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Original Article

Prevalence of *RDB*, *RSa*, *Hinc* and *Xmn* polymorphisms and HBBS11D haplotypes in patients with thalassemia minor

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ABSTRACT

Beta-thalassemia is one of the most common genetic diseases with autosomal recessive inherited patterns in the world and is one of the most common diseases in Iran that exists in all age and sex groups. Determining gene mutations in this disease can be effective in controlling and treating the disease. The present study determined the frequency of polymorphisms of *Hinc*, *RSaI*, *RDB*, and *Xmn* in patients with beta-thalassemia minor in Ardabil province. Fifty three beta-thalassemia patients referred to the genetic department of Imam Khomeini Hospital were studied. Blood samples were taken to determine the type of gene mutation. PCR samples were genetically evaluated to determine genetic mutations using RDB-Sequence-RFLP-Haplotype methods. A total of 53 samples were examined, of which 56.6% were male and the rest were female. The most positive cases in the first and second ranks were related to *XmnI* and *AvaII* enzymes with 73.5% and 60.3%, respectively. The most common mutation extracted in the studied samples with 14 cases (26.4%) was IVS2.1. Among the most common mutations extracted by the RDB method was related to IVS 1.2 with 26.4%. The results of the present study showed that the distribution of genetic mutations in the studied samples can be different from other places. Also, by performing targeted genetic counseling, it is possible to control and prevent the disease in the future.

1. Introduction

Thalassemia is a genetic disease in which hemoglobin loses its natural structure. Thus, the phenomenon of ineffective hemoglobin production occurs in the body, as a result of which defective hemoglobin is not able to provide proper oxygen to the organs of the body. So there is no general hemoglobin deficiency, but abnormally high hemoglobin has risen [1]. Hemoglobin is an oxygen transporter in red blood cells. Hemoglobin contains two different proteins called alpha and beta[2].

If the body is not able to produce enough protein, blood cells will not be fully formed. They will not be able to carry enough oxygen, resulting in a type of anemia that begins in childhood and lasts until the end of life. Although thalassemia is not a single disorder, a group of disorders affects the human body similarly. It is essential to understand the differences between different types of thalassemia [3]. In thalassemia, depending on whether the production of alpha chain or beta chain is reduced, they are divided into two general categories, thalassemia alpha, and beta-thalassemia, respectively.

Each of this Thalassemia is divided into subtypes based on the outbreak's severity.

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Under normal conditions, the alpha and beta chains production ratio is equal, but in thalassemia, this ratio is disturbed depending on the type of chain involved. In beta-thalassemia, extra alpha chains, and thalassemia alpha, many beta chains are deposited as ineffective chains, accompanied by extravascular hemolysis of red blood cells[4, 5]. In severe cases of thalassemia, hemolysis occurs by imposing increased hematopoiesis in the bone marrow and, consequently, bone deformation in the patient [6]. Also, extra-medullary hematopoiesis in these patients causes enlargement of the spleen and liver and enlargement of the lymph nodes [7]. In severe cases, thalassemia is a blood transfusion-dependent disease in which people die without receiving blood due to severe anemia until age 5 [4]. However, repeated blood intake causes iron to accumulate in various tissues, including the liver and heart, resulting in functional failure and eventual death. However, with the help of iron chelators, the effects of iron deposition in various tissues of patients with thalassemia major can be partially reduced [8].

Because HbA production and complete chain expression occur after birth, beta-thalassemia occurs 4-6 months after birth. Still, due to the presence and expression of alpha chains in fetal hemoglobin, alpha thalassemia manifests itself during the fetal period [7]. Iran, like other countries in the neighborhood, has many thalassemia significant patients. More than two million carriers live in Iran, and the frequency of beta-thalassemia varies considerably from region to region, with the highest frequency (10%) in the Caspian Sea and the Persian Gulf cities. The prevalence of the disease in other regions is between 4-8%. In southern Iran, Fars province, the frequency of genes is high, about 8-10%. Due to the high proportion of consanguineous marriages and their consequences in the gene pool, the rate of severe forms of the disease has increased [9]. The proportion of consanguineous marriages in Iran is 38%, of which 29% are between cousins, and (firstborns). So, even screening and detecting rare mutations are important in our population [9]. Also, based on the results of several articles, it has been reported that thalassemia is common in Iran along the Persian Gulf and the Caspian Sea side cities, Kohgiluyeh and Boyer-Ahmad, Fars, Kerman, Isfahan and Sistan, and Baluchestan.

The number of patients with this disease in these provinces is higher than the global and national rates. Haplotyping, using markers linked to the beta-globin gene, adds extra precision to any early diagnosis and is essential for any pre-pregnancy genetic diagnosis for beta-thalassemia. Diagnosis is made by two direct (determination of mutation) and indirect (study of gene continuity including RFLP, STR, and VNTR) methods for various thalassemia cases. This study aimed to determine the frequency of Hinc, RSa, RDB, and Xmn polymorphisms and D11HBBS haplotypes, which have more sensitivity in diagnosing the beta-thalassemia, in patients with beta-thalassemia minor in Ardabil province.

2. Materials and Methods

2.1. Study Place and sampling

For this study, the necessary coordination was made with the genetics department of Ardabil city hospital and the Homa Genetic Laboratory in Ardabil. The study population consisted of 53 beta-thalassemia patients selected based on the type of beta gene mutation in the test sheet. Then, 10 mL of whole blood was poured into a tube containing 1 mL of EDTA with a concentration of 0.5M, and after recording the information on it, it was kept at -20 ° C until the test time.

2.2. DNA extraction from blood

DNA was extracted using a Sinaclon-Iran column extraction kit in the following steps:

-The Lysis solution was left at 37 ° C for 10 min and gently shaken.

-700 µL of whole blood was mixed with 400 µL of Lysis solution and vortexed for 15 to 20 s. Then 500 µL of Precipitation solution was added to it, and after 5 s the vortex was centrifuged at 12000 g for 10 min.

-Return the microtube to a paper towel, gently empty the solution, and then return.

-Add 1 mL of washing buffer to the plate, and after vortexing for 5 min, centrifuge at

12000 g and empty. This step was repeated twice.

-Empty the wash buffer entirely and leave it at 65 ° C for 5 min to dry.

-Add 50 µL of Solvent buffer to completely dissolve the precipitate. Shaken and then placed at 65 ° C for 5 min.

2.3. Evaluation of purity and concentration of extracted genomic DNA

To evaluate the quality of the extracted DNA, 5 µL of the extracted DNA and the gene size indicator (100 bp) were taken on a 1% agarose gel (w/v) and simultaneously electrophoresed at 120 V for 5 min. For staining, instead of using ethidium bromide dye and preventing its toxic and harmful effects, Sina Clone DNAsafe dye was used (1 µL per 5 µL of the sample). Then, the gel was placed in a UV-Doc device to observe the bands and photographed.

2.4. Preparation of TBE buffer and agarose gel

In this study, we need x TBE5 buffer for making the gel and electrophoresis. For this purpose, firstly we used three traces, boric acid and EDTA powders. We produced 5X buffer and then diluted it in a ratio of 1: 4.

2.5. Determination of amplified fragment size in PCR

A size indicator (Ladder) is required to detect the band size obtained by PCR and ensure the desired part's amplification.

These markers are commercially available and show a different range of bands, which would be chosen according to the size of the desired part. This marker is loaded next to PCR products in one of the wells.

2.6. Quantitative evaluation of extracted DNA

The concentration of the DNA-containing solution will be obtained in an amount of light absorbed at 260 nm by using a spectrophotometer. By using this instrument we calculate the concentration of DNA in the solution and then measured will the protein contamination of the solution containing the DNA by reading the optical absorption of the solution at a wavelength of 280 nm and obtaining an absorption ratio of 260 nm to 280 nm. If this ratio is less than 1.8, the amount of contamination with proteins or aromatic substances (such as phenol) in the sample is higher and values greater than 2 indicate the possibility of contamination of the DNA-containing solution with RNA.

2.7. Selection of primers

After purifying the samples and obtaining genomic DNA, it is necessary to amplify the part of this genomic DNA that contains the desired fragment. For this purpose, the PCR technique was used. The first step in performing PCR is the design of the primer. The primer used in this research and ordered to Sina Clone Company for synthesis.

Table 1. The used primers sequences and characteristics for doing PCR

Gene Name	Primer sequences	PCR (bp)	Temperature
Hind II/ε	Forward:TCTCTGTTTGATGACAATTC Reverse:AGTCATTGGTCAAGCGCTGACC	760	55
Xmn/Gγ	Forward:AACTGTTGCTTTATAGGATTT Reverse:AAGGAGCATATTGATAACCTCAGC	657	55
Hind III/Gγ	Forward:AGTGCTGCAAGAAGAACAACTACC Reverse:CTCTGCATCATGGGCAGTGAGCTC	326	65
Hind III/Aγ	Forward:ATGCTGCTAATAGCTTCATTAC Reverse:TCATTGTGTGATCACTCAAGCAG	635	65
Hind II/5ωβ	Forward:TCCTATCCATTACTGTGTCCGCTAA Reverse:ATTGTCTTATTCTAGAGACGATTT	795	55
Ava II/ωβ	Sequence as for Hind 5' RFLP	795	55
Hind II/3ωβ	Forward:GTACCTCATACTTTAAGTCCTAACT Reverse:TAAGCAAAGATTATTTCTGGCTCTCT	913	55
RxI/β	Forward:AGACATAATTTATTAGCATTGCTG Reverse:CCCCTTCCTAATGACATGAACTTAA	1200	55
Ava II/β	Forward:GTGGTCTACCTTGGACCAGAGG Reverse:TTCGTCTGCTTCCCAATCTAACT	328	65
HinfI/β	Forward:GGAGGTTAAAGTTTTGCTATGCTGTAT Reverse:GGGCCTATGATAGGGTAAT	474	55

2.8. Performing polymerase chain reaction

The PCR program was given to the thermocycler according to the table 1. After PCR, enzyme digestion will determine the presence or absence of enzyme breakdown sites. Enzymatic digestion of the PCR product with each of the enzymes will be performed by adding 5 units of the enzyme (0.5 µL of enzyme) and 1µL of buffer to a 5 µL vial of DNA sample (PCR product), and the volume will be reached to10 microliters with 2 µL of double-distilled water.

The vial containing the enzymatic digestion reaction mixture with the enzymes associated with each marker will be placed overnight at 37 ° C to complete the enzymatic digestion.

Then the enzymatic digestion products will be examined by agarose electrophoresis.

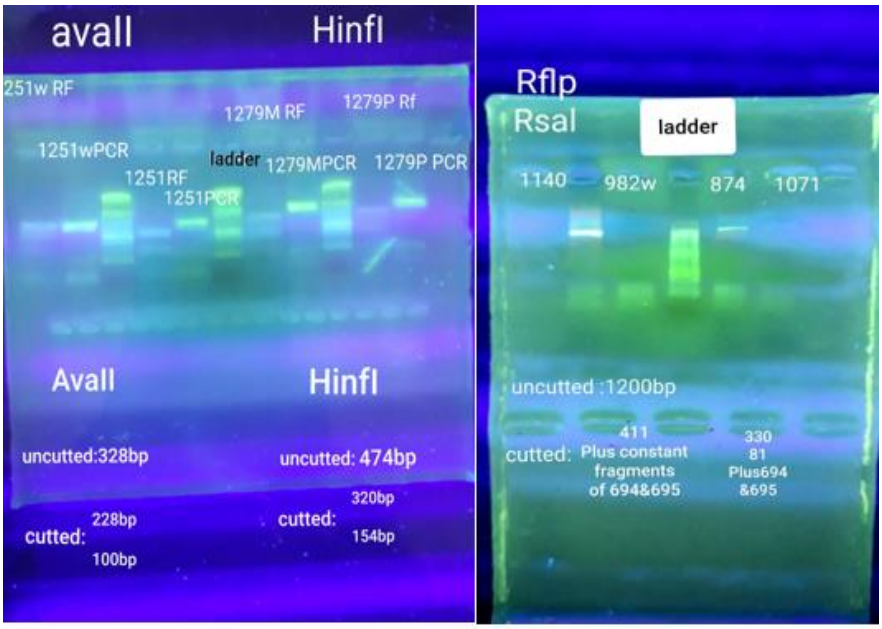

Fig. 2. The picture of the PCR gel

2.9. Evaluation of beta-thalassemia based on haplotype method

In this method, the disease is examined in the family, and patients related to the disease are tested using the Hap Screen Kit. The uses for this method are a haplotype kit called KBC Hap Screen HBB Kit 50ng, made in Iran by Kosar Biotechnology Company, and the analysis of these patients was based on their STRs.

2.10. Sequence Method

To evaluate the sequences, protein sequencing, transcription, and gene amplification were performed by PCR method with primers defined according to the PCR program, and sequences were checked.

2.11. Reverse Dot Blot Method

The Vienna lab kit made in Austria was used to perform this method.

2.12. Steps taken to prepare PCR

First put all vials include an HsTag DNA Polymerase vial with red lid, Tag Dilution Buffer vial with a black lid, Amplification Mix A1 vial with a yellow lid, Amplification Mix A2 with white lid, Amplification Mix B vial with a green lid, and the DNA extraction solution on ice under the Workstation. Poured 1ML of Hs Tag into a microtube and added 15ML of Dilution Tag.

Vertex shortly to mix them. Next, took 3 microtubes, wrote patient code A1 on the first microtube, wrote patient code A on the second one, and patient code B on the third microtube. Then pour 5ML of the mixture (Hs Tag Tag Dilution +) we have prepared into each of the microtubes. Then add 5ML of Mix A1 into microtubule A1, 5 ml of Mix B into microtube B1, 5ML of MixA2 into microtubule A2. Then pour 5ML of the DNA extraction product into the A1, A2, and B microtubes. We vertex and spin the microtubes shortly and enter the program into the thermocycler with PCR program with specific values. After PCR, the PCR product is stored at 2-8 ° C. Electrophoresis gel was used to analyze the PCR product.

2.13. RDB Steps

Set the temperature of the Bain Marie machine to 45 ° C and place the Hybridization Buffer and Wash SolutionA inside of it to reach 45 ° C. take TeststripA and TeststripB and write the patient's code on them and put each one in a Tray well.

Inside **TrayA:** Pour 20ML DNAT + 10ML PCR product A1 + 10ML PCR product A2 and pipette several times to mix (do not touch Teststrip).

Inside **TrayB:** Pour 10ML DNA + 10ML PCR product B and pipette several times to mix and then let it stay at room temperature for 5 min.

1ML of the Hybridization Buffer, which is already been heated to 45 C, is poured into each of the trays, and the top mixture is dissolved and the strips are placed in the solution. Place the tray in the Bain Marie machine at 45 ° C for 30 min and regularly shake at 50 Rpm. After 30 min, empty the solution with a sampler. Pour the previously heated 45°C ML1 Wash Solution A into each of the Trays and empty it after 10 s.

Pour 1ML Wash Solution A again and put it at 45°C for 15 min, shake it regularly and empty it, and pour 1ML Wash Solution A again.

3. Results

Among thalassemia patients referred to Ardabil city Hospital, 53 patients were eligible to enroll in the study and had a diagnosis of beta-thalassemia. 23 (43.4%) were female and 30 (56.6%) were male.

In the studied samples, the most positive cases in the first to third ranks were related to *Xmn I*, *Ava II*, and *Hind II-1* with 73.5% and 60.3%, and 52.8 % respectively (Figure 1). Among the cut cases, the most cases with 94.1% and 90% were related to *HindIII2* and *HinfI* enzymes, respectively. Among the uncut cases, the most cases with 91% and 59% were related to *RsaI* and *XmnI* enzymes (Table 2). The most common mutations extracted in the studied samples with 14 cases (26.4%) and 5 cases (9.3%) were related to Ivs2/1[G>A]/ Wt and Ivs2/745[C>G]/Wt; respectively. Among women, the most common mutation was related to Ivs2 / 1 [G> A] / Wt with 7 cases (30.4%) and Ivs2 / 745 [C> G] / Wt with 3 cases (13%) and among men the most common mutation was related to Ivs2 / {G> A] / wt with 7 cases (23.3%) (Table 3).Among the common mutations extracted by the Reverse Dot Blot method, the highest mutation with 14 cases (26.4%) was related to Ivs2/1[G> A]/wt (Figure 2).

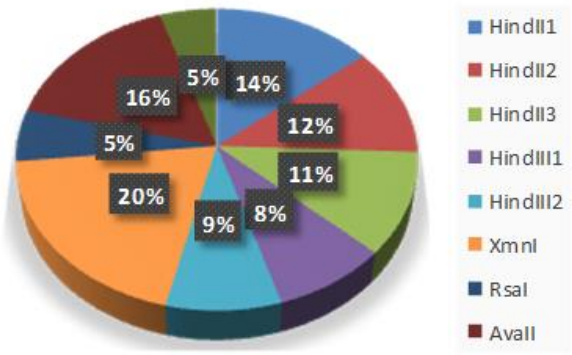

Fig.1. Frequency of positive PCR cases from blood samples of people with beta-thalassemia.

Table 2. Frequency distribution of positive cuts in RFLP enzyme

Name	Cutted	Un-Cutted
	n (%)	n (%)
HindII1	19(67.9)	9(32.1)
HindII2	14(60.9)	9(39.1)
HindII3	14(63.6)	8(36.4)
HindIII1	9(53)	8(47)
HindIII2	16(94.1)	1(5.9)
XmnI	16(41)	23(59)
RsaI	1(9)	10(91)
AvaII	18(56.3)	14(43.7)
HinfI	9(90)	1(10)

Table 3. Frequency distribution of gene mutations extracted in the studied samples

Name of mutation	Men	Women
	n (%)	n (%)
-30[T>A]/WT	1(3.3)	0
Codon126[GTG>GGG]/WT	1(3.3)	1(4.3)
Codon15[TGG>TGA]/WT	0	1(4.3)
Codon16[-C]/WT	1(3.3)	0
Codon35[TAC>TAA]/WT	0	1(4.3)
Codon36/37[T]/WT	1(3.3)	0
Codon39[C>T]/WT	1(3.3)	0
Codon5[-CT]/WT	0	2(8.6)
Codon8/9[+G]/WT	1(3.3)	0
Ivs1-25[25BP del]/WT	1(3.3)	0
Ivs1/1/WT	0	1(4.3)
Ivs1/130[G>C]/wt	2(6.6)	0
Ivs1/2[C>T]/wt	1(3.3)	1(4.3)
Ivs1/2[t>C]/wt	1(3.3)	1(4.3)
Ivs1/3[C>T]/wt	1(3.3)	1(4.3)
Ivs1/5[G>C]/wt	0	1(4.3)
Ivs1/6[T>C]/wt	3(10)	0
Ivs2/1[G>A]/wt	7(23.3)	7(30.4)
Ivs2/745[C>G]/wt	2(6.6)	3(13)
Ivs2[G>A]/wt	1(3.3)	1(4.3)
Codon8[-AA]/wt	1(3.3)	2(8.6)
Ivs1/110[G>A]/wt	4(13.3)	0
Total	30(100)	23(100)

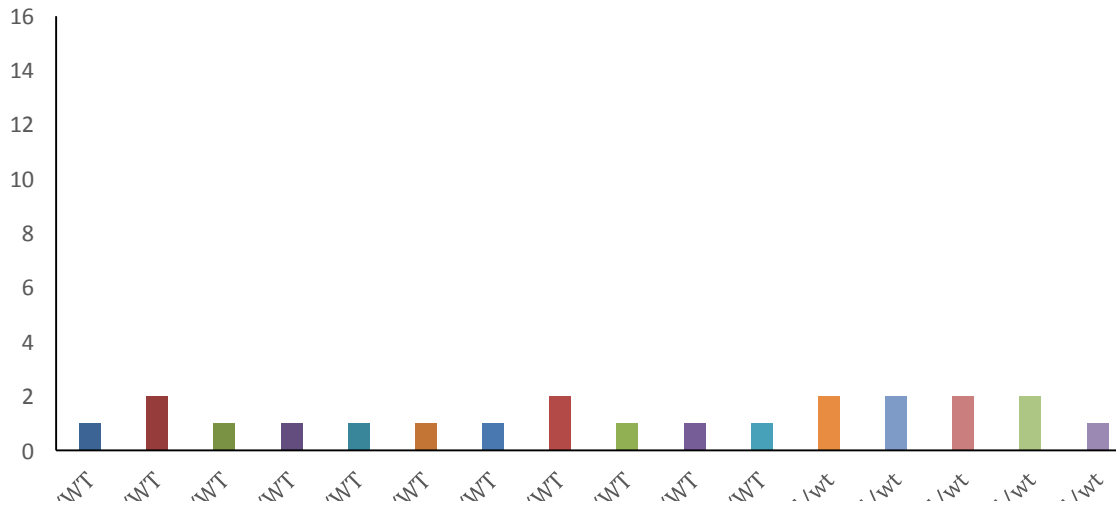

Fig. 2. Frequency distribution of gene mutations extracted by Reverse Dot Blot

4. Discussion

A haplotype is a combination of genes of the same order or alleles at different locations on a chromosome that are transferred together. A haplotype may contain one location, multiple locations, or a complete chromosome. The number of Loci recombination will determine the haplotype's structure and length [10]. In other words, a haplotype is a set of single nucleotide polymorphisms (snippets) on a chromosome of chromosome pairs that are statistically related.

It seems that by using these correlations and identifying a small number of alleles of a haplotype block, it is possible to unambiguously identify all polymorphic locations in that particular region. Such information is valuable in research into the genetic factors of common diseases and has been researched in the HapMap International Project for Humans. Restriction Fragment Length Polymorphism or RFLP is a type of mononucleotide polymorphism that restriction enzymes or Res can detect. These enzymes detect specific DNA sequences and make cuts in that sequence or surrounding areas. Changing any nucleotide in this sequence can destroy the enzyme recognition site. Therefore, DNA cleavage indicates the presence of the first allele, and non-cleavage indicates the presence of the second allele. RFLP is also the name of the technique used to cut DNA by a restriction enzyme. Beta-thalassemia is one of the most common diseases characterized by features such as microcytosis and hypochromia or paleness. This disease is very challenging in countries with limited treatment and support resources [11, 12]. Genetic diagnosis of patients can be very effective in managing the recurrence of the disease in families through prenatal diagnosis or pre-implantation diagnosis.

Numerous factors that can help diagnose the disease quickly and accurately should be considered in multiple studies [13]. Gender has been discussed in some studies as an influential and determining factor in this field. Various studies have examined the prevalence and complications of the disease based on gender. In a study, The prevalence and severity of thalassemia symptoms were different between females and males. In this study, it was mentioned that gender differences in thalassemia affect not only the prevalence of symptoms but also the severity of symptoms so that males experience more severe symptoms than females and show a higher frequency of the disease [14].

It was related [15] that other clinical complications of thalassemia have been pointed out, and in a conclusion, females are resistant to some symptoms of beta-thalassemia. Also, among the 53 patients in the present study, 23(43.4%) were female, and 30 (56.6%) were male.

It was related [16] that in Palestine, it was reported that between 200 female and 200 male patients participating in the study, the

symptoms presented by the number and structure of blood cells in men and women were significantly different. As a result of the research, the researchers suggested that the production of indicators and formulas related to gender can be an essential approach to reducing the variance of the results. In other words, in this study, it was found that the management of disease may be different in males and females, and it is necessary to determine the difference in the percentage of involvement between men and women in demographic studies and consider it in therapeutic guidelines.

It was studied [17] 195 patients with beta-thalassemia in Iraq and reported that men were more likely to develop beta-thalassemia than women. This result is consistent with the findings of the present study. In a study, In Mazandaran province, in 2018, the demographic characteristics of beta-thalassemia patients were examined. Among 1725 beta-thalassemia patients, 51.5% were female and 48.5% were male. This finding was not consistent with the results of the present study [18].

The beta-globin gene cluster is located on human chromosome 11 and contains 5 genes. Different methods have detected beta-thalassemia mutations located in this region. Among the methods for identifying these mutations, indirect methods can be pointed out that in these methods, haplotypes of RFLP polymorphic regions are evaluated using restriction enzymes and mutations buried in the heart of these haplotypes are tracked and identified. Of the sites of restriction fragment length polymorphism, or RFLP polymorphism, examined in the present study, the highest frequency was related to the three polymorphism sites of *XmnI* with 73.5%, *AvaII* with 3.3 60%, and Hind II1 with 52.8% [19, 20]. These polymorphisms were observed in 39, 32, and 28 patients. Detection of this frequency was done by PCR and sequencing. Also, in the RFLP process, the frequency of cleaved and non-cleaved alleles in *HindII1* polymorphism was 67.9%, 32.1.1%, in *AvaII*, 56.3%, 43.7%, in *XmnI*41, 33%, and in *HindIII2*, 94.1% and 5.9%, respectively. Each cleaved or non-cleaved allele can form a distinct haplotype in which there is a linkage imbalance, and they are passed on to continuous generations with a specific mutation. Continuity imbalance means that consecutive genes on chromosomes do not separate during meiosis due to the lack of a recombination site in the distances between these genes. Suppose a mutation is located in this region. It that case, it can be inherited for many generations in the presence of these haplotype markers, and therefore the presence of mutations in transporters can be indirectly detected. So, by identifying the haplotype with a specific mutation in a person, the existence of the mutation can be confirmed. Also, in a study conducted by Teh *et al*. [21], 219 patients with beta-thalassemia major were analyzed to determine the associated haplotype. Five HindIII and HincII loci were identified as polymorphism markers and 4 haplotypes associated with thalassemia were identified.

It was related [22] that In Korea, haplotypes associated with thalassemia were explored using RFLP polymorphisms. The prevalence of this disease is very low in Korea. In this study, evaluations were performed on 3 families by PCR, RFLR, and continuity studies.

It was related [23] that in Turkey, beta-thalassemia mutations included IVSI-110 (G> A), FSC8 / 9 (+ G), IVSIL-1 (G> A), IVSI-5 (G> C), IVSI-1 (G> A), IVSI-6 (T> C) and FSC8 (-AA) were evaluated. The patients included 22 unrelated patients with beta-thalassemia major and 72 healthy individuals. RFLP polymorphisms were investigated by PCR and RFLP. *HincII* polymorphisms in 5 'beta gene, *HindIII* in 5' G gamma gene, *HindIII* in IVS-II gamma gene, *HincII* in 3 'fake beta gene, *AvaII* in the beta gene, and *HinfI* in 3' beta gene were evaluated. The study's results revealed common haplotypes associated with beta-thalassemia with a frequency of 28.6% to 8.3%[23]. It was related [24] that two important mutations in the French population related to beta-thalassemia were examined. Also, 7 polymorphism sites in the beta-globin gene cluster were evaluated in 43 unrelated patients. The results showed that the predominant mutations in this population were meaningless types and there were no common mutations in the eastern and Middle

Eastern regions in this population. In this study, haplotypes with a strong association with the found mutations and haplotypes with a weak association with these mutations were identified [24]. The prevalence of beta-thalassemia is very high in Iran. Therefore, several studies have been performed to identify mutations and haplotypes associated with this disease. The distribution of many haplotypes, like mutations, is ethnic and regional.

It was related [25] that 150 patients with beta-thalassemia and 50 healthy individuals with RFLP polymorphisms were studied. Also, three abundant mutations named c.315G> A, c.93-21G> A, and c.92 + 5G> C were examined. The polymorphism markers used in this study included HindIII for the first mutation, which identified both the G and An alleles (*HindIIIG* and *HindIIIA*). Other markers were *AvaII* and BamHI. The results showed that 50% of beta-thalassemia patients were associated with these mutations. The most common haplotype of this study was observed in 39.33% of patients and 46% of normal individuals [25]. It was identified [26] that 20 different mutations in the beta-globin gene associated with beta-thalassemia disease. They analyzed the locus patterns of *HindIII*, *HinfI*, *HincII*, *RsaI*, and *AvaII* polymorphisms in 257 alleles of the Informative family. 17 different haplotypes were identified. One of these haplotypes is strongly associated with the most common beta-thalassemia mutation found in this study. Another haplotype was identified in continuity imbalance with the second common mutation [26].

5. Conclusion

The results of the present research study demonstrated that the distribution of genetic mutations in the samples of the present study can be different from other places. To better identify these mutations based on existing techniques, future studies can be performed on samples with a larger number, detailed experiments, and targeted genetic counseling to control and prevent the disease in the future. Identification of mutations causing beta-thalassemia in different populations significantly impacts the type of disease management and birth control of other involved children. Due to the presence of haplotypes concerning beta-thalassemia mutations, the founder's influence can be seen in many societies. Thus, haplotypes in a particular population often have a traceable pattern. Ancestor mutations may be buried in these haplotypes. Therefore, studying haplotypes and discovering their relationship with specific mutations can help in identify mutations indirectly. So that if there is a patient with beta-thalassemia in a family and the examination of haplotypes in the new fetus of the family shows similar haplotypes of the sick person; it should be considered that it is possible that the fetus would be involved even without direct detection of mutations. And finally, further evaluations with higher techniques must confirm the existence of the familial mutation.

It is recommended that the project be replicated in a more significant number of patients to further ensure that mutations are in continuity imbalance with specific haplotypes, that more mutations be added to the study, and that more RFLP markers be added to the study for further study.

Conflict of interest

None of the authors have any conflict of interest to declare

Consent for publications

All authors approved the final manuscript for publication.

Availability of data and material

The authors have to declare that they embedded all data in the manuscript.

Authors' contributions

SSI,SH and FA help in study design, doing, FA and MV help in manuscript draft writing, study design, SSI , MV and FA help in data collection, FA help in data analysis and manuscript writing, MV and SH help in sampling and data collection.

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Ethical consideration

This study design was approved by the research committee of the Islamic Azad University, Ahar Branch, Tabriz, Iran and registered by code IR.REC.IAU.AHAR.1399.9310 in the research unit.

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