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A First Study on Three HBB Gene Intronic SNPs: Molecular Profiling of β-Thalassemia Major in Southern Iraq

Amar Mousa Mandal[1,2]*, Aseel Shakir Mahmood[1]
[1] *Biotechnology, Science, University of Baghdad/ Baghdad/ Iraq.*
[2] *Science department, Basic Education, University of Al-Muthanna / Al-Muthanna / Iraq*

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Abstract

Genetic association between HBB genetic variations and β-thalassemia is the key factor for management and counseling β-thalassemia. This innovative investigation aims to explore the connection between three intronic SNPs in the *HBB* gene rs7946748 (64G>A), rs7480526 (71A>C), and rs1451581925 (386A>T) and clinical outcomes in β-thalassemia major patients from Southern Iraq. Using PCR amplification and Sanger sequencing, 100 participants (50 patients and 50 controls) were analyzed. The study revealed a strong correlation between the AA genotype and the A allele of rs7480526 and a heightened risk for β-thalassemia and notable alterations in hematological parameters. Furthermore, rs7946748 and rs1451581925 were linked to increased ferritin levels and elevated liver enzyme markers, indicating iron overload and hepatic stress in transfusion-dependent cases. Linkage disequilibrium (LD) analysis demonstrated significant associations between the GAT and AAT haplotypes, as well as disease susceptibility. In conclusion, the findings indicate AA (OR 2.53;95%cl 1.12-5.70, and p value= 0.4*) genotype and A allele (OR 2.09:95% Cl 1.17-3.71 and p value = >0.01**) of rs7480526, along with haplotype analysis reveal co-inheritance patterns that deepen understanding of genotype-phenotype relationships and disease susceptibility.

Keywords: β-thalassemia, Sanger sequencing, *HBB* gene's SNPs, genotype-phenotype relationship, linkage disequilibrium.

أول دراسة شاملة لثلاث متغايرات جينية داخلية في جين *HBB* وتأثيرها على البيتا–ثلاسيميا الكبرى في جنوب العراق.

عمار موسى مندل [1,2]*, اسيل شاكر محمود[1]
[1]التقنيات الاحيائية، العلوم، جامعة بغداد، بغداد، العراق
[2]قسم العلوم، كلية التربية الأساسية، جامعة المثنى، المثنى، العراق

الخلاصة:

الارتباط الجيني بين التغيّرات الجينية في جين *HBB* وبيتا– ثلاسيميا هو العامل الرئيسي لإدارة وتقديم الاستشارة الطبية للمصابين ببيتا– ثلاسيميا. استهدفت هذه الدراسة وللمرة الأولى العلاقة بين ثلاثة من

* Email:Ammar.Musa2306@sc.uobaghdad.edu.iq

متعددات الاشكال الجينية في جين بيتا غلوبين (*HBB*) وهي [(64G>A) rs7946748، rs7480526 (71A>C))، و [(386A>T) rs1451581925) وبين النتائج السريرية لمرضى بيتا–ثلاسيميا الكبرى في جنوب العراق. من خلال استخدام تقنية تفاعل البلمرة التسلسلي التقليدي وتحليل تسلسل سانجر، تم تحليل 100 مشارك (50 مريضاً و50 من الأصحاء كمجموعة سيطرة). أظهرت الدراسة وجود ارتباط قوي بين النمط الجيني AA والأليل A في [rs7480526] وزيادة القابلية للإصابة ببيتا–ثلاسيميا، إلى جانب تغيرات ملحوظة في المعايير الدموية. كما تم ربط [rs7946748] و[rs1451581925] بزيادة في مستويات الفيريتين وارتفاع مؤشرات إنزيمات الكبد، مما يشير إلى الحمل الزائد بالحديد والضغط الكبدي الذي يتسم به المرضى الذين يعتمدون على نقل الدم. كما أظهر تحليل عدم التوازن الربطي (LD) وجود ارتباطات مهمة للأنماط الفردانية [GAT و [AAT مع قابلية الإصابة بالمرض. وفي الختام، تشير النتائج إلى أن النمط الجيني (النسبة الأرجحية 2.53؛ 95% فترة ثقة 1.12–5.70، وقيمة الاحتمال = 0.04*) AA والأليل A (النسبة الأرجحية 2.09؛ 95% فترة ثقة 1.17–3.71، وقيمة الاحتمال <0.01**) في [rs7480526] إلى جانب تحليل للأنماط الفردانية، يكشف عن أنماط التوارث المشتركة التي تعمق فهم العلاقات بين النماط الجينية والانماط الضاهرية وأيضا قابلية الإصابة بالمرض.

Introduction

Thalassemia is a widely recognized monogenic genetic condition that is primarily classified into two distinct forms: alpha-thalassemia (α-thalassemia) and Beta-thalassemia (β-thalassemia). The development of β-thalassemia is attributed to the partial or total inhibition of Beta-globin (β-globin) chain production, which results in a disproportionate ratio of alpha to beta chains. On a global scale, researchers have identified close to 100 different alleles of β -thalassemia, with approximately 40 of these variants responsible for over 90% of the worldwide cases [1]. β-thalassemia is a hereditary condition characterized by diminished β-globin chain synthesis, which results in anemia [2]. β-thalassemia is classified according to mutations, which are further grouped by their clinical severity: beta-plus thalassemia (β^+) refers to mild mutations resulting in a partial decrease in β-globin synthesis, whereas beta-zero thalassemia (β^0) signifies severe mutations that cause a complete absence or significantly reduced production of the β-globin chain [3]. β-thalassemia major is a critical genetic disorder defined by an almost complete lack of β-globin chains, necessitating lifelong blood transfusions and iron chelation therapy[4]. β -thalassemia poses a major public health challenge in Southern Iraq, driven by *HBB* gene mutations in affected individuals, emphasizing the critical need for detailed mutation profiling to enhance carrier screening and genetic counseling efforts [5]. The management of β-thalassemia major requires regular blood transfusions to maintain a sufficient level of mature red blood cells [6]. Alterations in the β-globin gene impair the synthesis of beta chains in hemoglobin, resulting in the disorder [7]. The prevalence of beta-thalassemia in Iraq ranged from 35.7 to 49.6 cases per 100,000 people between 2003 and 2018, indicating a higher prevalence than the stated figure of 37.1 in 2015 [8]. In recent studies, Iraqi researchers explored genetic polymorphisms as a dependable factor for ensuring precise outcomes [9]. The β-globin gene cluster plays a vital role in hemoglobin disorders like β-thalassemia. Mutations associated with β-thalassemia correspond to distinct haplotypes within this cluster, highlighting regional origins and genetic exchange with neighboring populations [10]. Recent research has identified that mutations in the β-globin gene result in either decreased β-globin production, termed (β^+), or the total absence of β-globin synthesis, classified as (β^0). Both conditions contribute to the development of β-thalassemia major. The genotypes linked to β-thalassemia major, arranged in descending order of severity, are $\beta^0\beta^0 > \beta^0\beta^+ > \beta^+\beta^+$ [11]. β-thalassemia results from mutations impairing the production of β-globin chains, with more than 400 causative mutations documented so far [12]. The connection between phenotype and genotype

continues to be a primary focus in clinical research; however, the experimental investigation of numerous mutations presents considerable challenges. In silico analysis, on the other hand, serves as a more practical solution, delivering quicker, simpler, and more cost-effective results while producing extensive data [13,14]. Sanger sequencing is highly recognized as the gold standard for β-thalassemia genetic testing, providing unmatched precision in identifying mutations within the β-globin gene [15]. The hemoglobin subunit beta gene (*HBB*), measuring about 1.6 kb, comprises three exons divided by two introns. Its regulation is directed by a proximal 5′ promoter and an enhancer situated 50 kb upstream of the β -globin gene [16]. Alterations in the *HBB* gene commonly involve substitutions, deletions, or insertions of one or more nucleotides within the gene or its adjacent regions, leading to anemia and diminished red blood cell production [17]. A single nucleotide variant in the intron of the β-globin gene has the potential to disrupt RNA splicing, which may contribute to the development of β-thalassemia [18,19]. A single-point mutation in an intron can result in aberrant splicing in β+ thalassemia, suggesting that intron removal could support the accurate splicing of subsequent introns [20,21]. A nucleotide variant within an intron of the β-globin gene can disrupt RNA splicing, potentially resulting in β-thalassemia major by altering gene expression and pre-mRNA processing [18]. Furthermore, intronic variants within the *HBB* gene may activate cryptic splice sites, interfering with mRNA transcription and playing a role in the onset of β -thalassemia major, the most severe manifestation of the disorder [22]. Humans have a limited ability to remove excess iron, making iron overload an almost certain result of regular blood transfusions [23]. Homozygosis or compound heterozygosis in the *HBB* gene often results in severe, transfusion-dependent anemia (β-thalassemia major), while the β-thalassemia trait (heterozygous type) generally leads to mild or moderate anemia. The molecular basis of β-thalassemia is rooted in variations, predominantly point mutations, within the coding regions of the β-globin (*HBB*) gene [24]. Investigating SNPs within the *HBB* gene reveals potential links between genetic mutations and phenotypic variation [25,26]. The Database of Single Nucleotide Polymorphisms (dbSNP) server (https://www.ncbi.nlm.nih.gov/projects/SNP/) was employed to evaluate the novelty of SNPs. Each SNP position was analyzed and annotated relative to the corresponding reference genome to verify its prior inclusion in the dbSNP database. Profiling mutations in the *HBB* gene for a specific population is vital for enhancing the efficiency, simplicity, and cost-effectiveness of clinical diagnosis [26] . Many diseases recently relied on SNP detection to give powerful diagnoses for many diseases [27]. This study was conducted to identify the most prevalent HBB mutations linked to β-thalassemia.

Methodology

Ethical committee approval.

The research was performed in conformity with the ethical principles outlined in the Helsinki Declaration. Involving human subjects and received formal approval from the Ethics Committee for Postgraduate Studies at the College of Science, University of Baghdad (CSEC/0724/0049; July 28, 2024).

Patients' enrollment

This study analyzed genetic variations across 100 loci (50 patients and 50 controls) within the *HBB* gene using PCR amplicon (665 bp). Samples were obtained from β-thalassemia major patients, aged 2–15 years, at AL Muthanna Health Department/ Women and Children Hospital/ Thalassemia Division. Thi-Qar Health Department / Genetic Blood Diseases Center. The study exclusively included thalassemia major patients dependent on regular blood transfusions, aged (6 months to 15 years old), with transfusion intervals ranging from (2 to 4) weeks. In addition, were included with parental consent for blood donation. Controls

were healthy volunteers aged 2 to 20 years without β-thalassemia history. All participants were Iraqi citizens, enrolled between March 2, 2024, and December 31, 2024.

Hematology, biochemical, and hemoglobin variables parameters

Hematological parameters: ABO and Rh typing (ABO Blood Group and Rh (Rhesus) Typing), Hg (hemoglobin), WBC (white blood cells), NEUT (neutrophils), LYMPH (lymphocytes), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), and PLT (platelets), were tested by an automated hematology analyzer (Sysmex XS-1000i) [28,29]. The CL-900i Chemiluminescence Immunoassay System by Mindray leverages chemiluminescence technology to deliver highly sensitive and specific ferritin detection [30]. The Hemoglobin Testing D-10 System utilizes hemoglobin electrophoresis to identify different hemoglobin types, such as hemoglobin A1 (HbA1), hemoglobin A2 (HbA2), and hemoglobin F (HbF) [31,32].

DNA extraction and validation processes

Whole blood specimens were obtained from both the patients and control groups [33]. Genomic DNA was extracted using the EasyPure® Blood Genomic DNA Kit (Cat. No. EE121-01, Transgene Co., China). The integrity of DNA was assessed through the implementation of agarose gel electrophoresis [34]. Then, it was subsequently quantified using a Nanodrop spectrophotometer in accordance with established protocols [35, 36].

Genotyping

Two sets of specific PCR primers were designed for the *HBB* gene (GenBank acc. no. NC_000011.10) using NCBI Primer BLAST [37]. The PCR aimed for full *HBB* coverage with overlapping amplicons and was optimized for oligonucleotide lengths, annealing temperatures, and GC content. The PCR amplicon (forward primer 5′-ACGATCCTGAGACTTCCACAC-3′ and reverse primer 5′-AGTCAGGGCAGAGCCATCTA-3′) is 665 bp long the experimental design encompassed the amplification of the 5′-untranslated region (UTR), exon 1, intron 1, and exon 2, in addition to the regulatory region upstream of intron 2. The polymerase chain reaction (PCR) technique initiated with an initial denaturation step at a temperature of 94 °C for a period of 5 minutes, followed by 30 cycles comprising denaturation at 94 °C for 30 seconds, annealing at 61 °C for 60 seconds, and elongation at 72 °C for 30 seconds, concluding with a final extension at 72 °C for 5 minutes. Amplified product specificity was verified through agarose gel electrophoresis, and Sanger sequencing was conducted thereafter [38]. Chromatogram data were visualized, aligned, and trimmed by BioEdit ver. 7.1 against GenBank account. No. NC_000011.10. All identified SNPs were validated through manual inspection of the electropherograms using the Snapgene viewer tool, and non-standard SNPs were eliminated according to recommended protocols [39]. The SNPs detected in each sequenced sample were numbered according to their specific genomic positions within the PCR amplicons and their corresponding locations in the reference genome. Subsequently, the observed SNPs were evaluated to assess their possible novelty using the dbSNP (Single Nucleotide Polymorphism Database) server [40].

Statistical analysis

Data analysis was conducted utilizing SPSS-20 (version 20, IBM Corp., Armonk, NY, USA) to compute frequencies, percentages, and mean ± standard deviation (SD). The thresholds for statistical significance were established at $P < 0.05$ (indicating significance), $P < 0.01$ (indicating high significance), and $P \geq 0.05$ (indicating non-significance). The levels of HbA2 were reported as mean ± SD. Variations in means were evaluated employing

Student's t-test and ANOVA methodologies. SPSS-20 was further employed for the analysis of genotype and allele frequencies, utilizing chi-square tests to determine compliance with Hardy-Weinberg equilibrium. The WinPepi software was employed to calculate odds ratios and 95% confidence intervals for genotypes and alleles, whereas SHEsis software was utilized for the analysis of linkage disequilibrium and haplotype configurations.

Results and discussion

Assessment of clinical and demographic profiles.

The samples were carefully chosen to showcase noticeable differences in clinical traits between the cases and controls. This approach was designed to establish a solid starting point for analyzing the intended genotype-phenotype association. The comparative analysis between the patient and control groups highlighted important distinctions in demographic, clinical, and treatment-related attributes. Notably, the composition of the patient cohort showed a greater percentage of males (56.0%) relative to the control group (44.0%), although this discrepancy did not reach statistical significance ($p = 0.1$). Additionally, the mean age of patients (9.8 ±3.6 years) was lower than that of the controls (13.4±6.2 years), with a p-value of 0.06, indicating a trend toward significance without achieving statistical significance. Living conditions revealed a marked difference, as a larger proportion of patients lived in rural areas (58.0%) compared to controls (38.0%), a statistically significant finding ($p = 0.04$). Aligns with findings from population-based research indicating that socio-economic and geographic factors affect disease prevalence [41]. Consanguineous marriages were more prevalent among the patient group, with all patients showing varying degrees of familial marriage ($p<0.01$). Is consistent with findings that consanguineous marriages were prevalent in 96% of the parents of β-thalassemic patients, with 72% being first cousins. This high prevalence significantly correlates with the occurrence of thalassemia [42]. Clinical complications, including splenomegaly (42.0%), hepatosplenomegaly (30.0%), and related conditions, were exclusive to patients, showcasing significant disease burden ($p<0.01$). These complications indicate disease severity and often require regular blood transfusions [43]. Family history findings indicated that all carriers were within the patient group ($p<0.01$), suggesting a genetic predisposition [44]. The onset of blood transfusions, the frequency of these transfusions, and the administration of chelating drugs were significant factors: every patient required transfusions compared to none in the control group, with different intervals reported and 62.0% undergoing chelation therapy ($p<0.01$) [43], as shown in Table 1. These results emphasize the multifaceted clinical and genetic profile inherent in β-thalassemia patients.

Table 1 : - The Demographic and clinical characteristics of the investigated population.

Indices		Group (N=100)		P value
		Control n=50 (%)	Patients n=50 (%)	
Sex	Male	22 (44.0)	28 (56.0)	0.1NS
	Female	28 (56.0)	22 (44.0)	
Age (year)	Mean± S.D.	13.4±6.2	9.8±3.6	0.06NS
Living	Rural	19 (38.0)	29 (58.0)	0.04*
	City	31 (62.0)	21 (42.0)	
Consanguineous marriage	0.00	50 (100)	10 (20.0)	<0.01**
	1.00	0 (0.0)	12 (24.0)	
	2.00	0 (0.0)	18 (36.0)	
	3.00	0 (0.0)	10 (20.0)	
Complication	Non	50 (100)	9 (18.0)	<0.01**
	Splenomegaly	0 (0.0)	21 (42.0)	
	Hepatosplenomegaly	0 (0.0)	15 (30.0)	
	Splenectomy	0 (0.0)	3 (6.0)	
	Splenomegaly/colostomy	0 (0.0)	1 (2.0)	
	Splenectomy/hepatomegaly	0 (0.0)	1 (2.0)	
Family History	Non	50 (100)	0 (0.0)	<0.01**
	Carrier	0 (0.0)	50 (100)	
Age at the first blood Transfer (in a month)	Non	50 (100)	0 (0.0)	<0.01**
	1 month	0 (0.0)	1 (2.0)	
	2 months	0 (0.0)	21 (42.0)	
	3 months	0 (0.0)	15 (30.0)	
	4 months	0 (0.0)	8 (16.0)	
	5 months	0 (0.0)	4 (8.0)	
	6 months	0 (0.0)	1 (2.0)	
Transfusion interval (in a weak)	Non	50 (100)	0 (0.0)	<0.01**
	1 weak	0 (0.0)	0 (0.0)	
	2 weeks	0 (0.0)	15 (30.0)	
	3 weeks	0 (0.0)	27 (54.0)	
	4 weeks	0 (0.0)	5 (10.0)	
	5 weeks	0 (0.0)	3 (6.0)	
Receiving chelating drugs	No	50 (100)	19 (38.0)	<0.01**
	Yes	0 (0)	31 (62.0)	

Screening of Genetic Mutations via PCR

Thalassemia is widely regarded as a critical global health issue. In Iraq, Incidence rates for clinically significant thalassemia vary, ranging between 0.2 and 49.6 per 100,000 individuals. Of these, β-thalassemia is consistently the most frequently encountered type [8]. Alterations in the hemoglobin (Hb) gene can lead to either reduced or absent production of the alpha or β chains of the Hb protein, giving rise to different types of thalassemia. Therefore, examining the range of mutations in the Hb genes is essential to understanding the distribution and disease mechanisms of Hb variants [45]. Molecular characterization was carried out using conventional PCR technology to detect potential mutations in the *HBB* 1 gene. The PCR assay targeted the five-prime untranslated region (5′-UTR), exon 1, intron 1, exon 2, and a

portion of intron 2 by following the PCR protocol outlined in [46]. The methodology ensures consistent amplification conditions suitable for the targeted genetic analysis. The red and blue markers denote the start and end points of the PCR amplicon, a 665 bp fragment that covers most of the *HBB* gene, as shown in Figure. 1.a and b).

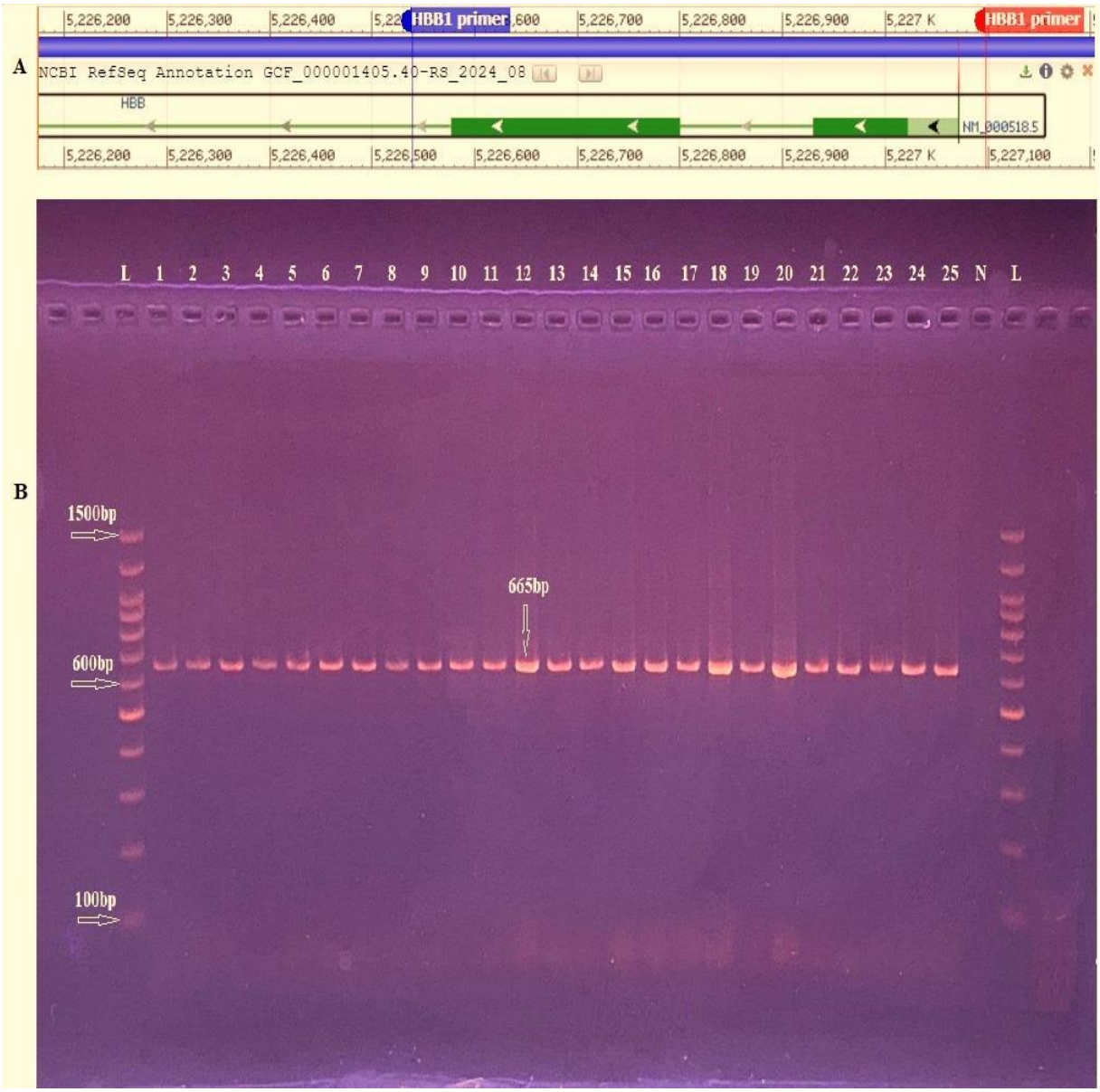

Figure 1: (a) An image illustrates the precise location of the PCR amplicon (665 bp) that encompasses most of the *HBB* gene. The depiction uses Red and Blue to mark the start and end points, respectively, of the *HBB* 1 (665 bp) amplicon. Part **(b)** shows an agarose gel electrophoresis of PCR products from 20 PCR assays using the *HBB* 1 primer, displaying an amplicon size of 665 bp. The primer's melting temperature was 61 °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.

Genetic Linkage and Disease Susceptibility

DNA sequencing is an essential tool for accurately identifying mutations and providing insights into genetic variations that may contribute to disease susceptibility [47]. Among the SNPs detected in both amplicons, two SNPs [rs7946748 (64G>A), rs7480526 (71A>C)] exhibited all three typical zygosity states, major homozygous, heterozygous, and rare homozygous. While [rs1451581925 (386A>T)] was observed only in the major homozygous and heterozygous states. The zygosity states of the SNPs were manually verified in their respective electropherogram files. The clarity and distinctiveness of the identified polymorphic peaks validated the sequencing procedures and confirmed the reliable identification of SNPs, as shown in Figure 2. a. By reviewing the dbSNP server, further details for these SNPs were identified with regard to their corresponding genomic sequences for both amplicons, 665 bp as shown in Figure 2. b. Data analysis reveals that there are three SNPs within the *HBB* gene rs7946748 (64G>A), rs7480526 (71A>C), and rs1451581925 (386A>T) for their association with β-thalassemia. Among the variants analyzed, rs7480526There 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 2. aligning with research indicating that specific HBB polymorphisms impact β-thalassemia risk by modulating gene expression or function [48]. Conversely, SNPs rs7946748 and rs1451581925 did not show significant associations, consistent with findings that not all *HBB* polymorphisms impact β-thalassemia uniformly across populations due to genetic background differences [49].

Table 2: Association analysis of the rs7946748, rs7480526, and rs1451581925 SNPs with the risk β-thalassemia

SNP thalassemia **ID**	**Patient** (n=50)	**Control** (n=50)	**Odds ratio**	**95%CI**	**`P-value**
64G>A (rs7946748)					
GG	3(6)	0 (0.0)	7.44	0.39-143.63	0.24 NS
GA	6(12)	10(20)	0.52	0.18-1.55	0.28 NS
AA	41(82)	40(80)	1.4	0.42+3.07	1.00 NS
G allele	12(12)	10(10)`	1.23	0.51-2.97	0.82 NS
A allele	88(88)	90(90)	0.81	0.34-1.97	0.82 NS
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
386A>T(rs1451581925)					
AA	0.1 NS	0.18-1.09	0.44	40(0.800)	32(0.640)
AT	0.1 NS	0.92-5.50	2.25	10(0.200)	18(0.360)
TT	-	-	-	0(0.000)	0(0.000)
A allele	0.1 NS	0.22-1.15	0.51	90(0.900)	82(0.820)
T allele	0.1 NS	0.87-4.51	1.98	10(0.100)	18(0.180)

Furthermore, this variability is reflective of findings that suggest not all *HBB* polymorphisms have a straightforward correlation with disease severity, highlighting the nuanced role of genetic modifiers in β-thalassemia. [50]. Furthermore, dbSNP validation confirmed these SNPs' within the *HBB* gene, highlighting their relevance for genetic screening and SNP genotyping in β-thalassemia risk assessment [45].

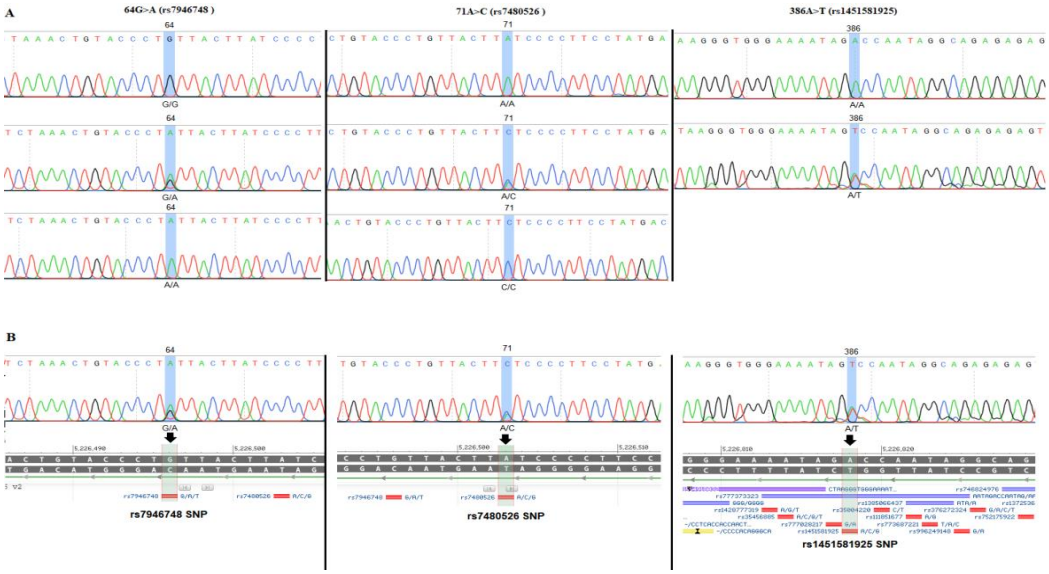

Figure 2: (a) This illustration presents the genotyping chromatograms and the genomic locations of SNPs rs7946748 (64G>A), rs7480526 (71A>C), and rs1451581925 (386A>T) within the *HBB* gene. It shows the pattern of the detected variants in the DNA chromatogram files targeting specific loci of the *HBB* amplicon. The identified variants are distinctly marked according to their positions in the PCR amplicons, with the symbol ">" denoting an insertion SNP. Part **(b)** of the figure involves the SNP novelty assessment for the *HBB* 1 amplicon using the dbSNP server, where the identified SNPs are indicated with black arrows on the corresponding figures.

Haplotype analysis

The analysis of linkage disequilibrium (LD) between SNPs rs7946748 (64G>A), rs7480526 (71A>C), and rs1451581925 (386A>T) within the *HBB* gene fragment reveals varying levels of LD strength. The LD plots revealed that the weakest linkage disequilibrium (LD) was between rs7946748 and rs1451581925, showing a D' of 10% and a squared correlation coefficient (r^2) of 0.008. On the other hand, the strongest LD was observed between rs7946748 and rs7480526, where a D' of 83% and an r^2 of 0.071 indicate a significant correlation, as shown in Figure 3. In relative terms, there were variable strengths of LD values between the observed SNPs with variable distributions of co-inheritance potentials among the detected SNPs in the amplified fragment of the *HBB* gene. The haplotype analysis showed varying associations between cases and controls. The A A A* haplotype (6.36% cases, 8.28% controls) had no significant association (Chi2 = 0.309, p = 0.578, OR = 0.738 [95% CI: 0.252–2.160]). The A-C-A haplotype was absent in some cases, preventing analysis. The G A A* haplotype (38.97% cases, 35.85% controls) also showed no significance (Chi2 = 0.132, p = 0.717, OR = 1.112 [95% CI: 0.626–1.976]). The G A T* haplotype (10.03% cases, 3.88% controls) approached significance (Chi2 = 2.813, p = 0.094, OR = 2.714 [95% CI: 0.812–9.078]). Both the G C A* (36.67% cases, 44.15% controls) and G C T* (2.33% cases, 6.12% controls) haplotypes were not significantly associated with the disease. However, the A A T* haplotype (5.64% cases, 0% controls) showed a significant association with cases (Chi2 = 5.706, p = 0.01), indicating a potential role in disease risk ,as shown in Table 3. Haplotypes, rather than genotypes at a single locus, might be regarded as the primary units of heredity and may allow us to understand better the function of polymorphic traits (i.e., SNPs) in disease susceptibility [51,52].

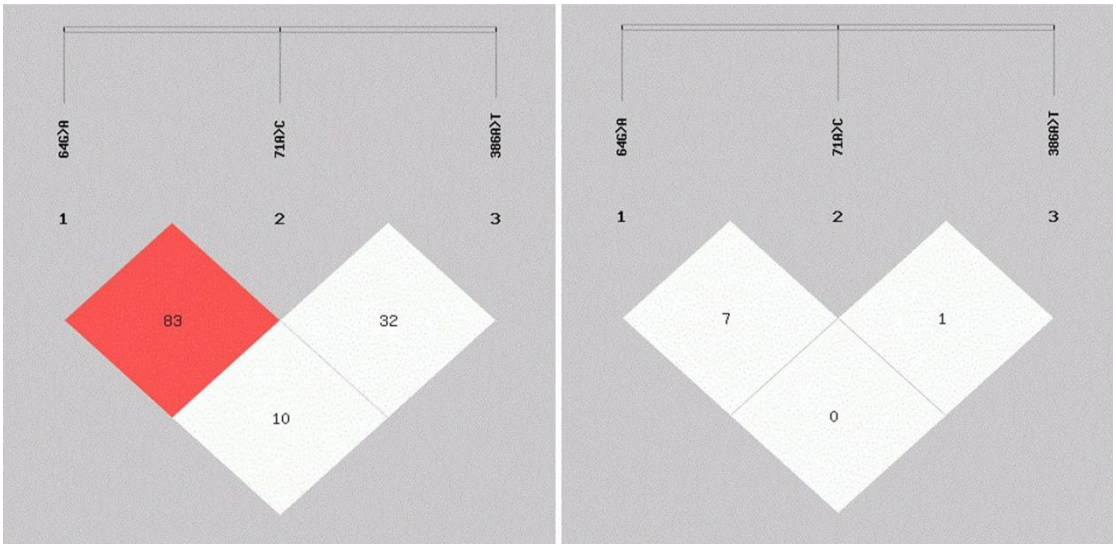

Figure 3: This figure depicts a Linkage Disequilibrium (LD) Plot of SNPs 64G>A, 71A>C, and 386A>T. This figure illustrates the linkage disequilibrium (LD) between three single nucleotide polymorphisms (SNPs): 64G>A, 71A>C, and 386A>T. The left panel represents the D' values between each pair of SNPs, with the intensity of the red shading indicating the strength of the linkage. The numbers inside the diamonds show the percentage of linkage between the SNPs. The right panel shows the r^2 values, representing the correlation of alleles between SNPs, where lighter shading corresponds to a lower correlation. The values below the plots summarize the D' and r^2 values between the three SNPs.

Table 3: Loci for hap-analysis: 64G>A, 71A>C, 386A>T

Haplotype	Case(freq)	Control(freq)	Chi2	Fisher's p	Odds Ratio	Odds [95%CI]
A A A*	6.36(0.064)	8.28(0.083)	0.309	0.578549	0.738	[0.252~2.160]
A C A	0.00(0.000)	1.72(0.017)	-			-
G A A*	38.97(0.390)	35.85(0.358)	0.132	0.716827	1.112	[0.626~1.976]
G A T*	10.03(0.100)	3.88(0.039)	2.813	**0.093517**	2.714	[0.812~9.078]
G C A*	36.67(0.367)	44.15(0.442)	1.4	0.236679	0.710	[0.402~1.253]
G C T*	2.33(0.023)	6.12(0.061)	1.846	0.174232	0.359	[0.077~1.667]
A A T*	5.64(0.056)	0.00(0.000)	5.706	**0.01**		-

The strongest LD is observed between rs7946748 and rs7480526, indicated by a (D' value of 83%) and (r^2 value of 0.071), suggesting a moderate association and potential co-inheritance between these variants. In contrast, the LD between rs7946748 and rs1451581925 is the weakest, with a D' of 10% and an r^2 of 0.008, reflecting minimal co-inheritance potential. This variability in LD values among the SNPs may reflect differences in evolutionary pressures or recombination events impacting these loci, which could influence their roles in β-thalassemia susceptibility [53]. Furthermore, the haplotype analysis does not show a significant association between cases and controls for the AA haplotype, while the GAT haplotype exhibits a trend toward significance, suggesting that certain SNP combinations may have differential impacts on β-thalassemia risk. These findings underscore the complexity of genetic contributions to β-thalassemia, where certain haplotypes may offer insight into disease susceptibility without yielding statistically significant associations individually[54]. Current research has not consistently identified the A A T and G A T haplotypes in general β-thalassemia studies. However, ancestral β-globin gene haplotypes, including those linked to the AHSP gene, have been shown to influence β-thalassemia

severity and outcomes, with haplotype diversity varying across regions. This aligns with the observed significance of the A-T haplotype in your findings, possibly indicating a localized genetic factor affecting β-thalassemia risk. Further studies on regional haplotype diversity may offer additional insights [10,55].

Multi-SNP Analysis in HBB Intronic Region

HBB. 64G>A (rs794674)

It was found that this SNP was previously deposited in the genome under the rs number rs7946748. This SNP is situated within the sequences of intron 2 of the *HBB* gene (https://www.ncbi.nlm.nih.gov/snp/rs7946748). The exact position of this SNP within the sequences of chromosome 11 was NC_000011.10:g.5226496G>A. This study aims to fill this gap by characterizing the rs7946748 SNP and assessing its association with β-thalassemia in a Southern Iraqi cohort, thereby contributing novel data to the understanding of genetic variability in this region. A relatively low frequency of the deposited rs7946748 SNP in the dbSNP database was notified, which was estimated to be 0. A=0.118179/17742 for the alternative allele, according to the ALFA database. These SNPs have equal frequencies in both groups (control and patients); their moderate frequency could imply that they play a modifier role in disease risk or progression rather than being the primary causative mutation. The clinical consequences have been evaluated, indicating that it has a benign or potentially benign impact. A distinct analysis of specific HbS polymorphisms demonstrated a significant correlation between these SNPs and their protective effects [56]. A parallel study similarly described rs7946748 as a framework within the β-globin gene among thalassemia major patients in Northern Iran. These frameworks hold the potential for use in tracking mutant alleles in prenatal diagnostic programs [57]. Its clinical significance is benign (dbSNP Database, "rs794674 [Homo sapiens]." National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/snp/rs794674.)

HBB. rs7480526 (71A>C)

Another critical element to ponder is that this analysis constitutes the unprecedented in its nature evaluation of the rs7480526 SNP found within the *HBB* gene in β-thalassemia major patients living in Iraq. Although numerous SNPs and mutations within the *HBB* gene have been explored on a global scale, including within Middle Eastern demographics, a significant deficiency exists in the literature concerning this specific SNP in the Iraqi population. Prior research has primarily focused on different mutations, leaving rs7480526 unexplored in terms of its frequency, linkage disequilibrium, and association with clinical outcomes in β-thalassemia. By analyzing this SNP, our study fills a critical gap, providing novel insights into the genetic variability of β-thalassemia in southern Iraq . The findings of this study not only expand the current understanding of *HBB* gene polymorphisms but also underscore the critical role of characterizing local genetic variants in deepening our comprehension of the disease's manifestation and progression in diverse populations. Concerning the detected 71A>C SNP in the *HBB* 1 amplicons, dbSNP showed that this SNP was previously deposited in the genome under the rs number rs7480526. This SNP is also located in the intron2 sequences of the *HBB* gene (https://www.ncbi.nlm.nih.gov/snp/rs7480526). The exact position of this SNP within the genomic sequences of chromosome 11 was NC_000011.10:g.5226503A>C. A high frequency of the deposited rs7480526 SNP in the dbSNP database was revealed, which was estimated to be 0.441891 for the alternative allele according to the GenomAD database. Due to this high frequency in the study group, this is in accordance with the previously mentioned information [58]. The clinical implications have been determined, indicating that it exerts a benign or likely benign influence. (National Center for Biotechnology Information (NCBI)). dbSNP, rs7480526 [Homo sapiens], https://www.ncbi.nlm.nih.gov/snp/rs7480526. *HBB. rs1451581925(386A>T)*

An additional pivotal aspect to consider is that this study represents a novel assessment of the rs7480526 SNP located within the *HBB* gene in individuals afflicted with β-thalassemia major residing in Iraq. 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 situated within the intronic 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 database. Due to the absence of any previous information on the frequency of rs1451581925, this SNP was not cited in any published study. Therefore, no previous knowledge of this variant is available. The clinical consequences have been evaluated, implying that they yield a threatening or non-threatening impact. (National Center for Biotechnology Information (NCBI). dbSNP, rs1451581925 [Homo sapiens], https://www.ncbi.nlm.nih.gov/snp/rs1451581925.

Genotype and phenotype connections in relation to established mutations

Determining the link between phenotype and genotype within a clinical framework is a fundamental aim of traditional research [59]. The findings showed that all identified mutations were associated with β-thalassemia major, and the affected individuals demonstrated variability in the disease phenotypes [60]. The homozygous type recorded the highest infection rate at 117%, with the genetic mutations distributed as follows: 44% for 64G>A (rs7946748), 41% for 71A>C (rs7480526), and 32% for 386A>T (rs1451581925). In contrast, heterozygous ß-thalassemia constituted 33% of cases, with the mutations distributed as 6% for 64G>A (rs7946748), 9% for 71A>C (rs7480526), and 18% for 386A>T (rs1451581925), as shown in Table 4. Most patients diagnosed with ß-thalassemia present with homozygous mutations, while a smaller subset shows heterozygous mutations. The milder presentation in heterozygous cases can be linked to the mutation's genetic location and the presence of a functional allele that compensates for the defective one. In less severe monogenic disorders, the mutation rate at the β-globin locus remains the most consistent and predictive marker of the disease phenotype. However, the intricate interaction between environmental and genetic factors introduces additional complexity in delineating the causal links between genotype and phenotype [61].

Table 4: The effect of mutations on genotypes patients with β-thalassemia major

β-Thalassemia mutation	Homozygous		Heterozygous	
	N	%	N	%
64G>A (rs7946748)	44	(44%)	6	(6%)
71A>C (rs7480526)	41	(41%)	9	(9%)
386A>T (rs1451581925)	32	(32%)	18	(18%)
Total	117		33	

The analysis of SNPs rs7946748, rs7480526, and rs1451581925 highlights significant associations between genotype variations and critical hematological parameters in β-thalassemia, underscoring the influence of genetic modifiers on disease severity, as shown in Table 5. Specifically, rs7480526 (71A>C) is linked to a low value for (HGB) and also for (RBC) counts in patients compared to controls ($p < 0.05$), aligning with the anemic profile typical of β-thalassemia, where ineffective erythropoiesis impairs red blood cell production [62]. Increased (MCV) and (MCH) in patients further underscore the presence of ineffective erythropoiesis. Moreover, ferritin levels, reflecting iron overload, were significantly elevated

in patients across all SNPs, particularly in rs7946748 and rs1451581925 ($p < 0.001$), due to the repeated blood transfusions required to manage chronic anemia [53]. Additionally, raised liver enzymes (ALT and AST) in rs7946748 and rs1451581925 (with AST at $p < 0.05$) suggest hepatic stress. This is likely a result of iron accumulation in the liver, a frequent complication in individuals with transfusion-dependent β-thalassemia[63] [64]. Elevated (WBC) counts associated with rs7480526 ($p < 0.05$) point to an increased inflammatory response or infection susceptibility, frequently observed in β-thalassemia due to immune dysregulation. Notably, variations in hemoglobin fractions were observed, with elevated (HbF) and altered HbA2 levels in rs7480526 and rs1451581925 ($p < 0.05$). Increased (HbF) levels can alleviate the severity of β-thalassemia by compensating for the globin chain imbalance caused by reduced β-globin production, thus improving anemia and ineffective erythropoiesis associated with the condition. Higher HbF has been shown to alleviate symptoms by compensating for deficient β-globin production [65]. Altogether, these findings reveal the role of these SNPs in influencing β-thalassemia pathophysiology, with rs7480526 emerging as a potential indicator of disease severity due to its associations with anemia, iron overload, and liver dysfunction, providing insights for targeted management and personalized treatment strategies. The foremost obstacle of this research is the small number of samples used in this study, which reduce the statistical power to detect true associations and increase the likelihood of false negative errors. This limitation makes it difficult to generalize findings or establish robust links between genetic variants and disease susceptibility. Additionally, limited sample sizes can hinder subgroup analysis and lead to inflated odds ratios due to sampling variability, thereby impacting the interpretability and reproducibility of the findings. While the association has not reached traditional thresholds of statistical significance, the higher frequency of specific genotypes in β-thalassemia patients underscores the need for further investigation into the role of this SNP in disease susceptibility.

Table 5 : The association of the identified SNPs with the hematological and biochemical parameters in the investigated population

No.	**Parameters**	**Controls**		**Patients**			**Controls**		**Patients**			**Controls**		**Patients**		
1.	**64G>A (rs794674 8)**	**GG (n=40)**		**GG (n=41)**			**GA (n=10)**		**GA (n=6)**			**AA (n=0)**		**AA (n=3)**		
		Mean	**S.D.**	**Mean**	**S.D**	**P**	**Mean**	**S.D**	**Mean**	**S.D**	**P**	**Mean**	**S.D**	**Mean**	**S.D**	**P**
	HGB	7.81	1.38	2.75	0.45	0.321	7.430	1.617	2.453	0.541	0.794	-	-	2.750	0.260	NA
	RBC	2.71	0.46	8.12	3.40	0.375	2.732	0.495	10.963	9.562	0.603	-	-	13.550	7.383	NA
	WBC	8.34	4.73	7.73	1.38	**0.023**	10.575	5.051	7.883	2.113	**0.025**	-	-	7.500	0.361	NA
	NEUT	48.15	9.01	47.73	9.12	0.181	47.090	11.564	49.650	11.505	0.058	-	-	47.400	13.533	NA
	LYMPH	39.59	7.28	39.42	8.50	0.147	38.650	11.725	39.600	6.312	0.727	-	-	38.767	10.485	NA
	MCV	77.31	5.85	76.52	4.97	**0.002**	76.820	5.507	81.883	8.923	0.948	-	-	77.300	6.022	NA
	MCH	27.71	1.99	27.47	2.03	**0.024**	27.020	2.103	28.383	2.097	0.598	-	-	27.367	1.801	NA
	MCHC	35.95	2.67	35.97	2.61	0.423	35.160	0.880	34.767	1.305	0.922	-	-	35.433	1.021	NA
	PLT	308.48	137.35	303.88	143.59	**0.00**	358.500	227.721	403.500	225.16	**0.015**	-	-	348.000	211.547	NA
	ALT	36.60	30.53	32.68	29.77	**0.00**	29.109	30.578	61.195	27.504	**0.046**	-	-	15.997	16.653	NA
	AST	46.50	21.29	43.75	21.21	**0.00**	44.511	28.466	66.873	26.449	**0.038**	-	-	36.740	14.682	NA
	ALP	164.09	67.78	165.69	66.68	**0.00**	153.860	36.663	139.333	40.102	**0.008**	-	-	157.633	35.440	NA
	UREA	25.97	7.20	25.10	7.31	**0.037**	22.510	5.641	25.267	6.826	0.344	-	-	27.667	2.650	NA
	Creatinine	0.63	0.11	0.61	0.10	**0.002**	0.600	0.067	0.617	0.041	**0.012**	-	-	0.733	0.153	NA
	Ferri	222	879	226	867	**0.**	247	874	266	49	**0.**	-	-	167	1455.1	NA

	tin	7.86	.61	3.25	.24	**00**	2.680	.094	8.567	2.11	**00**			8.733	0	
	HbF	82.32	5.41	81.59	5.62	**0.00**	77.780	5.224	79.950	7.393	**0.00**	-	-	81.967	0.777	NA
	HbA1	11.24	5.31	11.73	5.44	**0.00**	13.790	4.512	11.667	5.499	**0.003**	-	-	12.133	1.124	NA
	HbA2	2.45	0.59	2.60	0.64	**0.041**	2.800	0.650	2.133	0.367	0.294	-	-	2.300	0.173	NA
.2	**Parameters**	**Controls**		**Patients**			**Controls**		**Patients**			**Controls**		**Patients**		
	71A>C (rs7480526)	**AA (n=15)**		**AA (n=26)**		**P**	**AC (n=18)**		**AC (n=9)**		**P**	**CC (n=17)**		**CC (n=15)**		**P**
		Mean	**S.D.**	**Mean**	**S.D**		**Mean**	**S.D**	**Mean**	**S.D**		**Mean**	**S.D**	**Mean**	**S.D**	
	HGB	7.500	1.531	2.622	0.469	0.616	8.067	1.449	2.907	0.454	0.228	7.582	1.300	2.763	0.428	0.412
	RBC	2.737	0.514	8.107	4.119	0.757	2.743	0.478	11.566	7.796	0.258	2.666	0.407	8.305	3.237	0.561
	WBC	9.458	3.565	7.450	1.499	0.664	9.049	4.669	8.589	0.958	0.004	7.923	5.984	7.707	1.377	0.010
	NEUT	46.300	9.622	49.092	9.096	0.349	47.006	10.088	45.356	10.717	0.087	50.376	8.641	47.493	9.609	0.440
	LYMPH	40.293	9.796	38.277	8.494	0.602	39.244	8.622	40.878	5.904	0.783	38.776	6.542	40.460	9.099	0.312
	MCV	75.767	5.579	76.942	4.961	0.018	77.228	4.807	77.744	9.351	0.997	78.476	6.717	77.367	4.547	0.086
	MCH	27.540	2.451	27.696	1.973	0.018	27.489	1.972	27.444	2.750	0.783	27.682	1.726	27.427	1.680	0.089
	MCHC	36.373	2.046	36.069	2.486	0.192	35.672	2.841	35.389	1.473	0.992	35.400	2.322	35.547	2.851	0.994
	PLT	349.333	161.023	293.692	121.767	0.009	307.111	176.689	408.667	214.37	0.000	303.294	138.783	307.333	167.556	0.049
	ALT	39.169	38.914	39.260	32.184	0.000	26.168	22.588	35.621	34.811	0.001	40.964	28.580	27.574	24.380	0.005
	AST	46.965	25.885	47.300	24.201	0.002	39.704	16.222	48.272	25.540	0.036	52.124	24.727	42.733	18.690	0.021
	ALP	189.887	86.253	176.908	73.274	0.025	155.317	37.968	155.489	31.903	0.002	144.594	53.520	140.207	51.031	0.003

	UREA	26.740	6.729	25.635	7.757	0.606	24.367	4.691	26.378	5.817	0.428	24.953	9.181	24.000	6.502	0.031
	Creatinine	0.633	0.072	0.631	0.123	0.186	0.606	0.106	0.600	0.050	0.001	0.624	0.125	0.613	0.092	0.018
	Ferritin	2239.240	861.019	2276.435	981.698	0.000	2335.572	970.857	2270.100	851.90	0.000	2247.77	830.314	2281.520	738.291	0.000
	HbF	81.660	5.447	81.681	5.302	0.000	81.194	5.967	80.933	6.568	0.000	81.429	5.765	81.240	5.988	0.000
	HbA1	10.947	5.603	11.219	5.538	0.000	11.306	6.053	12.389	3.806	0.000	12.918	3.852	12.273	5.596	0.000
	HbA2	2.707	0.668	2.550	0.589	0.382	2.567	0.562	2.367	0.543	0.713	2.312	0.584	2.567	0.710	0.019
3.	**386 A>T (rs145158 1925)**	**AA (n=40)**		**AA (n=32)**			**AT (n=10)**		**AT (n=18)**			**TT (n=0)**		**TT (n=0)**		
		Mean	**S.D**	**Mean**	**S.D.**	***P***	**Mean**	**S.D**	**Mean**	**S.D.**	***P***	**Mean**	**S.D**	**Mean**	**S.D.**	***P***
	HGB	7.750	1.471	2.786	0.436	0.114	7.660	1.262	2.590	0.483	0.930	-	-	-	-	NA
	RBC	2.710	0.472	8.699	4.916	0.293	2.738	0.423	8.948	4.803	0.559	-	-	-	-	NA
	WBC	8.253	3.965	7.859	1.156	0.035	10.934	7.236	7.506	1.813	0.068	-	-	-	-	NA
	NEUT	48.723	9.196	46.828	9.643	0.110	44.810	10.315	49.917	9.027	0.205	-	-	-	-	NA
	LYMPH	39.160	8.566	40.316	7.960	0.575	40.360	6.975	37.772	8.651	0.154	-	-	-	-	NA
	MCV	76.415	5.268	78.213	6.071	0.027	80.410	6.661	75.439	4.716	0.307	-	-	-	-	NA
	MCH	27.313	1.978	27.772	1.952	0.017	28.600	1.896	27.211	2.121	0.460	-	-	-	-	NA
	MCHC	35.820	2.480	35.628	2.737	0.228	35.670	2.357	36.078	1.805	0.933	-	-	-	-	NA
	PLT	318.675	159.625	306.719	159.309	0.000	317.700	159.403	339.389	157.80	0.181	-	-	-	-	NA
	ALT	31.706	26.048	37.035	28.653	0.000	48.671	42.750	31.657	33.806	0.022	-	-	-	-	NA

	AST	44.658	21.676	49.723	21.666	0.000	51.893	26.372	39.672	23.371	0.022	-	-	-	NA
	ALP	160.393	53.630	166.625	72.039	0.000	168.640	93.923	153.894	41.560	0.005	-	-	-	NA
	UREA	24.418	6.757	26.013	6.578	0.040	28.720	7.279	23.972	7.732	0.133	-	-	-	NA
	Creatinine	0.620	0.109	0.622	0.094	0.001	0.620	0.079	0.617	0.120	0.242	-	-	-	NA
	Ferritin	2239.668	857.136	2253.081	860.407	0.000	2425.430	977.656	2319.022	924.55	0.001	-	-	-	NA
	HbF	81.735	5.388	82.025	5.381	0.000	80.130	6.678	80.328	6.055	0.000	-	-	-	NA
	HbA1	11.278	5.002	11.859	4.658	0.000	13.620	5.931	11.544	6.242	0.001	-	-	-	NA
	HbA2	2.495	0.627	2.475	0.683	0.007	2.630	0.566	2.606	0.467	0.102	-	-	-	NA

Conclusion

This study represents the first investigation in Southern Iraq analyzing the association of three intronic SNPs (rs7946748, rs7480526, and rs1451581925) in the *HBB gene* with β-thalassemia major. The findings demonstrate that A greater frequency of the A allele was observed in the research groups. Reinforcing its role in disease susceptibility and considered a risk factor; furthermore, the rs7480526 variant is significantly associated with lower hemoglobin levels and disease severity, emphasizing its potential role as a genetic marker profiling β-thalassemia risk in the Iraqi population. Elevated ferritin levels observed in patients with RS7946748 and RS1451581925 highlight the ongoing challenge of iron overload due to transfusion dependency. The study's LD and haplotype analyses revealed complex inheritance patterns, suggesting that these SNPs contribute to the genetic landscape influencing β-thalassemia. These insights underscore the need for integrating genetic profiling into clinical practices to inform more targeted and effective patient management strategies. Further research involving larger cohorts and mechanistic studies is recommended to enhance our understanding of these SNPs' roles in β-thalassemia pathophysiology.

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Ethical responsibilities of authors Ethics

The protocol and biomedical experiments conducted in this study adhered to the Helsinki Declaration, which outlines ethical principles for research involving human participants. The study received approval from the Ethical Committee of the College of Science at the University of Baghdad, with the reference number CSEC/0724/0049, issued on July 18, 2024.

Conflict of Interest

Conflict of Interest: The authors declare that they have no conflicts of interest.

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