Molecular characterization of beta-thalassemia mutations in Ninawa governorate

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ABSTRACT

Objectives: There are currently more than 200 known mutations affecting the beta globin gene; about 20 mutations account for 90 % of beta globin gene mutations in the world, and each ethnic population has its own unique and frequency of beta globin mutations. Prenatal diagnosis requires identification of the mutation spectrum in each population, and then it is possible to do direct identification for these mutations in the majority of the population. The aim of this study is to characterize the spectrum of beta globin gene mutations in Ninawa governorate patients with beta- thalassemia major.

Patients and methods: Twenty four thalassemic patients were included; they were transfusion dependent and they were diagnosed and registered in thalassemia center of Ninawa governorate. After DNA extraction from venous blood and PCR based DNA amplification, the allele's characterization was achieved by reverse hybridization to specific oligonucleotide probe designed to detect 22 beta-thalassemic mutations.

Results: Out of the forty eight alleles studied, 42 (87.5%) alleles were characterized, while 6 (12.5%) alleles were undetermined.

Eight alleles causing beta-thalassemia in Ninawa governorate were identified in these patients, and these alleles with their frequencies were:

IVS 1.110 (G>A)(27.08%), IVS 1.6 (T>C)(14.5%), cod 8 (-AA)(12.5%), cod 39 (C>T)(12.5%), IVS 2.1 (G>A)(12.5%), cod 44 (-C)(4.16%), IVS 1.5(G→C)(2.08%) and Cod 5(-CT)(2.08%).

More than 1/3 of the families have more than one affected sibling.

Eleven (45.83%) patients had homozygous alleles, and 12 (50%) patients with compound heterozygous alleles; In 1 (4.16%) patient the genotype was unknown.

Keywords: Thalassemia, beta globin genes mutations, Ninawa, oligonucleotide, reverse hybridization.

الخلاصة

مقدمة: حاليا تم اكتشاف ما يزيد عن ٢٠٠ نوع من الطفرات الوراثية التي تحصل في جين بيتاكلوبين والتي تؤثر على تصنيع سلسلة بيتا كلوبين في عدة مراحل من التصنيع وبالتالي تؤدي الى أنماط سريرية متعددة لمرض الثلاسيميا. ان عملية تحديد نوع الطفرات التي تؤدي الى فقر دم البحر المتوسط في مجتمع ما هي المرحلة اللازمة الأولى لإنشاء برنامج السيطرة على المرض.

الهدف من الدراسة: تحديد أنواع الطفرات الوراثية المسببة لمرض الثلاسيميا الكبرى نوع بيتا في مرضى محافظة نينوى.

المرضى وطرق العمل: شملت هذه الدراسة ٢٤ مريضا، وكل مريض مسجل في مركز الثلاسيميا في محافظة نينوى على انه مصاب بالثلاسيميا الكبرى نوع بيتا ويتم له إجراء نقل الدم بشكل متكرر.

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بعد استخلاص الحامض النووي (الدنا) من الدم الوريدي للمريض، تم إجراء تفاعل البلمره المتسلسل للحامض النووي (الدنا)، وبعد ذلك تم تحديد نوع الطفرة في الحامض النووي بطريقة التهجين العكسي مع مسبارات خاصة مكونة من عدد من القواعد النتروجينية مخصصة للكشف عن ٢٢ طفرة مسببة للثلاسيميا نوع بيتا.

النتائج: من مجموع ٤٨ اليل مسبب للثلاسيميا ، تم تحديد ٤٢ الليل (٨٧,٥%) وبقي ٦ اليل (١٢,٥%) لم يتم تحديدها.

تم تحديد ٨ أنواع من الطفرات في مرضى محافظة نينوى، وهي مع نسبة تواجدها كما يلي :

IVS 1.110 (G>A)(27.08%), IVS 1.6 (T>C)(14.5%), cod 8 (-AA)(12.5%), cod 39 (C>T)(12.5%), IVS 2.1 (G>A)(12.5%), cod 44 (-C)(4.16%), IVS 1.5(G→C)(2.08%) and Cod 5 (-CT)(2.08%).

العوائل التي أنجبت مريضا واحدا شكلت (٦٢,٥%) بينما العوائل التي أنجبت أكثر من مريض شكلت (٣٧,٥%).

أحد عشر (٤٥,٨٣%) مريضا كانوا يحملون اليلات متجانسة واثنا عشر (٥٠%) مريضا كانوا يحملون اليلات غير متجانسة بينما في مريض واحد (٤,١٦%) لم يتم التعرف على تركيبته الجينية.

مفاتيح الكلمات: ثلاسيميا، فقر دم البحر المتوسط، طفرات جين بيتا كلوبين، نينوى .

Thalassemias are a heterogeneous group of genetic disorders of hemoglobin synthesis, all of which result from a reduced rate of production of one or more of the globin chains of hemoglobin [1]. It is the world's most common monogenic disorder [2,3].

Most individuals who are homozygous or compound heterozygous for beta thalassemia have thalassemia major, a severe, life threatening anemia that requires regular blood transfusion for survival. Some patients, however, are less severely affected with a milder non-transfusion dependent disorder referred to as thalassemia intermedia [3, 4].

Since the treatment of thalassemia major is still unsatisfactory and costly and the disease is ultimately fatal, prenatal diagnosis has become an important option for couples at risk of producing an affected fetus.

Genetic counseling, population screening, prenatal diagnosis and the option of termination of affected pregnancies remain the mainstay strategy in the control of beta thalassemia major. Many studies have confirmed and shown the benefits of a thalassemia prevention program [3,5,6].

There are currently more than 200 known mutations in the beta globin gene; about 20 mutations account for 90 % of beta globin gene mutations in the world and each ethnic population has its own unique and frequency of beta globin mutations. Prenatal diagnosis requires identification of the mutation spectrum in each population, then it is possible to do direct identification for these mutations in the majority of the population using monoplex and multiplex amplification refractory mutation system (ARMS) or dot blotting [7- 9].

Characterization of beta thalassemia mutations have been carried out in countries around Iraq ; in Saudi Arabia [10], in Syria[11], in Jordan[12], in Kuwait[13], in Turkey[14], and in Iran[15] .

In Iraq a limited study has been carried out in Dohuk governorate region [16].

The aim of this study is to characterize the spectrum of beta globin gene mutations in Ninawa governorate patients with beta-thalassemia major who are registered in thalassemia center, using PCR - based DNA diagnostic techniques.

Patients and methods

This study was conducted during the period: from Faberuary 2007 to December 2007.

Patients

Twenty four thalassemic patients were included in this study, and they were randomly selected and according to the following criteria: The patients were diagnosed and registered as thalassemia major in Thalassemia Center in Ibn Al-Atheer Hospital in Ninawa Governorate. The diagnosis in the Thalassemia Center depends on clinical presentation, blood counting parameters (Hb, PCV and MCH), findings in blood film stained by Leishman stain and Hb electrophoresis.

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The patient has history of one blood transfusion or more per month for at least two months. A case sheet for each patient, including: name, sex, date of birth, age of presentation ….. etc had been prepared.

Methods

For each patient two ml of venous blood was withdrawn from a vein in the antecubital fossa and was put in a 2.5 ml EDTA tube (AFMA-Dispo-Jordan) and subjected to the following investigations:

1. DNA extraction: carried out in the Laboratory of Bone Marrow Transplant center in Children Welfare Teaching Hospital, of Medical City, Baghdad-Iraq, using Wizard Genomic Purification Kit (Promega Corporation-USA), according to the method described by the manufacturer [17].
2. DNA amplification and mutation identification: carried out in Italy by Dott. Marcello Morgutti (Servizio di Genetica, IRCCS "Burlo Garofolo", via dell 'Istria 65/1, 34100 TRIESTE, Tel.:0403785424, Fax: 0403785540, e-mail: morgutti@ burlo.trieste.it) using β-Globin StripAssay™ kit (ViennaLab Labordiagnostika GmbH-Austria- e-mail: info@viennalab.co.at) which intended for the identification of β-globin gene mutations based on polymerase chain reaction (PCR) followed by reverse hybridization to specific wild and mutant oligoprobes designed to detect 22 β–thalassemia mutations (ViennaLab Labordiagnostika GmbH) .

The assay cover 22 beta globin mutations as following: -87(C→G), -30(T→A), Codon 5(-CT), hemoglobin C (HbC), Hemoglobin S (HbS), Codon 5(-A), Codon 8(-AA), Codon 8/9(+G), Codon 22(7bp del), Codon 30(G→C), IVS 1.1(G→A), IVS 1.2(T→A), IVS 1.5(G→C), IVS1.6 (T→C), IVS 1.110(G→A), IVS 1.116(T→G), IVS 1-25(25bp del), Codon 36/37(-T), Codon 39(C→T), Codon 44(-C), IVS 2.1(G→A), IVS 2.745(C→G)
(ViennaLab Labordiagnostika GmbH)

Statistical analysis

Data analysis had been made by the use of statistical package (Epi-info version 6). The data was presented by frequency distribution, and means and standard deviation (SD) were made for selected variables.

Results

Twenty four patients with 48 alleles from 24 unrelated families resident in Ninawa governorate were studied (table 1). Twenty three patients were of Arabic origin for father and mother, while one patient (no.18) was of Kurd origin for father and mother.

The following criteria were considered in addition to the characterization of the alleles:

1. Sex: 8/24(33.3 %) patients were males and 16/24(66.6%) were females.
2. Age: the mean of age was 9.58 years (SD+/- 4.79) with range of 1-19 years.
3. Other affected siblings: 15/24 (62.5%) families have only one affected individual while 9/24(37.5%) families have more than one affected individual.
4. Location: 12/24(50%) patients were from Mosul (center of Ninawa) and 12/24(50%) patients were from the towns outside the center (Mosul) and as following: 4 patients from Tal-afar , 2 patients from Badosh and 1 patient from each of the towns : Bartalah, Baashika, Rabeaa, Kaiarah, Al-Hamdaniah and Alkosh (table 1).
5. Genotype: 11/24 (45.83%) of patients have homozygous alleles and 12/24 (50%) patients with compound heterozygous alleles (c.heterozygous) In 1/24 (4.16%) the genotype was unknown.
6. Characterization of the alleles: 42/48 (87.5%) of the alleles were characterized, while 6/48 (12.5%) of alleles were not determined.
7. Types of mutations: 8 types of mutations were determined, and these are: IVS 1.110 (G>A), IVS 1.6 (T>C), cod 8 (-AA), cod 39 (C>T), IVS 2.1 (G>A), cod 44 (-C), IVS 1.5(G→C) and Cod 5(-CT).
8. Frequency of the mutations (table 2):

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- IVS 1.110 (G>A) constitutes 27.08% (13/48) of alleles, and 76.92% (10/13) of these alleles present in homozygous state, that is in 5 patients, while 23.07% (3/13) of the alleles present in c. heterozygous state, that is in 3 patients.
- IVS 1.6 (T>C) constitutes 14.5% (7/48) of alleles, and 28.57% (2/7) of these alleles present in homozygous state, that is in one patient, while 71.42% (5/7) of these alleles present in c. heterozygous state, that is in 5 patients.
-cod 8 (-AA) constitutes 12.5%(6/48) of alleles, and 33.33% (2/6) of these alleles present in homozygous state, that is in one patient, while 66.66% (4/6) of these alleles present in c. heterozygous state, that is in 4 patients.
- cod 39 (C>T) constitutes. 12.5% (6/48) of alleles, and 66.66% (4/6) of these alleles present in homozygous state, that is in 2 patients, while 33.33% (2/6) of the alleles present in c. heterozygous state, that is in 2 patients.
- IVS 2.1 (G>A) constitutes 12.5% (6/48) of alleles, and 66.66% (4/6) of these alleles present in homozygous state, that is in 2 patients, while 33.33% (2/6) of the alleles present in c. heterozygous state, that is in 2 patients.
- cod 44 (-C) constitutes 4.16% (2/48) of alleles, and 100% present in c. heterozygous state, that is in two patients.
- IVS 1.5 (G→C) constitutes 2.08% (1/48) of alleles, and it presents in one patient as c. heterozygous state.
- Cod 5 (-CT) constitutes 2.08% (1/48) of alleles, and it presents in one patient as c. heterozygous state.
- undetermined alleles constitute 12.5 %(6/48) of alleles, 2 alleles in one patient and four alleles distribute in 4 patients, each with a determined allele.

Table (1): Results of thalassemia patients from Ninawa.

Code no.	Allele no. 1	Allele no. 2	Sex	Age year	Other Affe.	Locus
1	IVS 1.110 (G>A)	IVS 1.110 (G>A)	M	15	-	Mosul
2	IVS 1.110 (G>A)	IVS 1.110 (G>A)	F	5	-	Rabeaa
3	IVS 1.110 (G>A)	IVS 1.110 (G>A)	M	17	-	Mosul
4	IVS 1.110 (G>A)	IVS 1.110 (G>A)	F	15	1	Mosul
5	IVS 1.110 (G>A)	IVS 1.110 (G>A)	F	12	-	Mosul
6	cod 8 (-AA)	cod 8 (-AA)	F	6	-	Mosul
7	IVS 1.6 (T>C)	IVS 1.6 (T>C)	F	8	1	Tal-afar
8	cod 39 (C>T)	cod 39 (C>T)	F	13	-	Mosul
9	cod 39 (C>T)	cod 39 (C>T)	F	1	-	Tal-afar
10	IVS 2.1 (G>A)	IVS 2.1 (G>A)	M	6	-	Al-Hamdaniah
11	IVS 2.1 (G>A)	IVS 2.1 (G>A)	M	7	-	Mosul
12	cod 8 (-AA)	IVS 1.6 (T>C)	F	5	1	Alkosh
13	cod 8 (-AA)	IVS 1.6 (T>C)	M	8	3	Kaiarah
14	cod 5 (-CT)	IVS 1.6 (T>C)	F	9	-	Badosh
15	cod 8 (-AA)	IVS 1.110 (G>A)	M	11	2	Mosul
16	IVS 1.110 (G>A)	IVS 2.1 (G>A)	F	2	-	Tal-afar
17	IVS 1.6 (T>C)	cod 44 (-C)	F	13	-	Tal-afar
18*	IVS 1.5 (G>C)	IVS 2.1 (G>A)	M	6	-	Baashika
19	cod 8 (-AA)	cod 39 (C>T)	M	13	1	Mosul
20	cod 44 (-C)	not determined	M	15	3	Mosul
21	IVS 1.6 (T>C)	not determined	M	19	1	Mosul
22	IVS 1.110 (G>A)	not determined	F	6	-	Mosul
23	cod 39 (C>T)	not determined	F	12	1	Bartalah
24	not determined	not determined	F	6	-	Badosh

*patient of Kurd origin, M: Male, F: Female, other affe.: other affected individual in the family

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Table (2): Types of mutations, number of alleles and their frequency.

Mutation	Number of alleles	Mutation %
IVS 1.110 (G>A)	13	27.08
IVS 1.6 (T>C)	7	14.5
cod 8 (-AA)	6	12.5
cod 39 (C>T)	6	12.5
IVS 2.1 (G>A)	6	12.5
cod 44 (-C)	2	4.16
IVS 1.5 (G→C)	1	2.08
Cod 5 (-CT)	1	2.08
Not determined	6	12.5
	48	99.90

Discussion

In this study eight β-thalassemic mutations have been characterized in patients from Ninawa governorate. The patients included, were from different towns in the governorate. figure (1). This is the first study that delineates the β-thalassemic mutations in Ninawa governorate.

The eight mutations which have been characterized in the studied group determined that the main β-thalassemia mutations in Ninawa are of Mediterranean origin; with few mutations were of Kurdish and Asian Indian origins; The mutations, IVS 1.110 (G>A), IVS 1.6 (T>C), cod 8 (-AA), cod 39 (C>T), IVS 2.1 (G>A), and cod 5 (-CT) are included in the Spectrum of β-thalassemia mutations in Mediterranean peoples [18-21] and these mutations constitutes 81.2% of all types of mutations in the studied group.

The mutation cod 44 (-C), a Kurdish mutation [18] constitutes only 4.16% while the Asian Indian mutation IVS 1.5 (G>C) [18] constitutes 2.08% of all mutations (table 2).

The undetermined alleles constitute 12.5%, so we would expect that other mutations to β-thalassemia exist in this region. Beside that, this percentage of undetermined alleles was, also expected, as, such percentage was obtained in the surrounding areas; the undetermined alleles in Duhok was 11.5%, in Syria 12.8%, in Turkey 9.5% and in Iran 19.1% [16]. Direct DNA sequencing is one of the methods which can be used for determining such rare mutations [3].

Ninawa is one of the largest governorates in Iraq and although the Arabs form the main ethnic group, many other ethnic groups are present as Kurds, Turks and Ashour. The intermarriages and interrelations between these groups explains the relatively large number of mutation types determined in the studied group, as well as explaining the 12.5% of alleles which are still undetermined.

On the other hand, we tried to make the studied group representative for the state of beta-thalassemia alleles in Ninawa, with the patients included being from many towns around the center (Mosul), (figure 1 and table 1).

All the mutations characterized in this study have been previously described in other populations: The mutation IVS 1.110 (G>A) (create new splice site) is the most common cause of beta-thalassemia in Mediterranean countries, especially eastern Mediterranean region, but reaches lower frequencies in countries around the Arabian Gulf [22,23].

The IVS 1.6 (T>C) (consensus change mutation) was originally found in a Portuguese patient and subsequently in Greek Cypriots. It presents with relatively high frequency in most Mediterranean Arab Countries. Typically the clinical course of patients with this mutation is mild in nature [22,23].

The mutation cod 8 (-AA) (frameshift mutation) was originally detected in a Turkish patient [22] and it is the commonest mutation in Azerbaijan [24], but it is of low frequency in countries around Iraq and in Arab countries except Saudi Arabia, where it reaches 10% [23].

The mutation cod 39 (C>T) (nonsense mutation) has been found in Sardinia, Greece and Turkey and it was found in all Arab Countries, but with highest frequencies in Western Arab Countries such as Tunisia, Algeria and Morocco [22,23,25,26].

The mutation IVS 2.1 (G>A) (splice junction mutation) has been found in Mediterranean peoples (Greece, Italian and Tunisian) and recorded in most of the Arab countries with high frequency in north Jordan (20%) and it is

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the most common mutation in Kuwait and Iran [18,23].

The cod 44 (-C) (frameshift mutation) is a mutation of Kurdish origin, but it was detected in the countries of Arab peninsula [23].

The cod 5 (-CT) (frameshift mutation) is a Mediterranean mutation, and it was found in all Arab Mediterranean Countries except Algeria [23].

The IVS 1.5 (G>C) (consensus change mutation) is interesting because it was previously found in Chinese and Asian Indian populations, with its occurrence also in the Mediterranean region [23].

In this study, the results showed that the mutation IVS 110 (G>A) is the most common mutation in thalassemic patients in Ninawa governorate, a finding similar to that obtained in the surrounding countries (Turkey, Syria, Jordan and Saudi Arabia) in which, this mutation constitutes the most common mutation; beside that the three most common mutations are similar in Ninawa and Turkey (table 3).

Such finding can be explained by the following:

First: The overwhelming dominance of Ottoman Empire on Iraq in the past and for nearly four centuries had led to intermarriages and admixtures between Arabs and Turks especially in Ninawa (Nineveh), resulting in mutations in this governorate showing a high degree of similarity with the mutations in Turkey.

Second: The patients included in this study were of Arab origin (except one patient who was of Kurdish origin); while the majority of people in Syria, Jordan, and Saudi Arabia, are, also of Arab origin and some people in these countries are related to the same tribes or they have association through intermarriages or migration of residence.

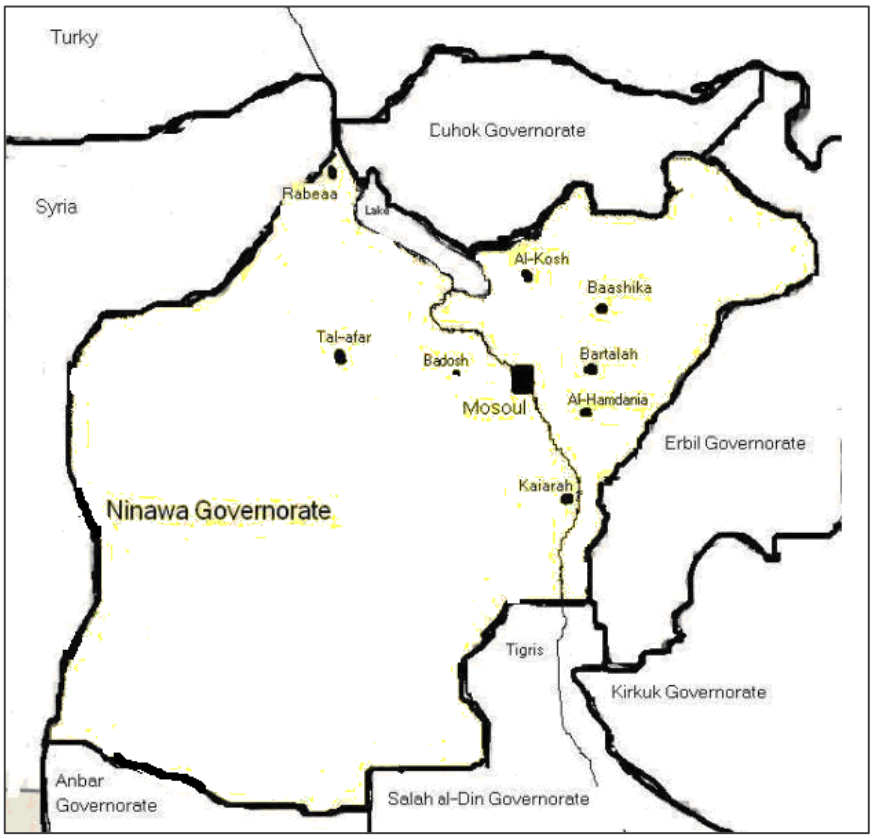

Figure (1): Shows the cities of Ninawa governorate, from which the patients were included in the study in addition to the center (Mosul).

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Table (3): Shows the frequency of the mutations in Ninawa and surrounding countries ordered according to their frequency.

Ninawa	Turkey	Syria	Jordan	Saudi Arabia
IVS 1.110 (27%)	IVS 1.110 (41.4%)	IVS 1.110 (24%)	IVS 1.110 (25%)	IVS 1.110 (22%)
IVS 1.6 (14.5%)	IVS 1.6 (10.6%)	IVS1.1 (17%)	IVS 2.1 (15%)	cod 39 (20%)
cod 8 (12.5%)	cod 8 (5.7%)	cod 5 (8.5%)	IVS 2.745 (14.2%)	IVS 2.1 (15%)
cod 39 (12.5%)	IVS 1.1 (5.3%)	cod 39 (6.4%)	IVS 1.1 (10.0%)	IVS 1-25 (14%)
IVS 2.1 (12.5%)	IVS 2.1 (4.9%)	IVS 2.1 (4%)	IVS 1.5 (5.5%)	cod 8 (10%)

On the other hand, the results showed difference between the most common mutation in Ninawa and the eastern surrounding area that is Duhok and Iran in which the most common mutation is IVS 2.1 (G>A) [16]; in Duhok this mutation followed by the Kurdish mutation cod 44 which was relatively uncommon in Ninawa (table 4).

Such difference can be explained by the difference in the origin of the people, where the people in Duhok and north Iran are of Kurd origin.

The main difference between the results of this study and what was obtained in the surrounding areas is the absence of the mutation IVS 1.1 which was detected in all areas around Ninawa {in Syria 17%, Jordan 10%, Saudi Arabia 7%, Kuwait 7.3%, Iran 2.9% and in Turkey 5.3% [12,23,16]}, and this difference can be explained, partly, by the small size of the sample.

This study demonostrated that 45.83% of patients have homozygous alleles and as the consanguineous marriages within certain ethnic communities having a high incidence of beta-thalassemia have led to an increase in homozygous patients within that particular community[22], so we expect that consanguineous marriage is responsible for such figure of homozygosity.

This study demonstrated another problem, which is the continuous birth of affected children in the same family; the results of this study showed that 37.5% of families in the studied group have more than one affected child, so initiation of a preventive program for beta-thalassemia is a cornerstone in the management of this disease.

Many experiences confirm that the best way for the management of thalassemias is through the introduction of a preventive program which includes population screening, genetic counseling, prenatal diagnosis and options of termination of affected pregnancies. This was demonstrated in Cyprus, Greece and many countries worldwide. The characterization of the common beta-thalassemia mutation in specific community is perquisite for such program [3, 18, 22, 27-29].

So characterization of the common mutations carried out in this study will provide a sound foundation on which to base a preventive program for thalassemia in Ninawa governorate, and, also, these findings will facilitate the improvement of medical services such as carrier screening, genetic counseling and prenatal diagnosis.

The relatively high frequency (45% of patients) of homozygous alleles obtained in this study can be explained by the consanguineous marriages in our communities.

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Table (4): Shows the frequency of the mutations in Ninawa and Duhok and Iran ordered according to their frequency.

Duhok	Iran	Ninawa
IVS 2.1 (18.3%)	IVS 2.1 (33.9%)	IVS 1.110 (27%)
cod 44 (12.5%)	IVS 1.5 (7.6%)	IVS 1.6 (14.5%)
cod 5 (10.6%)	cod 8/9 (4.8%)	cod 8 (12.5%)
cod 39 (8.7%)	IVS 1.110 (4.8%)	cod 39 (12.5%)
IVS 1.1 (8.7%)	cod 8 (4.5%)	IVS 2.1 (12.5%)

Conclusions

1. Eight β-globin alleles causing β-thalassemia syndrome in Ninawa governorate were characterized; most of these mutations are of Mediterranean type.
2. The frequency of these mutations was as follow:
   IVS 1.110 (G>A) (27.08%), IVS 1.6 (T>C) (14.5%), cod 8 (-AA) (12.5%), cod 39 (C>T) (12.5%), IVS 2.1 (G>A) (12.5%), cod 44 (-C) (4.16%), IVS 1.5(G→C)(2.08%) and Cod 5(-CT) (2.08%).
3. More than 1/3 of the families have more than one affected sibling.

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Appendix

استمارة المعلومات الخاصة بتحديد
الطفرات الوراثية فى مرضى الثلاسيميا

الموقع: رقم الملف
الاسم الثلاثي واللقب الجنس التولد

العشيرة:الاب القومية:الاب الديانة:الاب
الام الام الام
صلة الاب بالام
السكن الحالي السكن الاصلي
اخوة مصابين اخوات مصابات

Presentation:
Age of presentation date of presentation
Clinical features:

Investigations:
Blood group
Hb g/dl PCV % WBC count /cmm
Blood film:

Electrophoresis:
Hb-F Hb-A_2 Hb-A Hb-S

History of transfusion
Date of 1st transfusion: Date of last transfusion:
Frequency of transfusion: Leucocytes filter:

Present state:
Clinical features:

Spleen cm bcm endocrine: high weight puberty
Heart: Hypertrophy, Cardiomyopathy, Heart failure. Liver
Blood film:

Serum ferritine

The Mutation:

التاريخ

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