Some Molecular Characterization of β-Thalassemia Major In Koya City

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Abstract—β-thalassemia is an important health problem in Kurdistan region and clinical characterization of β-thalassemia problems revealed in Koya City, and it requires more investigation and screening of β-thalassemia patients and proper control programs, about 90 children suffering by different types of thalassemia in Koya City. The present study aims to investigate some molecular characterization and most common mutations that affect β-thalassemia patients. About 10 milliliters of venous blood sample was taking from 43 β-thalassemia patients before the scheduled blood transfusion using a disposable syringe used for DNA extraction and then molecular studies using a polymerase chain reaction-amplification refractory mutation system technique. The most common mutations in the B-thalassemia major patients were IVSII-1, codon 8/9, cd 44, and IVS1-5, among 43 patients that is representing 83.7% of β-thalassemia patients and IVSII-1 mutation was a most repeated one among all mutations.

Index Terms—Amplification refractory mutation system-polymerase chain reaction technique, β-Thalassemia, Molecular characterization, Mutations.

I. INTRODUCTION
Thalassemia is one of the most common genetic disorders on a worldwide. It represents the main cause of chronic hemolytic anemia in the middle east [1]. β-thalassemia major is the most important among the thalassemia syndromes because it is so common and usually produces severe anemia [2]. β-thalassemia major was characterized by progressive anemia manifested during the 2nd 6 months of life, associated with splenomegaly and chronic hemolytic anemia that sustain life, and individuals with β-thalassemia major usually present within the first 2 years of life with severe anemia, requiring regular red blood cells transfusions [1]. In β-thalassemia major, impaired biosynthesis of the β-globin leads to accumulation of unpaired alpha-globin chain, shortened red cell life span, and iron overload causing functional and physiological abnormalities in various organ systems [3]. Hemoglobin genetically is controlled by two developmentally regulated multigene clusters: The α-like globin cluster on chromosome 16 and the β-like globin cluster on chromosome 11 [4].

Thalassemia syndromes arise from mutations that affect every step in the pathway of β-globin gene expression, transcription, mRNA processing, mRNA translation, and post-translational integrity of the β-polypeptide chains [5]. Most types of β-thalassemias are due to point mutations, and deletion mutations found in rare cases, many mutations associated with β-thalassemia either reduce β-globin gene expression (β-type) or completely suppress β-globin gene (β-type) [6].

In Iraqi resident, the eight most frequent mutations are IVS-II-1 (G-to-A), codon.44 (-C), codon5 (-CT), IVS-I-1 (G-to-A), IVS-I-6 (T-to-C), codon 39 (C-to-T), codon 8/9 (+G), and IVS-I-5 (G-to-C), and twelve mutations of a total of 2000 screened mutations were detected by polymerase chain reaction-amplification refractory mutation system (PCR-ARMS) technique in the Dohuk of northern Iraq [7]. Amplification of specific-primer based on the matched primer is more efficient at annealing and directing extension than mismatched primer, the target DNA is amplified in two separate reactions using a common forward primer and either one of two reverse allele-specific primers, one complementary to the mutant sequence and the other to the wild DNA sequence. The presence of the mutant allele will generate a PCR product in the tube containing the mutation-specific primer and vice versa [8].

II. PROCEDURE
A total of 43 β-thalassemia major patients, who were manage at the hospital of Shaheed Dr. Khaled in Koya City, with ages between 1 and 19 years (median age of 9 years). Patients were include 22 males and 21 females, 10 ml of blood sample take from patients before scheduled of blood transfusion by using a disposable syringe and 5 ml of it frozen in EDTA anticoagulant tubes, then genomic DNA isolated from the completely fresh blood collected in EDTA anticoagulant tubes for molecular studies by Appling Wizard genomic DNA purification kits.
A. Primers Selection

A. Primer sets which are selected for ARMS analysis of mutations are shown in Table I [9].

B. Sequences of primers which are used for ARMS analysis

- Internal control primers:
  - Forward: ‘5--CAACGTATGCTCTT TGCACC-3’
  - Reverse: ‘5--GAGTCAAGGCTAGAGAT GCA GGA-3’

- Common primer:
  - Forward: ‘5--ACCTCACCCTGTGGAGGC GCC AC-3’
  - Reverse: ‘5--CCCTCTCTATGACACTA TA-3’

B. Primer Dilution

All primers were supplied by Promega Company as a lyophilized product of different picomols concentrations. Promega Company protocol was adopted for primers dilution and suspension using deionized water to reach a final concentration for 10 picomols/μl of suspension.

C. ARMS Components

Optimization of ARMS-PCR reaction was accomplished after several trials; thus, the following mixtures are adopted: Deionized water, Green master mix, internal control primer A, internal control primer B, common primer C or D, wild or mutant primer, and DNA sample which were prepared according to demanded procedure.

D. ARMS-PCR Protocol

For each mutation, a number of samples were testing according to phenotypes of the disease for both patient and control groups. Two ARMS-PCR reactions (two tubes) were performing for each sample: One for identifying the presence of the normal allele (using the normal primer) and the other for the presence of mutant allele (using the mutant primer). The molecular analyses of the samples were repeating to cover the seven types of β-thalassemia mutations using a specific ARMS primer set for each mutation (28).

ARMS-PCR Programmed:
To detect the mutations: IVSII-1, codon 8/9 the ARMS-PCR program (1) adopted was prepared according to demanded procedure. For codon 44 and IVSII-5 mutations, the ARMS-PCR program (2) adopted was prepared according to demanded procedure [4,10].

E. ARMS-PCR Control Reaction

To reveal the hazardous cross-contamination, and to check the activity of ARMS-PCR components, two external controls were used: Water and positive control samples. Negative and positive results were obtain, respectively [11].

F. Analysis of ARMS-PCR Products

The ARMS-PCR products and the ladder marker were resolved by electrophoresis. 7 μl of the product is loaded on 2% agarose gel (2 g agarose/100 ml ×0.5 TBE buffer with ethidium bromide solution [0.5 μg/ml]) and run at 70 V for 1 h, and bands are visualized on UV transilluminator and then photographed using photo documentation system [12].

III. Results

A. Codon 8/9 (+ G) Detection

The fragment size of internal control primers product was 861 bp., Codon 8/9 (+ G), and 225bp ARMS-PCR products observed as shown in Fig. 1. ARMS-PCR products of cd 8/9 mutation turn on a 2% agarose gel. All samples contain an internal control band (861 bp). Lane 1, DNA ladder marker (100 bp), Sample A, contains an amplified product in the wild (W) but lacks it in the mutant (M) primer. Sample B contains an amplified product in both wild (W) and mutant (M) primers, assigning the individual to heterozygous genotype. Sample C contains an amplified product in the mutant (M) primers indicating to the homozygous genotype (Fig. 1).

B. Cd44 Detection

Cd44, 445 bp ARMS-PCR products were observed as shown in Fig. 2. ARMS-PCR products Cd44 of mutations turn on a 2% agarose gel. All samples contain an internal control band (861 bp). First lane for DNA marker (100 bp), Sample A, contains an amplified product in both wild (W) and mutant (M) primers, assigning the individual to heterozygous genotype. Sample B contains an amplified product in the mutant (M) primers indicating to the homozygous genotype, and also, Sample C contains an amplified product in the mutant (M) primers indicating to the homozygous genotype (Fig. 2).

C. IVSII-5 Detection

IVSII-5, 285 bp ARMS-PCR products were observed as shown in Fig. 3. ARMS-PCR products of IVSII-5 mutations turn on a 2% agarose gel. All samples contain an internal

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sequence (5'-3')</th>
<th>Common primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSII-1(AC) Wild</td>
<td>5'-AAAGAAAACATGTCAGGCTCCA TAG ACT ACT GAC-3'</td>
<td>C</td>
</tr>
<tr>
<td>IVSII-1(AT) Mut</td>
<td>5'-AAAGAAAACATGTCAGGCTCCA TAG ACT GAT-3'</td>
<td>C</td>
</tr>
<tr>
<td>Codon8(9)(CT) Wild</td>
<td>5'-CTCTGCCCACAGGAGGATC TAA CGG CAC ACT-3'</td>
<td>C</td>
</tr>
<tr>
<td>Codon8(9)(CT) Mut</td>
<td>5'-CTCTGCCCACAGGAGGATC TAA CGG CAC ACC-3'</td>
<td>C</td>
</tr>
<tr>
<td>IVS-1-5 (AC) Wild</td>
<td>‘5-CTCTTAAAACGAGCTTGAACTGGAGGATC TAA CGG CAC ACT-3'</td>
<td>C</td>
</tr>
<tr>
<td>IVS-1-5 (AG) Mut</td>
<td>‘5-CTCTTAAAACGAGCTTGAACTGGAGGATC TAA CGG CAC ACT-3'</td>
<td>C</td>
</tr>
<tr>
<td>Cd44(GG) Wild</td>
<td>5'-ACATGAGGAGAGCTTGAACTGGAGGATC TAA CGG CAC ACT-3'</td>
<td>D</td>
</tr>
<tr>
<td>Cd44 (GA) Mut</td>
<td>5'-ACATGAGGAGAGCTTGAACTGGAGGATC TAA CGG CAC ACT-3'</td>
<td>D</td>
</tr>
</tbody>
</table>
control band (861 bp). First lane for DNA marker (100 bp). Sample A, contains an amplified product in both wild (W) and mutant (M) primers, assigning the individual to heterozygous genotype. Sample B contains an amplified product in the wild (W) but lacks it in the mutant (M) primer, and Sample C contains an amplified product in the mutant (M) primers indicating to the homozygous genotype sample (Fig. 3).

D. IVSII.1 Detection

IVSII.1, 634 bp ARMS-PCR products were observed as shown in Fig. 4. ARMS-PCR products IVSII.1 of mutations turn on a 2% agarose gel. All samples contain an internal control band (861 bp). First lane for DNA marker (100 bp). Sample A contains an amplified product in the mutant (M) primers indicating to the homozygous genotype, Sample B contains an amplified product in both wild (W) and mutant (M) primers, assigning the individual to heterozygous genotype. Sample C contains an amplified product in the wild (W) but lacks it in the mutant (M) primer (Fig. 4).

E. Genotype of Patients According to the Identified Variants

Table II and Fig. 5 present the genotype of patients according to the identified variants. More than half (75%) of the patients showed a homozygous genotype. Homozygotes for IVS II-1 mutation represented (11) = 30.5% of patients, homozygotes for Cd44 mutation represented (7) = 19.4% of patients, homozygotes for codon 8/9 mutation represented 16.6% of the patients, and homozygotes for IVS1-5 mutation represented (3) = 8.3%, whereas heterozygous genotype for

Fig. 3. Polymerase chain reaction-amplification refractory mutation system products of IVS1-5 β-thalassemia mutation on 2% agarose gel at 70 V for 1 h and half. Sample A, heterozygous genotype, Sample B, wild genotype, and Sample C, homozygous genotype. Marker: Ladder marker DNA 100 bp. cd: Codon, W: Wild, M: Mutant.

Fig. 4. Polymerase chain reaction-amplification refractory mutation system products of IVSII.1 β-thalassemia mutation on 2% agarose gel at 70 V for 1 h. Sample A, Homozygous genotype, Sample B, heterozygous genotype, Samples C, wild genotype. Marker: Ladder marker DNA 100 bp. cd: Codon, W: Wild, M: Mutant.

Fig. 5. The genotype of patients in study populations.
IV. DISCUSSION

Total characterized cases are 36 among 43 patients. It is found that 36 cases were β-thalassemia representing 83.7%, the most common mutation identified was IVSII.1 in 8 patients about 32.5%, and the finding that the IVS-II mutation is the most frequent mutation identified in our patients is not unexpected, since it was found to be the most frequent mutation in earlier studies from Duhok and Erbil and in almost all Iranian studies particularly those performed in Northwestern Iran [13]. IVSII.1 was most frequently encountered mutation in a previous study on parents of thalassemic children in Dohuk [6]. In Western Iran, the region nearest to our region, the prevalence of IVSII.1 was even much closer to our study [14]. However, our figures were much higher than those reported from Turkey where the overall prevalence of this mutation is 4.7%, and it was even lower in the East Anatolia region bordering Iraq at 3.4% [15].

The second most common mutation is codon 44 in 10 patients, about 23.2%, which has been labeled as Kurdish mutation [16], which was also the second most common mutation in previous study on obligate carriers of β-thalassemia by Al-Allawi, et al. (2006), who demonstrated Cd44 mutation in 12.5% of their 104 patients. The following two common mutations in our studies are codon 8/9 in 8 patients about 18.6% and IVS1-5 in 4 patients about 9.3%. These results confirmed by the reports of who carried out a study to determine the molecular basis of β-thalassemia in the Dohuk region of Iraq [6].

The frequent mutations codon 8/9 (+G) and IVS1-5 (G-to-C) were 7.7% and 6.7%, respectively, in Dohuk, and previous studies in Erbil and Sulaimania showed that mutation was the third most common mutation among Kurdish populations. This mutation frequency is higher than that reported from Turkey at 4% overall. Its frequency in Iran is even much lower at 1.7% overall and 0.5% in Western Iran but reaches as high as 9.1% in northwestern Iran. This mutation was considered among the oldest mutations in the Mediterranean region, more so in its Western part, and it is thought to be of Roman origin [16].

Genotype of patients identified according to the variants, more than (75%) of the patients showed a homozygous genotype. Homozygotes for IVS II-1 mutation represented 11 = (30.5%) patients, homozygotes for Cd44 mutation represented 7 = (19.4%) patients, homozygotes for codon 8/9 mutation represented 16.6% of the patients, and homozygotes for IVS1-5 mutation represented 3 = (8.3%). Similar high percentages of homozygous β-thalassemia patients were found in other populations of Arab and other developing countries [17]. Many study were reported that most B-thalassemia patients are homozygous. In United Arab Emirates, the study of which included 427 patients and reported homozygous and β-thalassemia in about 60% of the patients. The high degree of homozygosity in our patients reflects a high degree of consanguinity among the parents of thalassemic patients. Therefore, births from consanguineous marriages are more frequently homozygous for various alleles than those from no consanguineous marriages [18].

V. CONCLUSION

The most common mutation identified was IVSII, followed by Cd44, codon 8/9) and IVS1-5. Genotype of patients according to the identified variants, more than half of the patients showed a homozygous genotype, it is a reliable technique to diagnosis mutations in β-thalassemia disorder.

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