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Article

Clinical, Biochemical, and Molecular Characterization of HBB Gene Variants in Iraqi Patients with β-Thalassemia Major

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Abstract: β-Thalassemia major is an inherited blood disorder that affects hemoglobin, caused by mutations in the HBB gene. This disease results in chronic hemolytic anemia and dependence on blood transfusions for life. Although the frequency of β-thalassemia major is high in Iraq, there are only a small number of extensive studies that have looked at the relationship between molecular studies, clinical studies, and biochemical studies in this population. The purpose of this study was to identify mutations in the HBB gene of patients diagnosed with β-thalassemia major and to assess the relationships between mutations and both the clinical/biochemical characteristics of patients. 50 transfusion-dependent β-thalassemia major patients and 50 healthy controls. Clinical characteristics were collected for all subjects. All subjects underwent liver function tests, kidney function tests, hemoglobin electrophoresis, and serum ferritin tests. Mutations within the HBB gene were identified using two overlapping polymerase chain reaction (PCR) products, followed by Sanger sequencing, and compared bioinformatic tools. When compared to healthy controls, patients demonstrated significantly higher serum ferritin levels, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and fetal hemoglobin (HbF), with significantly lower HbA1 levels ($P < 0.05$). Additionally, serum ferritin levels were found to correlate with the transfusion interval, presence of clinical complications, and location of residence. Upon molecular characterization, three genetic variants were identified within the HBB gene: rs1451581925 (386A>T), rs35492035 (59C>G), and rs1609812 (334G>A). Among them, a rare missense variant predicted to replace alanine with proline (p.Ala136Pro) was rs35492035 and rs1609812 were significantly associated with β-thalassemia major susceptibility ($P = 0.049$) respectively. The metabolic manifestations observed in Iraqi suffering from β-thalassemia major were associated with a set of biochemical alterations and a wide range of HBB gene variants, supporting the influence of and value of rare coding variants to improve the genetic characterization of β-thalassemia in Iraqi population.

Keywords: β-Thalassemia Major, HBB Gene, Sanger Sequencing, Missense Mutation, Iron Overload

Introduction

Thalassemia is a genetic hemoglobin condition resulting from an abnormal production process in one or more types of globulin chains. Thalassemia is characterized by the following three things: ineffective production of red blood cells; ongoing low blood iron level(s); and low capacity for blood to carry oxygen. One of the many types of thalassemia is called β-thalassemia. β-thalassemia results from a genetic mutation in the β-globin (*HBB*) gene located on chromosome 11p15.5, causing either a decrease (β^{+}) or complete lack (β^{0}) of production of β-globin chains. Because all types of thalassemia disrupt the ability to produce normal hemoglobin, there are many ways that various forms of thalassemia can appear in humans [1] . This disease is considered a major public health problem worldwide, including southern Iraq, where previous studies have shown an increase in patients with β-thalassemia and other disorders of hemoglobin [2]. There are over 300 different genetic mutations in the *HBB* gene, which creates a broad genetic diversity and a commensurate broad spectrum of clinical severity between affected people, and underscores the need for continued investigation into genotype-phenotype relationships for individuals from different geographical areas [3] .

The *HBB* gene has undergone a variety of genetic alterations: single nucleotide substitutions, insertion, deletions and splice-site variations. All of these alterations cause a disruption in the normal process of β-globin synthesis which affects erythropoiesis. This ultimately results in the inability to produce red blood cells because of either homozygous or compound heterozygous individuals will show a much more severe clinical phenotype as compared to heterozygous carriers [4]. The development of bioinformatic tools has allowed for the identification and characterization of many *HBB* variants, which have been put into databases, for example the Database of single Nucleotide Polymorphisms (dbSNP) and are useful for annotating variants, classifying SNPs, and conducting genotype - phenotype association studies for precision medicine [5].Mutations at the splice site can disturb normal β-globin development and disrupt normal development of red blood cells through impacting normal erythropoiesis [6]. Some mutations in the intron could activate cryptic splice sites, resulting in abnormal processing of mRNA and reduced production of β-globin chains [7] . In general, there is a direct correlation between the type, site, and zygosity of mutations in *HBB* with the clinical severity of β-thalassemia and individuals that are homozygous typically show a more severe phenotype than heterozygous carriers[8] . There has been substantial improvement in the ability to identify and characterise *HBB* variants with the advent of bioinformatics and the use of databases such as the Database of Single Nucleotide Polymorphisms (dbSNP) are critical resources for variant annotation, SNP classification, and for genotype–phenotype associations studies related to precision medicine [9]. Patients with β-thalassemia major have very variable disease severity and clinical presentation, despite having the same underlying genetic disorder (i.e., a mutation in the *HBB* gene). The severity of the disease and the clinical presentation are heterogeneous and can differ significantly among individuals due to many factors that can influence disease severity, including the types of mutations in the *HBB* gene, the extent of transfusion requirements, the amount of iron overload from transfusions, the presence or absence of additional modifying factors, and other genetic factors (which means they can all affect the hematological and biochemical profiles of individuals with β-thalassemia major in different ways). As a result, there is increased emphasis on evaluating genotype–phenotype relationships to better understand the progression of the disease, predict clinical outcomes, and optimize patient management [10][11] . Routine lab tests are essential to monitoring patients who have β-thalassemia major as well as evaluating problems that may arise from the disease. The amount of serum ferritin in the body is an indirect measurement of how much iron has been built up from too many blood transfusions. In addition, blood tests that measure liver and kidney function provide important information on how these organs have been affected by repeated blood transfusions and exposure to excess iron. The hemoglobin electrophoresis is very helpful for diagnosis and monitoring because it shows how much HbF, HbA1, and HbA2 are present, which gives valuable information about the degree of severity of disease and the effectiveness of treatment [12].

Materials and Methods

Study Design and sampling

This study was of a case-control nature and included 100 individuals, 50 transfusion-dependent beta-thalassemia patients, provided samples for analysis of plasma and serum of 50 unaffected age-matched controls. The 50 beta-thalassemia individuals recruited were (28 male and 22 female) should be considered for possible participation in clinical trials or clinical care — these patients met the established criteria for transfusion-dependent beta-thalassemia major and exhibited laboratory evidence of hematologic abnormalities. Beta-thalassemia patients had been regularly transfused at intervals of once every (2-4 weeks) and met all of the following criteria to participate in the project transfusion-dependent beta-thalassemic major diagnosis severe hypochromic anemia [Hb <] and mean corpuscular volume (MCV <75 fL); and (3) HbF >3.5% (per hemoglobin electrophoresis) with reported lives. Blood samples were collected between March 2024 and December 2024 from patients and control groups at the Children and Women Hospital of Al-Muthanna Health Department and the Centre for Genetic Blood Disease in Thi-Qar Health Department, Iraq. Data on demographic characteristics and medical reports were collected from patients through standardized structured interviews by trained research assistants. All patients provided written informed consent from all participant parent or guardian to provide the blood sample prior to its collection. A sterile 5 mL peripheral blood sample was collected under aseptic conditions from each participant into vacuum tube with EDTA [13]. In addition to providing two mL of whole blood for analysis of the blood cells, a separate 3 mL of whole blood was then centrifuged to separate the serum portion, which was then divided into aliquots to be stored at −20°C for later biochemical testing [14] . Participants were advised they may withdraw from the study at any point and will not suffer any repercussions.

Hematology, biochemical, and Hemoglobin electrophoresis Assays

To perform blood tests (hematological analysis) for blood types [ABO and Rh], hemoglobin [Hg], white blood cells [WBC], The number of each type of white blood cell including neutrophils [NEUT], lymphocytes [LYMPH], mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], mean corpuscular hemoglobin concentration [MCHC], and platelets [PLT], were assessed with an Automated Hematology analyzer (BC-3000plus, Mindray, China) biochemical parameters, including creatinine, urea, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), were measured using the BS240 automated analyzer (Mindray, China) [15] . Serum ferritin levels were determined using the Chemiluminescence Immunoassay System (CL-900i, Mindray, China) [16].Hemoglobin types, including HbA1, HbA2, and HbF, were elevated by using the Hemoglobin Testing D-10 System [17].

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. Both purity and integrity of the extracted DNA were evaluated through 1% (w/v) agarose gel electrophoresis, while DNA quantification was identified by using a Nanodrop spectrophotometer (Biodrop, UK).

HBB Gene Amplification, Sequencing, and Variant Identification

The *HBB* gene-specific PCR primers were designed by using the NCBI Primer BLAST tool based upon the reference sequence (Accession No. NC_000011.10) found in GenBank. There were two sets of overlapping primer pairs to allow for total coverage and amplification of the target area. Afterward, PCR conditions were optimized for effective and specific amplification of the sequences of interest. [18]. The *HBB* gene's possible variations were intended to be captured by these overlapping PCR amplicons. The first amplicon ranges from a forward primer (5'-ACGATCCTGAGACTTCCACAC-3') and reverse primer (5'-AGTCAGGGCAGAGCCATCTA-3') of 665 base pairs (bp), including 5' UTR, exon 1, intron 1, and exon 2, and this amplicon also included a portion of the region upstream of intron 2. The second amplicon was at 881 bp in length and contained a forward primer (5'-TGGACAGCAAGAAAGCGAGC-3') and a reverse primer (5'-CATCAGTGTGGAAGTCTCAGGAT-3') and included the downstream region of intron 2, exon 3, and part of the 3' UTR of the *HBB* gene. PCR amplification was performed at optimized conditions to maximize specificity and efficiency for

amplification of the intended amplicon sequence during the thermal cycling process following the manufacturer's directions as depicted in (Table 1). Using 1.5% (w/v) agarose gel electrophoresis, PCR products were evaluated to determine the integrity and specificity of the PCR products, as well as to allow quantification of the PCR amplification and validation of whether the PCR amplified the target sequence correctly. The PCR amplification products were further validated by sequencing using Sanger methodology, according to standard protocols, with chromatograms assessed with BioEdit (version 7.1), using visualization tools, alignment and trimming against the GenBank reference sequence (Accession No. NC_000011.10). Single nucleotide polymorphisms (SNPs) identified from the sequence analysis have undergone manual validation through sequence electropherograms using SnapGene Viewer to ensure the SNPs were accurately detected. Non-standard SNPs were removed according to established protocols in order to maintain data accuracy and reliability[17] Each sequenced sample has been assigned a numeric classification based on the specific genomic location of detected SNPs found in the PCR target region and how they were located in the reference genome. Then, each SNP was assessed for novelty using dbSNP (single nucleotide polymorphism) to determine if the SNP had previously been described, or if it was a unique SNP[18] .

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 cycle)
Denaturation	94°C	30 seconds	
Annealing	60°C	30 seconds	(32 cycles)
Extension	72°C	60 seconds	
Final Extension	72°C	5 minutes	(1 cycle)
Hold	4°C	∞	

Sequence Analysis and SNP Identification

The Sanger dideoxy method was used to sequence all PCR amplified fragments according to the standard protocols at Macrogen Inc. (Seoul, Korea). The resulting electropherogram files were processed by trimming them and then aligning them to the *HBB* gene reference sequence using BioEdit software (7.1; Madison, USA). All identified SNPS were visually assessed by comparing with the electropherogram and each SNP was checked for novelty against the dbSNP database with the SnapGene Viewer tool (https://www.ncbi.nlm.nih.gov/snp/). Moreover, 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 was analyzed with the program SPSS Version 20 (IBM Corp., Armonk NY, USA), where frequency, percentages and mean ± standard deviations were calculated. A p value of less than 0.05 was considered statistically significant while a p value of less than 0.01 was considered of very high statistical significance. Differences in HbA2 levels presented as mean ± SD were compared to Student's t-test and analysed with ANOVA.

Results and Discussion

Clinical and Demographic Profile Analysis

The data revealed that βTM patients possessed significantly inferior clinical profiles compared to non-βTM control participants in terms of laboratory findings and many other clinical parameters including hematological and biochemical values, and complications. The sex distribution of the control and patient groups did not differ greatly (male: 44% female: 56%; p = 0.1); additionally, while βTM patients had a mean age of (9.8 ± 3.6 years), controls possessed an average age of (13.4 ± 6.2) years (p = 0.06), indicating that neither sex nor age had a significant effect on the presentation of disease. This finding confirms earlier studies conducted in Indian and Iraqi populations which reveal similar trends regarding βTM patients' clinical presentations [19]. Age may also affect some aspects of β-thalassemia

management (β-TM); for instance, an Iranian study found that older patients were more likely to suffer from impaired growth (for each additional year of life, there is a 1.57 increased chance of this happening) [20]. Different groups had very different living conditions. Patients were mostly in rural areas (58%) while there were significantly more control subjects in urban areas (62%). Hence, the differences (p=0.04) underscore the effect of socio-economic and geographic variables on disease management. In rural areas, patients have less access to healthcare, which often leads to poorer health outcomes and therefore higher ferritin levels and more complications. A study from Iran had similar results pointing to the importance of living environments in the management of β-thalassemia illness [21]. All subjects reported different levels of consanguinity in regard to marriage; there was no evidence for consanguinity in control subjects ($p < 0.01$). Consanguinity has a significant effect on the genetic transmission of β-thalassemia and is a particularity in countries such as northern Iraq and Iran where there are higher levels of intermarriage. The study reveals the need for both genetic counseling and community awareness programs to reduce incidences and the severity of the disease [22]. 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%)	

There were complications reported in 82% of patients with splenomegaly in 42%, hepatosplenomegaly in 30% of patients and 6% of patients having unnecessary splenectomy procedures compared to controls who had no complications at all ($p < 0.01$). The primary factor contributing to these complications was excessive levels of ferritin due to iron deposits from regular blood transfusions received. Splenectomised patients had the highest levels of ferritin (3000.00 ng/ml), indicating the need for targeted chelation therapy to avoid severe complications. In addition, a study conducted in Thailand demonstrated that splenectomised patients with β-thalassaemia had an increase in ferritin compared to non-splenectomised patients [23]. Blood transfusions were essential for the great majority of subjects, beginning mainly at two months (n=42%) and at three months (n=30%). The data indicates that individuals with β-thalassemia major have many systemic and clinical problems and will likely require specific management strategies to help them with their disease.

Profiling of Hematological and Biochemical Markers

Patients diagnosed with β-thalassemia major exhibited considerable metabolic impairment in the liver, as evidenced by the biochemical assessment performed on these subjects and their control counterparts. Serum levels of alanine aminotransferase (ALT) were reported to be significantly elevated in β-thalassemia major patients (35.09 ± 30.37 IU/L) compared to healthy controls (27.00 ± 8.58 IU/L) (P

= 0.02). The levels of aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were also noticeably increased in β-thalassemia major patients (48.10 ± 22.58 IU/L and 163.04 ± 62.61 IU/L, respectively) compared to those of the control group (24.10 ± 8.60 IU/L and 99.02 ± 19.47 IU/L, respectively), with highly significant statistical value ($P < 0.01$) associated with both enzyme markers. This consistent association supports the concept of liver dysfunction in β-thalassemia major patients due to chronic blood transfusion therapy and iron overload from previous studies. These results emphasize the need for routine assessment of liver function tests and iron status in patients with β-thalassemia major to minimize the possibility of long-term organ damage and improve the potential for positive patient outcomes [12]. In study comparisons of patients with Beta-Thalassemia versus healthy control subjects, renal function tests showed decreased levels of blood urea nitrogen [BUN] (25.27±6.00 mg/dl compared to 31.14±10.10 mg/dl) and creatinine (0.62±0.10 mg/dl compared to 0.75±0.17 mg/dl) indicating potential for deterioration in kidney function correlated with disease progression or resultant from complications due to blood transfusion, though some literature demonstrates that renal function is markedly preserved among patients with varying expressions of Beta-Thalassemia [24]. The findings of the study showed that during blood transfusions and chelation treatment, serum creatinine decreased and urea increased significantly in patients with thalassemia. There was also a significant ($p < 0.001$) increase in urea, uric acid, and creatinine levels in thalassemic patients compared to healthy individuals based on previous reports [25]. Hemoglobin electrophoresis is crucial in assessing fetal hemoglobin (HbF) levels in beta thalassemia patients [11]. Hemoglobin electrophoresis showed markedly elevated HbF levels in patients (83.41±5.63%) compared to controls (0.67±0.15%, $p<0.01$), reflecting increased erythropoietin production and enhanced erythropoiesis in healthy controls [26]. Compared to controls (96.65±0.86%, $p<0.01$), hemoglobin (HbA1) levels were significantly decreased in patient populations (12.74±5.22%), indicating that there was an impairment of hemoglobin production and also a persistency of fetal-like hemoglobin (HbF), which are two main pathophysiologic mechanisms of the clinical manifestations of β-thalassemia [27]. In terms of HbA2 levels, no statistically significant difference was observed between the β-thalassemia major patients (mean value of 2.52 ± 0.61%) compared to the control group (mean value of 2.68 ± 0.48%), as determined by the level of significance ($P = 0.14$). These results validate previous studies which demonstrated that HbA2 levels of both groups were comparable [28]. Differences between patients and controls included significantly decreased red blood cell counts ($2.71 \pm 0.48 \times 10^{12}$/L) and hemoglobin concentrations (7.73 ± 1.41 g/dL) in patients; the corresponding means for controls are $4.52 \pm 0.53 \times 10^{12}$ and 12.78 ± 1.69 g/dL respectively ($p < 0.01$). In addition, MCV of patients was smaller than controls while both MCH & MCHC were increased, indicating a reduction in the production of new erythrocytes [29]. The immune dysregulation seen in patients' transfusion-dependent, as demonstrated by significant increases in lymphocyte numbers (39.40±8.22% vs. 27.55±7.14%) and decreases in neutrophil counts ($47.94±9.45 \times 10^9$/L vs. $62.62±7.81 \times 10^9$/L). Results showed a small increase in relative platelet count; however, this was also not statistically significant ($p=0.19$). Similarly, these findings were consistent with observations made from an Indian study of β-thalassemia major patients [30]. as shown in (Table 3).

Table 3. 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
Liver function tests			
ALT (IU/L)	35.09±30.37	27.00±8.58	0.02*
AST(IU/L)	48.10±22.58	24.10±8.60	<0.01**
ALP (IU/L)	163.04±62.61	99.02±19.47	<0.01**
Kidney function test			
UREA (mg/dL)	25.27±6.00	31.14±10.10	<0.01**
Serum creatinine (mg/dL)	0.62±0.10	0.75±0.17	<0.01**
Hemoglobin electrophoresis test			
HbF (%)	83.41±5.63	0.67±0.15	<0.01**

HbA1 (%)	12.74±5.22	96.65±0.86	<0.01**
HbA2 (%)	2.52±0.61	2.68±0.48	0.14 NS
Blood counts			
RBC (10^{12}/L)	2.71±0.48	4.52±0.53	<0.01**
HGB (g/dL)	7.73±1.41	12.78±1.69	<0.01**
MCV (fL)	77.21±5.74	79.39±9.17	<0.01**
MCH (pg)	27.57±2.01	26.26±3.04	<0.01**
MCHC (g/dL)	36.79±2.43	34.15±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	62.62±7.81	<0.01**
LYMPH (%)	39.40±8.22	27.55±7.14	<0.01**
PLT ((10^9/L))	320.48±157.94	286.08±77.54	0.19NS

Biochemical analysis demonstrated the systemic impact of β-thalassemia major, with patients showing significantly elevated ferritin levels (2281.82±875.17 ng/mL) compared to controls (27.46±3.64 ng/mL, p<0.01), as shown in (Table 4). attributed to regular transfusions and iron overload.

Table 4. Ferritin serum level in patients with major bate thalassemia compare with controls

Test (UNITE)	Patients (N=50) Mean± S.D.	Control (N=50) Mean± S.D.
Ferritin (ng/ml)	2281.82±875.17	27.46±3.64
N.R. =		
p-value =<0.01**		

Patients with β-thalassemia major had far greater amounts of serum ferritin compared to the control group (P < 0.01). The patient group having blood transfusions every 3 weeks showed the highest average serum ferritin level (2419.05 ± 815.32); those patients receiving blood transfusions every 2 weeks had almost the same average serum ferritin level (2407.37 ± 726.10); however, patients receiving blood transfusions at 4-week intervals had the lowest average ferritin concentration (1364.98 ± 107.77), while those receiving blood transfusions at 5-week intervals had intermediate serum ferritin levels (1863.70 ± 118.03). as shown in (Table 5). The average ferritin level of control patients (27.46 ± 3.64) was significantly less than the patient subgroups. In conclusion, shorter transfusion periods resulted in higher concentrations of serum ferritin, which is indicative of greater amounts of iron accumulating in blood transfusion-dependent patients, as has previously been reported [31] .

Table 5. Ferritin serum level and Transfusion interval to patients with major bate thalassemia compare with controls

Group	Transfusion interval (weak)	N	Ferritin ( ) Mean± S.D.	p-value
Controls	-	50	27.46±3.64	<0.01**
Patients	2 weak	15	2407.37±726.10	
	3 weak	27	2419.05±815.32	
	4 weak	5	1364.98±107.77	
	5 weak	3	1863.70±118.03	

Serum ferritin levels in β-thalassemia major patients were significantly elevated compared to the control group regardless of where they lived (P < 0.01). The mean ferritin levels for participants in the control group from rural areas were higher (35.49 ± 2.72), than those from urban areas (22.53 ± 9.30). Likewise, mean serum ferritin concentrations for patients from rural areas (2424.91 ± 734.00) were higher than urban patients (2072.30 ± 1002.00) as shown in (Table 6). Furthermore, it was noted that ferritin levels were elevated for both patients and controls residing in rural areas; however, rural β-

thalassemia major patients had the highest overall ferritin levels. Patients living in rural areas in this study also had higher serum ferritin levels than those living in urban settings. Differences in health care, follow-up, and adherence to iron-chelation therapies may have contributed to the high levels of ferritin among the rural patients. Similar findings have been noted by [32] , who demonstrated that urban residence was associated with better treatment adherence and emphasized the need to improve healthcare accessibility in rural regions to optimize disease management.

Table 6. Ferritin serum level and living of patients with major bate thalassemia compare with controls

Group	Living	N	Ferritin ( ) Mean± S.D.	P-Value
Control	Rural	19	35.49±2.72	<0.01**
	City	31	22.53±9.3	
Patients	Rural	29	2424.91±734	
	City	21	2072.30±1002	

Ferritin levels were also increased for patients experiencing any complications, and patients with splenomegaly (2306.70±633.97 ng/mL) and those with hepatosplenomegaly (2646.55±730.45 ng/mL) had increased ferritin levels compared to those that did not have complications (1504.14±100.63 ng/mL) in terms of the mean serum ferritin level. Patients who had a splenectomy had the highest ferritin levels, with a mean ferritin level of 3000.00 ng/mL, indicating that these patients had a serious iron overload. These results show that patients need individualized management, chelation therapy, and close monitoring for the effective management of iron-related complications [33]. as shown in (Table 7).

Table 7. 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	
Patients	NON	9	1504.14±100.63	<0.01**
	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 *HBB*1 665 bp and *HBB2* 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 three SNPs were identified in the samples, located at various loci within the *HBB* gene. One SNP were detected in the 665 bp amplicon alignments, while two 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 .

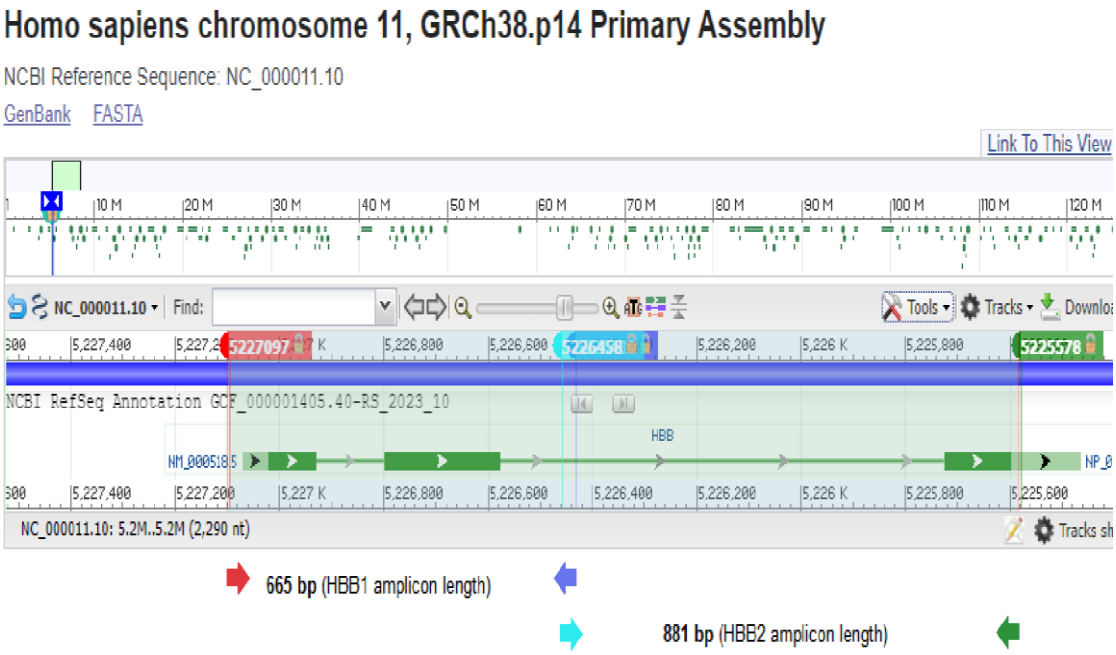

Figure 1. The exact position of the retrieved two PCR amplicons (665 bp and 881 bp) that covered the majority of *HBB* genes. The red and blue arrow refers to the starting and end points of the *HBB1* (665 bp) amplicon while the cyan and green arrow refers to the start and end points of the *HBB2* (881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

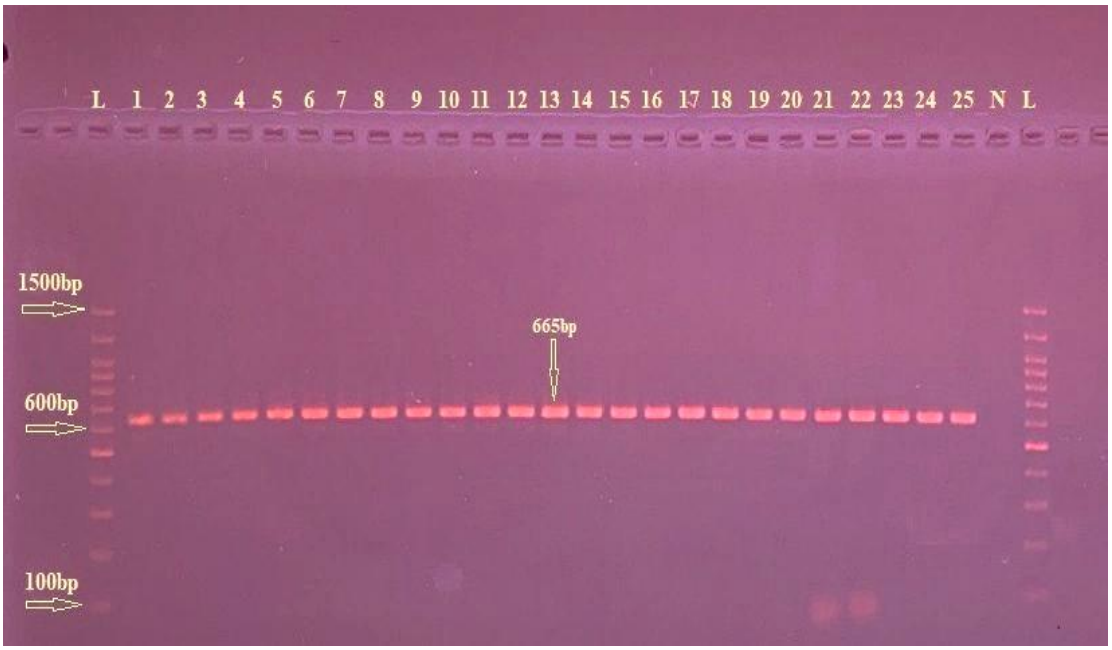

Figure 2. Shows an agarose gel electrophoresis of PCR products from 25 PCR assays using the *HBB* 1 primer, displaying an amplicon size of 665 bp. The primer's melting temperature was 60 °C, and the gel conditions were 1.5% agarose, initially run at 110 volts for 15 minutes before reducing to 75 volts 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.

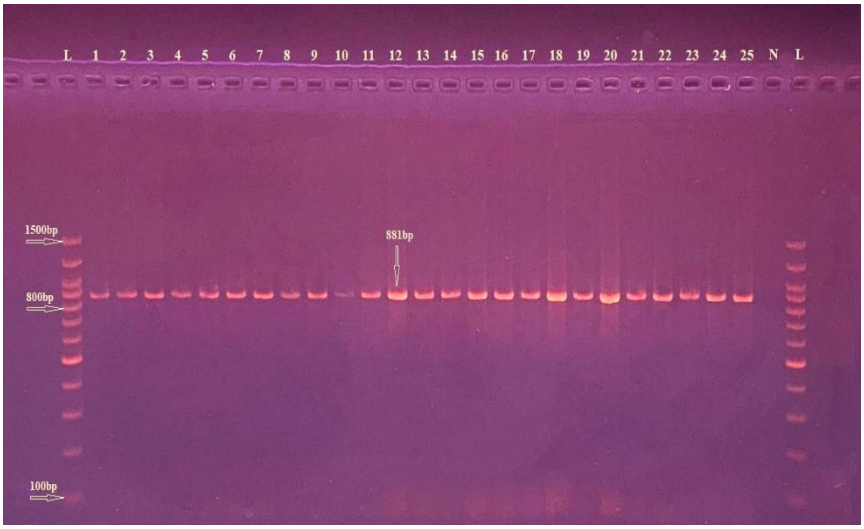

Figure 3. Shows an agarose gel electrophoresis of PCR products from 25 PCR assays using the *HBB* 2 primer, displaying an amplicon size of 881 bp. The primer's melting temperature was 58 °C, and the gel conditions were 1.5% agarose, initially run at 110 volts for 15 minutes before reducing to 75 volts 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 (386A>T) within the 665 bp amplicons (Figure 5 A). And the two SNPs (59C>G and 334G>A) within the 881 bp amplicons were manually verified in their respective electropherogram files (Figure 5 B). 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.

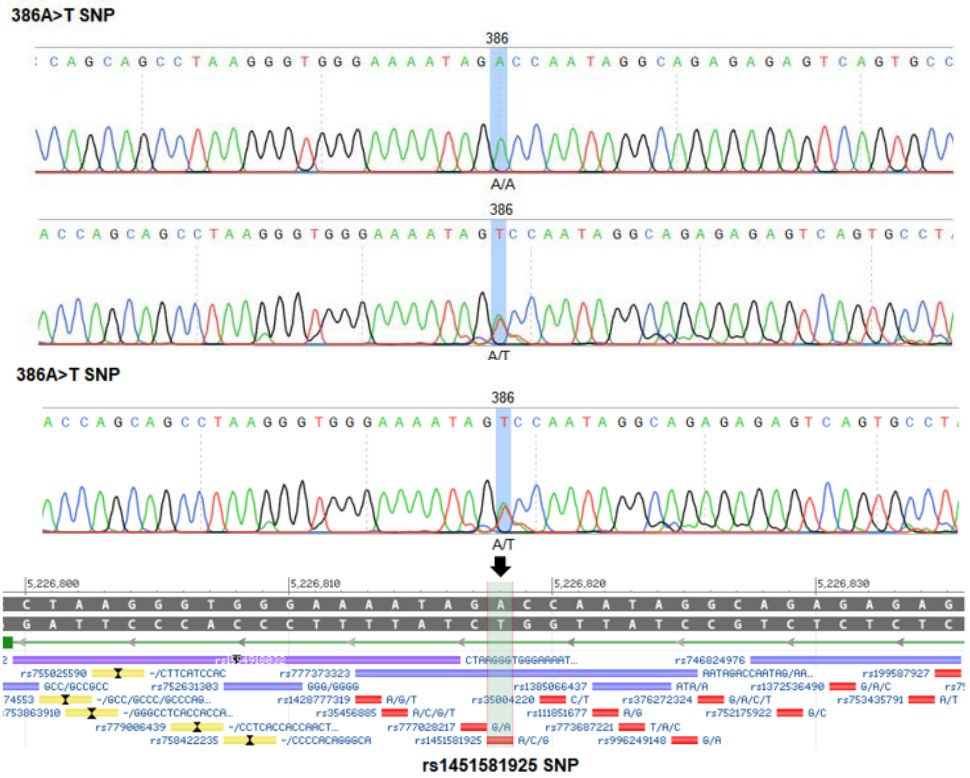

Figure 5 (A). The *HBB* gene variants were characterized based on the genotyping chromatograms, genomic localization, and dbSNP-derived novelty evaluation. The figure shows the

chromatograms of a representative set of *HBB*1 SNPs identified through Sanger sequencing. The locations of the SNPs (e.g., including 386A>T (rs1451581925) in the *HBB*1 amplicon) that had been detected relative to their corresponding genomic coordinate are shown, where ">" indicates a nucleotide substitution, and "ins" indicates an insertion variant. The novelty evaluation of the identified variants for *HBB*1 was determined using the dbSNP database. SNPs that have been previously identified have their accession numbers referenced in the dbSNP database, and the black arrows in the corresponding dbSNP browser images denote the corresponding loci for detection.

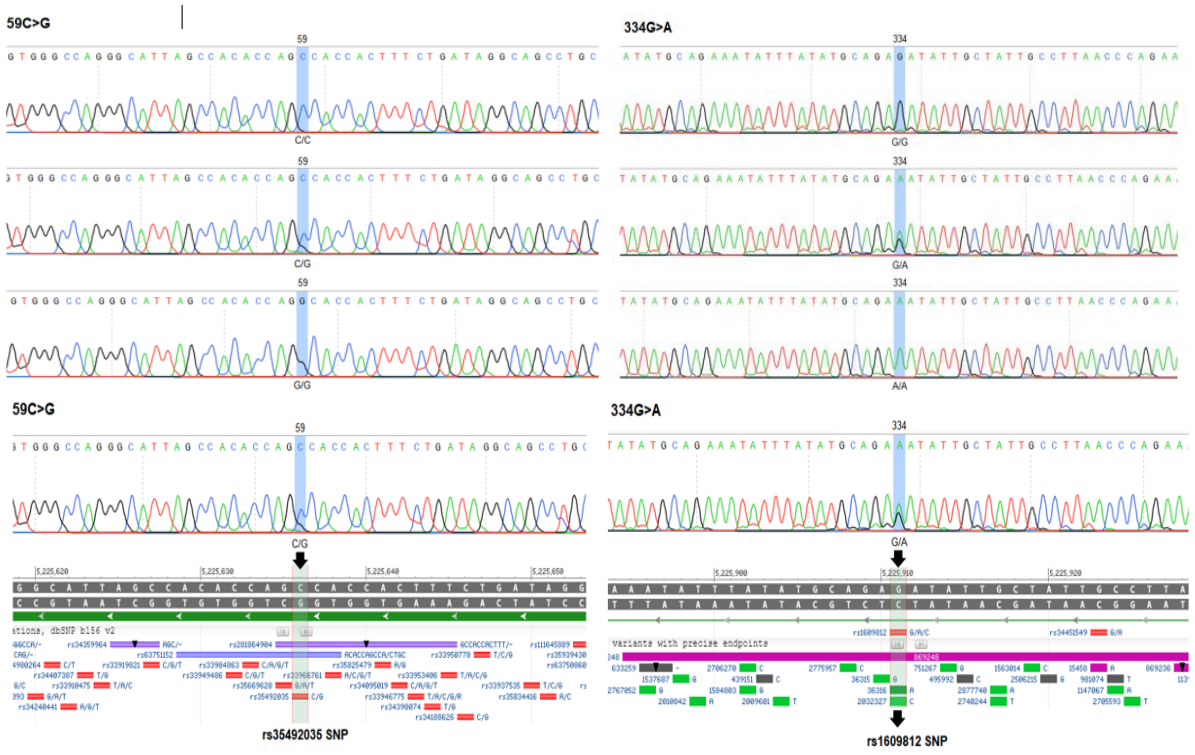

Figure 5 (A). Sanger sequencing chromatograms from genotyping showed that the *HBB* gene variants had genotypic locations in the genome. The detected SNPs were identified by location within *HBB*2 amplicons, which included 59C>G (rs35492035) and 334G>A (rs1609812). The variants are indicated as directly related to their genomic location with the use of the symbol ">" to specified the nucleotide substitution, and "ins" was used to specify an insertion variant. To assess the novelty of each identified *HBB* variant, SNPs were assessed using the dbSNP database, and the SNPs that have already been published are identified in the dbSNP website as associated with their respective dbSNP accessions, however the loci assessed in this study can be identified by a black arrow in the respective screenshots from the dbSNP browser.

For the 665 bp amplicons, the intronic SNP 386A>T located at the intron, is cataloged as rs1451581925. For the 881 bp amplicons, the intronic SNPs 59C>G and 334G>A are cataloged in dbSNP as rs35492035 and rs1609812, respectively.

Genetic association analysis

There were no significant variations in the genotype and allele frequencies for 386A>T (rs1451581925) and 59C>G (rs35492035) between the β-thalassemia major patients and the control groups ($P > 0.05$). There was a significant difference in the genotype frequency of the heterozygote GA variant.频 Between β-thalassemia major patients (16.0%) and control groups (26.0%) there was a significant difference in variant genotype frequency ($P = 0.049$, OR = 2.00; 95% CI: 0.68–220.52) but no significant differences were seen in genotype or allele frequency for any of the other variants of interest.

Overall, the results suggest that the allele distributions of the three SNPs investigated are comparable between patients and controls as evidenced by (Table 8).

Table 8. 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
386A>T (rs1451581925)		`			
AA	32(0.640)	40(0.800)	0.44	0.18-1.09	0.1 NS
AT	18(0.360)	10(0.200)	2.25	0.92-5.50	0.1 NS
TT	0(0.000)	0(0.000)	-	-	-
A allele	82(0.820)	90(0.900)	0.51	0.22-1.15	0.1 NS
T allele	18(0.180)	10(0.100)	1.98	0.87-4.51	0.1 NS
rs35492035 (59C>G)		`			
CC	33(66.0)	28(56.0)	1.53	0.68-3.40	0.4 NS
CG	17(34.0)	19(38.0)	0.84	0.37-1.89	0.8 NS
GG	0(0.0)	3(6.0)	0.13	0.01-2.59	0.2 NS
C allele	83(83.0)	75(75.0)	1.63	0.82-3.24	0.2 NS
G allele	17(17.0)	25(25.0)	0.61	0.31-1.22	0.2 NS
334G>A (rs1609812)		`			
GG	37(74)	37(74)	Ref.	(0.20-1.44)	0.32
GA	8(16)	13(26)	2.0	(0.68-	**0.049***
AA	5(10)	0(0)	12.2	220.52)	0.43
G allele	82(82)	87(87)	0.68	(0.311.47)	0.43
A allele	18(8)	13(13)	1.47	(0.68-3.17)	

Concerning the detected 386A>T SNP in the *HBB*1 amplicons, dbSNP showed that this SNP was previously deposited in the genome under the rs number rs1451581925. This SNP is located in the intron sequences of the *HBB* gene (https://www.ncbi.nlm.nih.gov/snp/rs1451581925). The exact position of this SNP within the genomic sequences was NC_000011.10:g.5226818A>G. However, no frequency database was found to be available for this SNP in the dbSNP. Due to the absence of any previous information on the frequency of rs1451581925, this SNP was not cited in any published study, and, therefore, no previous knowledge is available on this variant.

Concerning the detected 59C>G SNP in the *HBB*2 amplicons, dbSNP showed that this SNP was previously deposited in the genome under the rs number rs35492035. This SNP is located in the coding sequences of the *HBB* gene (https://www.ncbi.nlm.nih.gov/snp/rs35492035). The exact position of this SNP within the genomic sequences was NC_000011.10:g.5225636C>G. Due to the positioning of this SNP in the coding region of the *HBB* gene, it is found that the substitution of cytosine with guanine occurred in the amino acid residue alanine that is located in position 136 in the entire sequence of *HBB*. This alteration causes a substitution of Ala136 to Pro136 (NP_000509.1:p.Ala136Pro). An extremely low frequency of the deposited rs35492035 SNP in the dbSNP database was inferred, which was estimated to be 0.00003 for the alternative allele according to the PAGE-STUDY database. Due to this low frequency of rs35492035, this SNP was cited in only one study that assessed the putative effect of rs35492035 on some metabolic disorders in the body [34].

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 [35] .

The small sample size utilized in this research is one of the most significant weaknesses of this work; this limits the possibility of reaching statistical significance, and as a result, may create an obscuring effect for true associations, increase the probability of type II error, and decrease population generalizability of our results, ultimately making it difficult to determine definitive relationships between molecular analysis results, hematologic and biochemical differences, and susceptibility to disease. For the same reasons that smaller sample sizes reduce the chances of being able to look at subgroups (through subgroup analyses) and positively skew odds of being present based on likelihood, the small sample size of this study may limit the reliability, interpretability, and reproducibility of our outcomes; thus necessitating larger and more varied cohort populations for future similar studies.

Conclusion

The objective of this study was to characterize in detail β-thalassemia major using an integrated laboratory approach, including both clinical and laboratory findings, and the use of sequencing of the HBB gene of affected patients. Patients had evidence of significant hematological and biochemical abnormalities, with markedly increased serum ferritin and increased liver enzyme levels indicating chronic transfusion therapy and iron overload. The molecular analysis of HBB variants identified three HBB mutations which included the rare missense mutation rs35492035 (p.Ala136Pro), and intronic mutations rs1451581925, and rs1609812. Although most of the identified variants were not significantly associated with susceptibility to disease, the rs1609812 AA genotype was significantly associated with the development of β-thalassemia major, suggesting that this variant may be relevant as a genetic marker among the studied population. This study provides additional information on the spectrum of HBB variants identified in Iraqi patients and underscores the importance of using both molecular sequencing in conjunction with clinical and biochemical evaluations of patients in order to provide an accurate genetic characterization of disease and appropriate management of patients. Future studies should include larger patient cohorts and functional analyses of variants identified in this study to confirm that the mutations are significant in determining the severity of the disease, as well as their role in the genotype–phenotype relationship.

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