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Original Article

The Study of *BCL11A* Gene in Patients with Beta-Thalassemia Major and Intermedia by Random Amplephed Polymorphism DNA in Iraq

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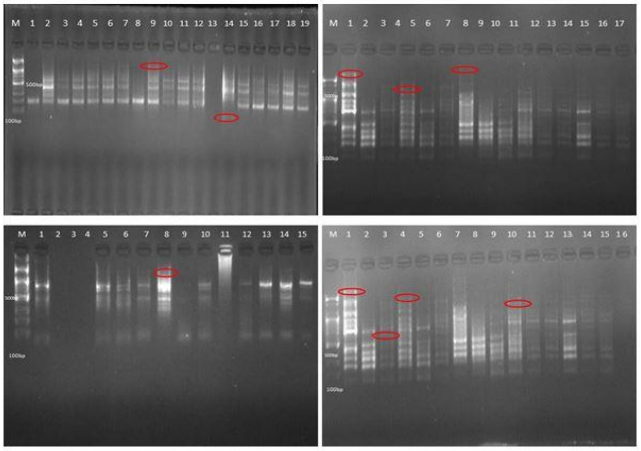
ABSTRACT

Thalassemia Autosomal recessive disorders occur due to mutations that lead to a decrease or absence of b-globin chains because mutations occur in the *BCL11A* gene being a potential therapeutic target to increase fetal hemoglobin levels in patients to reduce the severity of thalassemia symptoms. The study was designed to evaluate the relationship between the *BCL11A* gene polymorphism and its effect on the clinical features of patients with beta thalassemia major and intermedia. The whole blood DNA was extracted and an amplified gene was used as a template instead of the DNA genome in the RAPD technique PCR-RAPD-PCR, four primers (*APAA11, APU15, APAA17, and APD18*) to detect genetic polymorphism of *BCL11A* gene. The results showed that the number of bands in β-thalassemia major patients is more than the control group; there is also a difference in their molecular weights according to the type of the used primer. The results showed that there were statistically significant differences at the probability level of P<0.05 in the total number of bands generated when using each primer separately between the patients and the control group.

GRAPHICAL ABSTRACT



BCL11A control *BCL11A* patients

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Introduction

Thalassemia is a genetic blood disorder where the body produces an abnormal form of hemoglobin. It occurs when both parents are carriers of the disease or are affected by it so that they can pass it on to the next generation because thalassemia is the autosomal recessive [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]. It is transfusion-dependent anemia that ultimately resulted in iron overload, which is shown by an elevated serum ferritin level. This ongoing iron buildup causes the gradual organ damage and malfunction (liver, heart, and endocrine glands) [3]. The body's iron stores are influenced by the serum ferritin levels [4]. Due to a rise in iron load, thalassemia major patients typically pass away between the ages of 24 and 16 years. If iron chelation is not utilized to treat iron overload brought on by lifelong transfusion-dependent anemias such as beta-thalassemia intermedia, it typically leads to the deadly heart toxicity in the second decade of life [5]. Anemia is caused by low levels of hemoglobin, which is the primary intracellular protein for RBCs, as well as blood loss, or the fast death of blood cells [6]. There are genetic variants or variables affecting some clinical problems associated with the b-thalassemia phenotypes but they are not mapped to the b-globin cluster. These comprise variations at the hemoglobin fetal (Hb F) are related to the quantitative trait loci (QTL) like the HBS1L-MYB intergenic region, *BCL11A* gene [7, 8]. *BCL11A* (B-cell lymphoma/leukemia 11A) position on chromosome (2p16.1) encodes its transcription factor. At least isoforms of the *BCL11A* gene have been noted, which share identical exons one and two. *BCL11A* is primarily expressed in the brain and major hematopoietic cells such as the early T-cell progenitors, hematopoietic stem cells, B cells, and common lymphoid progenitors, but it is only weakly expressed in T-lymphocytes [9]. *BCL11A* performs its functions in brain/multiple cell lineages/fetal-to-adult hemoglobin switching; *BCL11A* indirectly or directly regulates the downstream targets' expression. Because of its critical function in fetal-to-adult hemoglobin switching in erythroid biology, *BCL11A* has recently gained increased research. In human erythroid cells, it was found that *BCL11A* acts as a crucial factor for the silence of the Gamma-globin gene and can decrease fetal hemoglobin (HbF) while promoting adult hemoglobin (HbA) [10]. According to several studies, *BCL11A* interacts with the repressor element-1, lysine-specific demethylase 1, and silencing transcription factor corepressor 1 to silence the γ-globin [11]. It has been discovered that KLF1 can influence the *BCL11A* expression in a way that indirectly regulates Gamma-globin, adding a greater support for the development of clinical therapeutic medicines leveraging this regulatory network [12]. RNA interference has also been demonstrated to enhance the HbF production by chemically drugs targeting *BCL11A* [13]. Recently, target genes can be safely and accurately edited thanks to the advantages of CRISPR-Cas9 technology [14]. These findings provide patients with beta-hemoglobinopathies with a therapeutic approach by using autologous stem cell editing and transplantation. All of the aforementioned data point to *BCL11A* as a potential treatment gene for beta-hemoglobinopathies [15].

In RAPD PCR, a single arbitrary primer is used in a PCR reaction, resulting in the amplification of many distinct DNA products. The standard RAPD technique employs short synthetic oligonucleotides (10 bases in length) of random sequences as arbitrary primers to amplify nanogram amounts of total DNA by polymerase chain reaction (PCR) at low annealing temperatures [16].

The study was designed to evaluate the relationship between the *BCL11A* gene polymorphism and its effect on the clinical features of patients with beta thalassemia major and, secondly, the *BCL11A* gene being a potential therapeutic target to increase fetal hemoglobin levels in patients to reduce the severity of thalassemia symptoms.

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

The 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.

Genomic DNA isolation and column purification

The gSYNC TM DNA Extraction Kit from Geneaid Company (Taiwan) was used to extract DNA from whole blood according to the protocol procedure attached to the kit and which is 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 measuring the DNA amount by the nanodrop device, which ranged from 1.80 to 1.98 ng/ml and the absorption ratio A 260/280 was within the obtained amount.

Polymerase chain reaction

PCR Mixture For a 25 µl reaction volume: (2 µl DNA template, 2 µl reverse primer, 2 µl forward primer, 12.5 µl Taq G2 ® green master mix, and 6.5 µl nuclease free water) were added to the PCR tubes. The components of PCR tubes were perfectly combined by spinning them down and up with a pipette, and then centrifuged for 10 seconds or more with a Mini spin Microcentrifuge. The PCR tubes were placed in the TECHEN thermocycler. The PCR reaction contained 35 cycles of the following steps: initial denaturation (5 minutes at 95 °C), DNA denaturation (30 sec at 95 °C), primer annealing *BCL11A* (45 sec at gradient 60-62 °C), primer extension (1 minute at 72 °C), and the final extension (10 min at 72 °C). The PCR product was electrophoresis in 2% agarose gel and 1X TBE buffer with ethidium bromide 3%, at 75 V for 40 min (Table 1) [17].

PCR-RAPD-PCR technique-random amplified polymorphic DNA (RAPD)

The volume of reagents used in RAPD (20 µl): 10 µl Nuclease free water, 2.5 µl DNA genome, 2.5 µl of each primer, 5 µl AccuPower® ProFi Taq PCR PreMix, and Master Mix (Bioneer). The mixture was inflated in a Techne prime thermocycler with the Tables 2 and 3 [18]. The RAPD-PCR product observations were carried out by Gel DOC UV Transilluminator.

Statistical analysis

Data statistical analysis was carried out by SPSS version 26. Chi-square was used to display important statistics and significant differences with a P<0.05 probability level [19].

Table 1: The sequence of *BCL11A* primer, length, and GC%

Gene	Sequences		Length (Base)	Size (bp)	References	GC%
BCL11A (rs766432)	F	5′-TTGTTTCGCTTTAGCTTTATTAAGGTACAA- 3′	30 bp	135 bp	17	33.93%
	R	5′-GACGTGTTCTGTATCTTGATTTTGGT- 3′	26 bp			

Table 2: PCR program of RAPD for all primers

Samples characteristic	NaCl 0,9%	Thiamine	Ascorbic acid	Combination
Age (year, mean ± SD)	48,8± 18,4	52,3± 16,8	53,3± 11,7	50,7±11,1
Gender Male, n (%) Female, n (%)	 10 (38,5) 15 (61,5)	 12 (50,0) 13 (50,0)	 11 (50,0) 14 (50,0)	 20 (68,8) 5 (31,3)
MAP, mmHg (mean ± SD)	94.8 ± 17.0	97,6 ± 25,4	91,4 ± 16.0	92,4 ± 16.0
Lactate, acid	2,0 (1-10,0)	1,3 (1,0-5,5)	1,0 (1-10,0)	2,0 (1,0-4,0)
NLR, median (min-max)	10,6 (2,5-30,3)	14,4 (0,2-86,8)	13,9 (1,6-69,3)	10,7 (1,4-64,6)

Table 3. The type of RAPD primers used in the experience

Primers	Length	GC %	Sequences
OPAA11	10 base	80	ACCCGACCTG
OPU15	10 base	70	ACGGGCCAGT
OPAA17	10 base	70	GAGCCCGACT
OPD18	10 base	60	GAGAGCCAAC

Results and Discussion

The results showed that the number of bands in β-thalassemia major patients is more than the control group. There is also a difference in their molecular weights according to the type of primer used. The RAPD technique are used because it is easy, fast, low-cost, and does not require large amounts of DN [20, 21] since traditional RAPD technology has some disadvantages such as randomness, low output, and poor reproducibility [22]. PCR-RAPD-PCR was used in a previous study [18] and this technique is based on polymorphism in the amplified gene by using PCR. It was sued to study genetic alteration and analyze genetic instability to understand molecular events in the blood of patients with beta thalassemia (Figure 1).

The results of the PCR-RAPD-PCR for the *BCL11A* gene showed that the total bands in β-thalassemia major patients were (303 bands), while in the control group there were (97 bands), as dsplayed in Figure 2. The reason for using the amplified gene instead of the DNA genome as a template is because the DNA genome contains a coding region for proteins (Exons) and a non-coding region (Introns), while the amplified genes in our study contain a coding region for proteins, thus may reduce this procedure or limit the randomness of the RAPD technique [18]. The OPAA17 primer showed the highest number of bands, where the total bands reached 86 (28.3%), while the OPU15 primer gave the highest total of 27 bands (27.8%) in the control group, the results showed when using the four primers that there were statistically significant differences at the significance level of $P<0.05$ between patients and the control group, as presented in Table 4.

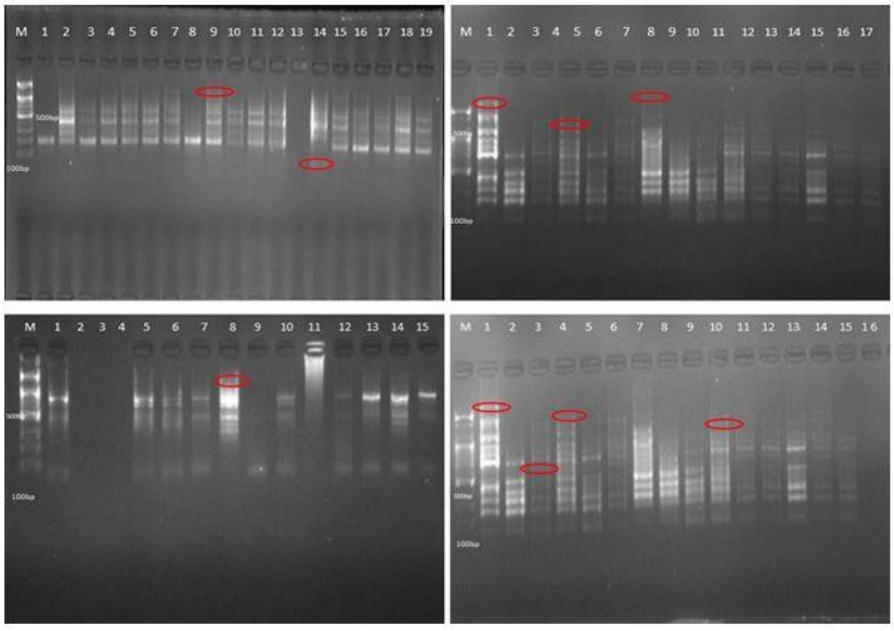

Figure 1: PCR- RAPD-PCR generated by (OPAA 11, OPU15, OPAA17, and OPD 18) four primers. The first lane from the right (M) is a DNA ladder marker 25-300bp in size, and the other lanes are PCR-PAPD bands on 2% agarose gel electrophoresis

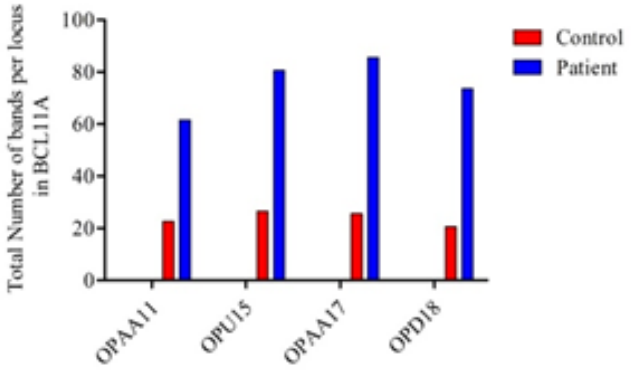

Figure 2: The statistical numbers, the percentages of the total monomorphic, and polymorphic and unique bands of the *BCL11A* gene between patients and the control group

Table 4: In patients and control groups, a total number of various markers in the *BLC11A* gene

Material	Patients *BCL11A*	Control *BCL11A*	Chi-Squares	P-Value
OPAA11	62	23	14.553	P < 0.0001
OPU15	81	27	21.400	P < 0.0001
OPAA17	86	26	25.186	P < 0.0001
OPD18	74	21	22.499	P < 0.0001

The results can be shown by PCR-RAPD-PCR for the size of the band's fragments. In patients, the largest was in the *BCL11A* gene (1900-2000) bp by using OPAA11, OPU15, and OPAA17 primers, and the smallest size was 30 bp by using the OPU15 primer, for the control, the largest size fragment was 2000 bp when using the OPU15 primer, while the smallest size fragment was 30 bp when using the same primer, as depicted in Figure 3.

The results for polymorphic bands in the *BCL11A* gene in patient samples showed the total 6 bands with a percentage polymorphism of 7.7%. The primer OPU15 gave the highest number of these bands was 4 with a percentage polymorphism of 4.9%, while the primer OPD18 did not give any polymorphic bands. In the control samples, the total number of polymorphic bands was 5 and the percentage polymorphism was (20.5%) (Figure 4). Likewise, the OPAA 11 primer gave the highest number of these bands was 3 with percentage polymorphism 13.04 %, while the OPD18 primer did not give any polymorphic bands, as listed in Table 5.

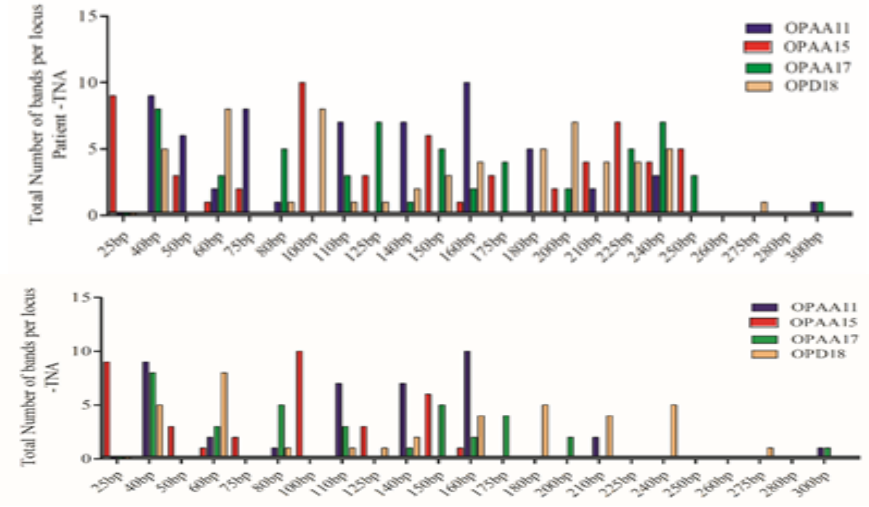

Figure 3: The distribution of the fragment sizes in the *BCL11A* gene β- thalassemia patients and control

Table 5: In patients and control group, the percentage of polymorphic bands of various markers of the *BLC11A* gene

Material	Patients *BCL11A*	Control *BCL11A*	Chi-Squares	P-Value
OPAA11	16.7	60	0.480	Not significant
OPU15	66.7	20	0.571	Not significant
OPAA17	16.6	20	0.002	Not significant
OPD18	0	0	-	-

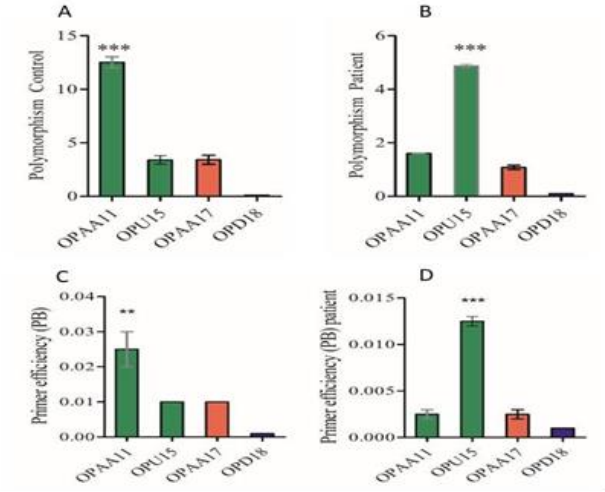

Figure 4: A, B) The comparison between polymorphism four primers of control and patient in the *BCL11A* gene of β- thalassemia major; (C, D) Primer efficiency in the *BCL11A* gene of β-thalassemia major patients and controls

The primer efficiency in the *BCL11A* gene in patient samples was 0.013, the primer discriminatory power was (66.66%), and it was 0.03 in the *BCL11A* gene in control samples. The primer efficiency value ranges from 0 to 1 and is identified as the primer's capacity to produce polymorphisms. The primer's efficiency is demonstrated by its ability to produce the highest proportion of polymorphic bands as compared with the sum number of amplified bands. The ability to demonstrate polymorphism between normal and patient individuals is an important feature of an efficient primer (Figure 5) [23].

The primer discriminatory power was (60%). There are noticeable differences in the discriminatory power of each primer between the control group and the patients in the BCL11A gene. The reason for this is due to the different number of polymorphic bands, because the calculation of the discriminatory power depends on the number of polymorphic bands for each primer divided by the total of polymorphic bands for all primers. Polymorphisms are generally caused by point mutations resulting from translocations, inversions, single nucleotide substitutions, DNA section duplication, rearrangements involving insertions or deletions, and mistakes in tandemly repeated DNA replication [24]. the lost and modification bonds intensely are due to one or more of the following events; changes due to point mutations, genome rearrangements, or DNA damage [25].

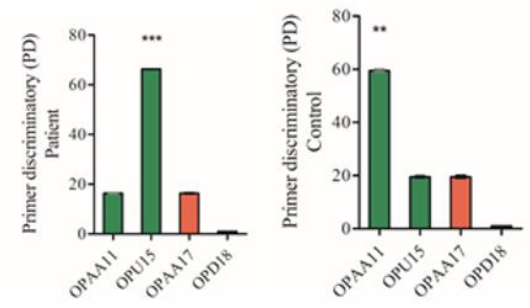

Figure 5: Primer discriminatory power in the *BCL11A* gene of β- thalassemia major patients and controls

The results for unique bands in the *BCL11A* gene in patients showed a total of band 10 with a percentage uniqueness of 13.4%. The OPD18 primer gave the highest number of these bands 4 with percentage uniqueness of 5.4%, while the primers OPAA 11, OPU15, and OPAA17 gave a number of these bands 2 with percentage uniqueness of 3.2%, 2.4%, and 2.3%, respectively. In the control, the total number of unique bands 13, and the percentage uniqueness was 55.2%, so OPAA 11 and OPD18 primers gave the highest number of these bands 4, with percentage uniqueness of 17.3% and 19.04%, respectively. The OPAA17 primer provides two bands with percentage uniqueness of 7.6%, as presented in Table 6. The variation in the total of bands between beta-thalassemia patients and control group could be due to the nucleotides count of these primers, genotype of beta thalassemia patients, and the number of identical sites of primers in beta thalassemia genes changed by mutations and translocations. This will influence on the template interaction sites of primers and leads to an increase or loss of bands. The difference in the number of bands and the change in their intensity in the RAPD-PCR technique is related to alterations in genetic material (Figure 6) [26].

Table 6: In patients and control group, the percentage of unique bands of various markers of the *BLC11A* gene

Material	Patients *BCL11A*	Control *BCL11A*	Chi-Squares	P-Value
OPAA11	20%	30.7%	0.064	Not significant
OPU15	20%	23.1%	0.005	Not significant
OPAA17	20%	15.3%	0.011	Not significant
OPD18	40%	30.7%	0.066	Not significant

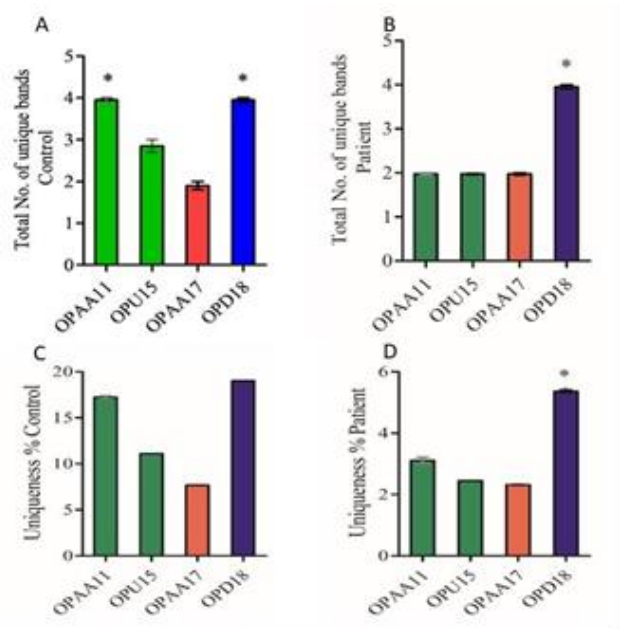

Figure 6: A, B) A total number of unique bands controls and patients of four primers in the *BCL11A* gene. C, D) Percentage uniqueness of each primer in *BCL11A* gene controls and patients

The results for monomorphic bands in the *BCL11A* gene in patients showed the total of these bands 17, with a percentage monomorphism of 22.6%. The primer OPD18 gave the highest number of these bands 7 and percentage monomorphism of 9.4%, while the primers OPAA 11 and OPU15 gave the lowest number of these bands 3 and percentage monomorphism 4.8% and 3.7%, respectively. In the control group, the total number of monomorphic bands was 13, and the percentage of monomorphism was 54.4%. Therefore, OPAA11 and OPAA17 primers gave the highest number of these bands 4, with the percentage of monomorphism 17.3% and 15.3%,

respectively, while the OPU15 primer gave only two bands with percentage monomorphism 7.4%, as indicated in Table 7 and Figure 7.

Table 7: In patients and control group, the percentage of monomorphic bands of various markers of the BLC11A gene

Material	Patients *BCL11A*	Control *BCL11A*	Chi-Squares	P-Value
OPAA11	17.65	30.8	0.135	Not significant
OPU15	17.65	15.3	0.004	Not significant
OPAA17	23.5	30.8	0.047	Not significant
OPD18	41.2	23.1	0.270	Not significant

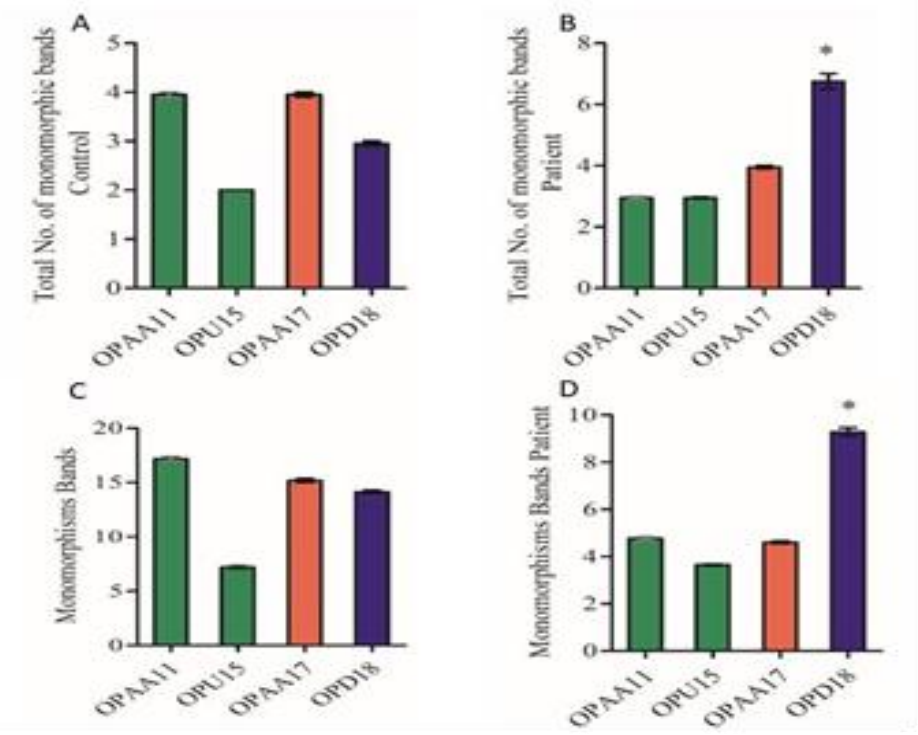

Figure 7: A, B) Total number of monomorphism bands of controls and patients of four primers in *BCL11A* gene. C, D) Comparison between monomorphism bands of control and patient in the *BCL11A* gene of β- thalassemia major

Genetically, it shows a contrasting pool of fragments in its genotypes due to the point mutations at oligonucleotide sites. Also, when there is a distance between the termini sequences that changes due to the deletion or insertion mutation events leads to the genetic polymorphism [27].

Although there is a clear difference in the total band preparation between patients and the control group for the *BCL11A* gene, the statistical analysis showed that there were no statistical differences at the probability level of P<0.05 by using chi-square where the P-value was (0.621), as reported in Table 8. There are noticeable differences in the molecular size of fragments generated from primers between patients and control groups in BCL11A. Polymorphism also includes variations in molecular weights of the amplified bands caused by various types of mutations and translocations, causing movement shifts of bands and possibly the addition of new bands (Table 8) [28].

RAPD-PCR was discovered to be a powerful molecular genetic technique for detecting genetic variability and similarity in different populations and genetic polymorphism (Figure 8) [29, 30].

Table 8: Statistical analysis for monomorphic, polymorphic, and unique bands of the *BCL11A* gene between patients and control group

Gene	Types of band	Overall total band	Total No. of band (patient)	Total No. of band (control)	X^2	P-value
BCL11A	Polymorphic band	11	6	5	0.954	0.621
	Unique band	23	10	13		
	Monomorphic band	30	17	13		
Total		64	33	31		
Significance *P <0.05,**P <0.01,***P <0.005, NS=No significance P >0.05						

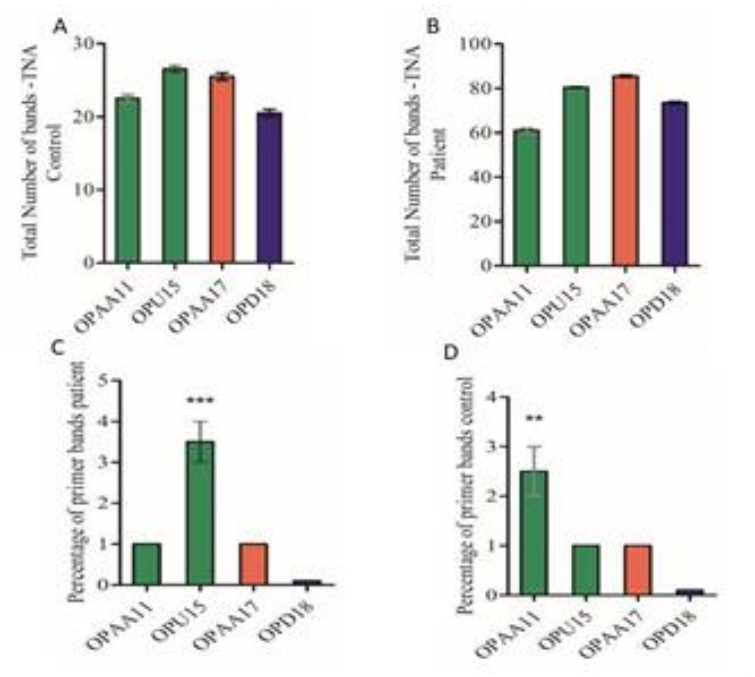

Figure 8: A, B) Statistical numbers and percentages of the total monomorphic, polymorphic, and unique bands of the *BCL11A* gene between patients and the control group; C, D) A total number of polymorphic bands in the *BCL11A* gene between patients and control group

Conclusion

The results of this study 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 distinguish β-thalassemia genetically.

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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.

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