

SPECTRUM OF β -THALASSEMIA MUTATIONS IN VARIOUS ETHNIC REGIONS OF IRAN

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ABSTRACT

Objective: Thalassemia is a group of inherited disorders characterized by reduced or absent amounts of hemoglobin, the oxygen-carrying protein inside the red blood cells. β -Thalassemia, one of the most widespread genetic disease in the world, is a common autosomal recessive disorder generally caused by point mutations in the β -globin gene that is located as a cluster on the short arm of chromosome 11 (OMIM: MIM # 141900). The objective of this study was to identify spectrum of Beta Thalassemia mutations in various ethnic regions of Iran.

Methodology: We extracted and differentiated the Iron deficiency patients with the help of 10 discrimination indices (Mentzer Index, England and Fraser Index, Srivastava Index, Green and King Index, Shine and Lal Index, red blood cell (RBC) count, red blood cell distribution width, red blood cell distribution width index (RDWI), Mean Density of Hemoglobin per Liter of blood (MDHL) and Mean Cell Hemoglobin Density (MCHD)) from beta-thalassemia patients.

Results: In a total of 1098 carriers (1045 beta-thalassemia and 53 iron deficiency), we detected different β -thalassemia mutations in the studied subjects of 5 different ethnic regions from Iran. β -Thalassemia was diagnosed based on complete blood counts, Hb electrophoresis, and ASO-Hybridization in patients from each area separately at Research center of Thalassemia and Hemoglobinopathies, Ahwaz Jondishapur University of Medical Sciences, Shafa Hospital, Iran. This study has shown that the most common mutation for each region was IVS-II-1 (G \rightarrow A) (34%) in Khuzestan, IVS I (3' end)-25 bp (28.7%) in Booshehr, IVS II- 1 (G \rightarrow A) (41.5%) in Fars, IVS-II-1 (G \rightarrow A) (31.8%) in Isfahan, IVS I-5 (G \rightarrow C) (44.8%) in Sistan- Baloochestan, respectively.

Conclusions: The presence of such a high frequency of various local mutants alleles is strong support for role of non-isolating genetically areas. In likelihood, both founder effect and natural selection caused by migration from neighboring areas have complemented each other to produce the high frequency of unique alleles within each region.

KEYWORDS: β -Thalassemia, Iron deficiency, Differential indices, β -globin gene, IVS (intervening sequence), CD (Codon), OMIM.

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INTRODUCTION

The thalassemias are widespread with about 5% of the world population affected by it. It is most prevalent around the Mediterranean Sea i.e. countries like Greece, Italy, Turkey and North African countries. It is also seen in Saudi Arabia, Iran, Afghanistan, Pakistan India and South East Asian countries like Thailand and Indonesia. Iran, a country spread over

1,648,000km two wide, has a large number of thalassemia major patients like many other countries in the region.¹ β -thalassemia is very rare in Iran. The gene frequency of β -thalassemia, however, is high and varies considerably from area to area, having its highest rate of more than 10% around the Caspian Sea and Persian Gulf. The prevalence of the disorder in other areas is between 4-8%. In Isfahan, a city built around the river Zayandeh-Rood in the central part of Iran, the frequency rises again to about 8%. In the Fars Province, in southern Iran, the gene frequency is also high and reaches 8-10%.¹

Beta-Thalassemia, one of the most widespread genetic disease in the world, is a common autosomal recessive disorder generally caused by point mutations in the β -globin gene that is located as a cluster on the short arm of chromosome 11.²⁻⁴ More than 200 different mutations affecting diverse levels of beta-globin genes expression have so far been identified.^{3,5,6} Different strategies of classification individuals genotypes by β -globin gene cluster and cloning nucleotides sequencing lead to identification of several mutations in Mediterranean,⁷ Asian Indians,^{8,9} American Blacks¹⁰ and Chinese.¹¹ The global distribution indicates a high prevalence in a belt around the earth, which is around the 40th parallel in the Mediterranean area but eastwards moves further south, reaching the equator in Indonesia.

More than two million carriers of β -thalassemia live in Iran. Since the Iranian populations are mixture of different ethnic groups, it is necessary to determine the frequency and distribution of mutations in the different parts of the country. For this purpose, we studied β -thalassemia chromosomes of total 1098 affected patients (1045 beta-thalassemia and 53 iron deficiency) by using the ASO-Hybridization. We detected frequency of different β -thalassemia mutations in the studied subjects of five different ethnic regions from Iran including Isfahan, Sistan- Balochestan, Khuzestan, Booshehr and Fars.

METHODOLOGY

β -Thalassemia was diagnosed based on complete blood counts, Hb electrophoresis, and ASO-Hybridization of total 1045 beta-thalassemia carriers at Research Center of Thalassemia and Hemoglobinopathies, Ahwaz Jondishapur University of Medical Sciences, Shafa Hospital, Iran. The hematological data indicates the low MCV (Mean Corpuscular Volume) and low MCH (Mean Corpuscular Hemoglobin) values and high or borderline Hb A2 fraction in heterozygous state. Due to the prevalence of thalassemia in Iran, a descriptive cross-sectional study was conducted to determine a more valid variable for screening minor thalassemia patients.

After initial abnormal blood count as described above, since iron deficiency is the other explanation for low MCV or MCH, we used different indices for differential diagnosis between these two disorders and found the Mean and Standard deviation of hematological values as shown in Table-I. We calculated 10 discrimination indices such as Mentzer Index,¹² England and Fraser Index,^{13,14} Srivastava Index,¹⁵ Green and King Index,¹⁶ Shine and Lal Index,¹⁵ Red blood cell (RBC) count, red blood cell distribution width and red blood cell blood distribution width index (RDWI),¹⁷ MCHD Index,¹⁸ MDHL Index.¹⁹ Other two indices include Red blood cell count (RBC) and red blood cell distribution widths (RDW)^{20,21} were obtained with Counter. We could differentiate the beta-thalassemia patients (1045) from iron deficiency (53) with the help of indices results successfully in total 1098 subjects.

DNA Isolation: 5ml Peripheral blood samples were collected in tubes containing EDTA. We used Viena Lab Kit.

DNA Extraction: DNA was extracted from the blood sample cells by using the Viena Lab Kit. This Kit contains two parts i.e. Lysis Solution and GENXTRACT Resin. The DNA extraction procedure was: (i) put 100 μ l blood sample in micro tube, add 1ml Lysis Solution, incubate 15 minutes in room temperature, spin down at 3000 rpm for 5 minutes, (ii) add 1ml Lysis

Solution to extracted supernatant, incubate 5 minutes in room temperature, spin down at 12000 rpm for 10 minutes. (iii) add 200 μ l GenXtract to supernatant, incubate 20 minutes at 56°C (iiV) incubate 10 minutes at 98°C and spin down at 3000 rpm for 5 minutes. Finally, we extracted supernatant that involves pure DNA and use it in next step.

PCR Amplification: In vitro amplification of genomic DNA was performed with HotStarTaq Master Mix Kit Qiagen by PCR technique in Gene Amp PCR System 2400 thermal-cycler (Mastercycler 5330), using different sets of primers including whole structural and untranslated regions of β -globin gene, described by Kanavakis et al.²² The amplification cycle consisted of: An initial denaturation at 95°C for 15 min was followed by 35 cycles of a three-step cycling protocol (95°C for 1 minutes, 60°C for 1 minutes, and 72°C for 90 seconds) and a final elongation step at 72°C for 8 min. PCR reaction composition using HotStarTaq Master Mix were prepared in mixtures of 2.5units HotStarTaq DNA Polymerase, 1 \times PCR buffer, 200 μ mm of each dNTP, 0.1–0.5mm of each primer, 1ml Template DNA.

Hybridization: Hybridization of PCR products was done using different solutions including DNAT (1.6% NaOH) and Hybridization Buffer by mix 10 μ l of DNAT with 10 μ l of PCR product, add 1ml Hybridization Buffer at 45°C and keep it on shaker for 5 minutes.

Washing: After 30 minutes washing process was started with Wash solution A, three times each time for 15 minutes at 45 °C on shaker.

Chromatography: Chromatography was applied to the bands using solutions including Wash solution B, Conjugation Solution, and Color Developer. Method applied by adding 1ml Conjugation Solution to the samples, removes the Conjugation Solution after 15 minutes and adds 1ml of Wash solution B twice. Finally apply 1ml of Color Developer solution for 15 minutes in dark place.

RESULTS

Analysis of Mutation: This study was initiated by screening genomic DNA sample of the patients in order to find different mutations and concluded the development of PCR follows by hybridization. Blood samples were collected from 1045 patients (260 less than 10 years and 785 more than 10 years) of five different areas of Iran. DNA was isolated and the samples were subjected to gene amplification. The primers were selected on the basis of location. The amplified DNA samples were screened for the presence or absence of different mutations. Denatured DNA samples were spotted onto nylon membranes and hybridized to oligonucleotide probes for frame-shift mutation in corresponding sequences. At the next stage, DNA samples carrying unknown alleles were screened for common mutations.

Table-I: The data shows the frequency of different type of Beta-gene mutations among ethnic regions of interest in Iran and Previously Reported population which include: B1, B3, B5, AND B6: Mediterranean; B4: Asian Indian, SE Asian, Melanesian; B2: Iranian; B7: Asian Indian, Japanese. Description of letters in table are: B1: IVS I-I (G \rightarrow T A); B2: IVS II-I (G \rightarrow T A); B3: IVS I-110(G \rightarrow T A); B4: IVS I-5 (G \rightarrow T C); B5: IVS I-6 (T \rightarrow T C); 6: IVS II-745(C \rightarrow T G); B7: IVS I (3' end)-25 bp; B8: CD 8/9; B9: Unknown.

| Ethnic region | Province | Beta globin gene mutations | | | | | | | | | Total |
|---------------|--------------|----------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|----------|-------|
| | | B1 | B2 | B3 | B4 | B5 | B6 | B7 | B8 | B9 | |
| Sothwest | Khuzestan | 16(5.5) | 96(34)* | 45(15.7) | 11(3.8) | 26(9) | 0 | 0 | 38(13.3) | 54(18.7) | 286 |
| South | Booshehr | 24(11.7) | 38(18.5) | 0 | 19(9.2) | 0 | 0 | 59(28.7)* | 20(9.7) | 45(22.2) | 205 |
| Sothwest | Fars | 74(41.5)* | 19(10.6) | 34(19.1) | 0 | 0 | 0 | 0 | 19(10.6) | 32(18.2) | 178 |
| Central | Isfahan | 14(7.6) | 58(31.8)* | 36(19.7) | 0 | 0 | 0 | 0 | 52(28.5) | 22(12.4) | 182 |
| Southeast | Baloochestan | 6(3) | 66(34) | 0 | 87(44.8)* | 0 | 20(10.3) | 0 | 0 | 15(7.9) | 194 |
| Type | | β^o | β^+ | β^+ | β^+ | β^+ | β^+ | β^o | β^o | | |
| Total(No.) | | 134 | 277 | 115 | 117 | 26 | 20 | 59 | 129 | 168 | 1045 |

* indicates most common mutations among each ethnic group

β- Globin Mutations: In our study several known thalassemia mutations with different frequency in various areas has been found (Fig-1). The most common known mutations with higher frequency were detected in each ethnic region of interest. Results have shown that most frequent mutation includes Mediterranean^{7,23-25} Asian, Indian and Japanese^{8,9} and SE Asian, Melanesian.^{26,27} We found that the IVS II- 1(G ā' A) mutation was the most common β-thalassemia defect with frequency of 26.5% (277/1045), followed by IVS I-I (G ā' A) (12.8%), CD 8/9 (12.3%), IVS I-5 (11.2%), IVS I - 110 (11%) among all five provinces (Table-I).

We also calculated the related frequency for each area separately (Table-I). The most common mutation for each region was IVS-II-1 (G ā' A) (34%) in Khuzestan, IVS I (3' end)-25 bp (28.7%) in Booshehr, IVS II- 1(G ā' A) (41.5%) in Fars, IVS-II-1 (G ā' A) (31.8%) in Isfahan, IVS I-5 (G ā' C)(44.8%) in Sistan-Baloochestan, respectively(Table-I).

DISCUSSION

Iran is a country which has a population with a different ethnic identity and different languages. The people who live in different parts speak different languages, but the common language is Persian. As we saw mutation in β-globin gene will lead to thalassemia.

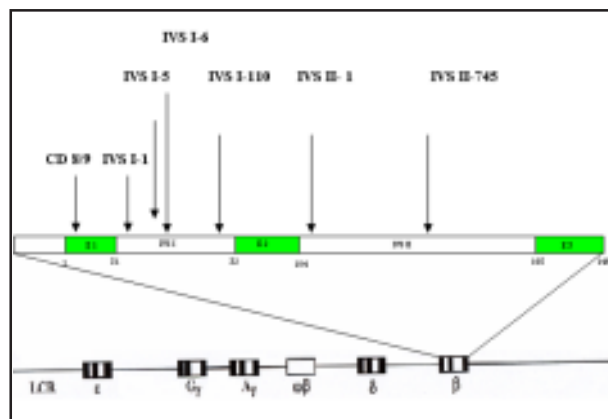


Fig 1. β-globin gene scanning for mutation identification of β- thalassemia. The fragments represent the PCR amplification area of â-globin gene; IVS I and IVS II- are intervening sequences (introns) 1 and 2. CD- is codon.

Although, all this mutations were found in coastline areas but their geographic distribution has special characteristic properties. In most parts of the world, a small number of thalassemia mutations predominant and the most common ones tend to be those that are geographically the most widespread and presumably also the oldest. For instance, in China and Southeast Asia, four alleles account for 91% of the genes³⁰ and in the Mediterranean Basin, six mutation account for 92% of the genes.³¹ However, Mutation 619 bp deletion is predominant in India or mutations IVS I-110 & IVS II-1 are most predominant in Arab populations.²⁸ Because of Wars and trade off between different countries in the past years there was exchange of genetic materials between different populations.

We attempted to detect the most frequent β-thalassemia mutations in Khuzestan and Booshehr (Southwest and Southeast regions), Isfahan and Fars (Central Part). Different authors have reported various mutations that are most prevalence in Khuzestan. In a work done



Fig-2: The origin of mutant alleles with high frequency within different parts of Iran. The most frequent beta-gene mutations in each ethnic region indicated individually.

by Karimi et al., on 87 patients with β -thalassemia intermedia using ARMS technique, they have detected four different mutations involves IVS II-1, IVS I-110, IVS I-1, and CD 8/9. They claimed that these mutations are the most frequent in Iran and IVS II-1(24%) is most predominant in Khuzestan.²⁹ In a similar work on 1217 patients with β -thalassemia the most predominant mutation in North part of Iran was IVS II-1(34%) and in South part was IVS I-5(%).³⁰

Najmabadi et al. have studied β -globin mutations and claimed that the most common β -globin mutations is IVS I-130 (G-C), which was identified in six subjects from the North of Iran, three subjects from the Southwest, as well as in one DNA of unknown geographical origin.³¹ Karimi et al. have reported the highest prevalence mutations of IVS-II-1, IVS-I-110, IVS-I-1 and CD 8/9. They claimed that the IVS-II-1 defect, being the most frequent in south of Iran, was present at the highest rate (24%).³² We have found that most predominant in South was IVSII-I (34%) follows by IVS I-110(15.7%) and CD 8/9(13.3%). Previous research work showed most predominant mutation in Pakistan³³ is IVS I-5(37%) therefore this mutation is most predominant in Sistan-Baloochestan (44.8%)(Southeast of Iran) because it is a neighborhood area to Pakistan. In a work done on thalassemia patients in Hormozgan (Southern part of Iran) claimed that most predominant mutation there is IVS I-5(69%) followed by IVS II- 1(9.6%).³³

Research work done in 8 Gulf (Arab) Countries showed that most predominant mutations are IVS I-110 and IVS II-1 followed by IVS I-5, CD39, CD6, IVS I(3' end)-25 bp del.³⁴ Our finding was similar to the work done in Kuwait which showed 6 mutations are most predominant involve IVS II-1, IVS I-6, CD39, IVS I-110, CD8, IVS I-1(all give 64%) and followed by another 2 involve CD44, CD 36/37 (Kurd, Iranian types) that give 10% of the population.³⁵ We detected different β -thalassemia mutations in the studied chromosomes and 5 different areas from Iran which showed IVS-II-I (G \rightarrow A) was the predominant mutation found

in all ethnic regions. The most common mutation for each region was IVS-II-1 (G \rightarrow A) (34%) in Khuzestan, IVS I (3' end)-25 bp (28.7%) in Booshehr, IVS II- 1(G \rightarrow A) (41.5%) in Fars, IVS-II-1 (G \rightarrow A) (31.8%) in Isfahan, IVS I-5 (G \rightarrow C)(44.8%) in Sistan-Baloochestan, respectively(Table-I). The presence of such a high frequency of various local mutants alleles confirms support for a role of non-isolating genetically areas. In likelihood, both founder effect and natural selection caused by migration from neighboring areas have complemented each other to produce the high frequency of unique alleles within each region (Fig-2). The results presented here can be used as a basis of prenatal diagnosis of β -thalassemia.

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