A SPECTRUM OF B-THALASSEMIA MUTATIONS IN SULAIMANI PROVINCE OF IRAQ: IDENTIFICATION OF NOVEL MUTATIONS

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ABSTRACT

Background

β-thalassemia is a most common hereditary disease where the patients suffer from decreased or absence of beta-globin chain synthesis, which leads to hemolytic anaemia and other complications. Very little data about beta-globin mutations in the Kurdish population is available to date.

Objective

This study aimed to provide a more precise picture of the β-thalassemia mutations spectrum and to estimate their frequencies.

Methods

A cohort of 100 β-thalassemia patients was tested to detect mutations in the beta-globin gene's regions (3 exons and two introns) using molecular techniques (polymerase chain reaction and Sanger sequencing).

Results

In this study, a total of 31 beta-thalassemia mutations were identified. The results showed that IVSII-666 C>T and IVSII-16 G>C were predominant over other mutations, with 59% of thalassemic patients having these mutations. Other common mutations found, in order of decreasing frequency, were Cd2 T>C, IVSII-74 T>G, IVS1-110 G>A, IVSI-5 G>C, IVSII-1 G>A, IVSII-81 C>T. The remaining mutations were uncommon and accounted for a few cases. More importantly, five novel beta-thalassemia mutations, namely, IVSII-13 G>A, IVSII-14 A>C, IVSII-17 delC, IVSII-68_69 dupG, and Cd2/3 +C, were discovered which have not been previously reported in other populations.

Conclusion

The results obtained in this study can be used as a guide before prenatal diagnosis and during premarital screening of β-thalassemia in the Kurdish population.

Keywords: *β-Thalassemia, Mutation, Beta globin gene, Polymerase chain reaction, Sanger sequencing.*

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INTRODUCTION

β-thalassemia is an autosomal recessive genetic disorder that is characterised by a decreased (β+) or absent (β0) synthesis of the beta-globin chains of haemoglobin due to mutations in the beta-globin gene (*HBB*) on chromosome 11. Moreover, β-thalassemia is a prevalent form of thalassemia and is associated with severe anaemia and other complications, such as bone marrow hyperplasia and splenomegaly [(1)]. At the clinical level, β-thalassemia can be grouped into three main types: β-thalassemia trait (minor) (β+ or β0), intermedia (β+/β+; β+/β0), or major (β0/ β0).

β-thalassemia minor is a silent carrier caused by a single defective gene. β-thalassemia major is caused by two beta-globin gene defects that lead to severe anaemia and other complications. Patients diagnosed with β-thalassemia major need lifelong blood transfusions and other medical support with iron chelating agents to combat iron overload. β-thalassemia intermedia is characterised by moderate anaemia, and the majority of affected patients do not require regular blood transfusions [(2,3)].

To date, more than 200 beta-globin gene mutations have been detected all over the world. Beta-globin gene mutations present heterogeneity at the molecular level.

The spectrum and frequency of mutations vary among ethnic groups and geographical locations. Annually, the incidence of beta-thalassemia is estimated to be one in 100,000 worldwide [(1,4,5)]. High incidence has been reported in the Mediterranean, Middle East, Indian subcontinent, and Southeast Asia [(6)]. The cultural practices of marrying within the same caste, ethnicity, and consanguineous marriage are considered important contributors to the high incidence of β-thalassemia in the Middle East's population [(7)]. Although the Kurds are among those populations who are at a high risk of β-thalassemia, there is no known database about the pattern and frequency of beta-globin mutations. For this reason, this study aims to determine the frequency and types of the most common and rare β- thalassemia mutations in the Kurdish population in Sulaimani province, Iraq.

PATIENTS AND METHODS

A total of 100 unrelated patients with known β-thalassemia (54 males and 43 females), identified depending on haematological parameters at Thalassemia and Congenital Blood Disorders Center, Sulaimani province, Kurdistan region, Iraq, were included in this study. All 100 subjects were from unrelated families and lived in Sulaimani province.

Blood samples were collected from patients during their attendance for blood transfusion. Sex, age, related medical examinations, and medical histories were recorded in an electronic medical record information system. This study was approved by the Medical Ethics Committee of the General Directorate of Health in Sulaimani province.

Methods

Blood samples were collected with EDTA anticoagulants. According to the manufacturer's instructions, the DNA was extracted from the blood samples using an Addprepp Genomic DNA extraction kit (Addbio, Korea). All DNA samples were subjected to *in vitro* amplification by polymerase chain reaction to amplify the entire region of the beta-globin gene (3 exons and two introns) using two sets of primers (Table 1) as described previously [(8)].

The standard 20 µl PCR reaction mixture was prepared, and it consisted of 1X add TaqMaster mix (Addbio, Korea), ten pmol forward primer, ten pmol reverse primer, and ten ng template DNA. The standard PCR conditions used for set I primers include initial denaturing temperature (3 min at 95°C), followed by 30 cycles of 30 sec at 95°C, 30 sec at 58°C (annealing temperature), 30 sec at 72°C (extension temperature), with a final extension of 3 min at 72°C. The thermal cycling of set II primers was performed under conditions similar to those of set I primers.

Ten (10µl) of the products were loaded on 1% agarose gel with ethidium bromide solution in 1X TBE buffer, and the gel was run at 100 volts for 30 min to confirm the successful amplification of the beta gene. The bands were visualised on a UV transilluminator and photographed using a photo documentation system (Figure 1). The PCR products were purified using a PCR GenepHlow™ Gel/PCR Kit (Geneaid, Taiwan). Purified PCR products were sequenced at Microsynth Seqlab (Gottingen, Germany).

RESULTS

One hundred patients who had clinically manifested β-thalassemia were included in this study to detect the type and frequencies of their mutations. A total of 31 mutations of the beta-globin gene were detected and comprised null mutations (β0) as well as mild mutations (β+) that caused the reduced production of

the β-globin chain (Figure 2). As shown in Table 2, the most frequent mutations were IVSII-666 C>T and IVSII-16 G>C, each of which was seen in 59% of the cases. These mutations were followed in decreasing frequency by Cd2 T>C, IVSII-74 T>G, IVS1-110 G>A, IVSI-5 G>C, IVSII-1 G>A, IVSII-81 C>T, with 54%, 21%, 16%, 14%, 14%, and 12% of thalassemic patients having these mutations, respectively. Other common mutations and the percentages of patients having these mutations included CAP + 20 C>T (9%), IVSII-745 C>G (8%), Cd8/9+G (8%), Cd39 C>T (7%), IVSI-6 T>C (6%), Cd5-CT (4%), Cd8-AA (4%), IVSII-44 T>A (3%), IVSI-1 G>A (2%), IVSI-6 T>G (2%), IVSI-40 T>C (2%). The remaining mutations were detected in 1% of the cases. More importantly, uncommon and rare mutations were detected, including IVSII-13 G>A, IVSII-14 A>C, IVSII-17 delC, IVSII-68_69 dupG and Cd2/3 +C.

The results indicated that the molecular basis of β-thalassemia in the Kurdish population is very heterogeneous, and the location of mutations within the beta-globin gene varies. In 5'UTR, CAP + 20 C>T mutation was found, and 21 mutations were located in intron regions, representing 67.74% of all mutations. The remaining mutations, representing 29.01%, were located in the Exon region, and these mutations were frameshift, missense, nonsense, and silent mutations.

The clinical significance of the mutations was studied (Table 2). Some mutations in the beta-globin gene are described as pathogenic mutations that affect beta-globin chain production. Among uncommon and rare mutations, only Cd2/3 +C is a pathogenic mutation that causes frameshift mutation.

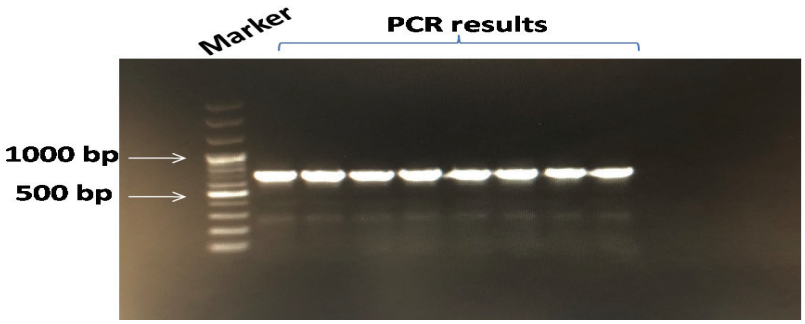

Figure 1. PCR amplification results for beta-globin gene on agarose gel electrophoresis (1%).

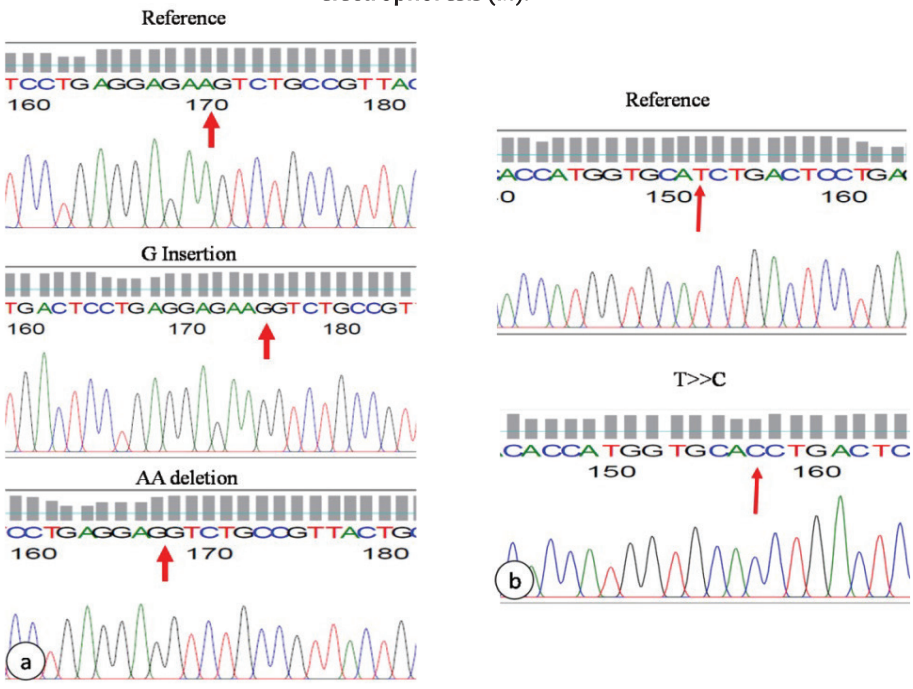

Figure 2. Sanger sequencing technique to detect Beta-globin: a. the two mutations detected include G insertion and double A deletion, b. Thymine is substituted by cytosine.

Table 1 The set of primers used to amplify the beta-globin gene

Primer	Nucleotide (5' to 3')	Position*	Product size (bp)
Set I: Forward primer	AGAAGAGCCAAGGACAGGTACG	61991-61990	760
Set I: Reverse primer	TGCAATCATTCGTCTGTTTCCC	62730-62751	
Set II: Forward primer	TCCCTAATCTCTTTCTTTCAGG	63190-63211	660
Set II: Reverse primer	TTTTCCCAAGGTTTGAACTAGC	63829-63849	

***Position according to the GeneBank accession number U01317**

Table 2. Beta-globin mutations in the β-thalassemia patients.

Mutations					
Common name	**HGVS**	**Unique identification**	**Percentage among patients (%)**	**Location within gene**	**Clinical significance**
CAP + 20 C>T	c.-31 C>T	rs63750628	9	5`UTR	Uncertain significance
IVSI-1 G>A	c.92+1 G>A	rs33971440	2	Intervening sequence 1	Pathogenic
IVSI-1 G>T	c.92+1 G>T	rs33971440	1	Intervening sequence 1	Pathogenic
IVSI-5 G>C	c.92+5 G>C	rs33915217	14	Intervening sequence 1	Pathogenic
IVSI-6 T>C	c.92+6 T>C	rs35724775	6	Intervening sequence 1	Pathogenic
IVSI-6 T>G	c.92+6 T>G	rs35724775	2	Intervening sequence 1	Pathogenic
IVSI-40 T>C	c.92+40 T>C	rs375672426	2	Intervening sequence 1	Benign
IVS1-110 G>A	c.93-21 G>A	rs35004220	16	Intervening sequence 1	Pathogenic
IVSI-128 T>C	c.93-3 T>C	rs34527846	1	Intervening sequence 1	Pathogenic
IVSI-130 G>C	c.93-1 G>C	rs33943001	1	Intervening sequence 1	Pathogenic
IVSII-1 G>A	c.315+1 G>A	rs33945777	14	Intervening sequence 1	Pathogenic
IVSII-13 G>A	c.315+13 G>A	Novel	1	Intervening sequence 2	Benign
IVSII-14 A>C	c.315+14 A>C	Novel	1	Intervening sequence 2	Benign
IVSII-16 G>C	c.315+16 G>C	rs10768683	59	Intervening sequence 2	Benign
IVSII-16_17dupC	c.315+16_17insC	rs18475493131	1	Intervening sequence 2	Uncertain significance
IVSII-17 delC	c.315+17 del C	Novel	2	Intervening sequence 2	Benign
IVSII-68_69dupG	c.315+68_69insG	Novel	1	Intervening sequence 2	Benign
IVSII-44 T>A	c.315+44 T>A	rs1352476647	3	Intervening sequence 2	Benign
IVSII-74 T>G	c.315+74 T>G	rs7480526	21	Intervening sequence 2	Benign
IVSII-81 C>T	c.315+ 81 C>T	rs7946748	12	Intervening sequence 2	Benign
IVSII-666 C>T	c.316 -184 C>T	rs1609812	59	Intervening sequence 2	Benign
IVSII-745 C>G	c.316-106 C>G	rs34690599	8	Intervening sequence 2	Pathogenic
Cd2 T>C	c.9 T>C	rs713040	54	Exon (silent mutation)	Benign
Cd15 G>A	c.47 G>A	rs63750783	1	Exon (Nonsense mutations)	Pathogenic
Cd39 C>T	c.118 C>T	rs11549407	7	Exon (Nonsense mutations)	Pathogenic
Cd48 T>C	c.146 T>C	rs33952850	1	Exon (Missense mutations)	Others
Cd2/3 +C	c.9_10 ins C	Novel	1	Exon (Frameshift mutations)	Pathogenic
Cd5-CT	c.17_18 del CT	rs34889882	4	Exon (Frameshift mutations)	Pathogenic
Cd8-AA	c.25_26 del AA	rs35497102	4	Exon (Frameshift mutations)	Pathogenic
Cd8/9+G	c.26_27 ins G	rs35699606	8	Exon (Frameshift mutations)	Pathogenic
Cd20 +G	c.62_63 ins G	rs1554918165	1	Exon (Frameshift mutations)	Likely pathogenic
Undetected	-	-	3	-	

DISCUSSION

β-thalassemia is one of the most common genetic disorders, and many defects in the beta-globin gene characterise it. Several studies on the frequency of mutations associated with β-thalassemia among Iraqi Kurds have been carried out previously [9,10]; unfortunately, no large-scale investigation has been performed to illuminate the status of beta-globin gene mutations. The present study detected a wider spectrum of β-thalassemia mutations among the Kurdish population residing in the Sulaimani province.

In the current study, 31 different mutations of the beta-globin gene have been characterised. Two benign mutations (IVSII-666 C>T and IVSII-16 G>C) were the most frequent mutations identified in our patients, which were seen in 59% of the cases. This result contrasts with previous studies, particularly those conducted in the North of Iraq, where the latter mutations were not observed [9]. High rates of other mutations, including Cd2 T>C, IVSII-74 T>G, IVS1-110 G>A, IVSI-5 G>C, IVSII-1 G>A, IVSII-81 C>T, were recorded in the present study. Similar to the previous mutations, benign mutations (Cd2 T>C, IVSII-74 T>G and IVSII-81 C>T) have not been found in other Iraqi Kurdish studies; these β-globin gene mutation spectrum discrepancies were probably due to the testing strategy and the population selected.

IVS1-110 G>A pathogenic mutation is more frequent among Mediterranean populations [11, 12]. In the current study, 16% of the patients were found to have this mutation. A similarly high rate of IVS1-110 G>A mutation was also found among other ethnicities in some cities in Kurdish areas of Iraq and Turkey [13-15]. Moreover, high frequencies were also observed in northwestern and Kurdish Iranians [16-18]. A group of Tunisian researchers reported that the frequency of IVS1-110 G>A was 10.8%, and they suggested that this mutation might have been introduced by the Turkish and Phoenician influence [18].

IVSI-5 G>C presents with relatively high frequency in most Arabian Gulf countries, including Saudi Arabia (10%), Kuwait (18.8%), Bahrain (16.7%), the United Arab Emirates (55%), and Oman (61.6%) [19]. Additionally, the highest frequency of this mutation (21.6%) was found in a study conducted in an Iraqi city located in the Arabian Gulf [20]. In contrast to the previous findings, this pathogenic mutation occurred in 14% of the cases, and the same result was found among Iraqi Kurds and Arabs living in the northern and central areas of Iraq [10, 21]. This mutation has Asian Indian and Southeast Asian origins [22].

Another important observation is that similar to many other studies conducted on an Iraqi Kurdish population, which found IVSII-1 G>A to be the predominant mutation [9,10], the frequency rate for this pathogenic mutation was also high in this study, and 14% of thalassemic patients were found to have it.

Almost all studies were conducted in Turkish Azeri, Northwestern Iran, West Iran, and Southwest Iran and Iranian Kurds reported the latter mutation as the most common beta-globin gene defect [23-25]. Moreover, it has been previously reported as having the highest rate in other populations in the surrounding countries, such as Jordan, Syria, Kuwait, and Saudi Arabia [19]. According to the above data, this mutation can be described as Mediterranean.

Other pathogenic mutations were found in this study (IVSII-745 C>G and Cd8/9+G), with 8% of subjects having these mutations. The first of these was not detected previously, while the latter frameshift mutation was detected at high frequencies in other Iraqi reports [10,21]. IVSII-745 C>G is a Mediterranean mutation that was found to occur at the highest frequency in Jordan [4]. On the other hand, it was detected at low frequency in Mediterranean Arab countries including Syria (1.4%), Lebanon (1%), Israeli Arab (6%), Egypt (5.6%), Tunisia (4.4%), and Algeria (0.9%) [19]. The Cd8/9+G mutation is of Asian-Indian origin, and one Iranian study showed that this mutation is less common among the Sistani Iranian population [25]. Kerman city in Iran recorded it at 4.9% [26]. The differences in the frequency rates of the mutations might be due to the Iraqi and Iranian populations being mixtures of different ethnic groups. In the north of Iraq, there is a large Kurdish population, while in the west and south of Iraq, Arab ethnicities are more predominant.

We found a notable difference in the regional frequency of a nonsense mutation, i.e., Cd 39 C>T, compared with the previous Iraqi Kurdish studies in Sulaimani and Erbil cities. This dissimilarity was also noticed among the Iraqi Arab populations. For instance, this mutation recorded low frequency in Wasit city, while it was the most frequent mutation in Karbala city [14, 27]. This variation might be explained as due to the different ethnic groups living in Iraq.

It was suggested that this pathogenic mutation was very common in Mediterranean and Arabian countries, including Algeria, Tunisia, Morocco, Tunisia, Syria, Saudia Arabia, Iraq, and Jordan [4]. Another pathogenic Mediterranean mutation is IVSI-6 T>C, which was found in 6% of the patients in the present study. In contrast with our results, this was found to be the most common allele mutation among Kurds in the North of Iraq [28-30]. Two frameshift mutations (Cd5-CT and Cd 8 (-AA)) were less frequent among our patients. The first of these was also found in all Arab Mediterranean and Arabian Gulf Countries [19]. Meanwhile, Cd 8 (-AA) is a Turkish mutation with low frequency in Iraq and Arab countries, except for Saudi Arabia, where a rate of 10% has been reported [19].

In contrast with our results, other research found it was the fourth most common allele mutation among Kurds in the North of Iraq [28, 30]. This study revealed that our patients' pathogenic mutations of beta-globin gene mutations such as IVSI-1 G>A, IVSI-128 T>C, and Cd15 G>A were less frequent. In contrast, the other less frequent pathogenic and non-pathogenic mutations (IVSII-44 T>A (3%), IVSI-6 T>G, IVSI-40 T>C, IVSI-1 G>T, IVSI-130 G>C, Cd48 T>C, Cd20 +G) have never before been documented in the Iraqi Kurdish population.

In conclusions, in the current study, using the sequencing technique and a large cohort of patients provided more details about the spectrum and frequency of the common and rare pathogenic β-thalassemia mutations in the Sulaimani province. The study's molecular analysis of the beta-globin gene to identify common and pathogenic mutations provides a guideline to help organise a program to prevent β-thalassemia disease.

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