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Study of β-Thalassemia Mutations Using the Polymerase Chain Reaction-Amplification Refractory Mutation System and Direct DNA Sequencing Techniques in a Group of Egyptian Thalassemia Patients

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ABSTRACT

The aim of this study was the molecular characterization of β-thalassemia (thal) mutations in a group of 95 Egyptian thalassemic patients from Fayoum in Upper Egypt, Cairo, Alexandria and Tanta in Lower Egypt and the Nile Delta. To identify these anomalies the polymerase chain reaction-amplification refractory mutation system (PCR-ARMS) was used, complemented by direct DNA sequencing for uncharacterized cases.

In 80 of the 95 patients, the b-thal mutation was detected by PCR-ARMS. The most common allele encountered in our study was IVS-I-6 (T→C) (36.3%); the second most common mutation was IVS-I-110 (G→A) (25.8%). In addition, we report three homozygous cases for the promoter region –87 (C→G) allele with a frequency of 3.2%. DNA sequencing of uncharacterized cases (14 cases, 15 alleles) revealed six cases (six alleles) of codon 27 (G→T), and three cases (three alleles) of the IVS-II-848 (C→A) mutation. Codon 37 (G→A) in the homozygous state was found in one patient with positive consanguinity. The frameshift codon 5 (–CT) mutation was detected in two of our uncharacterized cases. The codon 15 (TGG→TGA) mutations was detected in one patient (one allele, 0.5%). All studied cases were fully characterized by this strategy.

Screening for β-thalassemic mutations using ARMS-PCR for the seven most frequent alleles in Egypt succeeded in determining the β-globin genotype in 84.2% of our patients (91.6% of the expected alleles). To improve the efficiency of routine screening, the PCR-ARMS mutation panel should be updated to include the reported rare alleles. Direct DNA sequencing is an additional way to allow a full characterization of β-thal patient in Egyptian population.

Key Words: *β-Thalassemia mutations - DNA sequencing - β-Thalassemia in Egypt.*

INTRODUCTION

β-Thalassemia (thal) is a group of inherited disorders characterized by a reduced β-globin chains synthesis. Around 7% of the world's population is affected by the disease which is highly prevalent in tropical and subtropical regions including the Mediterranean, Southeast Asia and Southern China **[1,2]**. At the molecular level, more than 190 mutations affecting the β-globin gene are associated with this disease **[2]**. However, the spectrum and frequency of these mutations vary among different populations. Immigration plays a major role in both the distribution and the extent of mutation variations within each country **[3]**. Previous studies done for the molecular characterization of β-thal in Egypt have screened the most common Mediterranean mutations with recovery of a varying percentage of uncharacterized alleles **[4,5]**.

The aim of this study was the molecular characterization of β-thal mutations in a group of Egyptian thalassemic patients originating from Fayoum in Upper Egypt, Cairo, Alexandria and Tanta in Lower Egypt and the Nile Delta, using a polymerase chain reaction-amplification refractory mutation system (PCR-ARMS) technique fitted for a panel of common Mediterranean b alleles and complemented by DNA se-

quencing of the β-globin gene(s) to find out the accuracy of such a technique in our population and to identify new alleles.

SUBJECTS AND METHODS

Our study was conducted on 95 thalassemic patients, 52 patients from the New Children's Hospital Haematology Unit at Cairo University, 22 patients from the Pediatric Hematology Unit at Tanta University, 12 patients from the Haematology Unit at Alexandria University and four from the Pediatric Outpatient Clinic at Fayoum University. (Author: is this correct?).

All the thalassemic patients had their clinical history recorded including their parents' relationship (consanguinity), frequency of blood transfusions and history of splenectomy. Prior to sampling for DNA analysis, patients or their parents gave written consent in agreement with regulation.

Laboratory Investigations:

Routine hematological investigations included a complete blood count CBC, reticulocyte count and hemoglobin (Hb) electrophoresis using cellulose acetate membranes (Helena Laboratories, Beaumont, TX, USA) in Tris-EDTA borate buffer, pH 8.4.

Molecular Investigations:

DNA was extracted from 2mL EDTA anticoagulated whole blood using QIAamp DNA Blood Mini Kit from Qiagen (Hilden, Germany) according to manufacturer's instructions. We used PCR ARMS for each mutation in the following manner: The patient's sample was amplified using a set of three primers fitted to amplify either the wild type or mutated allele as described by Newton et al. **[6]**. Seven common Mediterranean mutations were detected [IVS-I-110 (G→A), IVS-I-6 (T→C), IVS-I-1 (G→A), IVS-II-1 (G→A), IVS-II-745 (C→G), –87 (C→G) and codon 39 (C→T)] **[4,5,7,8]**. All uncharacterized alleles were identified using direct sequencing using the following protocol **[9]**. The whole β-globin gene was amplified in two fragments using the following primers **[10,11]**: fragment A: 21: RSA/β 5'-AGA CAT AAT TTA TTA GCA TGC ATG-3' (forward); 22: 5'-ACA TCA AGG GTC CCA TAG AC-3' (reverse); fragment B: 16: 5'-CAG TCA AGG CTG AGA GAT GCA GGA-3' (reverse); 30: 5'- ACC TCA CCC TGT GGA GCC AC-3' (forward) and internal sequencing primers. In total, 100μL reaction volume was treated according to the following conditions: Initial denaturation was done at 95ºC for 4min., then 35 cycles of 1min. at 94ºC, 2min. at 58ºC, and finally, 2min. at 72ºC, followed by extension for 10min. at 72ºC using a 9600 Thermal Cycler (Perkin Elmer Life Science Corporation?, Boston, MA, USA). The PCR product was then purified using the QIA quick PCR purification kit (Qiagen) prior to cycle sequencing reaction using an ABI PRISM™ 310 automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The sequencing primers design **[10,11]**. Forward primers: P30→5'-ACC TCA CCC TGT GGA GG AC-3'; Exit B-F→5'-CTA GCA ACC TCA AAC AGA CAC-3'; IntB-F→5'-CT GAG GAG AAG TCT GCC GTT-3'; Seq. F→5'-CAT GAG TGT GGA AGT CTC AG-3'; P15-F→5'-ATG ATA CAA TGT ATC ATG CCT CTT TGC ACC ATT CTA-3'; P13→5'-TGG ATT CTG CCT AAT AAA A C-3'; Seq. H-F→5'-AAG GCT GGA TTA TTC TGA GT-3'. Reverse primers: Int. B-R→5'-TCC GAC GAC CAC CAG ATG-3'; Ext. B-R→5'-G AAC CTG GGT CTC CAA GAA ACT-3'; P22→5'-CA GAT ACC CTG GGA ACT ACA-3'; P20→5'-TCA AAT CTT ACC CTT TGT CTG CTT-3'; P20→5'-TCA AAT CTT ACC CTT TGT CTG CTT-3'; Seq. G-R→5'-GAG ACT CTA TGT AAT TCA TTG-3'; Seq. C-R→5'-TGT GTC AGA CGG ATC ATG TA-3'; Seq. B-R→5'-CGA TTA TCG TCG ATG TTA GG-3'; Seq. A-R→5'-ACC ACA CCG ATT ACG GGA CC-3'; Seq. K-R→5'-ACG GAT ACG GAA TAA GTA GG-3'; P16-R→5'-AGG AAC GTA GAG AGT CGG AAC TGA G-3'.

RESULTS

The patients' clinical data and molecular results are indicated in Tables (1,2), respectively. The PCR-ARMS analyses succeeded in identifying 75% of the expected thalassemic alleles. The 15 partially or entirely (one patient) uncharacterized patients were identified using direct DNA sequencing. The results of the sequencing step are given in Table (2). At the end of the sequencing step all thalassemic alleles were characterized. Allele frequency of the β-thal mutations found in this study is indicated in Table (3).

Table (1): Summary of the clinical data.

Age in months	Mean: 95.25±63.74; median 72 months
Male to female ratio	1.3:1
Positive consanguinity	51.9%
Frequency of blood transfusion per year	11.88±6.16
Splenomegaly	73.7%
Splenectomy	25.2%

Table (2): Results of β-Thalassemia genotypes using PCR-ARMS and seven common mediterranean mutations complemented by DNA sequencing.

Genotype using PCR-ARMS	n	%
IVS-I-6(T→C)/IVS-I-6(T→C)	23	24.20
IVS-I-110(G→A)/IVS-I-110(G→A)	16	16.80
IVS-I-1(G→A)/IVS-I-1(G→A)	13	13.60
IVS-I-110(G→A)/IVS-I-6(T→C)	6	6.30
IVS-II-745(C→G)/IVS-II-745(C→G)	4	4.20
–87(C→G)/–87(CÆG)	3	3.20
IVS-I-6(T→C)/IVS-I-1(G→A)	5	5.30
IVS-I-6(T→C)/IVS-II-1(G→A)	4	4.20
IVS-I-110(G→A)/IVS-I-1(G→A)	3	3.20
IVS-I-1(G→A)/IVS-II-745(C→G)	1	1.05
IVS-II-745(C→G)/codon 39(C→T)	2	2.10
Totally uncharacterized patients	1	1.05
Partially uncharacterized patients	14	14.7
Genotype of Uncharacterized Cases at the End of DNA Sequencing Results		
Codon 37(G→A)/codon 37(G→A)	1	1.05
IVS-I-110(G→A)/codon 27(G→T)	3	3.20
IVS-I-6(T→C/codon 27(G→T)	2	2.10
IVS-I-110(G→A)/Hb S [b6(A3)Glu→Val, GAG→GTG]	1	1.05
IVS-I-6(T→C)/Hb S [b6(A3)Glu→Val, GAG→GTG]	1	1.05
IVS-I-110(G→A)/IVS-II-848(C→A)	2	2.10
IVS-I-1(G→A)/codon 27(G→T)	1	1.05
IVS-I-110(G→A)/frameshift codon 5(–CT)	2	2.10
IVS-I-6(T→C)/codon 15(TGG→TGA)	1	1.05
IVS-II-745(C→G)/IVS-II-848(C→A)	1	1.05
Totally uncharacterized patients	0	0.00
Partially uncharacterized patients	0	0.00

Table (3): Allel frequency of β-Thalassemia mutations.

Mutation	n	Allele frequency (%)
IVS-I-6 (T→C)	66	36.3
IVS-I-110 (G→A)	49	25.8
IVS-I-1 (G→A)	39	19.0
IVS-II-745 (C→G)	12	6.4
Codon 27 (G→T) [Hb Knossos, β27(B9)Ala→Ser, GCC→TCC]	6	3.2
–87 (C→G)	6	3.2
IVS-II-848 (C→A)	3	1.6
Frameshift codon 5 (–CT)	2	1.0
Codon 39 (C→T)	2	1.0
Codon 37 (G→A)	2	1.0
Codon 15 (TGG→TGA)	1	0.5

DISCUSSION

The molecular basis of β-thal were extensively studied in the Mediterranean region and Arab countries allowing the set-up of mutation detection protocols fitted for the common mutations present in these various regions. In Egypt, most protocols used a PCR-ARMS method for detecting seven common β-thal mutations **[4,5,7,8]**. However, uncharacterized cases may represent up to 23%. Reported percentages of uncharacterized cases were 13.8% **[12]**, 11.8% **[13]** and 23% **[8]**. In our study, coverage of the PCR-ARMS technique was 91.6% alleles (175/190 expected alleles). We were able to find the genotype of 80/95 (84.2%) of the studied cases, with only 15.8% of partially characterized cases (14/95 cases) and one completely uncharacterized case. Using direct DNA sequencing as a second step, we characterized all 16 missing alleles.

The frequency of the β-thal mutations reported in this study was more or less similar to the limited number of previous reports about b-thal mutations in Egypt **[4,5,8,12]**. However, a few exceptions do exist. The most common allele encountered in our study was IVS-I-6 (T→C) with a frequency of 42 cases and 66 alleles (36.3%). This is a relatively higher frequency when compared to several other studies carried out on Egyptian patients. Waye et al. **[13]** reported a frequency of 13.6%, Weatherall and Clegg **[1]** reported a frequency of 15.1%. On the other hand, the IVS-I-110 (G→A) mutation is considered to be the most common mutation by most studies, with frequencies up to 41% **[4]**; this mutation was the second most common one (25.8%) found in our study. In addition, we reported three homozygous cases for the promotor region –87 (C→G) allele with a frequency of 3.2%. In the study by Hussein et al. **[4]**, a frequency of 1.4% was reported for this allele, while Weatherall and Clegg **[1]** reported this mutation to occur with a frequency of 0.8%.

Using direct DNA sequencing **[9]** for undetected cases, we were able to find some rare β-globin gene mutations. Six cases (six alleles) showed a mutation at codon 27 (G→T) [Hb Knossos, b27(B9)Ala→Ser, GCC→TCC], three cases (three alleles) were found to have the IVSII-848 (C→A) mutation, and one case displayed the rare codon 37 (G→A) mutation. This

mutation changes the codon 37 (TGG), coding for tryptophan, into TGA which is a stop codon, thus terminating translation **[14]**. Codon 37 (G→A) was found in one homozygous case (two alleles); this patient was an offspring of a consanguineous marriage, thus explaining the homozygosity for this rare allele. The frameshift codon 5 (–CT) mutation was detected in two patients. The last mutation, codon 15 (TGG→TGA), was detected in one patient (one allele, 0.5%). To the best of our knowledge, this mutation has not been reported in Egypt before this study. At the end of the direct DNA sequencing step no β-globin alleles were uncharacterized as two patients were found to be compound heterozygotes for Hb S [β6(A3) Glu→Val, GAG→GTG] and a β-thal mutation.

In conclusion, mutation panels used for routine PCR-ARMS screening for β-thal mutations should be updated to include the reported rare alleles in different studies. Complementing PCR-ARMS with direct DNA sequencing can minimize the possibility of uncharacterized cases, and thus enhance molecular characterization studies of β-thal.

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