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Original Article

Association of *BCL11A* Gene Polymorphism in Human Cells of Thalassemia Patient by Evaluation of Amplification Refractory Mutation System (ARMS)

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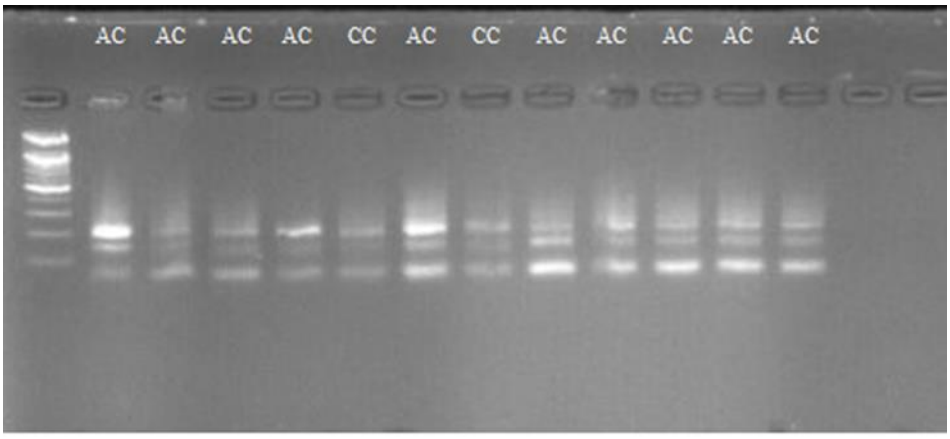
ABSTRACT

Thalassemia is an autosomal recessive disorder. It occurs due to mutations that lead to a decrease or absence of β-globin chains. In human erythroid cells, it was found that *BCL11A* acts as a crucial factor for the silence of the γ-globin gene and can decrease fetal hemoglobin (HbF) while promoting adult hemoglobin (HbA). This study was designed to evaluate the relationship between the *BCL11A* gene polymorphism and its effect on patients with β-thalassemia major and, secondly. The whole blood DNA was extracted and an amplified gene was used for the ARMS-PCR technique. The results revealed the presence of two alleles A and C as well as three genotypes AA, AC, and CC in β-thalassemia. ARMS-PCR was examined by frequencies 0.3, 0.6, and 0.1, respectively, as well as in control 0.1, 0.8, and 0.1, respectively. The frequency of the two alleles A and C was investigated in patients A (56), C (44), as for the control A (52) and C (48). The nucleotide sequencing was done for this gene. The *BCL11A* gene polymorphism rs766432A > C was found in all patients at the (A65664C) site, according to the sequencing results.

GRAPHICAL ABSTRACT



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Introduction

Thalassemia is a genetic blood disorder in which the body produces an abnormal form of hemoglobin [1]. A series of congenital anemias known as thalassemia is caused by a defect in the synthesis of one or more globin subunits from normal human hemoglobin [2]. Anemia is caused by low levels of hemoglobin, which is the primary intracellular protein for red blood cells (RBCs) and the blood loss or the fast death of blood cells [3]. *BCL11A* (B-cell lymphoma/leukemia 11A) position on chromosome (2p16.1) encodes its transcription factor [4].

The RNA interference has also been demonstrated to enhance the HbF production by chemically drugs targeting *BCL11A* [5]. Recently, the target genes can be safely and accurately edited thanks to the advantages of CRISPR-Cas9 technology [6]. These findings provide patients with β-hemoglobinopathies with a therapeutic approach by using the autologous stem cell editing and transplantation. All of the aforementioned data point to *BCL11A* as a potential treatment gene for beta-hemoglobinopathies [7]. The Amplification Refractory Mutation System (ARMS) and a variety of polymerase chain reaction (PCR) methods are commonly used to identify genetic polymorphisms in the β-globin gene. Among these the PCR methods, are the amplification refractory mutation system and the amplification refractory mutation system (ARMS) [8]. The ARMS analysis is based on primer-specific polymerase amplification with a set of primers complementary to the most common polymorphism in a population under study [9]. The ARMS analysis, also known as the allele-specific PCR method, employs two PCR reactions, one containing primers specific for the wild allele and the other containing primers specific for the mutant allele. A band from the normal reaction corresponds to the wild allele, bands from the mutant reaction correspond to the mutant allele, and bands from both reactions correspond to the heterozygous allele when gel electrophoresis is performed [10, 11]. This study was designed to evaluate the relationship between the *BCL11A* gene polymorphism and its effect on the clinical features of patients with β-thalassemia major.

Materials and Methods

Study sites

The study was conducted in the Genetic Engineering Laboratory, Department of Biology, College of Science, Misan University.

Blood samples collection

Samples were collected from the Center of Hereditary Hematology of the Maysan Health Directorate in Maysan Province, southern Iraq, in November 2021. The study group consisted of 140 samples, 100 samples were patients with thalassemia and 40 samples were a control group with no family history of β-thalassemia major. All of them were frequently visited by the Center for Genetic Blood Diseases in Maysan. These patients had a mean age of 15.3 years old. The blood was collected from the median cubital vein and placed in an EDTA tube that was marked and stored at -20 °C.

Genomic DNA isolation and column purification

The gSYNC TM DNA Extraction Kit from Geneaid Company (Taiwan) was used to extract DNA from the whole blood according to the protocol procedure attached to the kit and included in the appendix. The DNA was extracted from the samples and the presence of the DNA genome was confirmed by electrophoresis on 1% agarose, and then the amount of DNA was measured by the nanodrop device. The absorbance ratio of A260/280 was obtained in the range of 1.70 to 1.96.

Sample preparation for BCL11A tetra-primer ARMS

The *BCL11A* tetra-primer ARMS was carried out by using Tag 2X master mix red (Ampliqon/Denmark) and two pairs of *BCL11A* primers (*BCL11A*_A_Fwd primer, *BCL11A*_A_Rev primer, and *BCL11A*/mutation_C_Fwd primer, *BCL11A*/mutation_C_Rev primer) were mixed in 25 µl PCR reaction. The sequence of *BCL11A* primer is presented in Table 1 and *BCL11A*

reaction mix preparation is summarized in Table 2.

BCL11A tetra-primer ARMS genotyping

In this tetra-primer, ARMS inner primers, which were allele-specific, were utilized to create a DNA fragment containing the *BCL11A* polymorphism's special allele. While the outer primers are employed to create the outer control band A-allele for homozygous, two DNA bands are synthesized; 135 bp long for the inner A-allele band and 193 bp for the outer control band. In C -allele for homozygous, two DNA bands are synthesized; 116 bp long for the inner C-allele band and 193 bp long for the outer control band. Three DNA bands are produced for heterozygous alleles and they are synthesized; the outer control band is 193 bp, the inner A-allele band 135 bp, and the inner C allele band 116 bp long. The tetra-primer ARMS amplicons were visualized on a 2.5% agarose gel [12].

Table 1: The sequence of *BCL11A* primer and length

Gene	Sequences		Size (bp)	References
BCL11A-A (rs766432)	F	5′-TTGTTTCGCTTTAGCTTTATTAAGGTACAA- 3′	135	12
	R	5′-GACGTGTTCTGTATCTTGATTTTGGT- 3′		
BCL11A-C (rs766432) (mutation)	F	5′-CCAAACAGTTTAAAGGTTACAGACAGACT- 3′	116	12
	R	5′-AAAATGAATGACTTTTGTTGTATGTAGAG- 3′		

Table 2: The volumes of the used reagents for *BCL11A* reaction mix preparation

Mixture	Volume (µl)
Sterile distilled water	9.5
PCR master mix	12.5
BCL11A _A_Fwd primer	0.5
BCL11A _A_Rev primer	0.5
BCL11A /mutation_C_Fwd primer	0.5
BCL11A /mutation_C_Rev primer	0.5
Genomic DNA template	1
Final volume	25

Thermal cycling conditions for BCL11A tetra-primer ARMS

The PCR mixture for a 25 µl reaction volume is listed in Table 3.

Statistical analysis

Data statistical analysis was carried out by (SPSS version 26). Chi-square was used to display important statistics and significant differences with $P < 0.05$ probability levels [13]. The expected genotype of Hardy-Weinberg equilibrium was examined manually, and then by Michael H.Court's (2005-2008) online calculator, and also the difference from HWE was accomplished by using SPSS version 26 and Michael H. Court's (2005-2008) online calculator (2005-2008). MedCalc statistical software (version 20.0111) was used to calculate the odds ratios (ORs) and the confidence intervals (CIs) for genotypes and alleles (https://www.Medcalc.net) [14].

Results and Discussion

The results showed the presence of two alleles A and C and three genotypes AA, AC, and CC in β-thalassemia major, as depicted in Figures 1 and 2.

Table 3: *BCL11A* and *BCL11A* -M thermal cycle conditions (ARMS)

PCR steps	Temperature (°C)	Time	Cycles
Initial denaturation	95	2 Min	1
Denaturation	95	30 Sec	30
Annealing	55	30 Sec	
Extension	72	45 Sec	
Final extension	72	10 Min	1
Final hold	4	10 Min	-

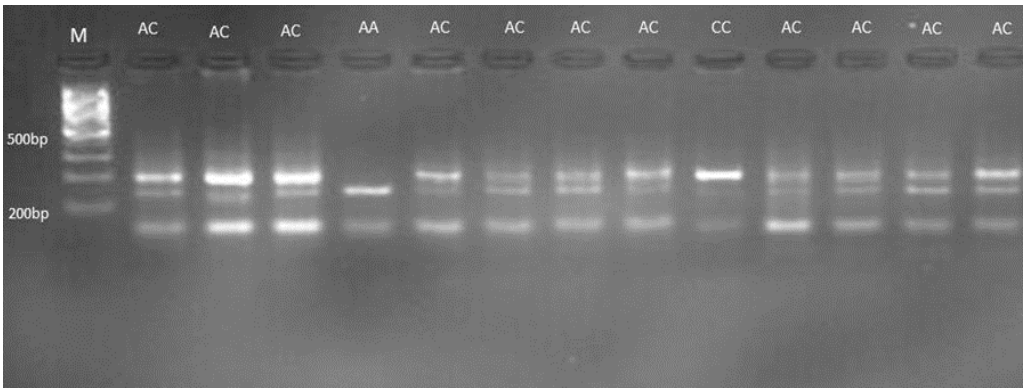

Figure 1: ARMS-PCR of *BCL11A* gene in control samples on 2% agarose gel electrophoresis, 75 volt (5 min) than 90 volt (5 min) and 120 volt (20 min)

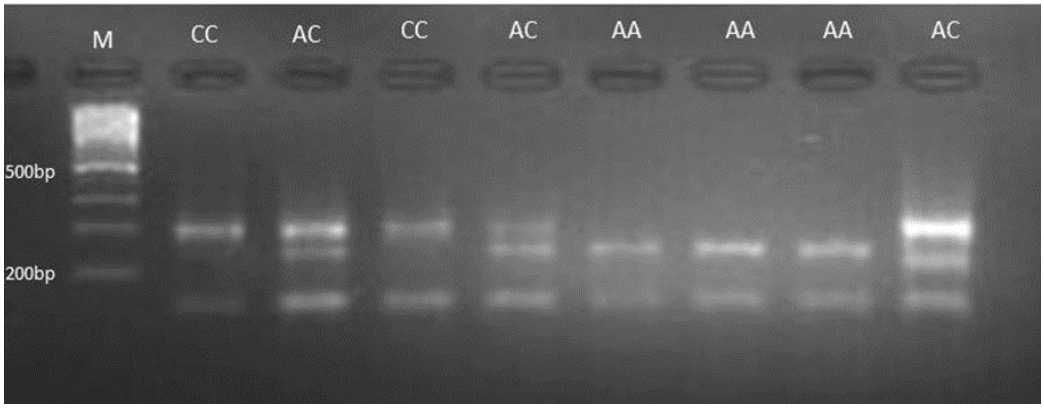

Figure 2: ARMS-PCR of *BCL11A* gene in β-thalassemia major patients on 2% agarose gel electrophoresis, 75 volt (5 min) than 90 volt (5 min) than 120 volt (20 min)

The *BCL11A* locus is a quantitative trait loci (QTL) that has a high persistence role for the HbF level [15]. Inducing Hb F in β-thalassemia is an extremely promising strategy for reducing disease severity [16]. This is because of their potential use in developing targeted therapeutic approaches for β-thalassemia, γ-globin, and Hb switching modifier genes [17]. Individuals who have severe hemoglobinopathies caused by β-globin chain disorders, such as β-thalassemia intermedia and major, typically, clinical phenotypes are inversely related to the degree of HbF expression retention [18]. The results of ARMS PCR analysis for the *BCL11A* gene showed that three genotypes, AA, AC, and CC were in β-thalassemia major patients' samples with frequencies of 0.3, 0.6, and 0.1, respectively, and in control 0.1, 0.8, and 0.1, respectively. The frequency of the two alleles A and C in patients with A (0.56) and C (0.44) as for the control A (0.52) and C (0.48), is illustrated in Table 4 and Figure 3.

Table 4: Genotype and allele frequencies in the *BCL11A* gene of β- thalassemia major patients and control

Gene	Genotype	Genotype frequency (patient)	Genotype frequency (control)	Alleles	Alleles frequency (patient)	Alleles frequency (control)
BCL11A	AA	0.3	0.1	A	0.56	0.52
	AC	0.6	0.8	C	0.44	0.48
	CC	0.1	0.1	-	-	-

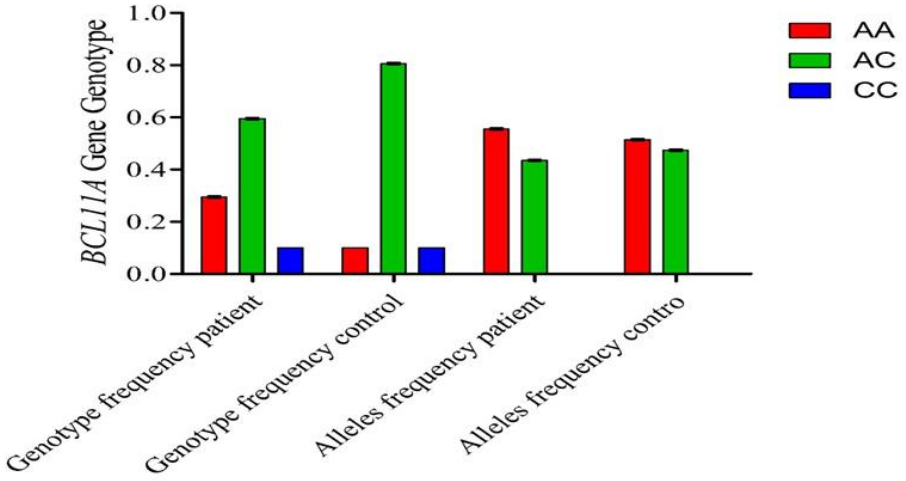

Figure 3: Genotype and allele frequencies in the *BCL11A* gene of β- thalassemia major patients and control

Only three loci with the common polymorphisms account for a significant portion of the variation in HbF levels. The β-globin gene cluster, an intergenic interval among the HBS1L and MYB genes (HMIP), and *BCL11A* are among these loci. The results of using ARMS-PCR showed that there are two alleles A and C with three genotypes AA, AC, and CC which are consistent with the previous studies [19, 20]. The results of the frequency distribution of the A and C alleles of the *BCL11A* gene showed that their distribution percentage was not equal among the β-thalassemia major patient and control samples, where the A allele percentage in patients was 56%, while in the control it was 52% and the percentage of the C allele was in the patient at 44 % and in the control, it was 48%, as summarized in Table 5.

According to our study, the results of the frequency distribution showed no significant differences P=0.634 at the probability level of P<0.05 between β-thalassemia major patients and control samples, as demonstrated in Table 6 and Figure 4.

The statistical analysis of the results by using the chi-square showed that there are no significant differences between the genotype distribution of β-thalassemia major patients and controls under the P<0.05 probability level, where it reached a p-value 0.220, as displayed in Table 7 and Figure 5.

Table 5: Genotype and alleles percentage of *BCL11A* gene polymorphism among patients and control group

Gene	Genotype	Patients %	Control %	Alleles	patients %	Control %
BCL11A	AA	10(25)	5(13)	A	45(56)	42(52)
	AC	25(62)	32(80)	C	35(44)	38(48)
	CC	5(13)	3(7)	-	-	-

Table 6: Allele frequency of *BCL11A* Gene among β-thalassemia major patients and control samples

Gene	Alleles	Patients (%)	Control (%)	Odd ratio	CI (95%)	P-value
BCL11A	A	45	42	1.1633	0.6241 to 2.1682	0.634
	C	35	38			
Significance: *P<0.05, **P<0.01, and ***P<0.005, NS=No significance (P>0.05).						
X^2: Chi-square and H.W.E Hardy-Weinberg equilibrium (if P<0.05, it is not consistent with H.W.E).						

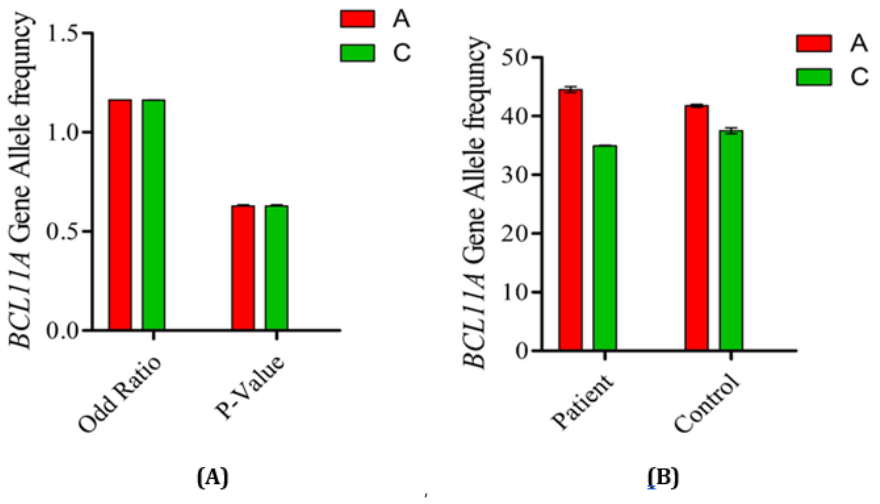

Figure 4: (A) Genotype and alleles percentage of *BCL11A* gene polymorphism among patients and control group (B) Odd ratio and P-value of *BCL11A* Gene among β-thalassemia major patients and control samples

Table 7: The statistical analysis of the genotype frequencies of the *BCL11A* gene among β-thalassemia major patients and control group

Gene	Genotype	Total	Patints	Control	X^2	P-value
BCL11A	AA	15	10	5	3.026	0.220
	AC	57	25	32		
	CC	8	5	3		
Significance: *P<0.05, **P<0.01, and ***P<0.005, NS=No significance (P>0.05).						
X^2: Chi-square and H.W.E Hardy-Weinberg equilibrium (if P<0.05, it is not consistent with H.W.E).						

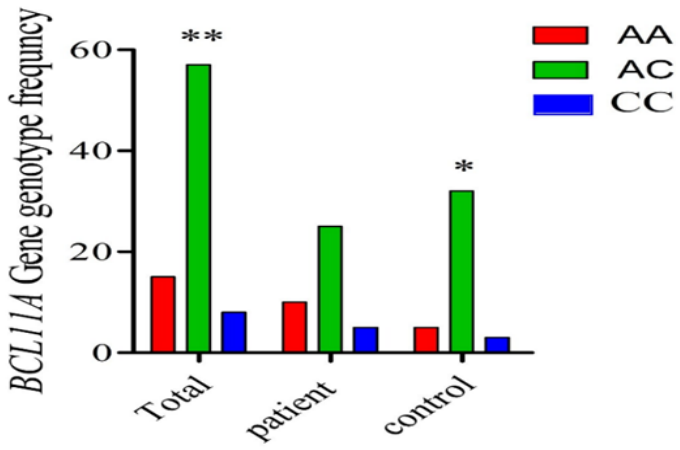

Figure 5: The numbers of the total AA, AC, and CC genotype frequencies of the *BCL11A* gene between β-thalassemia patients and control group by using the Chi-square test

By using the Hardy-Weinberg equilibrium law, the results of the *BCL11A* gene showed a statistically significant difference between the expected and the observed for the control group, with P=0.0001 value at its P<0.05 probability level. This means that control group is not subject to a Hardy-Weinberg distribution, as represented in Table 8 and Figure 6.

Table 8: The excepted and observed frequencies under Hardy-Weinberg equilibrium in the *BCL11A* gene of the control group

Gene	Genotype	Multi-population (observed)%	Multi-population (Expected)%	X^2 (H.W.E)	P-value
BCL11A	AA	5(12.5)	11	14.593	0.0001***
	AC	32(80)	20		
	CC	3(7.5)	9		
Significance: *P<0.05, **P<0.01, and ***P<0.005, NS=No significance (P>0.05).					
X^2: Chi-square and H.W.E Hardy-Weinberg equilibrium (if P<0.05, it is not consistent with H.W.E).					

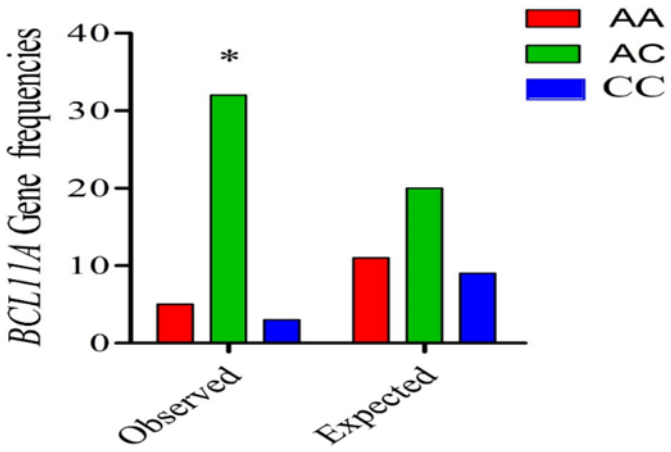

Figure 6: The excepted and observed frequencies under Hardy-Weinberg equilibrium in the *BCL11A* gene of the control group

The deviation from the Hardy-Weinberg equilibrium in the control sample is due to the frequency of consanguineous marriage in these societies, and this possibility is very likely in our society.[21] Another possibility for deviation from the Hardy-Weinberg equilibrium is the small size of the control sample, as the Hardy Weinberg equilibrium is very sensitive to the low frequencies of alleles in the homozygous (Chen, 2010). While in the patient group, the results showed an agreement with the Hardy-Weinberg equilibrium due to the absence of statistically significant differences between the frequency of the expected and observed alleles. Matching with Hardy-Weinberg equilibrium, it indicates that the alleles are inherited independently and no allele is dropped out during genotyping [23]. The results of genetic analysis of the ARMS-PCR technique for the *BCL11A* gene in β-thalassemia major show three genotypes, which are AA, AC, and CC. The AA genotype appeared in a percentage in β-thalassemia major patients of 25% and 12.5% of control samples, and there was no significant difference between the control sample and the patient. The P-value was 0.159 at the probability level P<0.05 and the odd ratio value was 2.333 and the confidence interval was between 0.717 and 7.586. The AC genotype in 80% of control sample was compared with the 62% of thalassemia major patient samples and there was no significant difference where the p-value was 0.08 at P<0.05 probability level, the odd ratio was 0.4167 and the confidence range was between 0.1525 and 1.138. The genotype CC appeared in 7.5% of β-thalassemia major patients and 12.5% of the control group. Therefore, there

was no significant difference between the two samples of the patient and the control, where its value was P = 0.46 at the probability level of P<0.05, the odd ratio was 1.7619, and the value of the confidence interval was between 0.391 and 7.929, as illustrated in Table 9 and Figure 7.

Under the dominant model in this study, the results did not reveal a statistically significant difference between the β-thalassemia major patients and the control samples where, P=0.5991 at P<0.05 as the probability level, as illustrated in Table 10.

Under the recessive model in this study, the results did not indicate a statistically significant difference between the β-thalassemia major patients and the control samples where, P=0.439 at P<0.05 as the probability level, as presented in Table 11.

Table 9: The genotype distribution and frequency of the *BCL11A* polymorphism in patients and controls

Gene	Alleles	Patints (%)	Control (%)	OR	CI (95%)	P-value
BCL11A	AA	10(25)	5(13)	2.333	0.717-7.586	0.159
	AC	25(62)	32(80)	0.4167	0.1525-1.138	0.08
	CC	5(13)	3(7)	1.7619	0.391-7.929	0.46
Significance: *P<0.05, **P<0.01, ***P<0.005, NS=No significance (P>0.05).						
X^2: Chi-square, H.W.E: Hardy-Weinberg equilibrium (if P<0.05, it is not consistent with H.W.E).						

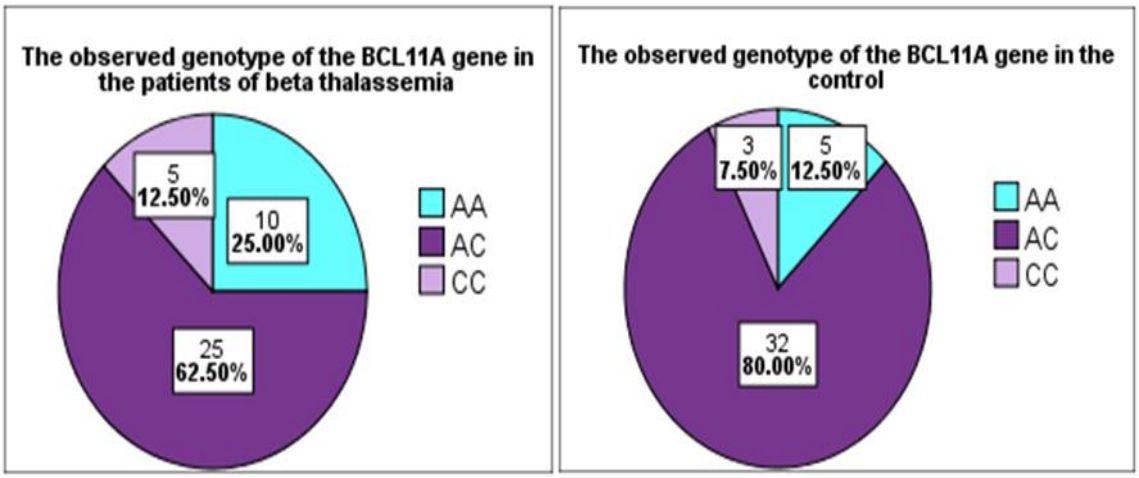

Figure 7: The observed genotype distribution and the frequencies of the *BCL11A* polymorphism in patients and controls

Table 10: Distribution of the *BCL11A* genotype in the patients and controls under *the* dominant model

Gene	Alleles	Patients %	Control %	OR	CI (95%)	P-value
BCL11A	AA	10	5	0.77	0.3047-1.985	0.5991
	AC +CC	30	35			
Significance: *P<0.05, **P<0.01, ***P<0.005, NS=No significance (P>0.05).						
X^2: Chi-square and H.W.E: Hardy-Weinberg equilibrium (if P<0.05, it is not consistent with H.W.E).						

Table 11: Distribution of the *BCL11A* genotype in the patient and control under the recessive model

Gene	Alleles	Patients %	Control %	Odd ratio	CI (95%)	P-value
BCL11A	AA+AC	35	37	0.567	0.1261-2.554	0.4605
	CC	5	3			
Significance: *P<0.05, **P<0.01, ***P<0.005, NS=No significance (P>0.05).						
X^2 : Chi-square and H.W.E: Hardy-Weinberg equilibrium (if P<0.05, it is not consistent with H.W.E).						

BCL11A (rs766432) A>C is partly associated with β-thalassemia major and its relative effect on the patients' phenotype as well as the presence of more than another SNP involved in the phenotypic events of the disease [20]. The previous studies reported that polymorphism of *BCL11A* (rs 766432) A>C β- thalassemia major patients was less effective in increasing the Hb F level than the other SNPs presented in *BCL11A* genes such as Xmn-1 and HBS1L-MYN (Figure 8) [12]. This study showed that the A allele percentage was higher in patients 56% than it was in controls 52%, but there were no significant differences at the level of P> 0.05. From the above-mentioned points, we note that the A allele distribution is identical to the its pattern observed in almost all the world's populations, including the population of white Americans, whites in Europe, and Asian populations (e.g., Japan, China, and India), where the A allele has a frequency range of 51-88% (Figure 9) [8]. The results of the nucleotide base sequence analysis indicate the alterations in the nitrogenous bases, more particularly in (63603, 63606, 63609-63610, 63610, 63610-63611, 63611, 63612, 63613, 63614, 63615, 63618-63619, 63621-63622, 63622-63623, 63666, 65611, and 65664) sites of the *BCL11A* gene as the base G changed to C (G63610C), (G65611C), and the base G changed to A (G63611A), as the base c changed to A (C63612 A), as the base A changed to C (A63615C), and as the base G changed to T (G65664T) (Table 12).

Table 12: Nucleotide changes and type of mutations, the resulting amino acid changes, and their impact on the translation process of *BCL11A* gene

Gene	Site of SNP / InDel polymorphism	Nucleotides (SNPs)	Amino acids	Types of mutation	Effect of mutation on translation	Accession no.	Triple code	Missense mutation %	Silent mautation %	Non-sense mutation %	Frameshift mutation %
BCL11A	63603	T	-	Deletion	Frameshift	GU324936.1	-	13	31	-	56
	63606	A	-	Deletion	Frameshift	GU324936.1	-				
	63609-63610	A	-	Insertion	Frameshift	GU324936.1	-				
	63610	G>C	G>R	Transversion	Missense	GU324936.1	GGC>CGC				
	63610-63611	C	-	Insertion	Frameshift	GU324936.1	-				
	63611	G>A	G>D	Transition	Missense	GU324936.1	GGC>GAC				
	63612	C>A	G>G	Transversion	Silent	GU324936.1	GGC>GGA				
	63613	A	-	Deletion	FrameShift	GU324936.1	-				
	63614	A	-	Deletion	FrameShift	GU324936.1	-				
	63615	A>C	K>N	Transversion	Missense	GU324936.1	AAA>AAC				
	63618-63619	C	-	Insertion	FrameShift	GU324936.1	-				
	63621-63622	A	-	Insertion	FrameShift	GU324936.1	-				
	63622-63623	A	-	Insertion	FrameShift	GU324936.1	-				
	63666	A>G	L>L	Transition	Silent	GU324936.1	TTA>TTG				
	65611	G>C	G>A	Transversion	Missense	NG-011968.1	GGC>GCC				
	65664	G>T	V>L	Transversion	Missense	NG-011968.1	GTA>TTA				

SNP: Single nucleotide polymorphism; InDel: Insertion /deletion polymorphism; C: Cytosine; T: Thymine; A: Adenine; G: Guanine; I: Isoleucine; S: Serine; A: Alanine; T: Threonine; D: Aspartic acid; G: Glysine; and L: leucine.

Figure 8: SNPs at the studied sites of the *BCL11A* gene

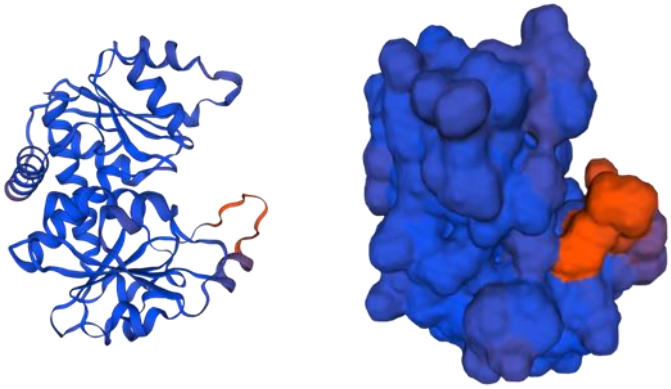

Figure 9: The 3D shapes of the *BCL11A* gene in β-thalassemia

Compared the alleles appearance and the genotypes frequency between control samples and patients, the results showed that the AC genotype revealed the clear differences, but without statistical significance with a value of p = 0.08, which was higher in the control sample by 80%, compared with 62% in the β- thalassemia major patient sample. Whereas, the homogeneous genotypes (AA and CC) were higher in patients, but not statistical, p>0.05 [24].

Conclusion

Our results proved the modification precision to the standard RAPD technique which produced bands in thalassemia patients more than in control. The primers at the RAPD level can be nominated to be a distinctive indicator of β-thalassemia, and thus these primers can be adopted to genetically distinguish β-thalassemia.

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Authors' contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

Conflict of Interest

We have no conflicts of interest to disclose.

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