Investigation of β-Globin Gene Mutations in Patients with β-Thalassemia in Şanlıurfa Province of Turkey

Türkiye'nin Şanlıurfa İlinde β-Thalassemia'lı Hastalarda β-Globin Gen Mutasyonlarının Araştırılması

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ABSTRACT Objective: The β-thalassemia (β-thal) is a diverse group of hemoglobin disorders characterized by a reduced or absent synthesis of the globin chains of hemoglobin (Hb) molecule. Hemoglobin beta gene (HBB) is responsible for synthesis of beta globin chain, and has many mutations, changing from a population to other. β-thal is an important health problem in the Şanlıurfa province placed in Southeastern Anatolia Region of Turkey. We aimed to investigate the frequency of the HBB gene NM_000518.4:c.93-21G>A (IVS1-110G>A or g.252G>A), c.25_26delAA (Cd8 -AA or g.75_76delAA), and c.315+1G>A (IVS2-1G>A or g.496G>A) mutations which were the most common in Turkey by using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR). **Material and Methods:** Blood samples were collected from 51 patients with β-thal (25 males and 26 females), and DNA was isolated by using salting out procedure. The normal and mutant alleles of the HBB gene c.93-21G>A, c.25_26delAA, and c.315+1G>A were amplified by using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method in two distinct PCR tubes. The PCR products were examined on the agarose gel for presence of DNA marker. **Results:** The frequency of the c.93-21G>A, c.25_26delAA, and c.315+1G>A mutant alleles in 28 of 51 patients was detected as 26.5%, 3.9%, and 3.9%, respectively. **Conclusion:** This study was the first research of β-thal mutations in this province, and showed that the c.93-21G>A mutation was the most frequent in this province among three mutations by ARMS-PCR method, and different from those reported in Central Anatolia, Aegean and Mediterranean regions of Turkey. This research may facilitate the implementations of genetic counseling and prenatal diagnosis in the population of Şanlıurfa.

Key Words: PCR, globins, mutation, beta-thalassemia, DNA mutational analysis

ÖZET Amaç: Beta-talassemiler, hemoglobin (Hb) molekülü globin zincirleri sentezinin azalması ya da sentez edilememesi ile karakterize edilen hemoglobin düzensizliklerinin farklı bir grubudur. Hemoglobin beta geni (HBB), beta globin zinciri sentezinden sorumludur ve çok sayıda mutasyon noktalarına sahiptir. Gen üzerindeki mutasyon oran ve çeşitleri bir toplumdan diğerine değişmektedir. β-talassemi, Türkiye'nin Güney Doğu Anadolu Bölgesinde yer alan Şanlıurfa ilinde önemli bir sağlık problemidir. Biz, Türkiye'de en yaygın olarak bilinen HBB geni NM_000518.4: c.93-21G>A (IVS1-110G>A veya g.252G>A), c.25_26delAA (Cd8 -AA veya g.75_76delAA) ve c.315+1G>A (IVS2-1G>A veya g.496G>A) mutasyonların frekanslarını amplifikasyon refrakter mutasyon sistemi-polimeraz zincir reaksiyonu (ARMS-PCR) kullanarak belirlemeyi amaçladık. **Gereç ve Yöntemler:** Kan örnekleri, β-talassemi'li 51 hasta çocuktan (25'i erkek ve 26'sı kız) toplandı ve DNA tuzla çöktürme yöntemi ile izole edildi. HBB geni c.93-21G>A, c.25_26delAA ve c.315+1G>A normal ve mutant allelleri iki ayrı PCR tüpünde ARMS-PCR metodu ile çoğaltıldı. PCR ürünleri, DNA markeri varlığında agaroz jeli üzerinde incelendi. **Bulgular:** Elli bir hastanın 28'inde c.93-21G>A, c.25_26delAA ve c.315+1G>A mutant allellerinin frekansları sırasıyla %26.5, %3.9 ve %3.9 olarak belirlendi. **Sonuç:** Bu çalışma, bu ilde β-talassemi mutasyonları üzerine yapılan ilk çalışmadır ve c.93-21G>A mutasyonun bölgede ARMS-PCR metodu ile üç mutasyon arasında en sık mutasyon olduğu ve bunun Türkiye'nin İç Anadolu, Ege ve Akdeniz bölgelerinden elde edilen sonuçlardan farklı olduğunu gösterdi. Bu araştırma, Şanlıurfa toplumunda genetik danışma ve prenatal teşhis uygulamalarını kolaylaştırabilir.

Anahtar Kelimeler: PCR, globinler, mutasyon, beta-talasemi, DNA mutasyonal analizi

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β-thalassemia (β-thal) is a common autosomal-recessive disorder resulting from over 200 different mutations of the *HBB* (Hemoglobin beta) gene, and characterized by either reduced (β^+-thal) or absent (β^0-thal) β-globin chain(s) synthesis.[1] The *HBB* gene is located on chromosome 11 (11p15), and consists of three exons and two introns.[2] The majority of these mutations on *HBB* gene cause defects in transcription, RNA splicing and translation because of frameshifts and nonsense codons or produce highly unstable b-globin products.[3]

β-thal runs a chronic course requiring repeated blood transfusions that usually leads to iron overload, and no other effective therapy is presently available.[4] In addition, if untreated, affected individuals manifest failure to thrive and shortened life expectancy.[5]

The frequency and spectrum of β-thal mutations vary among different countries, which include Turkey, Cyprus, Greece, Bulgaria, Iran, Syria, Azerbaijan, and Lebanon. The most common mutations seen in these countries were IVS1-110 (G>A), IVS1-1 (G>A), Codon 5 (-CT), Codon 39 (G>T), IVS1-6 (T>C), IVS2-1 (G>A).[6-16] In addition, some individuals with β-thal carry compound mutations in the Mediterranean populations.[17,18] Immigration and consanguineous marriages play a major role in both the distribution and the extent of mutation variations within each country.[19]

β-thal is a major public health concern in Turkey; throughout the country the β-thal carrier rate is rather high (2-10%), and mutation frequency determined on *HBB* gene in Turkey vary considerably from region to region.[7,17] The IVS1-110 (G>A) mutation (39.3%) is the most common β-thal deficiency in Turkey, followed in reducing order by IVS1-6 (T>C), frame shift codon 8 (-AA), IVS1-1 (G>A), IVS2-745 (G>C), IVS2-1 (G>A), codon 39 (C>T), frame shift codon 5 (-CT), all of which have frequency above 2%.[7,17,18] In order to control β-thal, a comprehensive study dealing with molecular diagnosis of mutation causing this disease is needed for carrier detection and establishment of prenatal diagnostic program.[20]

The population of the Şanlıurfa province in the Southeastern Anatolia Region of Turkey is about 383.870, and he total fertility rate is 4.83 in this province, whereas, 2.53 in Turkey as a mean. In addition, the rate of population growth is 3.6 percent per year, much higher than the national average of 1.8 percent.[21]

Conventional PCR-based techniques, including amplification refractory mutation system-polymerase chan reaction (ARMS-PCR), restriction endonuclease digestion analysis (RFLP), denaturing gradient gel electrophoresis, and direct sequencing have been applied for molecular diagnosis and carrier screening of thalassemia.[3,22,23]

We focused to investigate on the rate of the c.93-21G>A, c.25_26delAA, and c.315 + 1G >A mutations among individuals by using ARMS-PCR due to the high fertility rate and population growth in this province, and to detect the most frequent of these mutations in Turkey.

MATERIAL AND METHODS

PATIENTS AND DNA EXTRACTION

β-thal patients were diagnosed on the basis of complete blood count and Hb electrophoresis in 51 patients under the care of Hematology unit of the Hospital of Harran University from September 2007 to August 2007. The children consisted of 25 males and 26 females, ages ranging from two to 15 years (average ages: 7 ± 3.2). EDTA-blood samples were collected from these patients before blood transfusion and genomic DNA was extracted by the standard salting out procedure.[24] The isolated DNA was dissolved in deionised water in quantity of 20 ng/µl and in purity 1.7-2.0.

AMPLIFICATION REFRACTORY MUTATION SYSTEM - PCR (ARMS-PCR)

The *HBB* gene NM_000518.4: c.93-21G>A (IVS1-110G>A or g.252G>A), c.25_26delAA (Cd8 -AA or g.75_76delAA), and c.315+1G>A (IVS2-1G>A or g.496G>A) mutations were investigated by the ARMS-PCR technique as described previously.[3] The target DNA was 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, the other to the normal DNA sequence. An internal PCR control was used, which amplifies another region of the genome (861-bp) as positive control.

The ARMS-PCR reactions were carried out in a 10-ml reaction volume containing 1xPCR buffer (Fermentas, St. Leon-Rot, Germany), 50 ng of DNA, 0.2 μM of each primer (Bio Basic Inc, Ontario, Canada, Table 1), 3 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTPs, Fermentas) and 0.3 unit of Taq DNA Polymerase (Fermentas). The reactions for the three different mutations were performed under identical PCR thermal conditions, that is, an initial denaturation step at 94°C for 3 min, followed by 25 cycles of 94°C denaturation for 45 s, 65°C annealing for 1 min, 72°C extension for 2 min, and final extension at 72°C for 5 min. Following the PCR reactions, the PCR products were separated on 2% agarose gel, and analyzed with Alpha Imager System (AlphaInnotech, San Leandro, California USA). The PCR profiles on the gel were compared with a DNA ladder (100-1500 bp, Bio Basic Inc) (Figure 1, 2).

ETHICS

Faculty of Medicine institutional review board approved the study, and written informed consent was obtained from all patients. The study complied with world medical association declaration of Helsinki ethical principles for medical research involving human subjects.

RESULTS

We were able to identify mutations successfully in all the cases by using the ARMS-PCR technique (Figure 1 and 2). We characterized 35 (34.3%) alleles out of 102 for three mutations, i.e., c.93-21G>A, c.25_26delAA, and c.315 + 1G >A. The percentage prevalence of these mutations shows that c.93-21G>A is the most frequent mutation (26.5%) while the second common mutation in these alleles is c.25_26delAA with frequency 3.9% and the third common mutation is c.315+1G>A (3.9%). The results are summarized in Table 2. We observed six homozygous and fifteen heterozygous individuals with the c.93-21G>A, only four heterozygous individuals with c.25_26delAA, and one homozygous and two heterozygous individuals with the c.315-1G >A (Table 2). We compared our results with those obtained in other regions of Turkey (Table 3), and in other ethnic populations of neighboring countries (Table 4). We did not detect any compound mutations in this study. These three mutations could not be detected in 23 of 51 patients by using ARMS-PCR technique.

DISCUSSION

Each ethnic group has its own characteristic spectrum of mutations, with a few that are unique to a particular region together with several rare ones.[25] Recent molecular studies on Turkish *HBB* gene have revealed the presence of more than 30 different mutations associated with this disorder.[7,18] β-thal is a major public health concern in Turkey; for the country, the carrier frequency of β-thal is estimated to be 2.1%, but in certain regions, this figure increases to 10%.[7,17,18] In this study, we identified and characterized the molecular basis of β-thal in 28 of 51 patients using ARMS-PCR. There were to-

TABLE 1: The sequences of primers used in this study.[3]

Mutation	Primer sequences	PCR product (bp)
c.93-21G>A	5'-ACCTCACCCTGTGGAGCCAC-3' (common)	419
	5'-ACCAGCAGCCTAAGGGTGGGAAAATACACC-3' (normal)	
	5'-ACCAGCAGCCTAAGGGTGGGAAAATAGAGT-3' (mutant)	
c.25_26delAA	5'-CCCCTTCCTATGACATGAACTTAA-3' (common)	520
	5'-ACACCATGGTGCACCTGACTCCTGAGCAGA-3' (normal)	
	5'-ACACCATGGTGCACCTGACTCCTGAGCAGG-3' (mutant)	
c.315+1G>A	5'-ACCTCACCCTGTGGAGCCAC-3' (common)	634
	5'-AAGAAAACATCAAGGGTCCCATAGACTGAC-3' (normal)	
	5'- AAGAAAACATCAAGGGTCCCATAGACTGAT-3' (mutant)	
Internal control	5'-GAGTCAAGGCTGAGAGATGCAGGA-3' (1)	861
	5'-CAATGTATCATGCCTCTTTGCACC-3' (2)	

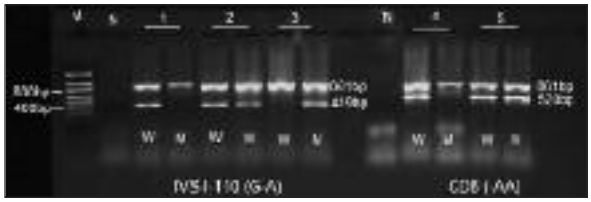

FIGURE 1: ARMS-PCR profiles of c.93-21G>A, and c.25_26delAA alleles of HBB gene.

Lane M, DNA ladder; lane N, negative control (water); the c.93-21G>A: lane 1, GG genotype (wild type, normal); line 2, GA genotype (heterozygote, carrier); lane 3, AA genotype (homozygote mutant); the c.25_26delAA: lane 4, wild type (normal); lane 5, heterozygote (carrier) (W: wild type allele, M: Mutant allele)

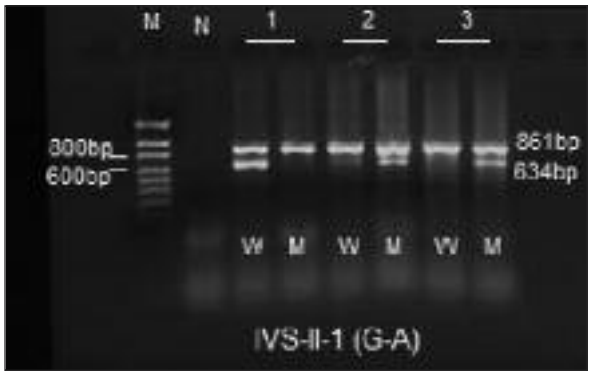

FIGURE 2: ARMS-PCR profile of c.315+1G>A allele of HBB gene.

Lane M, DNA ladder; lane N, negative control (water); lane 1, GG genotype (wild type, normal), lane 2: GA genotype (heterozygote, carrier); lane 3, AA genotype (homozygote mutant) (W: wild type allele, M: Mutant allele).

tally 35 mutant alleles (34.3%) for c.93-21G>A, c.25_26delAA, and c.315+1G>A on *HBB* gene.

The c.93-21G>A was diagnosed as the most common mutation (26.5%) in this province (Table 2). This was the first study of β-thal mutations from Şanlıurfa province. The frequency (26.5%) of c.93-21G>A was lower than the average of Turkey (39.3).[7] The highest frequency is recorded in the central (52.3%) and Western regions of Turkey (34.1-42.4%), and the lowest values in Eastern regions of Turkey (26.4-26.6%).[7,17,26] However, our patient group and others had already reported this mutation with different frequency rates in various regions of Turkey as 52.3% in the Central Anatolia, 42.4% in Aegean and Mediterranean Regions, 34.1% in Marmara Region, 31% in Black Sea Region, 27.1% in Eastern Anatolia Region, and 26.4% in the Southeastern Anatolia Region (Table 3). Our results were similar with those reported in the literature for Southeastern and Eastern Anatolia Regions of Turkey (Table 3). The ethnic identities of the latter regions seemed to be more preserved than the Western and Southern coastal parts of Turkey, which displayed greater heterogeneity. The c.93-21G>A mutation has been known to be a typical Eastern Mediterranean defect. The relative frequency of the c.93-21G>A mutation decreases rapidly along the Northeast to Southwest axis, reaching its maximum among Romania (31.3%), Turkish Cypriots (77%), Turkey (39.2%), Greece (42%), Bulgaria (23.0%), Iran (11.5%), Syria (24%), Azerbaijan (20.2%), and Lebanon (34.2).[5-14,17] Our results were similar to the results obtained in Syrian populations (Table 4).

TABLE 2: Frequencies of three different β-thal mutations in Şanlıurfa province.

Mutations	Phenotype	Number of alleles	Number of homozygous/heterozygous	Frequency (%)
c.93-21G>A	β^+	27	6/15	26.5
c.25_26delAA	β^0	4	0/4	3.9
c.315+1G>A	β^0	4	1/2	3.9
Total	-	35	7/21	34.3

TABLE 3: Comparison of the frequency of three different β-thal mutations among different regions of Turkey.

Mutations	Southeastern Anatolia * Region 117 (%)	East Anatolia Region 59 (%)	Aegean and Mediterranean Regions 198 (%)	Central Anatolia Region 111 (%)	Marmara Region 88 (%)	Black Sea Region55 (%)	Şanlıurfa province 102 (%)
c.93-21G>A	31 (26.4)	16 (27.1)	84 (42.4)	58 (52.3)	30 (34.1)	17 (31.0)	27 (26.5)
c.25_26delAA	5 (4.3)	5 (8.4)	9 (4.6)	7 (6.3)	7(8.0)	1 (1.8)	4 (3.9)
c.315+1G>A	3 (2.6)	2 (3.4)	11 (5.6)	5 (5.4)	3 (3.4)	3 (5.5)	4 (3.9)
Reference	(7)	(7)	(7)	(7)	(7)	(7)	this study

*Values indicate the number of alleles, and values in parentheses indicate rate of alleles.

The second most common mutation is c.25_26delAA (3.9%). On the other hand, frequency of the c.25_26delAA mutation was reported between 1.8% and 8.4%, in different regions of Turkey previously.[7] Our study and others had already reported this mutation with different frequencies in various regions of Turkey as 8.4% in the Eastern Anatolia Region, 8.0% in the Marmara Region, 6.3% in the Central Anatolia Region, 4.6% in the Aegean and the Mediterranean Regions, 4.3% in the Southeastern Anatolia Region, and 1.8% in Black Sea Region (Table 3). Our result was similar with those reported in the literature for the Southeastern, the Aegean and Mediterranean Regions of Turkey. In addition, the data of c.25_26delAA was consistence with the Bulgarian result (Table 4).

The third most common mutation in this region is c.315+1G>A mutation that constitutes about 3.9% (Table 2). On the other hand, this study and others had already reported this mutation with different frequencies in various regions of Turkey as 5.6% in the Aegean and Mediterranean Regions, 5.5% in the Black Sea Region, 5.4% in the Central Region, 3.4% in the Eastern Anatolia and the Marmara Regions, and 2.6% in the Southeastern Anatolia Region.[5] Our data were similar with those reported in the literature for the Eastern Anatolia and the Marmara Regions. However, the value obtained in Şanlıurfa province was lower than the Southeastern Anatolia Regions (Table 3). In the same way, the frequency of the β-thal allele c.315 + 1G >A in the neighboring countries' populations, was lower and higher. However, the frequency of this mutation was similar to Syrian result (Table 4). However, we could not obtain compound mutations in our patients.

Consanguineous marriages and population migrations appear as important factors causing the high frequency of this disease in Eastern and Southeastern Anatolia populations of Turkey.[7,17] There are a number of consanguineous marriages in Şanlıurfa population. In the same way, the total fertility rate in Şanlıurfa province is 4.83, whereas it is 2.53 in Turkey as a mean. In addition, the rate of population growth is 3.6 percent per year, much higher than the national average of 1.8 percent.[21]

Our results provide a foundation for prenatal genetic testing and genetic counseling that would be part of a thalassemia prevention program in the Şanlıurfa province. The application of the knowledge about mutation pattern was found to be beneficial since the mutations could be screened in the order in which they are present in our population. Hence, it would not only help to reduce the screening cost but also to promote early genetic counseling and prevention of an affected child.

In conclusion, ARMS-PCR method was a rapid and direct molecular technique in screening β-thal mutations in population surveys. There was also a need to investigate other known mutations in *HBB* gene in this region for prenatal analysis and genetic counseling.

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TABLE 4: Comparison of the frequency of three different β-thal mutations studied in different populations.

Mutation	Romania 32(%)*	Turkey 795(%)	Cyprus 188(%)	Greece 1179(%)	Bulgaria 801(%)	Iran 130(%)	Syria 146(%)	Azerbaijan 99(%)	Lebanon 520(%)
c.93-21G>A	10(31.25)	312(39.3)	131(69.9)	497(42.5)	184(23.0)	15(11.6)	35 (24.0)	20 (20.2)	178(34.2)
c.25_26delAA	-	43 (5.5)	<1	<1	31 (3.9)	-	<1	21 (21.2)	13 (2.5)
c.315+1G>A	-	37 (4.7)	-	104 (2.0)	7 (0.9)	36 (27.7)	6 (4.1)	5 (5.5)	45(8.6)
References	(5)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)

*Values indicate the number of alleles, and values in parentheses indicate rate of alleles.

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