

Association of HBB Gene Polymorphisms rs10768683, rs1609812 and rs334 with Transfusion-Dependent Beta-Thalassemia (TDT): A Case-Control Study

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Abstract

Background: While the primary causative mutations in the HBB gene determine the basic severity of β -thalassemia, secondary genetic modifiers within the β -globin gene cluster may influence phenotypic variability. This study investigated the association of three HBB polymorphisms rs10768683 (G>C), rs1609812 (A>G), and rs334 (T>A; HbS) with transfusion-dependent β -thalassemia (TDT) in southeastern Iran.

Methods: This case-control study enrolled 400 TDT patients and 400 healthy controls. Genotyping was performed using PCR-RFLP (rs1609812, rs334) and ARMS-PCR (rs10768683). Hematological, biochemical, and hormonal parameters were assessed. Associations were evaluated using age-adjusted logistic regression under multiple genetic models.

Results: Patients exhibited microcytic hypochromic anemia and severe iron overload (serum ferritin: 6234.43 ± 5582.97 ng/mL). The rs1609812 GG genotype conferred a nearly threefold increased TDT risk under both codominant 2 (OR = 2.85, 95% CI: 1.35–5.96, $p = 0.009$) and recessive models (OR = 2.75, 95% CI: 1.40–5.40, $p = 0.006$). Conversely, rs10768683 GC heterozygosity exhibited a protective effect under codominant 1 (OR = 0.75, $p = 0.029$), dominant (OR = 0.78, $p = 0.041$), and over-dominant models (OR = 0.80, $p = 0.036$). No significant association was found for rs334 across any genetic model ($p > 0.05$). All three polymorphisms deviated from Hardy-Weinberg equilibrium in both groups ($p < 0.001$).

Conclusion: This study demonstrates that HBB polymorphisms rs1609812 and rs10768683 are significantly associated with TDT susceptibility, with the GG genotype conferring risk and GC heterozygosity providing protection. These findings highlight the role of HBB-linked SNPs as genetic modifiers and support their potential utility in risk stratification.

Introduction

Among different protein types, hemoglobin (Hb) is a multifunctional protein that participates in a wide range of physiological activities, including catalytic functions. Nevertheless, its primary biological role is the transport of respiratory gases, particularly oxygen (O_2) from the lungs to peripheral tissues, where it binds and releases O_2 cooperatively^[1]. Structurally, hemoglobin consists of an iron-containing heme prosthetic group and four globin polypeptide chains: two alpha and two non-alpha subunits (β , δ , or γ)^[2,3]. Thalassemia is a common cause of microcytic anemia, resulting from a genetic defect in the production of alpha or beta chains of hemoglobin, leading to ineffective erythropoiesis, premature destruction of red blood cells (RBCs), and anemia^[4]. Alpha thalassemia major can lead to hydrops fetalis and death at birth, while beta thalassemia major presents early in life and usually requires lifelong blood transfusions before the age of two^[5]. Milder forms of alpha and beta thalassemia range from asymptomatic or mild anemia to cases requiring regular blood transfusions^[6]. About 5% and 1.5% of the world's population are carriers of alpha and beta thalassemia, respectively, who are usually asymptomatic^[7,8]. Beta thalassemia is caused by mutations in the beta globin gene on chromosome 11 and is more common in Asian, Mediterranean, and Middle Eastern populations^[4].

Beta thalassemia is clinically divided into three forms: minor (carrier, usually with mild or asymptomatic anemia), intermedia, and major (caused by homozygous or compound heterozygous mutations), with varying severity depending on transfusion dependence^[4]. The International Thalassemia Federation has proposed a more practical classification of Transfusion-Dependent Beta-Thalassemia (TDT) and Non-Transfusion-Dependent Beta-Thalassemia (NTDT), which is the basis for treatment decisions^[9]. TDT is characterized by severe hemolytic anemia and the need for regular blood transfusions from early childhood, and iron chelation therapy is also essential to prevent complications of iron overload^[7]. Life expectancy in these patients has increased from about 17 years in the 1970s to about 50 years in recent studies, due to improvements in transfusion and iron chelation therapy^[5]. TDT is a monogenic autosomal recessive disease caused by mutations in the HBB gene, which results in reduced or absent beta-globin chain production, globin chain imbalance, ineffective erythropoiesis, chronic anemia, and impaired iron metabolism^[10,11]. TDT can also be associated with structural hemoglobin variants such as HbS, HbC, and HbE^[12]. The global prevalence of the disease has increased in recent decades, partly attributed to population migration^[12,13]. In addition to current treatments, gene therapy and gene editing are being developed as promising therapeutic approaches^[14].

The β -globin gene cluster is located on the short arm of chromosome 11 (11p15.5) and contains five functional genes and one pseudogene^[15]. rs334 is a nonsynonymous SNP in the HBB gene on chromosome 11 that changes the codon GAG to GTC at amino acid 6, converting glutamic acid to valine, creating hemoglobin S (HbS). This mutation causes sickle cell disease in the homozygous state and sickle cell trait in the heterozygous state with a possible partial protection against malaria^[15,16]. In contrast, rs1609812 is an

intronic SNP (C < T) in the HBB gene that is located in a noncoding region, does not alter the protein sequence, and is mainly used as a genetic marker in population and haplotype studies and has no known pathogenic effect [17, 18]. Similarly, rs10768683 is an intronic polymorphism (G < C) in the HBB gene that does not cause an amino acid change and is used as a haplotype marker in HBB gene studies [17, 19]. In individuals with TDT, the clinical severity is not determined solely by the primary causative mutation in the HBB gene, but is also influenced by genetic modifiers including single nucleotide polymorphisms (SNPs) that affect gene expression, globin-chain balance, and hematologic parameters. For instance, the rs334 polymorphism, corresponding to the Glu6Val substitution in the β -globin chain; although classically associated with sickle-cell disease, illustrates how structural variation of the β chain may modulate the burden of globin imbalance and thereby influence the transfusion requirement in β -thalassemia [20]. The rs1609812 and rs10768683 variants, located within intronic or regulatory regions of HBB or its haplotype block, may serve as markers of particular haplotypic backgrounds or influence splicing/regulation of β -globin gene expression [12, 17].

Given that TDT is typified by markedly reduced or absent β -globin synthesis, leading to excess unpaired α -chains, ineffective erythropoiesis, and lifelong dependence on regular red-cell transfusions, the presence of polymorphic alleles that modify residual β -globin output, fetal-hemoglobin induction, or hemoglobin-chain stability can have meaningful clinical implications [21]. In this context, analyzing the association of rs334, rs1609812 and rs10768683 with metrics such as pre-transfusion hemoglobin concentration, transfusion interval and iron-overload markers enables us to explore whether these SNPs contribute to inter-individual variability in transfusion needs among TDT patients.

Materials and Methods

3.1. Study Design and Setting

This investigation was designed as a single-center, case-control study to evaluate the potential association between three functional polymorphisms within the *HBB* gene cluster rs334, rs1609812, and rs10768683 and the clinical phenotype of TDT. The study was carried out over a 13-month period, from April 2024 to April 2025, at Ali-Asghar Hospital, a major tertiary referral center for hemoglobinopathies in Zahedan, the capital of Sistan and Baluchestan province in southeastern Iran. This region is recognized for a particularly high prevalence of TDT, providing a well-defined population for a genetic association study. The research protocol was reviewed and approved by the Institutional Ethics Committee of Zahedan University of Medical Sciences under the reference number IR.ZAUMS.REC.1402.129 and was conducted in strict accordance with the principles of the Declaration of Helsinki. Voluntary, written informed consent was obtained from all participants or their legal guardians after a thorough explanation of the study's objectives and procedures, prior to any data or biospecimen collection.

3.2. Study Population

3.2.1. Case and Control Groups

The case group comprised 400 individuals with a confirmed diagnosis of TDT, recruited from the thalassemia ward of Ali-Asghar Hospital in Zahedan. Diagnosis was established through a combination of clinical evaluation, family history, and molecular diagnostic testing consistent with the standard criteria for TDT. The control group consisted of 400 healthy individuals with no personal or family history of hemoglobinopathies or other hematological disorders, recruited from the general population. Demographic characteristics, including age, were recorded for both groups.

3.2.2. Sample Size Calculation

A statistical power analysis was conducted to determine the appropriate sample size required to detect a statistically significant association between the rs334, rs10768683, and rs1609812 polymorphisms and TDT. Drawing on data from prior association studies, it was estimated that a minimum of 400 cases and 400 controls would provide 80% statistical power to detect a significant association (odds ratio ≥ 1.5) with the selected SNPs, using a two-sided significance threshold (α) of 0.05.

3.3. Data and Sample Collection

3.3.1. Clinical and Laboratory Parameters

To characterize the clinical status of the TDT patient cohort, a comprehensive set of demographics, hematological, biochemical, and hormonal data was systematically extracted from the electronic medical records of the patients at the time of enrollment. The demographic variable collected was participant age in years. The complete blood count (CBC) panel included the following indices: white

blood cell count (WBC, $\times 10^3/\mu\text{L}$), red blood cell count (RBC, $\times 10^6/\mu\text{L}$), hemoglobin (Hb, g/dL), hematocrit (Hct, %), mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), mean corpuscular hemoglobin concentration (MCHC, g/dL), red cell distribution width (RDW, %), and platelet count ($\times 10^3/\mu\text{L}$). The biochemical profile comprised fasting blood sugar (FBS, mg/dL), blood urea nitrogen (BUN, mg/dL), creatinine (Cr, mg/dL), the hepatic enzymes aspartate aminotransferase (AST, U/L) and alanine aminotransferase (ALT, U/L), calcium (Ca, mg/dL), and phosphorus (P, mg/dL). Additionally, a hormonal and iron metabolism panel was documented, including serum levels of thyroid-stimulating hormone (TSH, $\mu\text{IU/mL}$), parathyroid hormone (PTH, pg/mL), and ferritin (ng/mL). All laboratory measurements were performed in the hospital's central clinical pathology laboratory using automated, calibrated analyzers and standard clinical chemistry protocols. The full profile of these parameters extracted from both the patient and control groups, expressed as mean $\pm$ standard deviation (SD), is summarized in Table 1.

Table 1
Clinical and demographic characteristics of β -thalassemia major patients and controls.

Parameter evaluated	TDT (n = 400)	Control (n = 400)	p-value
Demographic			
Age (years)	16.06 $\pm$ 8.96	36.10 $\pm$ 11.13	< 0.001
Complete Blood Count (CBC)			
WBC ($\times 10^3/\mu\text{L}$)	7.11 $\pm$ 2.70	-	-
RBC ($\times 10^6/\mu\text{L}$)	3.69 $\pm$ 0.42	-	-
Hb (g/dL)	9.75 $\pm$ 1.39	-	-
Hct (%)	29.68 $\pm$ 3.92	-	-
MCV (fL)	80.95 $\pm$ 3.54	-	-
MCH (pg)	26.54 $\pm$ 1.33	-	-
MCHC (g/dL)	32.83 $\pm$ 1.36	-	-
RDW (%)	15.14 $\pm$ 2.34	-	-
Platelet ($\times 10^3/\mu\text{L}$)	255.84 $\pm$ 116.99	-	-
Biochemical Parameters			
FBS (mg/dL)	109.96 $\pm$ 29.34	-	-
BUN (mg/dL)	14.43 $\pm$ 3.84	-	-
Cr (mg/dL)	0.67 $\pm$ 0.18	-	-
AST (U/L)	45.10 $\pm$ 32.11	-	-
ALT (U/L)	47.49 $\pm$ 39.05	-	-
Ca (mg/dL)	8.94 $\pm$ 0.76	-	-
P (mg/dL)	4.58 $\pm$ 1.24	-	-
Hormonal & Iron Studies			
TSH ($\mu\text{IU/mL}$)	3.96 $\pm$ 1.49	-	-
Ferritin (ng/mL)	6234.43 $\pm$ 5582.97	-	-
PTH (pg/mL)	33.99 $\pm$ 17.57	-	-
Data are presented as mean $\pm$ standard deviation (SD). P-values were calculated using an independent-samples t-test. The dash (–) indicates that the parameter was not measured in the control group. Abbreviations: TDT, transfusion-dependent β -thalassemia; WBC, white blood cell count; RBC, red blood cell count; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; FBS, fasting blood sugar; BUN, blood urea nitrogen; Cr, creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Ca, calcium; P, phosphorus; TSH, thyroid-stimulating hormone; PTH, parathyroid hormone.			

3.3.2. Blood Sample Collection and Processing

For the genetic analysis, a 2 mL sample of peripheral venous blood was drawn from each participant in both the case and control groups. The sample was collected under aseptic conditions by a trained phlebotomist using a sterile vacutainer system into commercially available tubes containing ethylenediaminetetraacetic acid (K₂-EDTA) as an anticoagulant to prevent clotting. Immediately following collection, each blood sample was gently inverted 8–10 times to ensure thorough mixing with the anticoagulant. All blood samples were then labeled with unique de-identified barcodes to protect participant confidentiality and were promptly transported on ice to the molecular genetics research laboratory at Zahedan University of Medical Sciences for processing. Upon arrival, the whole blood samples were stored upright at – 80°C until the batch extraction of genomic DNA, with no prior freeze-thaw cycles. Written informed consent and ethical approval were obtained from all participants before the blood samples were collected.

3.4. Genetic Analysis

3.4.1. DNA Extraction

Genomic DNA was extracted from peripheral blood leukocytes using the standard *salting-out* method [22]. The quality and concentration of the extracted DNA were assessed using a *NanoDrop* spectrophotometer, yielding an average concentration of approximately 85 ng/μL for each sample.

3.4.2. SNP Selection and Primer Design

Based on a thorough review of the 1000 Genomes Project database, three SNPs within the *HBB* gene cluster were targeted: rs1609812 (intron variant, 5225911 A/G), rs10768683 (intron variant, 5226561 G/C), and rs334 (missense variant, 5227002 T/A). These SNPs were strategically selected due to their minor allele frequency (MAF) exceeding 0.1 in relevant populations, a threshold that ensures sufficient statistical power, combined with their functional potential or prior literature evidence suggesting an association with various clinical phenotypes and disorders. For each target SNP, specific sets of primers (forward and reverse) were meticulously designed using *Oligo 7* software to ensure optimal thermodynamic properties, high specificity, and the absence of secondary structures or primer-dimer formation. The oligonucleotide sequences were synthesized by *Metabion* (Germany) and were procured through the local supplier *Pishgam* Company (Tehran, Iran). The complete primer sequences, the corresponding genotyping methodology, restriction enzymes, expected product sizes, and optimized annealing temperatures for each SNP are detailed in Table 2.

Table 2
Primers and restriction enzyme specifications.

SNP	Consequence Position	Primer sequence	Genotyping method	Restriction enzyme	Product size (bp)
rs1609812 A/G	Intron Variant	F: CATTAGCCACACCAGCCAC	PCR-RFLP	<i>Pdml</i>	G: 317
	5225911	R: TTTCTGGGTTAAGGCAATAGAAATAT			A: 293 + 24
rs10768683 G/C	Intron Variant	RO: AACGTGGATGAAGTTGGTGGTGAG	ARMS	-	G/C: 426
	5226561	F(G): GAAGGGGAAAGAAAACATCAAGG F(C): GAAGGGGAAAGAAAACATCAAGC			
rs334 T/A	Missense Variant	F: GGAAAATAGACCAATAGGCAGAGAGAG	PCR-RFLP	<i>Ddel</i>	T: 193 + 44
	5227002	R: CACTAGCAACCTCAAACAGACAC			A: 237
SNP, Single Nucleotide polymorphism; bp, Base pair; F, forward; R, reverse; PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism					

3.4.3. Genotyping Protocols

Genotyping of the target SNPs in the *HBB* gene was performed using two distinct methods as follows: rs1609812 (A/G) and rs334 (T/A) were analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), while rs10768683 (G/C) was genotyped using the amplification refractory mutation system PCR (ARMS-PCR) method. For each SNP, PCR amplification was carried out in a total reaction volume of 15 μL. The reaction mixture consisted of 7.5 μL of PCR master mix, 0.8–0.9 μL of each forward and reverse primer (at concentrations according to the manufacturer’s protocol), 1 μL of genomic DNA template, and double-distilled water (DDW) adjusted to reach the final volume. The detailed composition of each reaction is provided in Table 3. The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 5 minutes, followed by 30–33 cycles of denaturation at 94°C for 30

seconds, annealing at the specific temperature for each SNP (61°C for rs1609812, 63°C for rs10768683, and 60°C for rs334) for 30 seconds, and extension at 72°C for 30 seconds. A final extension step was performed at 72°C for 5 minutes to conclude the amplification. For the PCR-RFLP assays of SNPs rs1609812 and rs334, the PCR products were subsequently digested with the appropriate restriction endonuclease. A volume of 0.1 µL of *Pdml* (for rs1609812) or *Ddel* (for rs334) was used per reaction and incubated at 37°C for 7 and 5 hours, respectively. No enzymatic digestion was required for rs10768683, as the ARMS-PCR method yields allele-specific amplicons directly. The digested (or undigested, in the case of rs10768683) products were then resolved by agarose gel electrophoresis. Electrophoresis was performed using 3% agarose gels for rs1609812 and rs334 at a constant voltage for 110 minutes, while a 2% agarose gel was used for rs10768683 for 100 minutes. The resulting DNA band patterns were visualized under ultraviolet (UV) light to determine individual genotypes. Table 3 provides a comprehensive summary of the detailed PCR amplification and electrophoresis conditions for *HBB* SNP genotyping.

Table 3 Detailed PCR amplification and electrophoresis conditions for HBB SNP genotyping.															
SNP	PCR amplification													electrophoresis	
	PCR Reaction Mix				Thermal Cycling Conditions						Enzyme Incubation			Gel Conditions	
Master Mix	F	R	DNA	DDW	95	94	Annealing temp.	72	72	Enz.	Time	Inco.	Gel % / Time		
rs1609812 A/G	7.5	0.8	0.8	1	4.9	5 min	30 s	61 °C	30 s	5 min	0.1 µL	7 h	37 °C	%3 /110	
rs10768683 G/C	7.5	0.8	0.8	1	4.9	5 min	30 s	63 °C	30 s	5 min	-	-	-	%2 /100	
rs334 T/A	7.5	0.9	0.9	1	4.7	5 min	33 s	60 °C	33 s	5 min	0.1 µL	5 h	37 °C	%3 /110	
SNP, single nucleotide polymorphism; Master Mix, PCR master mix; F, forward primer; R, reverse primer; DNA, genomic DNA template; DDW, double-distilled water; Annealing temp., annealing temperature; Enz., restriction enzyme; Inco., incubation (for enzymatic digestion); Gel %, agarose gel percentage.															

3.4.4. Quality Control

To verify the reliability and reproducibility of the genotyping results, 10% of the total samples were randomly selected and subjected to repeat analysis in a blinded fashion, with 100% concordance observed. Furthermore, negative control samples (containing master mix and DDW, but no DNA template) were included in each PCR run to detect any potential contamination.

3.5. Statistical Analysis

All statistical analyses were performed using SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA). Continuous demographic and clinical data are presented as the mean ± standard deviation (SD), and differences between the case and control groups were assessed using an independent-samples t-test. The distribution of genotype frequencies in the control group was tested for deviation from Hardy-Weinberg equilibrium (HWE) using the Chi-square (χ^2) test. Differences in genotypic and allelic frequencies between patients and controls were evaluated using either the Chi-square test or Fisher's exact test, as appropriate. To account for the significant difference in age between the case and control groups, binary logistic regression analysis was performed, with age included as a covariate in the model. Accordingly, all reported p-values, odds ratios (ORs), and 95% confidence intervals (CIs) for the association between each SNP and TDT susceptibility were adjusted for age. A two-tailed p-value of less than 0.05 was considered statistically significant for all tests.

Results

4.1. Demographic findings

The patient cohort is significantly younger (16.06 ± 8.96 years) than the healthy control group (36.10 ± 11.13 years). Hematological Profile: Patients exhibit a characteristic microcytic, hypochromic anemia. Red blood cell (RBC) count is low (3.69 ± 0.42 ×10⁶/µL), with significantly reduced hemoglobin (Hb: 9.75 ± 1.39 g/dL) and hematocrit (Hct: 29.68 ± 3.92%). The red cell indices confirm microcytosis (MCV: 80.95 ± 3.54 fL) and hypochromia (MCH: 26.54 ± 1.33 pg; MCHC: 32.83 ± 1.36 g/dL). Anisocytosis is present, as indicated by an elevated Red Cell Distribution Width (RDW: 15.14 ± 2.34%). The white blood cell (WBC) count is within a normal range (7.11 ± 2.70 ×10³/µL). Platelet count shows wide variation but is generally normal on average (255.84 ± 116.99 ×10³/µL). Biochemical Parameters: Fasting

blood glucose (FBS) is 109.96 ± 29.34 mg/dL, at the upper limit of normal. Renal function parameters are within normal ranges (BUN: 14.43 ± 3.84 mg/dL; Creatinine: 0.67 ± 0.18 mg/dL). Liver transaminases are mildly elevated (AST: 45.10 ± 32.11 U/L; ALT: 47.49 ± 39.05 U/L), suggesting possible hepatic iron overload or involvement. Serum calcium is at the lower end of normal (8.94 ± 0.76 mg/dL), while phosphorus is within the normal range (4.58 ± 1.24 mg/dL). Hormonal and Iron Studies: The most striking finding is extremely elevated serum ferritin (6234.43 ± 5582.97 ng/mL), indicative of severe iron overload due to chronic transfusion therapy. Thyroid-stimulating hormone (TSH) is within the normal reference range (3.96 ± 1.49 μ U/mL). Parathyroid hormone (PTH) level is also normal (33.99 ± 17.57 pg/mL). Table 1 shows the demographic characteristics of the patients.

4.2. Genetic Association Analysis of HBB Polymorphisms with β -Thalassemia Major

4.2.1 Analysis of SNP rs1609812 (A/G):

For SNP rs1609812, the most common genotype in both patients and controls was the heterozygous AG genotype (75.5% and 78.7%, respectively). The homozygous GG genotype was observed in 7.8% of patients, compared with 3.0% in controls. Both groups deviated significantly from HWE ($p < 0.001$). Age-adjusted analysis revealed a significant association under the codominant 2 model, with individuals with the homozygous GG genotype having a higher risk of β -thalassemia major than those with the AA genotype (OR = 2.85, 95% CI: 1.35–5.96, $p = 0.009$). This association was also significant under the recessive genetic model (GG vs. AA + AG: OR = 2.75, 95% CI: 1.40–5.40, $p = 0.006$). However, no significant associations were detected under the codominant 1 (AG vs. AA: OR = 1.12, 95% CI: 0.75–1.55, $p = 0.821$), dominant (AG + GG vs. AA: OR = 1.15, 95% CI: 0.80–1.65, $p = 0.581$), over dominant (AG vs. AA + GG: OR = 0.85, 95% CI: 0.64–1.18, $p = 0.277$), or allelic (G vs. A: OR = 1.15, 95% CI: 0.95–1.42, $p = 0.218$) models.

4.2.2 Analysis of SNP rs10768683 (G/C):

The distribution of SNP rs10768683 genotypes showed that the GG genotype was more frequent in β -thalassemia major patients (73.3%) than in controls (66.3%). A significant deviation from HWE was present in both groups ($p < 0.001$). The age-adjusted analysis indicated significant protective associations under specific models. In the codominant 1 model, the heterozygous GC genotype was associated with a reduced risk compared to the GG genotype (OR = 0.75, 95% CI: 0.55–1.02, $p = 0.029$). This protective effect remained significant in the dominant model (GC + CC vs. GG: OR = 0.78, 95% CI: 0.59–1.08, $p = 0.041$) and the over-dominant model (GC vs. GG + CC: OR = 0.80, 95% CI: 0.61–1.10, $p = 0.036$). No significant association was found for the homozygous CC genotype in the codominant 2 model (CC vs. GG: OR = 0.89, 95% CI: 0.54–1.52, $p = 0.548$) or the recessive model (CC vs. GG + GC: OR = 0.98, 95% CI: 0.58–1.65, $p = 0.793$). The allelic model showed a trend towards a protective effect for the C allele, though it was not statistically significant (OR = 0.82, 95% CI: 0.65–1.08, $p = 0.059$).

4.2.3. Analysis of SNP rs334 (T/A):

The genotypic distribution for SNP rs334 showed a predominance of the homozygous wild-type (TT) genotype in both β -thalassemia major patients (97.0%) and healthy controls (98.8%). The heterozygous (TA) and homozygous variant (AA) genotypes were observed at low frequencies in both groups. Significant deviation from the Hardy-Weinberg equilibrium (HWE) was noted in both cohorts ($p < 0.001$). Following age-adjusted logistic regression analysis, no statistically significant association was found between the rs334 polymorphism and β -thalassemia major across all tested genetic models. In the codominant models, the heterozygous state (TA) yielded an odds ratio (OR) of 2.60 (95% CI: 0.80–8.26, $p = 0.115$), while the homozygous variant state (AA) yielded an OR of 2.08 (95% CI: 0.21–22.59, $p = 0.559$). Similarly, non-significant results were obtained under the dominant (OR = 2.48, 95% CI: 0.88–7.05, $p = 0.091$), recessive (OR = 2.09, 95% CI: 0.23–22.35, $p = 0.568$), and over-dominant (OR = 2.58, 95% CI: 0.82–8.20, $p = 0.116$) models. The allelic model also did not show a significant association (OR = 2.40 for the A allele, 95% CI: 0.95–6.19, $p = 0.076$). Figure 1 shows the results of PCR and RFLP arms. Also, Table 4 shows allelic and genotypic distribution of *HBB* polymorphisms.

Table 4
Allelic and genotypic distribution of *HBB* polymorphisms.

SNP	TDT, n (%)	Control, n (%)	Genetic model	OR (95%CI) *	p-value*
rs1609812 A/G					
AA	67 (16.7)	73 (18.3)		1 (reference)	
				-	
AG	302 (75.5)	315 (78.7)	Codominant 1	1.12 (0.75–1.55)	0.821
GG	31 (7.8)	12 (3.0)	Codominant 2	2.85 (1.35–5.96)	0.009
HWE	< 0.001	< 0.001	Dominant	1.15 (0.80–1.65)	0.581
			Recessive	2.75 (1.40–5.40)	0.006
			Over dominant	0.85 (0.64–1.18)	0.277
A	436 (54.5)	461 (57.6)	Allelic	1 (reference)	
G	364 (45.5)	339 (42.4)	Allelic	1.15 (0.95–1.42)	0.218
rs10768683 G/C					
GG	293 (73.3)	265 (66.3)		1 (reference)	
GC	79 (19.7)	105 (26.2)	Codominant 1	0.75 (0.55–1.02)	0.029
CC	28 (7.0)	30 (7.5)	Codominant 2	0.89 (0.54–1.52)	0.548
HWE	< 0.001	< 0.001	Dominant	0.78 (0.59–1.08)	0.041
			Recessive	0.98 (0.58–1.65)	0.793
			Over dominant	0.80 (0.61–1.10)	0.036
G	665 (83.1)	635 (79.4)	Allelic	1 (reference)	
C	135 (16.9)	165 (20.6)	Allelic	0.82 (0.65–1.08)	0.059
rs334 T/A					
TT	388 (97.0)	395 (98.8)		1 (reference)	
TA	10 (2.5)	4 (1)	Codominant 1	2.60 (0.80–8.26)	0.115
AA	2 (0.5)	1 (0.2)	Codominant 2	2.08 (0.21–22.59)	0.559
HWE	< 0.001	< 0.001	Dominant	2.48 (0.88–7.05)	0.091
			Recessive	2.09 (0.23–22.35)	0.568
			Over dominant	2.58 (0.82–8.20)	0.116
T	786 (98.2)	794 (99.2)	Allelic	1 (reference)	
A	14 (1.8)	6 (0.8)	Allelic	2.40 (0.95–6.19)	0.076
SNP, Single-nucleotide polymorphism; CI, confidence interval; OR, odds ratio. HWE, Hardy Weinberg equilibrium; *p-value and OR (95%CI) were adjusted for Age. Codominant 1 and Codominant 2 indicate the heterozygous and homozygous codominant models, respectively. p < 0.05 is considered statistically significant.					

Discussion

The present case-control study was designed to investigate the potential association of three *HBB* gene polymorphisms rs10768683, rs1609812, and rs334 with TDT in a cohort from southeastern Iran. By comparing 400 TDT patients with 400 healthy controls and applying age-adjusted logistic regression models, we identified significant associations for two of the three tested variants. Specifically, the homozygous GG genotype of rs1609812 conferred a nearly threefold increased risk of TDT, while the heterozygous GC genotype of rs10768683 exhibited a modest protective effect. The rs334 (HbS) variant, although more prevalent in the patient group, did not reach

statistical significance. Beyond the genetic findings, the comprehensive clinical and laboratory characterization of the TDT cohort revealed the expected hallmarks of chronic transfusion-dependent anemia, iron overload, and multi-organ involvement. These demographic and biochemical data provide essential context for interpreting the genetic associations observed.

β -thalassemia is a monogenic disorder characterized by decreased or absent β -globin production, leading to chronic anemia, ineffective erythropoiesis, and, in its transfusion-dependent form, progressive iron overload and secondary organ damage [23, 24]. Globally, mutations in the HBB gene result in a spectrum of clinical severity, and it is well recognized that the clinical phenotype is influenced not only by the primary causative mutation but also by secondary genetic modifiers, including polymorphisms within the β -globin gene cluster itself [25]. In this context, the evaluation of HBB-linked SNPs such as rs1609812, rs10768683, and rs334 in TDT patients holds both scientific and clinical significance, as it may refine our understanding of phenotypic variability, assist in individualizing monitoring and treatment strategies, and potentially inform genetic counseling in populations with a high prevalence of β -thalassemia.

The demographic analysis revealed a highly significant difference in age between the TDT patients and the control group (16.06 ± 8.96 vs. 36.10 ± 11.13 years, $p < 0.001$). This discrepancy is consistent with the natural history of β -thalassemia major, wherein individuals are diagnosed in early childhood and remain under continuous care in pediatric and young adult thalassemia centers. The recruitment of patients from a specialized hospital ward inevitably skews the age distribution toward younger individuals, while the healthy control group, drawn from the general adult population, reflects a broader age range. To mitigate the potential confounding effect of this age disparity, all genetic association analyses were adjusted for age using binary logistic regression. This stringent statistical approach ensures that the reported odds ratios reflect genuine genotype-phenotype associations rather than artifacts of demographic imbalance. The hematological profile of the TDT cohort demonstrated the characteristic features of microcytic, hypochromic anemia despite regular transfusion therapy. The markedly reduced Hb (9.75 ± 1.39 g/dL) and Hct ($29.68 \pm 3.92\%$), combined with low MCV (80.95 ± 3.54 fL) and MCH (26.54 ± 1.33 pg), confirmed the persistence of anemia with suboptimal red cell indices. The elevated RDW ($15.14 \pm 2.34\%$) indicated significant anisocytosis, likely reflecting a mixture of transfused and endogenous red cells. Biochemical parameters provided further insight into the systemic consequences of TDT. Fasting blood glucose (109.96 ± 29.34 mg/dL) was at the upper limit of normal, consistent with the known risk of glucose intolerance and diabetes mellitus secondary to iron-induced pancreatic damage in this population. Mildly elevated liver transaminases (AST: 45.10 ± 32.11 U/L; ALT: 47.49 ± 39.05 U/L) suggested hepatic involvement, most likely attributable to iron overload, although transfusion-related viral infections cannot be entirely excluded. Serum calcium (8.94 ± 0.76 mg/dL) was at the lower boundary of normal, a finding that may reflect hypoparathyroidism related to iron deposition or vitamin D deficiency, both common in thalassemia. The most striking biochemical abnormality was the profoundly elevated serum ferritin level (6234.43 ± 5582.97 ng/mL), confirming severe iron overload secondary to chronic transfusion therapy. This finding underscores the critical importance of optimizing iron chelation therapy in this cohort. Notably, TSH and PTH levels were within normal ranges, suggesting preserved thyroid and parathyroid function in the majority of patients at the time of assessment. Together, these demographic and clinical characteristics provide a robust baseline profile of the study population, which is essential for interpreting the genetic association results.

The rs1609812 polymorphism, located in the intronic region (IVS2-666) of the HBB gene, has been previously characterized as a silent polymorphism that does not directly alter the β -globin amino acid sequence. However, it has been studied in surveys of β -globin gene cluster polymorphism frameworks and may serve as a marker of haplotypes in linkage disequilibrium with functionally significant variants [18]. In the present study, the heterozygous AG genotype was the most prevalent in both patients (75.5%) and controls (78.7%). Importantly, the homozygous GG genotype was significantly overrepresented in TDT patients (7.8%) compared to controls (3.0%). Age-adjusted logistic regression demonstrated that individuals carrying the GG genotype had a 2.85-fold increased risk of TDT under the codominant 2 model (GG vs. AA: OR = 2.85, 95% CI: 1.35–5.96, $p = 0.009$), and this association remained significant under the recessive model (GG vs. AA + AG: OR = 2.75, 95% CI: 1.40–5.40, $p = 0.006$). Genc et al. (2020), in a study conducted in southeastern Turkey on β -thalassemia trait carriers, investigated the relationship between five SNPs—including rs1609812—and fetal hemoglobin (HbF) levels. Using real-time PCR genotyping, they reported that, unlike several other tested polymorphisms that showed significant associations with increased HbF, rs1609812 did not demonstrate a statistically significant difference between β -thalassemia trait and control groups in the codominant model [26]. At first glance, the results of Genc et al. may appear to conflict with our findings. However, several crucial differences between the two studies must be emphasized. First, Genc et al. studied β -thalassemia trait carriers heterozygous individuals with generally mild or absent anemia whereas our study focused on transfusion-dependent patients representing the severe end of the clinical spectrum. Second, their outcome of interest was HbF level, a continuous modifier phenotype, rather than the dichotomous disease state. Third, the genetic background and HBB haplotype structure of the Turkish population may differ from that of southeastern Iran. It is plausible that the rs1609812 G allele, in the specific haplotypic context of the Zahedan population, is in strong linkage disequilibrium with a pathogenic β -thalassemia mutation, thereby acting as a risk marker for TDT. This interpretation aligns with the

understanding that polymorphisms like rs1609812 may modify disease severity by tagging haplotypic backgrounds that correlate with residual β -globin synthesis or the efficiency of globin-chain pairing [27,28]. Our finding of a recessive effect with only the homozygous GG state conferring risk suggests a dosage-dependent mechanism, possibly involving the interaction of two copies of a risk haplotype. The significant deviation from Hardy-Weinberg equilibrium in both groups for this SNP further supports the hypothesis of selection pressure or population substructure related to the high prevalence of HBB mutations in this region.

The rs10768683 variant (HBB c.315 + 16G > C; IVS-II-16, A_{val}I) is a high-frequency intronic polymorphism that has been extensively used as an informative marker for reconstructing β -globin haplotypes in population studies [27]. Recent haplotype analyses published after 2020 consistently employ this SNP to trace the genetic background of pathogenic β -thalassemia mutations and to infer linkage disequilibrium patterns [29]. In our study, the GG genotype was more frequent in TDT patients (73.3%) than in controls (66.3%). In contrast to rs1609812, the heterozygous GC genotype of rs10768683 was associated with a statistically significant protective effect against TDT. Specifically, individuals with the GC genotype had a 25% reduced risk compared to GG homozygotes under the codominant 1 model (OR = 0.75, 95% CI: 0.55–1.02, $p = 0.029$). This protective effect was corroborated under the dominant model (GC + CC vs. GG: OR = 0.78, $p = 0.041$) and the over-dominant model (GC vs. GG + CC: OR = 0.80, $p = 0.036$). The protective association of the C allele at rs10768683 is a novel and intriguing finding that warrants careful interpretation. It is important to clarify that rs10768683 itself is unlikely to be a causal variant directly influencing TDT risk. Rather, as an intragenic marker within intron 2 of HBB, it likely reflects a specific haplotypic framework. The C allele may be in linkage disequilibrium with genetic backgrounds that are either less frequently associated with severe β -thalassemia mutations or are linked to loci that upregulate compensatory mechanisms such as fetal hemoglobin production. Under the over-dominant model, heterozygosity at this locus appears to confer an advantage, reminiscent of the well-established heterozygote advantage of β -thalassemia trait itself against severe malaria. It is plausible that the GC heterozygous state at rs10768683 marks a balanced haplotypic combination that mitigates disease severity, possibly through a more favorable pattern of globin-chain gene expression. While the allelic model showed a non-significant trend ($p = 0.059$), the consistency of the protective signal across multiple genotype models strongly suggests a genuine biological phenomenon rather than a statistical artifact. These results align with the established utility of rs10768683 in haplotype-based genetic epidemiological mapping and underscore its potential value not as a direct causal variant but as a surrogate marker for disease-modifying haplotypes [29].

The rs334 variant, resulting in the Glu6Val substitution in the β -globin chain, is the canonical sickle cell allele (HbS). It is well characterized in genomic and clinical literature as a pathogenic missense variant with profound clinical consequences in homozygous and compound heterozygous states [30,31]. While rs334 is historically studied in sickle cell disease, its presence in β -thalassemia cohorts is of significant interest in regions such as southeastern Iran where both hemoglobinopathies coexist, and where compound heterozygosity (HbS/ β -thalassemia) represents a clinically important diagnosis [16,31,32]. In our cohort, the homozygous wild-type genotype (TT) was overwhelmingly predominant in both TDT patients (97.0%) and controls (98.8%). The heterozygous TA and homozygous AA genotypes were observed at low frequencies in both groups, with a borderline increase in the patient group. After age-adjusted logistic regression, the TA genotype yielded an OR of 2.60 (95% CI: 0.80–8.26, $p = 0.115$), and the allelic OR was 2.40 (95% CI: 0.95–6.19, $p = 0.076$). Although these odds ratios suggest a potential trend toward increased risk, none of the genetic models achieved statistical significance at the conventional $p < 0.05$ threshold. The failure to reach statistical significance is likely attributable to the low minor allele frequency in both groups, which substantially limits statistical power for detecting a true association. Larger sample sizes, or meta-analytic approaches pooling data from multiple centers, would be required to definitively establish or refute an association. Nevertheless, the presence of the A allele in TDT patients at a frequency higher than in controls warrants clinical vigilance; genotyping for rs334 in TDT cohorts remains essential to identify compound heterozygous HbS/ β -thalassemia individuals, who require distinct clinical management strategies and may exhibit a more severe phenotype [31]. Moreover, recent pleiotropic association studies have suggested that rs334 carrier status may influence risks for outcomes beyond hematological disease, underscoring the broader clinical importance of this polymorphism [30].

Our clinical laboratory data, particularly the extreme hyperferritinemia and mild hepatic enzyme elevations, highlight the ongoing challenge of managing iron overload in TDT, even in the era of modern chelation therapy. The association of the rs1609812 GG genotype with TDT risk, coupled with the clinical markers of severe disease, raises the question of whether this genotype correlates with more severe iron loading or higher transfusion requirements. Future analyses stratifying patients by genotype and examining quantitative traits such as serum ferritin, pre-transfusion hemoglobin, or annual transfusion volume could test the hypothesis that the rs1609812 GG genotype identifies a subset of patients predisposed to a more severe clinical course. Similarly, the protective signal observed for rs10768683 heterozygosity could be explored in relation to milder clinical indices. The borderline calcium levels and elevated FBS also underscore the multi-system impact of TDT and the importance of comprehensive endocrine surveillance.

Several limitations of this study should be acknowledged. First, the significant age difference between the case and control groups, although addressed through age-adjusted regression, represents a potential source of residual confounding. Recruitment from a pediatric and young adult thalassemia ward inherently limits the age distribution of the patient group. Second, the deviation from Hardy-Weinberg equilibrium observed for all three polymorphisms in both groups suggests the influence of population substructure, assortative mating, or selection pressure, which are well-recognized phenomena in populations with a high burden of hemoglobinopathies. This deviation necessitates cautious interpretation of the genetic association results, although stringent quality control measures and the consistency of findings across multiple genetic models provide reassurance. Third, the sample size, while powered to detect associations of moderate effect, may be insufficient to detect weak associations, particularly for low-frequency variants such as the rs334 A allele. Fourth, the absence of data on the specific primary HBB gene mutations underlying TDT in this cohort precludes an analysis of interaction effects between primary mutations and the studied polymorphisms. Future studies incorporating comprehensive mutation profiling alongside SNP genotyping would provide deeper insight into the clinical significance of these polymorphisms. Finally, as a single-center study conducted in southeastern Iran, the generalizability of the findings to other populations with different genetic backgrounds requires validation in independent cohorts. Based on these findings, we propose several avenues for future investigation. First, replication studies in larger, multi-center cohorts with comprehensive HBB mutation characterization are warranted to validate the associations of rs1609812 and rs10768683 with TDT. Second, functional studies exploring the potential regulatory role of rs1609812 within its intronic context could clarify whether this polymorphism directly influences HBB gene expression or splicing efficiency. Third, haplotype-based analyses combining the three studied SNPs with other markers across the β -globin gene cluster would provide a more complete picture of the haplotypic architecture associated with TDT risk and could identify specific risk and protective haplotypes. Fourth, longitudinal studies examining whether these polymorphisms correlate with quantitative clinical outcomes including transfusion frequency, iron burden (as measured by ferritin or MRI-derived liver iron concentration), and the onset of organ complications would be of direct clinical utility. Finally, given the geographic overlap of β -thalassemia and sickle cell disease, continued surveillance for the rs334 (HbS) variant in TDT populations is recommended to ensure the accurate identification of compound heterozygotes who may benefit from tailored management, including hydroxyurea therapy or early consideration of curative options.

Conclusion

In conclusion, this case-control study conducted in a high-prevalence region of southeastern Iran demonstrates that polymorphisms within the HBB gene cluster are significantly associated with transfusion-dependent β -thalassemia (TDT). Specifically, the homozygous GG genotype of rs1609812 confers a nearly threefold increased risk of TDT, implicating this intronic variant as a potential genetic marker of disease susceptibility, likely through linkage disequilibrium with pathogenic haplotypes. Conversely, the heterozygous GC genotype of rs10768683 exhibits a significant protective effect, suggesting that particular haplotypic backgrounds marked by this polymorphism may modulate disease severity. Although the rs334 (HbS) variant did not reach statistical significance, its elevated frequency in patients underscores the importance of routine screening in populations where β -thalassemia and sickle cell disease coexist. These findings advance the understanding of HBB-linked polymorphisms as modifiers of β -thalassemia phenotype and highlight the potential clinical utility of genotyping these variants for risk stratification, prognosis, and individualized patient management. Validation in larger, multi-ethnic cohorts with comprehensive molecular characterization is warranted to confirm these associations and to elucidate the underlying haplotypic and functional mechanisms.

Declarations

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Statement of Ethical Approval: The research protocol was reviewed and approved by the Institutional Ethics Committee of Zahedan University of Medical Sciences under the reference number IR.ZAUMS.REC.1402.416. A copy of the approval certificate is available at <https://ethics.research.ac.ir/IR.ZAUMS.REC.1402.129>.

Consent Statement: Voluntary, written informed consent was obtained from each participant or their legal guardian prior to enrollment in the study.

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Figures

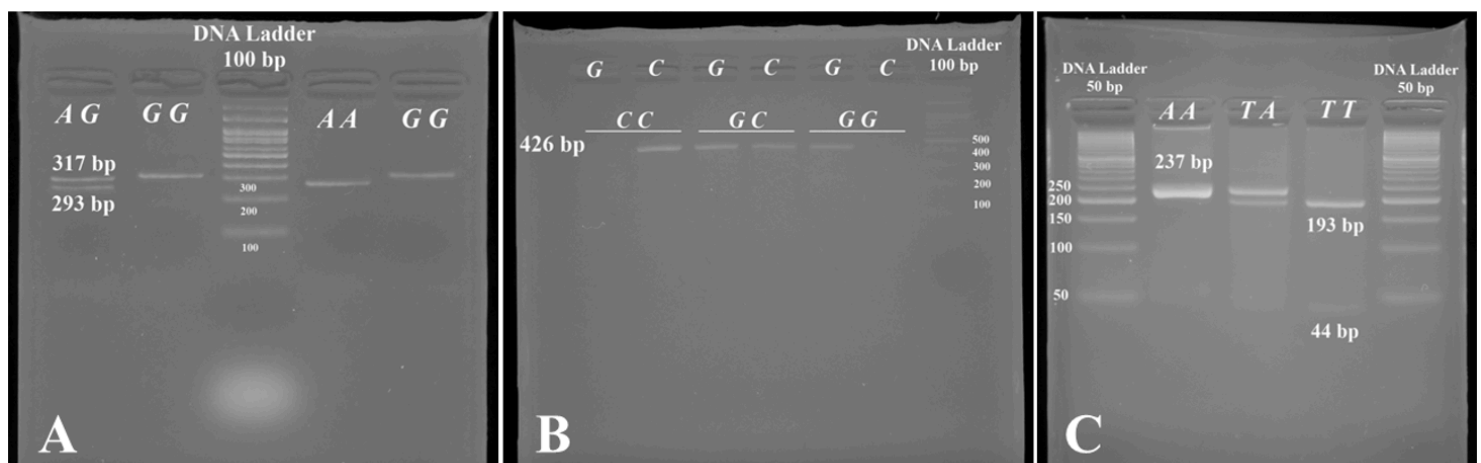


Figure 1

Gel electrophoretic separation of PCR products for genotypingHBB, rs1609812 A/G (A), rs10768683 G/C (B) and rs334 T/A (C) polymorphisms.