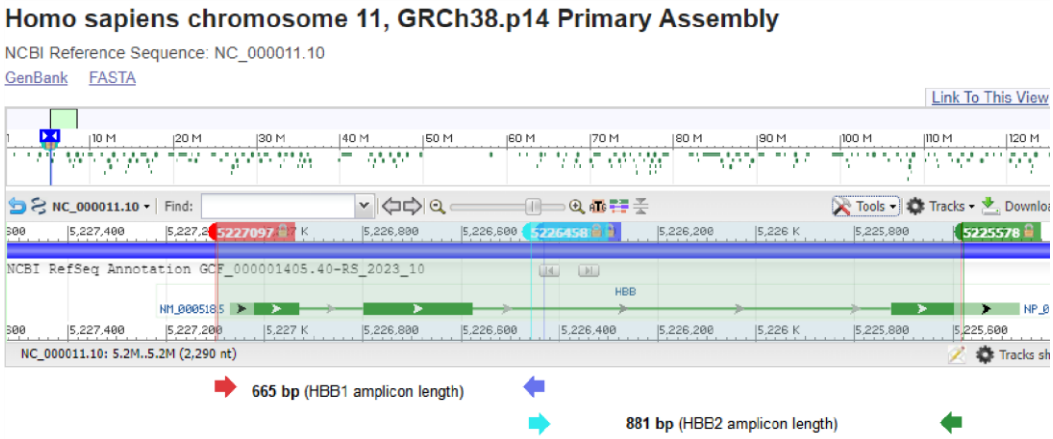
Complications further elevated ferritin levels, with splenomegaly (2306.70±633.97 ng/mL) and hepatosplenomegaly (2646.55±730.45 ng/mL) patients showing higher levels than those without complications (1504.14±100.63 ng/mL). Splenectomy patients recorded the highest levels (3000.00 ng/mL), indicating severe iron overload. These findings underscore the need for tailored management, chelation therapy, and regular monitoring to address iron-related complications effectively (41). as shown in (Table 5).

Table (5): Ferritin serum level and complications of patients with major bate thalassemia compare with controls

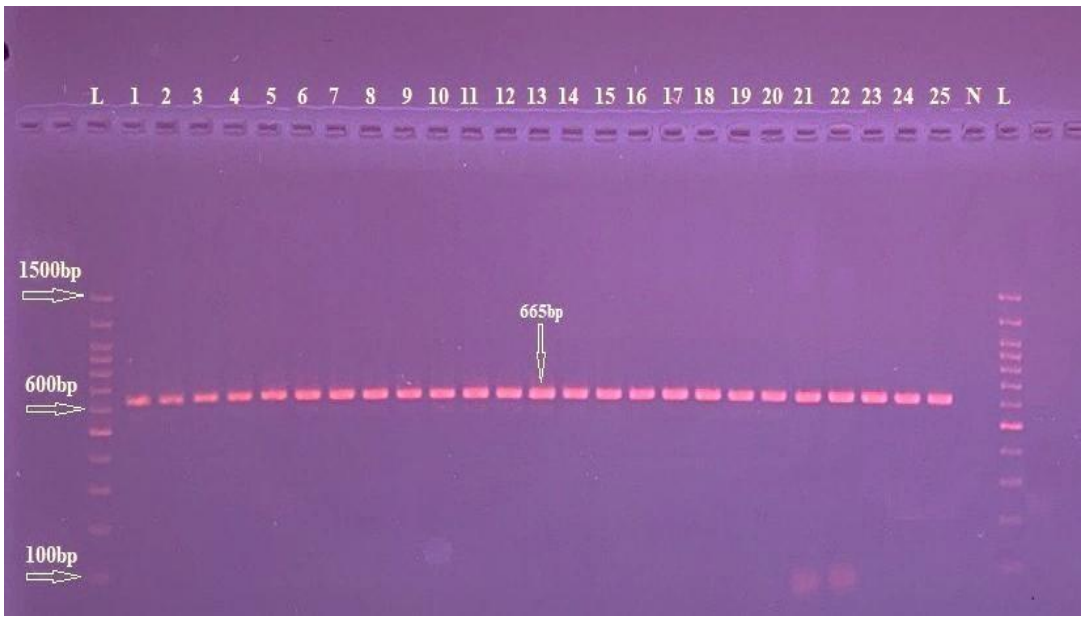
Group	Complications	N	Ferritin (ng/mL) Mean± S.D.	P-Value
Control	-	50	27.46±3.64	<0.01**
Patients	**NON**	9	1504.14±100.63	
	Splenomegaly	21	2306.70±633.97	
	Hepatosplenomegaly	15	2646.55±730.45	
	Splenectomy	3	3000.00±0.01	
	Splenomegaly/colostomy	1	1640.50±0.0	
	Splenectomy/hepatomegaly	1	3000.00±0.0	

Molecular analysis and SNPs identification

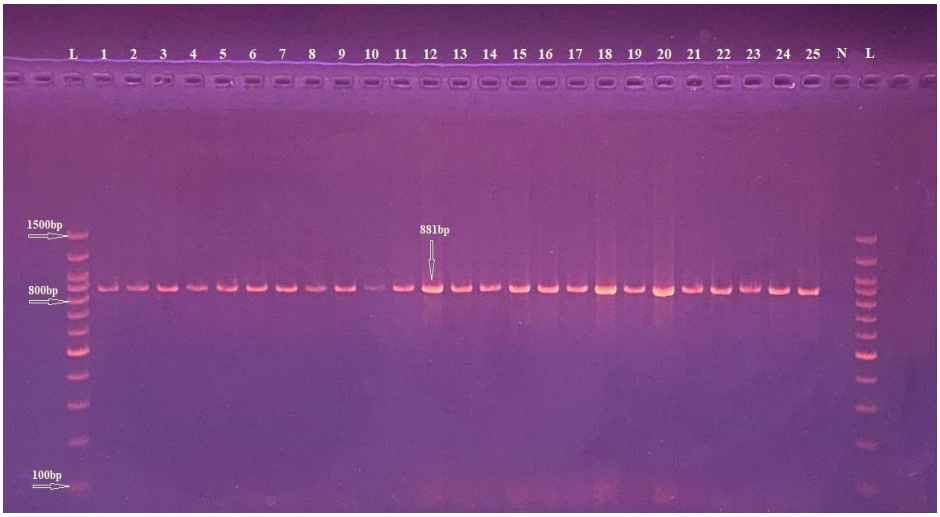
These overlapping amplicons were specifically designed to encompass all possible genetic variations within the *HBB* gene, ensuring comprehensive coverage of potential mutations and polymorphisms as shown in (Figure 1). Two specific PCR amplicons, with expected lengths of 665 bp and 881 bp, were successfully designed to encompass all coding regions of the *HBB* gene, as confirmed by gel electrophoresis as shown in (Figure 2) and (Figure 3). After subjecting these amplicons to Sanger sequencing, a total of thirteen SNPs were identified in the samples, located at various loci within the *HBB* gene. Six SNPs were detected in the 665 bp amplicon alignments, while seven SNPs were identified in the 881 bp amplicon alignments relative to the reference sequences of the *HBB* gene. Among the SNPs detected in both amplicons, some exhibited all three typical zygosity states – major homozygous, heterozygous, and rare homozygous – while others were observed only in the major homozygous and heterozygous states as shown in (Figure 5) (Suppl. Material).



Figure(1): The exact position of the retrieved two PCR amplicons (665 bp and 881 bp) that covered the majority of *HBB* genes. The red and blue arrow refers to the starting and end points of the *HBB1* (665 bp) amplicon while the cyan and green arrow refers to the start and end points of the *HBB2* (881 bp) amplicons.



Figure(2): shows an agarose gel electrophoresis of PCR products from 25 PCR assays using the *HBB* 1 primer, displaying an amplicon size of 665 bp. The primer's melting temperature was 60 °C, and the gel conditions were 1.5% agarose, initially run at 110 volts for 15 minutes before reducing to 75 volts for an additional 60 minutes. After running, the gels were stained with ethidium bromide and visualized under UV light. Lane L on the gel contains a DNA ladder ranging from 1500 to 100 bp, Lanes 1-25 show positive amplification results, and Lane N serves as a negative control.



Figure(3): shows an agarose gel electrophoresis of PCR products from 25 PCR assays using the *HBB 2* primer, displaying an amplicon size of 881 bp. The primer's melting temperature was 59 °C, and the gel conditions were 1.5% agarose, initially run at 110 volts for 15 minutes before reducing to 75 volts for an additional 60 minutes. After running, the gels were stained with ethidium bromide and visualized under UV light. Lane L on the gel contains a DNA ladder ranging from 1500 to 100 bp, Lanes 1-25 show positive amplification results, and Lane N serves as a negative control.

The zygosity states of the one SNP (71A>C) within the 665 bp amplicons and the two SNPs (94-95insG and 334G>A) within the 881 bp amplicons were manually verified in their respective electropherogram files (Figure 5 A). The clarity and distinctiveness of the identified polymorphic peaks validated the sequencing procedures and confirmed the reliable identification of SNPs. By reviewing the dbSNP server, further details for these SNPs were identified regarding their corresponding genomic sequences for both amplicons, 665 bp and 881 bp (Figure 5 B).

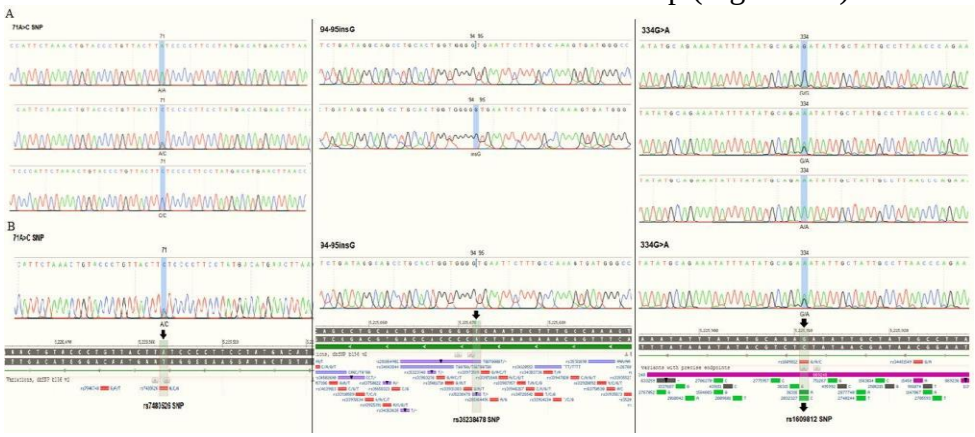


Figure (5): (A): This illustration presents the genotyping chromatograms and the genomic locations of : SNP rs7480526 (71A>C), within the *HBB1* gene and the genomic locations of SNPs 94-95insG and 334G>A within the *HBB2* gene. It shows the pattern of the detected variants in the DNA chromatogram files targeting specific loci of the *HBB1 and HBB2 amplicons*. The identified variants are distinctly marked according to their positions in the PCR amplicons, with the symbol "ins" denoting an insertion SNP and the symbol ">" denoting a substitution of SNP. Part (B) of the figure involves the SNP novelty assessment for the *HBB* 1and *HBB2* amplicons using the dbSNP server, where the identified SNPs are indicated with black arrows on the corresponding figures.

For the 665 bp amplicons, the intronic SNP 71A>C, located at the intron, is cataloged as rs7480526. For the 881 bp amplicons, the intronic SNPs 94-95insG and 334G>A are cataloged in dbSNP as rs35238478 and rs1609812, respectively.

Genetic association analysis

The relationship between the *HBB* gene and beta-thalassemia was assessed by analyzing the allelic and genotypic distributions of three identified SNPs using genotype-phenotype association analysis. Among the variants analyzed, rs7480526 There was a notable correlation with β-thalassemia. Individuals carrying the AA genotype were found to have an increased risk, with an odds ratio (OR) of 2.53 (95% CI: 1.12–5.70, a p-value of 0.04). Furthermore, the A allele appeared more frequently in patients than in the control group. Reinforcing its role in disease susceptibility ($p < 0.01$) as shown in (Table 6).

Table (6): Genotypes and allelic frequencies of the identified SNPs in the *HBB* gene and their association with the risk of beta-thalassemia.

SNP thalassemia ID	Patient (n=50)	Control (n=50)	Odds ratio	95%CI	`P-value
71A>C (rs7480526)		`			
AA	26(52.0)	15(30.0)	2.53	1.12-5.70	**0.04***
AC	9(18.0)	18(36.0)	0.39	0.16-0.97	0.07 NS
CC	15(30.0)	17(34.0)	0.83	0.36-1.91	0.83 NS
A allele	61(61.0)	48(48.0)	2.09	1.17-3.71	**<0.01****
C allele	39(39.0)	52(52.0)	0.59	0.34-1.03	0.08 NS
334G>A (rs1609812)		`			
	37(74)	37(74)	Ref.	(0.20-1.44)	
GG	8(16)	13(26)	2.0	(0.68-	0.32
GA	5(10)	0(0)	12.2	220.52)	**0.049***
AA	82(82)	87(87)	0.68	(0.311.47)	0.43
G allele	18(8)	13(13)	1.47	(0.68-3.17)	0.43
A allele					

Concerning the detected 71A>C SNP in the *HBB1* amplicons, dbSNP showed that this SNP was previously deposited in the genome under the rs number rs7480526. This SNP is also located in the intron sequences of the *HBB* gene (https://www.ncbi.nlm.nih.gov/snp/rs7480526). The identified SNP was precisely located within the genomic sequence of chromosome 11 at NC_000011.10:g.5226503A>C. Analysis of the dbSNP database indicated a high prevalence of the rs7480526 variant, with an alternative allele frequency of 0.441891, as reported in the GnomAD database. The observed high frequency of this SNP in the study population aligns with previously documented data, reinforcing its significance in genetic variation studies (42). Interestingly, one insertion SNP was detected between 94th and 95th positions of the *HBB2* amplicons (94-95insG SNP). The dbSNP server showed that this SNP was previously deposited in the genome under the rs number rs35238478. This SNP is located in the coding sequences of the *HBB* gene (https://www.ncbi.nlm.nih.gov/snp/rs35238478). The exact position of this SNP within the genomic sequences was NC_000011.10: g.5225672-5225673ins. Though this SNP was deposited in the dbSNP server as a deletion SNP, this study found that the detected polymorphism in the locus took the pattern of insertion. Due to the

positioning of this SNP in the coding region of the *HBB* gene, it is found that the insertion of guanine has occurred in the amino acid residue threonine that is located in position 124 in the entire sequence of *HBB*. This insertion of adenine causes a frameshift in the reading frame in the translation machinery of *HBB* (NP_000509.1:p.Thr124fs). Due to this frameshift, possible drastic consequences in the biological activity of *HBB* were expected for those patients with this SNP. In our study, only two individuals exhibited this deleterious frameshift alteration, while the other 98 individuals did not. An extremely low frequency of the deposited rs35238478SNP in the dbSNP database was inferred, which was estimated to be 0.00003 for the alternative allele according to the PAGE-STUDY database. No frequency of rs35238478 was deposited in the dbSNP, and this SNP was cited in only one study that assessed the putative effect of rs35238478 on some metabolic disorders in the body (43) . Conversely, analysis of the rs1609812 SNP revealed a significantly higher frequency of the minor homozygous AA genotype in the patient group (10%) compared to the control group (0%), suggesting an increased risk of developing beta-thalassemia ($p = 0.049$, OR = 12.2). as shown in (Table 6) This finding indicates a strong association between rs1609812 and the presence of this high-risk genotype in affected individuals. This intronic SNP rs1609812 has shown a significant association with beta-thalassemia, particularly among individuals with the rare homozygous rs1609812: AA genotype, who were found to have a higher risk of developing beta-thalassemia. This association aligns with findings in other studies, including reports of two Kelantan Malay individuals in whom this SNP was also linked to beta-thalassemia risk (44) . However, the role of rs1609812 in beta-thalassemia may vary across different populations. For instance, in a case involving a Portuguese infant with beta-thalassemia, this SNP was not reported to have any pathogenic effect (45). Additionally, in a study on a Southeast Turkish population, this polymorphism was not associated with higher levels of fetal hemoglobin, indicating no impact on the severity of beta-thalassemia (46). These controversial differences suggest that the impact of the rs1609812 may depend on variable genetic and possibly non-genetic factors specific to each population. The major limitation of this study is the small sample size, which diminishes statistical power, potentially obscuring true associations and increasing the likelihood of false-negative results. This constraint affects the generalizability of findings, making it more challenging to establish definitive relationships between molecular analysis, hematological and biochemical variations, and disease susceptibility. Furthermore, the restricted sample size limits the feasibility of subgroup analyses, potentially leading to inflated odds ratios due to sampling variability. Therefore, the reliability, interpretability, and reproducibility of the findings may be compromised, underscoring the need for larger, more diverse cohorts in future research.

Conclusion

This study provides a comprehensive analysis of the genetic and clinical profile of βthalassemia major in Southern Iraq, identifying critical *HBB* gene mutations that influence disease severity.

Significantly, SNPs rs7480526 (71A>C) and rs1609812 (334G>A) exhibited a strong association with β-thalassemia susceptibility, while the rare rs35238478 (94-95insG) indicated a possible role in disease expression. Hematological and biochemical evaluations confirmed severe anemia, disrupted hemoglobin composition, and significantly elevated ferritin levels, particularly among splenectomized patients, reinforcing the necessity for optimized transfusion regimens and iron chelation therapy. As a results, consanguineous marriages and rural residency were identified as major risk factors, highlighting the urgent need for genetic counseling to mitigate disease incidence. These findings offer essential insights into molecular diagnostics and personalized disease management, emphasizing the importance of advanced genetic screening and targeted therapeutic interventions to enhance patient outcomes.

Conflict of interest

It is hereby declared that the authors have disclosed no conflicts of interest related to this work.

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