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Identification of β-globin gene mutations among transfusion-dependent β-thalassemia patients

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Abstract:

BACKGROUND: β-thalassemias are widely distributed in Mediterranean and Middle Eastern countries, including Iraq. There are more than 400 transfusion-dependent β-thalassemia patients registered in the thalassemia center. β-thalassemia is a significant problem in Karbala as well as other regions of Iraq. The detection of the most frequent mutations is significant to the implementation of an effective preventive program in this area because of the significant burden it places on the local health authorities, patients, and their families.

OBJECTIVES: To define the most common mutations and their frequencies among patients with transfusion-dependent β-thalassemia and to evaluate the reverse hybridization strip assay method for the detection of β-thalassemia mutations.

PATIENTS, MATERIALS AND METHODS: Sixty transfusion-dependent β-thalassemia patients were recruited from the thalassemia center in Karbala. Blood samples were aspirated from each patient just before blood transfusions for CBC, reticulocyte count, DNA extraction, PCR amplification, and identification of the mutations by reverse hybridization technique using the β-Globin strip assay method.

RESULTS: A total of 60 patients with 120 chromosomes were studied, searching for the most common mutations causing β-thalassemia. Among the twelve identified mutations, the six most frequent mutations represented 79.16% of all β-globin defects. These mutations were IVSII-1 (30.83%), IVSI-110 (15.83%), Codon 5 (10.83%), Codon 44 (8.33%), IVSI-1 (6.67%), and IVSI-5 (6.67%). The detection rate of the method used in our population was 96.66%.

CONCLUSION: The most frequent mutations encountered were IVSII.1 and IVSI-110, while IVS 2.745 was the least common mutant allele. Reverse hybridization strip assay molecular techniques used in the current study provide an extremely quick, precise, and simple to carry out molecular diagnostic technique for the detection of β-thalassemia mutations.

Keywords:

Transfusion-dependent β-thalassemia, β-globin gene mutation, polymerase chain reaction

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Introduction

β-thalassemia develops from an imbalanced globin chain synthesis caused by the absence of or insufficient β-globin chain formation, resulting in an excess of α-globin chains. The precipitation of excess α-globin chains damages the red cell precursor membrane, resulting in apoptosis and severe intramedullary destruction of erythroid precursor cells in the bone marrow (ineffective erythropoiesis).[1]

β-thalassemia is mainly caused by point mutations or, more rarely, by deletions in the β-globin gene on chromosome 11.[2] All over the world, more than 350 β-thalassemia mutations have been reported in the IthaGenes database.[3] The most severe form of β-thalassemia is caused by the mutations

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in the β-globin gene that result in the absence of β-globin chains (β^0). Other mutations result in β^+ thalassemia by allowing the formation of β-globin chains in various proportions.[4]

With more than 400 patients registered in the thalassemia center in the province, β-thalassemia is a significant problem in Karbala as well as other regions of Iraq. In this study, we aim to explore the most frequent mutations and their frequencies among patients with transfusion-dependent β-thalassemia. It is imperative that an effective preventive program be implemented in this area because of the significant burden it places on the local health authorities, patients, and their families. Therefore it was essential to define the most common mutations and their frequencies among patients with transfusion-dependent β-thalassemia and to evaluate the reverse hybridization strip assay method for the detection of β-thalassemia mutations in this locality.

Patients, Materials and Methods

Patients

A cross-sectional study was conducted on 60 transfusion-dependent B-thalassemia patients of both sexes, all ages, and from all the geographical areas in Karbala governorate who were attending the thalassemia center for receiving blood, treatment, and follow-up. The present study was conducted over a period extending from June 2021 to March 2022. The diagnosis of the patients was based on history, clinical examination, blood counting parameters, blood film, and hemoglobin (Hb) electrophoresis. The high performance liquid chromatography (HPLC) results were recorded at the time of the first presentation. The patients' complete personal, family, and medical histories have been obtained from the patients and their file records with their informed consent. The study was approved by an Ethical Committee at the College of Medicine/ Al-Nahrain University, Baghdad, Iraq.

Methods

For each patient, 3 mL of Ethylene diamine tetra acetic acid (EDTA) blood samples was aspirated under strict aseptic techniques for complete blood count, reticulocyte count, DNA extraction, polymerase chain reaction (PCR) amplification, and identification of mutations by the reverse hybridization technique. Patient samples were collected just before blood transfusions.

DNA was extracted from peripheral blood leukocytes, and it was stored in a deep freezer (−70°C) until the time of hybridization. Extracted DNA was used for amplification by PCR.

All PCR reagents and DNA templates were kept refrigerated throughout, and all steps were performed until the start of the thermal cycling program on ice (0°C–4°C).

The amplified products were then hybridized to a test strip that contained oligonucleotide probes of both the wild type and mutant types, designed to identify 22 β-thalassemia mutations found in Mediterranean countries. The color of the bound biotinylated sequences was used to identify the affected alleles. The strip assay covers 22 β-globin mutations: −101 (C>T), −87 (C>G), −30 (T>A), codon 5 (−CT), codon 6 (G>A) HbC, codon 6 (A>T) HbS, codon 6 (−A), codon 8 (−AA), codon 8/9 (+G), codon 15 (G>A), codon 27 (G>T) Knossos, IVSI-1 (G>A), IVSI-5 (G>C), IVSI-6 (T>C), IVSI-110 (G>A), IVSI-116 (T>G), IVSI-130 (G>C), codon 39 (C>T), codon 44 (−C), IVSII-1 (G>A), IVSII-745 (C>G), and IVSII-848 (C>A). The DNA was extracted, amplified, hybridized, and detected according to the commercial kits and industry-standard procedures (β-Globin StripAssay MED™).

Statistical analyses

The statistical analysis of this study was performed using the SPSS software version 21 (IBM Corp., Armonk, NY) and Microsoft Excel 2013. Numerical data were described as mean and standard deviation. An analysis of variance was used for the comparisons among more than two groups, whereas categorical data are described as counts and percentages. The Chi-square test was used to describe the association between the variables. The lower level of accepted statistical significance is equal to or below 0.05.

Results

Among the 120 β-thalassemia chromosomes investigated, 12 different mutations were detected using the strip assay method. The mutations were IVSII-1 (G>A), IVSI-110 (G>A), codon 5 (−CT), codon 44 (−C), IVSI-1 (G>A), IVSI-5 (G>C), IVSI-6 (T>C), codon 39 (C>T), codon 8 (−AA), codon 8/9 (+G), IVS IVSI-116 (T>G), and IVSII-745 (C>G).

The majority of β-thalassemia mutations identified were of Mediterranean origin, with a few mutations of Kurdish and Asian Indian origin. The Mediterranean mutations were IVSII-1 (G>A), IVSI-110 (G>A), codon 5 (−CT), IVSI-1 (G>A), IVSI-6 (T>C), IVS 1-1 (G-A), codon 39 (C>T), codon 8 (−AA), IVS IVSI-116 (T>G), and IVSII-745 (C>G), and these mutations constitute 78.33% of all types of mutations in the studied group. The mutations IVSI-5 (G>C) and codon 8/9 (+G), an Asian Indian mutation, constitute 10% of all mutations, whereas the Kurdish mutation codon 44 (−C) constitutes only 8.33% of all mutations, as shown in Table 1.[5]

Table 1: Characteristics of the commonly observed β-thalassemia mutations in Karbala province, with the pathogenesis of each mutation

Mutation	Type	Origin	Description	Sequence alteration
IVSII-1	β^0	Mediterranean	RNA processing: Splice junction	[G>A]
IVSI-110	β^+	Mediterranean	RNA processing: Cryptic splice site	[G>A]
Codon 5	β^0	Mediterranean	RNA translation: Frameshift mutation	[−CT]
Codon 44	β^0	Kurdish	RNA translation: Frameshift mutation	[−C]
IVSI-1	β^0	Mediterranean	RNA processing: Splice junction	[G>A]
IVSI-5	β^0	Asian Indian, SE Asian	RNA processing: Consensus splice sites	[G>C]
IVSI-6	β^+	Mediterranean	RNA processing: Consensus sequence	[T>C]
Codon 39	β^0	Mediterranean	RNA translation: nonsense stop codon	[C>T]
Codon 8	β^0	Mediterranean	RNA translation: Frameshift mutation	[−AA]
Codon 8/9	β^0	Asian Indian, Japanese	RNA translation: Frameshift mutation	[+G]
IVSI-116	β^0	Mediterranean	RNA processing: Cryptic splice sites	[T>G]
IVSII-745	β^+	Mediterranean	RNA processing: Cryptic splice sites	[C>G]

Table 2: Frequency and distribution of different β-thalassemia mutations in 120 alleles from 60 transfusion-dependent β-thalassemia patients

Mutation	Total number of alleles	Number of homozygous	Number of heterozygous	Overall frequency (%)
IVSII-1 (G>A)	37	13	11	30.83
IVSI-110 (G>A)	19	3	13	15.83
Codon 5 (−CT)	13	4	5	10.83
Codon 44 (−C)	10	3	4	8.33
IVSI-1 (G>A)	8	2	4	6.67
IVSI-5 (G>C)	8	2	4	6.67
IVSI-6 (T>C)	5	0	5	4.17
Codon 39 (C>T)	5	2	1	4.17
Codon 8 (−AA)	4	1	2	3.33
Codon 8/9 (+G)	4	1	2	3.33
IVSI-116 (T>G)	2	0	2	1.67
IVSII-745 (C>G)	1	0	1	0.83
Uncharacterized	4	1	2	3.33
Total	120	32	56	100

The six most frequent mutations represented 79.16% of all β-globin defects. These mutations in the order of frequency were IVSII-1 (G>A) (37 alleles: 30.83%), IVSI-110 (G>A) (19 alleles: 15.83%), codon 5 (−CT) (13 alleles: 10.83%), codon 44 (−C) (10 alleles: 8.33%), IVSI-1 (G>A) (8 alleles: 6.67%), and IVSI-5 (G>C) (8 alleles: 6.67%). Another six mutations were sporadic or less frequent, including IVSI-6 (T>C), codon 39 (C>T), codon 8 (−AA), codon 8/9 (+G), IVSI-116 (T>G), and IVSII-745 (C>G) [Table 2].

The molecular method used in the current study had an overall detection rate of 96.66% because, out of a total of 120 examined alleles, we identified 116 alleles, while only 4 alleles out of 120 (3.33%) remained uncharacterized.

Among the 60 patients, there were 31 homozygous patients, 26 patients with compound heterozygous states, 2 patients identified only one mutation, and another mutation was undetermined, whereas 1 patient detected no mutation. The most frequent mutation in homozygous patients was IVSII-1, followed by codon 5, while the most frequent compound heterozygous mutation was IVSII-1/IVSI-110, codon 5/IVSI-110, IVSI-5/IVSI-110, and IVSI-6/IVSII-1 (three patients for each genotype) [Table 3]. Figure 1 shows some results of ViennaLab strips for the detection of β-thalassemia mutations.

Discussion

The 12 characterized mutations in the studied group revealed that the majority of β-thalassemia mutations in Karbala were of Mediterranean origin, with a few mutations of Kurdish and Asian Indian origin. The high frequency of Mediterranean mutations and the low frequency of Asian Indian mutations are similar to the findings obtained in Iraq[6-10] and surrounding countries, especially Syria, Jordan, and Saudi Arabia.[11-13]

The different β-thalassemia mutations observed in Karbala and the frequencies of these mutations are compared with the frequencies of the same mutations in other Iraqi and neighboring countries' studies, and we have made the following observations [Table 4].

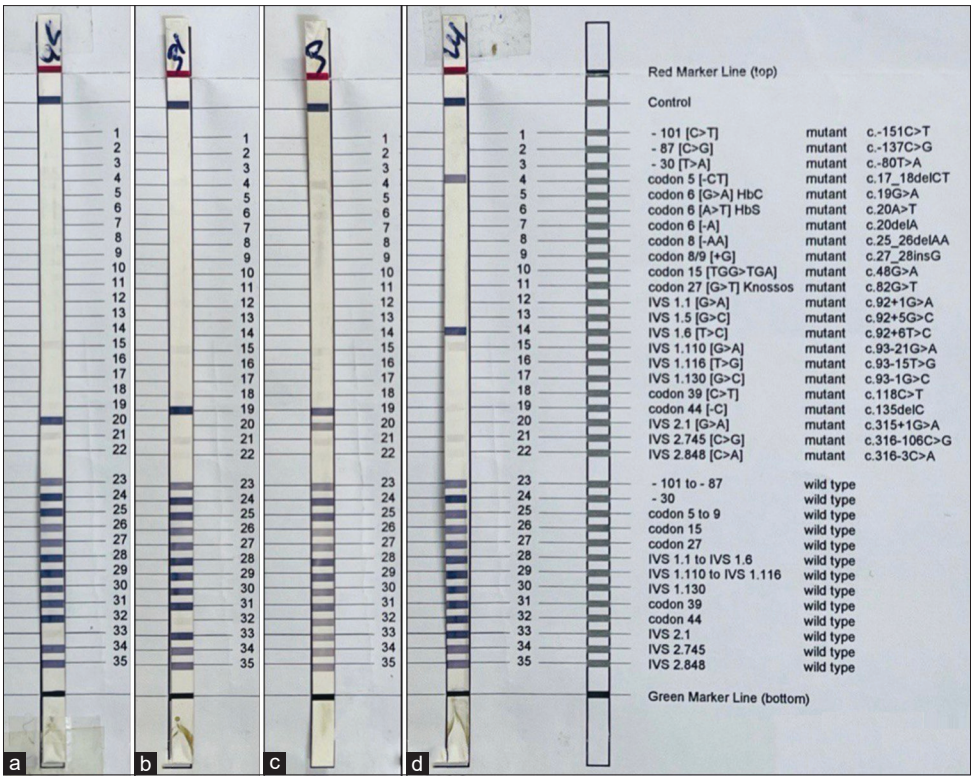

Figure 1: Results of the β-thalassemia mutations detected by ViennaLab strips used in the current study. (a) Homozygous IVSII-1 (G>A), (b) Homozygous codon 44 (−C), (c) Compound heterozygous codon 44 (−C)/IVSII-1 (G>A), (d) Compound heterozygous codon 5 (−CT)/IVSI-6 (T>C)

The mutation IVSII-1 (G>A) (splice junction mutation) is a null β^0-thalassemia allele, which is the most frequent mutation identified with a mean overall frequency of 30.83%. This mutation was predominant in Baghdad (29.03%), (18.3–28.7%) in the three Kurdish provinces in northern and northeastern Iraq, where it was the most frequently encountered mutation.[6,8] The IVSII-1 mutation was the most frequent mutation in Iranian patients,[14-17] as was the mutation recorded in most Arab countries, but such high rates are not shared by other Iraqi neighboring states, except for Eastern Saudi Arabia and Kuwait,[11,18,19] which supports the idea that the latter rates may also be attributable to gene flow from neighboring Iran.

The other most common mutation identified in the current study was IVSI-110 (G>A) (cryptic splice site mutation), a Mediterranean β^+ mutation, which was the second most frequent mutation encountered in the enrolled patients and also represented the second most frequent mutation in Baghdad[6] and the most common mutation in another Iraqi study.[20]

In contrast to many other Eastern Mediterranean populations, the IVSI-110 is the most prevalent mutation in neighboring Jordan, Turkey, Lebanon, and Syria,[11,12,21,22] and it reaches high frequencies in all Arab countries (12.0%–38.0%) but lower frequencies in countries around the Gulf (0.0%–2.0%).[11] Furthermore, the frequent distribution of the IVSI-110 mutation in Iraq and neighboring countries is in agreement with the possible ancient Greek origin of this mutation.[23] The frequency of the IVSI-110 mutation is the highest (77%) in Cyprus and gradually decreases as the countries are further South.[24]

Codon 5 (−CT) (frameshift mutation) has been considered a Mediterranean β^0 mutation, which was the 3rd most common mutation at 10.83% and was found in all Arab Mediterranean countries except Algeria.[11] It represented a low frequency in Saudi Arabia at 1.5%[19] and 3.2% in Syria.[25] On the other hand, the mutation was found in a significant proportion of patients in Dohuk[7] and Iranian Kurds,[14] but not from Turkey's East Anatolia region.[26]

Codon 44 (−C) (frameshift mutation) was another frequent mutation found in this study, which represented 8.33%. It is a β^0 mutation with an elevated prevalence in Iraq than in other countries around Iraq, and it was also detected in the countries of the Arab Peninsula.[11] The mutation was reported as the second most frequent mutation (12.5%) in the Duhok province[7] but was relatively sporadic in the two other Kurdish governorates to the East.[8,27] This mutation was considered a Kurdish mutation, with some authors hypothesizing that it arose in the Duhok region.[28] Because of the interactions between the people, the existence of this Kurdish mutation at high rates in Karbala is not unexpected.

IVSI-1 (G>A) (splice junction mutation) creates a β^0 allele and constitutes 6.67% of alleles in the current study; the frequency of the mutation was similar to that obtained in the Nineveh governorate.[20] The mutation was identified in Northern Iraq with a high frequency (17.7%)[8] and in Dohuk (8.7%),[7] while it was not detected in Baghdad.[6] It was recorded in most countries around Iraq at various frequencies.[11,12,19] The most common mutation in Palestinians was IVSI-1 (G>A), which was prevalent in 31.5% of the studied alleles.[29] The IVSI-1 mutation also has a high frequency in Lebanon (15.0%)[11] and Syria (14.7%),[25] with fewer occurrences in the Gulf countries (1.0% in Oman and 3.0% in Bahrain).[11]

Table 3: Distribution of β-globin genotypes among 60 transfusion-dependent thalassemic patients: Homozygotes and compound heterozygotes

Mutations' genotype	*n* (%)
Homozygous mutations	
IVSII-1 (G>A)/IVSII-1 (G>A)	13 (21.66)
Codon 5 (−CT)/Codon 5 (−CT)	4 (6.66)
IVSI-110 (G>A)/IVSI-110 (G>A)	3 (5)
Codon 44 (−C)/Codon 44 (−C)	3 (5)
IVSI-1 (G>A)/IVSI-1 (G>A)	2 (3.33)
IVSI-5 (G>C)/IVSI-5 (G>C)	2 (3.33)
Codon 39 (C>T)/Codon 39 (C>T)	2 (3.33)
Codon 8 (−AA)/Codon 8 (−AA)	1 (1.66)
Codon 8/9 (+G)/Codon 8/9 (+G)	1 (1.66)
Compound heterozygous mutations	
IVSII-1 (G>A)/IVSI-110 (G>A)	3 (5)
Codon 5 (−CT)/IVSI-110 (G>A)	3 (5)
IVSI-5 (G>C)/IVSI-110 (G>A)	3 (5)
IVSI-6 (T>C)/IVSII-1 (G>A)	3 (5)
IVSI-1 (G>A)/IVS I-110 (G>A)	2 (3.33)
Codon 44 (−C)/IVSII-1 (G>A)	2 (3.33)
Codon 44 (−C)/IVSII-745 (C>G)	1 (1.66)
Codon 5 (−CT)/IVSI-6 (T>C)	1 (1.66)
Codon 8 (−AA)/IVSI-1 (G>A)	1 (1.66)
Codon 44 (−C)/IVSI-110 (G>A)	1 (1.66)
Codon 8 (−AA)/IVSII-1 (G>A)	1 (1.66)
IVSI-6 (T>C)/IVSI-110 (G>A)	1 (1.66)
Codon 8/9 (+G)/IVSII-1 (G>A)	1 (1.66)
Codon 39 (C>T)/IVSI-116 (T>G)	1 (1.66)
Codon 5 (−CT)/IVSII-1 (G>A)	1 (1.66)
codon 8/9 (+G)/IVSI-116 (T>G)	1 (1.66)
IVSI-1 (G>A)/uncharacterized	1 (1.66)
IVSI-5 (G>C)/uncharacterized	1 (1.66)
Uncharacterized/uncharacterized	1 (1.66)
Total	60 (100)

The IVSI-5 (G>C) (consensus change mutation), which constitutes 6.67% of alleles, was similar to that reported in Dohuk by Al-Allawi *et al*.,[7] while it represented 8.5% in Nineveh,[20] 7.5% in Kirkuk,[10] and 3.22% in Baghdad.[6] The mutation is interesting because it was previously found in Chinese and Asian Indian populations, with its occurrence also in the Mediterranean region.[11] In the United Arab Emirate, the most frequent homozygous mutation was the IVSI-5 (G>C) (53.0%)[30] and Oman (62%),[11] while it was quite frequent in neighboring Kuwait and Saudi Arabia (18.8% and 23.2%, respectively).[11,19]

The majority of other Arab countries also had it; however, the frequency is noticeably lower in northern African countries. The latter is probably related to the fact that Mesopotamia served as an essential trade route between India and the Mediterranean at various times in history.[31]

IVSI-6 (T>C) (consensus sequence mutation) constitutes 4.17% of alleles. The result was nearly similar to that of the Baghdad and Kirkuk studies,[6,10] while the mutation represented 8.7% of all alleles characterized in Duhok[7] and was the second most frequent mutation in Nineveh (9.6%).[20] It is considered a Mediterranean β^+ mutation, initially observed in a patient from Portugal and subsequently in Greek Cypriots.[32] It is present at a relatively high frequency in most Mediterranean Arab countries,[11] and among Palestinians, it is one of the most prevalent

Table 4: Types and frequencies of β-thalassemia mutations identified in Karbala in comparison with other Iraqi and neighboring countries' studies

Mutations	Karbala	Bagh[6]	Kirkuk[10]	Nor.IQ[8]	Syria[11]	Jordan[12]	SA[19]	Kuw[11]	Iran[15]	Turkey[42]
IVSII-1 (%)	30.83	29.03	20	28.7	4	15	27.5	29	33.9	6.24
IVSI-110 (%)	15.83	17.74	16.25	1.2	24	25	22[11]	Reco	4.8	20.65
Codon 5 (%)	10.83	9.67	2.5	6.3	8.5	3.8	1.5	0	0.7	3.5[43]
Codon 44 (%)	8.33	14.51	2.5	8.3	0	0	1.5	1	2.6	0
IVSI-1 (%)	6.67	0	2.5	17.7	17	10	5.8	7.3	2.9	6.88
IVSI-5 (%)	6.67	3.22	7.5	2.4	0	5.5[11]	23.2	18.8	7.6	4.3[43]
IVSI-6 (%)	4.17	4.83	3.75	2.4	4	8.3	4.4	7.3	1.1	7.10
Codon 39 (%)	4.17	1.61	2.5	9.1	6.4	4.6	20.3	7.3	1.7	8.63
Codon 8 (%)	3.33	3.22	21.25	9.1	0.7	0	10[11]	3	4.5	4.30
Codon 8/9 (%)	3.33	1.61	12.5	9.1	1.4	0	2.5[11]	1.3	4.8	3[43]
IVSI-116 (%)	1.67	0	3.75	0	1.4	0	0	0	0	0
IVSII-745 (%)	0.83	11.29	0	0.4	1.4	14.2	1.5	0	Reco[44]	3.87

Bagh=Baghdad, Nor.IQ=North Iraq, SA=Saudi Arabia, Kuw=Kuwait, Reco=The mutation is recorded irrespective to (%)

worldwide at 48.5%.[33] The clinical manifestations of the homozygous IVSI-6 patients ranged greatly, from the transfusion-dependent thalassemia major phenotype to a nontransfusion-dependent thalassemia intermedia phenotype.[33]

Codon 39 (C>T) (nonsense mutation) is a Mediterranean β-mutation and constitutes 4.17% of alleles in the current study, with a higher frequency in Northern Iraq (9.1%),[8] while in Kirkuk and Baghdad, it represents 2.5% and 1.61%, respectively.[6,10] The mutation has been found predominantly in the Sardinian population, which accounts for 95.7% of the beta-thalassemia chromosomes,[34] and it was observed in all Arab countries, but with highest frequencies in the North African Arab countries such as Tunisia, Algeria, and Morocco,[11,35,36] and its frequency decreases when moving to the East,[11] except for high frequencies in Bahrain (24.2%) and Saudi Arabia (20.3%).[11,19]

Codon 8 (−AA) (frameshift mutation) is a Mediterranean β^0 mutation that constitutes 3.33% of all alleles characterized in our study. The result was in agreement with that reported in Baghdad and Duhok,[6,7] while in Kirkuk, it was the most frequent mutation (21.25%),[10] and in Erbil, it was the second most common with a frequency of 15%.[37] It was originally detected in a Turkish patient, and it is the most common mutation in Azerbaijan,[38] but it is of low frequency in Arab countries around Iraq except Saudi Arabia, where it reaches 10%.[11] In Turkey, the frequency was 5.6%,[39] while in Syria and Iran, the frequency of mutation was 6.2% and 5.94%, respectively.[16,25]

Codon 8/9 (+G) (frameshift mutation) is an Asian Indian β^0 mutation; it is found in all regions of India;[40] it is also established at low frequencies in all the countries around Iraq (except Jordan) but achieves its peak frequency in the Arab Peninsula countries.[11] It constitutes 3.33% of the alleles characterized in the current study. In Baghdad, the mutation represented 1.61%,[6] while in Erbil, the frequency of the mutation was 20%,[37] and in Kirkuk, it was 12.5%.[10] As we move West and North to Dohuk province and the East Anatolia region of Turkey, its frequency appears to decline.[39] In the other neighboring countries of the eastern Mediterranean, the mutation is less frequent or nonexistent.[11,12,19] The historic Silk Road, which passed through Iraq, may have had an impact on trade as it may have caused the codon 8/9 mutation to occur in our region.[27]

IVSI-116 (T>G) (cryptic splice site mutation) is a Mediterranean β^0 mutation and constitutes 1.67% of alleles. In spite of this mutation being a Mediterranean mutation, it was not reported in all middle and northern Iraq studies,[6-9] except in Kirkuk, where the mutation was represented at 3.75%.[10] The mutation was identified in Syria (1.2%),[25] while it was not identified in the other surrounding Arab countries.[11] Furthermore, it was reported in Turkey at a low frequency (1.6%).[26]

IVSII-745 (C>G) (cryptic splice site mutation) is the least common mutation in this study and constitutes 0.83% of alleles. It is a Mediterranean β^+ mutation detected in Mediterranean Arab countries but at a lower frequency,[11] but found at its highest frequency in Jordan at 14.2%[12] and 11.29% in Baghdad,[6] while the mutation was not presented in Dohuk.[7] The mutation is relatively rare, with a frequency of 2.3% in Iran[17] and 1.5% in Saudi Arabia.[19]

The data above showed that the different β-thalassemia mutations in the Iraqi population were geographically scattered throughout the Iraqi areas, which may be related to the history of malaria, which predisposes to a selection advantage against β-thalassemia predominance.[41] The majority of the mutations identified in this study are also present in neighboring countries but at a different frequency. This is consistent with research from other parts of the world that demonstrates the prevalence of gene flow as a result of population migration.[11]

Conclusions

Among the 12 identified mutations, IVSII-1 and IVSI-110 were the most frequent, while IVSII-745 was the least common mutant allele. Reverse hybridization strip assay molecular techniques used in the current study provide an extremely quick, precise, and simple to carry out molecular diagnostic technique for the detection of β-thalassemia mutations.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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