

MOLECULAR GENETIC CHARACTERIZATION OF β -THALASSEMIA AND SICKLE CELL SYNDROME IN THE ALBANIAN POPULATION

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ABSTRACT

β -Thalassemia (β -thal) is a major public health problem in Albania as it is in many Mediterranean countries. We determined the different β -thal alleles that are present in the Albanian population by using the temporal temperature gradient electrophoresis (TTGE) method because of its high throughput, cost-effectiveness, sensitivity and simplicity. DNA from blood of 68 patients with β -thal, 26 with sickle cell anemia or sickle cell β -thal, 54 parents of these patients and 14 heterozygotes related to these families. We found the IVS-I-110 (G>A), codon 39 (C>T), IVS-I-6 (T>C), IVS-I-1 (G>A) and codon 44 (–C) mutations that accounted for nearly 90% of the β -thal alleles. Their frequencies were similar to those found in other studies in the Albanian population. This method has permitted the detection of heterozygotes for β -thal in this population and offers a prenatal diagnosis with a probability of 90% accuracy.

Key words: β -Thalassemia (β -thal); Temporal temperature gradient electrophoresis (TTGE); Polymorphisms; Enzyme digestion.

INTRODUCTION

β -Thalassemia (β -thal) (OMIM 141900), the most common hereditary anemia in the Mediterranean region, results from over 200 causative mutations in the β -globin gene (HBB) [1]. These are responsible either for reduction (β^+) or complete absence (β^0) of the β chain and lead to a mild-to-severe hemolytic anemia [2]. Despite the molecular heterogeneity, the prevalent molecular defects are limited in number in each population that is at risk. Such mutations can be detected by a number of polymerase chain reaction (PCR)-based procedures. The most used methods are reverse dot-blot analysis or primer-specific amplification, with a set of probes complementary to the most common mutations in a particular population, and denaturing high performance liquid chromatography (HPLC) [3]. Should these methods fail to detect a mutation, sequence analysis can detect any mutation in the HBB coding region.

Like most other neighboring countries in the region, Albania is affected by thalassemia and hemoglobin (Hb) disorders. It is estimated that the overall carrier frequency of β -thal and sickle cell anemia is about 7-8%. Most patients are located in the provinces along the Adriatic and Ionian coasts where malaria used to be endemic [4]. The aim of this study was to determine the different β -thal alleles that are present in the Albanian population using a highly sensitive, cost-effective

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and suitable method for the high throughput screening of a large number of samples.

MATERIALS AND METHODS

DNA was obtained from the blood of 68 Albanian patients with β-thal, 26 with sickle cell anemia or sickle cell/β-thal, 54 parents of these patients and 14 heterozygotes related to these families. Most of the patients were from districts from southwest Albania. In 108 patients and heterozygotes, the sex ratio was 47 males to 61 females. Their age group ranged from 2 to 15 years old.

We used the TTGE method (temporal temperature gradient electrophoresis) for the detection of mutations and polymorphisms in the sequences of the β-globin gene as described by Shaji *et al.* [5]. This method was developed at the Laboratory of Biochemistry and Molecular Genetics, Henri Mondor Hospital (APHP), Creteil, Paris, France.

The DCode™ instrument (Bio-Rad Laboratories, Hercules, CA, USA) and TTGE technique are simple to use and have enormous potential for high throughput screening since gel casting is easy; the constant denaturing gel used eliminates the need to pour chemical gradient gels. DNA extraction was performed using the kit Nucleon BACC3, DNA (manufactured for Amersham BioSciences UK Ltd. by Tepnel Life Sciences, Manchester, UK).

The β-globin gene was amplified as seven fragments (A, B, C, D, E, F, and G) [6]. Fragments A-F contain the coding and non coding sequences of the β-globin gene, and fragment G can detect three gene polymorphisms [IVS-II-16 (C>G), IVS-II-74 (G>T) and IVS-II-81 (C>T)], while fragments B, C, and D contain the polymorphic sites at codon 2 (C>T), IVS-II-16 and IVS-II-666 (T>C), respectively (Figure 1) [5].

The PCR products were analyzed in 16-20 cm 6% polyacrylamide gels prepared in 1.5X Tris acetate-EDTA buffer containing 6 mol/L of urea. We loaded 10 μL of PCR product with 5 μL of 2X gel loading dye onto the gel. Electrophoresis was carried out at 130 V with constant temperature increments of 2°C/hour on the DCode™ mutation detection system (Bio-Rad Laboratories) that has an automatic thermal regulator. The temperature range for TTGE for each PCR fragment was determined by reducing the upper and lower temperatures by 12°C because each mol of urea lowers the melting temperature by 2°C. The temperature ranges for the different PCR fragments were: 57°C to 69°C for fragment A, 56°C to 70°C for fragments B and C, 48°C to 59°C for fragments D, F and G and 57°C to 69°C for fragment E.

Of the nine previously reported mutations common in the Albanian population, eight (96.5%) can be searched for on fragments B and C of the β-globin gene [4,7,8]. Thus, fragments G, B and C were analyzed first, followed by the remainder. Fragment G was used

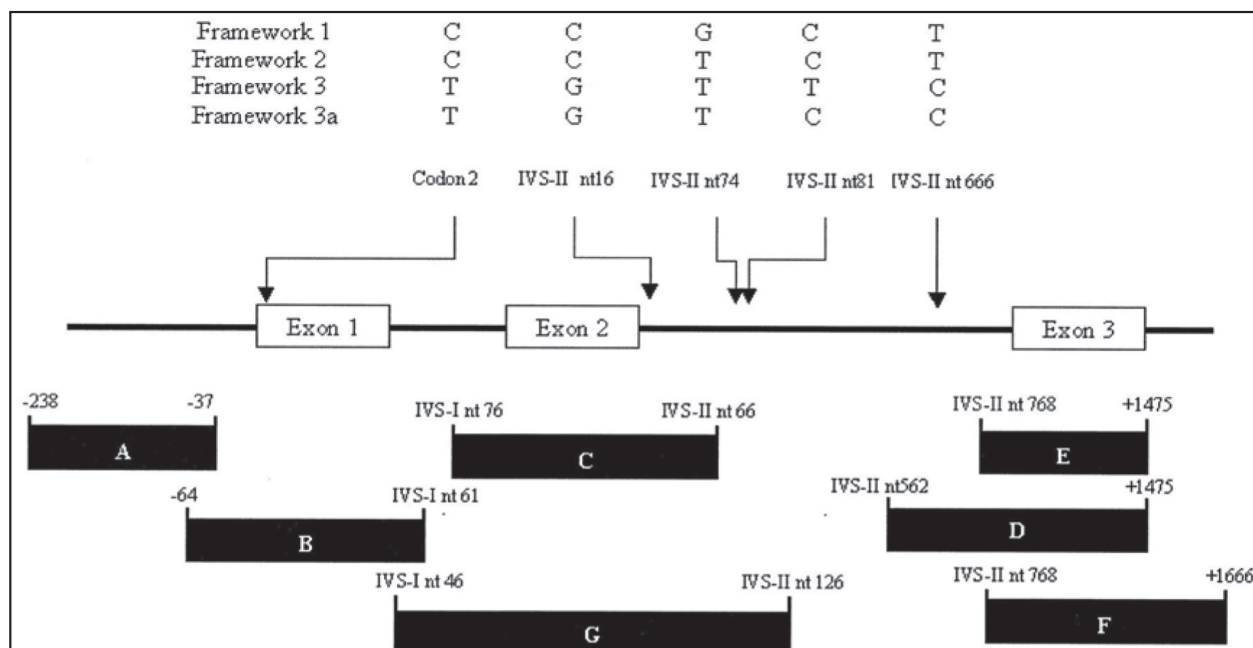


Figure 1. Fragments analyzed by TTGE and the locations of the polymorphisms within the β-globin gene

for the study of the frameworks and interpretation of the results of fragments B, C and D. Fragments B and C were used because they contain the more frequent mutations found in the Albanian population.

The PCR products from healthy controls were also loaded onto every TTGE gel. Because fragments B, C and D each contain one polymorphic site, DNA samples heterozygous for these polymorphisms (frameworks 1/3) were used as controls, whereas for fragments A, E, and F, a control from a healthy individual was used. Confirmation of the β -thal alleles was obtained following restriction endonuclease digestion.

RESULTS

Examples of the patterns produced by various mutations are presented in Figure 2. In all the samples studied from different families, the pathological alleles were identified and all the genotypes were determined. As a result, there were 68 patients with β -thal (of these, 16 were β -thal homozygotes and 52 were β -thal compound heterozygotes), 26 patients with sickle cell syndrome (of these, nine were β^S homozygotes and 17 were β^S/β -thal patients), and 11 β -thal heterozygotes and three Hb S [$\beta 6(A3)Glu \rightarrow Val$, $GAG \rightarrow GTG$] heterozygotes. Table 1 lists the homozygous and heterozygous genotypes of patients with β -thal and Table 2 lists the eleven β -thal mutations that were observed in 144 chromosomes.

The IVS-I-110 (G>A) mutation was the most frequent β -thal allele in the patients with β -thal major (β -TM), followed by IVS-I-6 (T>C), codon 39 (C>T), IVS-I-1 (G>A) and codon 44 (–C). Nearly 90% of the chromosomes tested carried one of these five alleles. All of these mutations have been observed before in the Albanian population, except for the IVS-I-2 (T>C) mutation which was detected in fragment B in a patient with β -thal with framework 1/1; this patient was a compound heterozygote for IVS-I-2/ codon 44. The mother, with framework 1/1, also displayed a fragment B TTGE pattern different from that of the control with framework 1/3. DNA sequencing of this fragment led to the characterization of the mutation (Figure 3).

DISCUSSION

The frequency of the 11 alleles detected in our study are compared with those seen in other studies in the Albanian population [4,7,8]. The results for the five

Table 1. Homozygous and compound heterozygous genotypes of the patients with β -thalassemia.

| β-Thalassemia Genotypes | |
|---|-----------------|
| Homozygotes | <i>n</i> |
| IVS-I-110(G>A)/IVS-I-110(G>A) | 12 |
| Codon 39(C>T)/codon 39(C>T) | 2 |
| IVS-I-6(T>C)/IVS-I-6(T>C) | 1 |
| IVS-I-1(G>A)/IVS-I-1(G>A) | 1 |
| Subtotal | 16 |
| Compound Heterozygotes | |
| IVS-I-6(T>C)/IVS-I-110(G>A) | 12 |
| IVS-I-6(T>C)/codon 39(T>C) | 10 |
| IVS-I-110(G>A)/IVS-I-1(G>A) | 5 |
| IVS-I-110(G>A)/codon 39(C>T) | 5 |
| IVS-I-110(G>A)/codon 44(–C) | 3 |
| IVS-I-6(T>C)/IVS-I-5(G>C) | 2 |
| Codon 39(C>T)/codon 44(–C) | 2 |
| IVS-I-110(G>A)/codons 82/83(–G) | 2 |
| IVS-I-110(G>A)/codon 5(–CT) | 2 |
| Codon 39(C>T)/IVS-II-1(G>A) | 2 |
| IVS-I-110(G>A)/IVS-II-1(G>A) | 1 |
| IVS-I-110(G>A)/IVS-II-745(C>G) | 1 |
| IVS-I-6(T>C)/codon 4(–C) | 1 |
| IVS-I-6(T>C)/codon 5(–CT) | 1 |
| IVS-I-1(G>A)/IVS-II-745(C>G) | 1 |
| IVS-I-1(G>A)/codon 39(C>T) | 1 |
| IVS-I-2(T>C)/codon 44(–C) | 1 |
| TOTAL | 68 |

most common alleles are more or less similar to those in other studies.

A total of 379 chromosomes with a β -thal mutation that were analyzed in all studies in the Albanian population revealed 13 mutations, five of which (IVS-I-110, codon 39, IVS-I-6, IVS-I-1 and codon 44) accounted for 92% of all β -thal chromosomes. The frequency data for the alleles present in Albania have been compared with those reported in neighboring countries [9-17].

Frequencies of the most common β -thal alleles (IVS-I-110, codon 39, IVS-I-6) were similar to those of the surrounding Balkan and Mediterranean countries. The IVS-I-110 mutation has its highest frequency in the east Mediterranean region (Cyprus, Lebanon,

Table 2. Frequency of β-globin alleles in patients with β-thalassemia and sickle cell syndrome.

| Alleles | % |
|-------------------|-------|
| IVS-I-110 (G>A) | 43.50 |
| IVS-I-6 (T>C) | 18.50 |
| Codon 39 (C>T) | 16.66 |
| IVS-I-1 (G>A) | 8.33 |
| Codon 44 (–C) | 4.16 |
| IVS-I-5 (G>C) | 2.08 |
| IVS-II-1 (G>A) | 2.08 |
| Codon 5 (–CT) | 2.08 |
| IVS-II-745 (C>G) | 1.38 |
| Codons 82/83 (–G) | 1.38 |
| IVS-I-2 (T>C) | 0.69 |

The total number of characterized β-thal chromosomes was 144.

Greece, Republic of Macedonia, Turkey), whereas the codon 39 mutation is found mainly in the west (Sardinia, Sicily, Spain). The mutation IVS-I-6, also known as the Portuguese β-thal, is a distant third. It is present in many populations without having an unusually high frequency in any particular country [4]. Other and rarer mutations have their origins in other populations, such as codon 44 in Kurdish Jews.

According to the method used for the detection of mutations in the β-globin gene in our study, TTGE appears to be a good choice in terms of throughput, cost-effectiveness, sensitivity and simplicity. In a population with a wide heterogeneity, use of hybridization with allele-specific probes or specific primers needs a long time and a great number of probes or primers. To exclude these problems, we adapted a new strategy for screening β-globin gene mutations in a short time. Temporal temperature gradient electrophoresis is a reliable method for determining new or rare mutations in a population study.

The molecular screening approach was performed in two steps: indirect scanning by TTGE followed by enzyme digestion. We were able to find a total of 11 mutations: IVS I-110, codon 39, IVS I-6, IVS I-1, codon 44, IVS-I-5 (G>C), IVS-II-1 (G>A), codon 5 (–CT), IVS-II-745 (C>G) and codons 82/83 (–G) by applying this effective approach. Besides these mutations, TTGE revealed one uncharacterized mutation, IVS I-2, which was then sequenced.

Common point mutations such as IVS-I-110, codon 39, IVS-I-6, IVS-I-1 and codon 44, were present in nearly 90% of β-thal alleles, which will facilitate a prenatal diagnostic program [18-21]. It makes possible the detection of the heterozygotes for β-thal in the Albanian population screening program and offers a prenatal diagnosis with a probability of 90% accuracy.

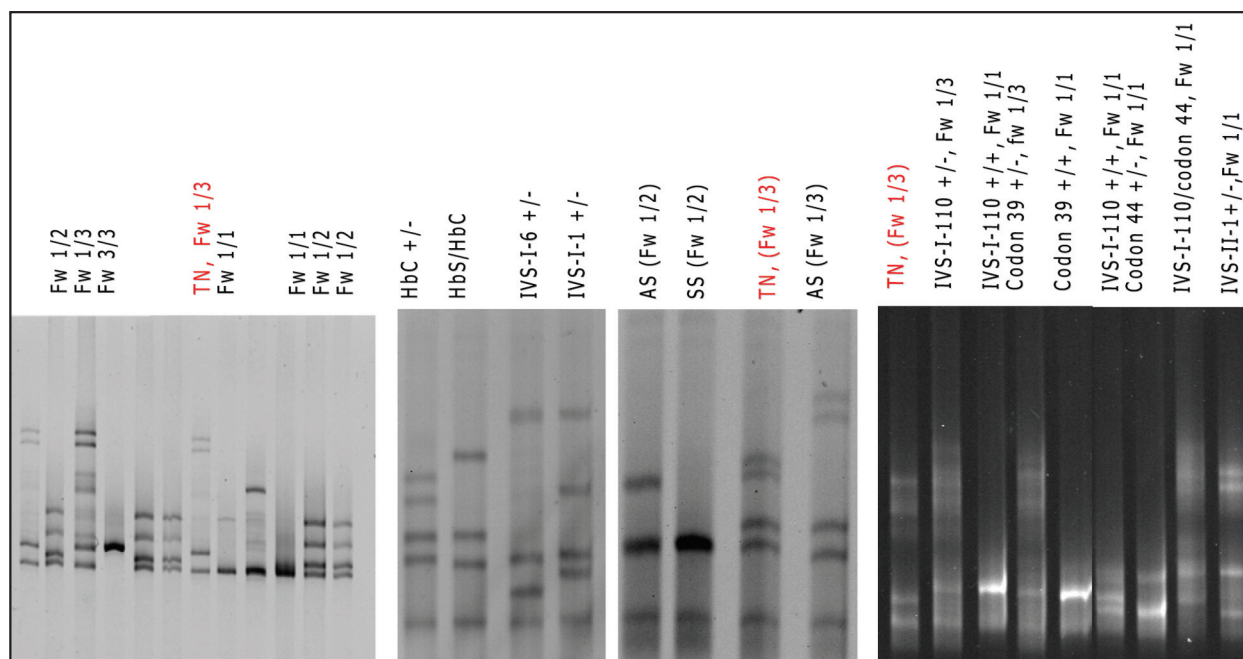


Figure 2. Patterns of different mutations analyzed by TTGE

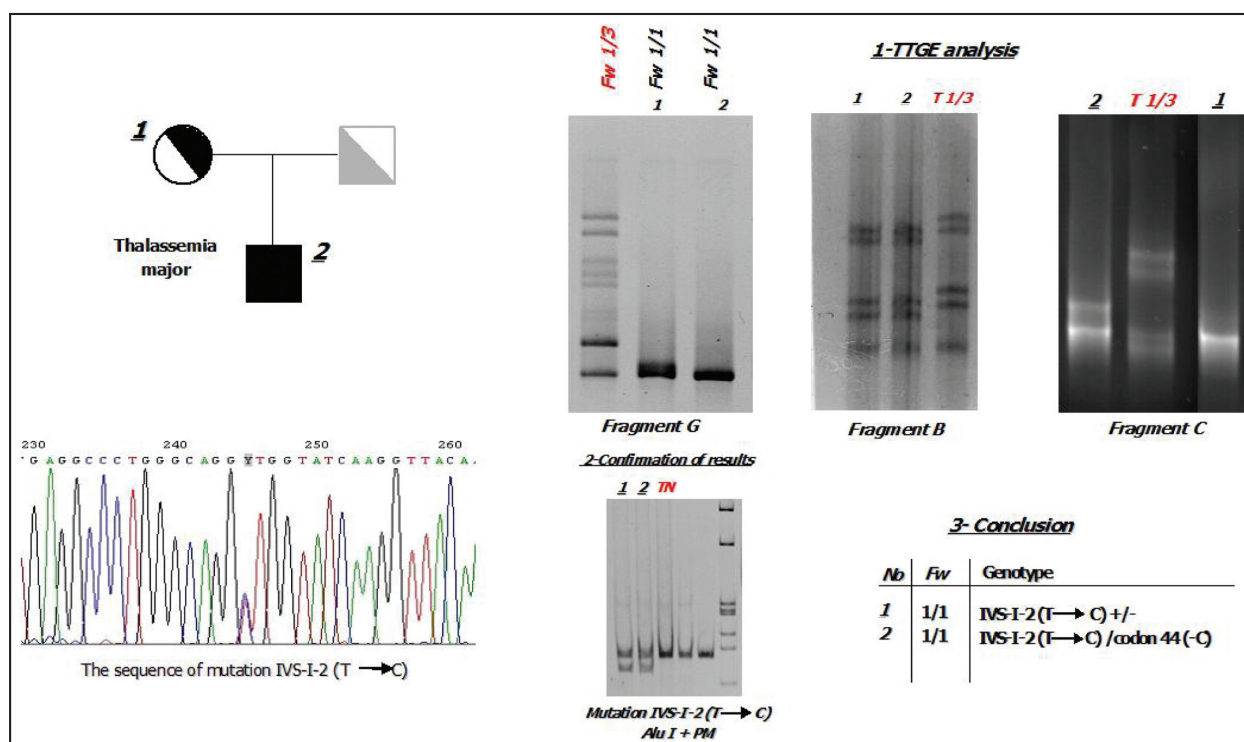


Figure 3. Diagnosis of β -thalassemia by TTGE analysis, enzyme digestion and DNA sequencing

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