

ALPHA-THALASSEMIA: DELETION ANALYSIS IN IRAN

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Abstract

Background-Alpha-thalassemia is one of the most prevalent hemoglobin disorders in the world. The molecular basis of α -thalassemia is deletions of variable lengths involving one or both α -genes at the α -globin gene cluster. Functional point mutations leading to inactivation of the α -genes are less frequent. So far, no comprehensive population screening for α -thalassemia has been performed in Iran and no molecular diagnostic services are available for this disease. As a result, a considerable number of patients with microcytic, hypochromic anemia and normal Hb A2 levels might be misdiagnosed as silent α -thalassemia. The aim of the present study was to determine the spectrum of common α -thalassemia mutations in Iran.

Methods-A total of 57 Iranian subjects were randomly chosen from a pool of patients with microcytic hypochromic anemia and negative α -thalassemia genotyping. They were tested for the 2 most frequent α -thalassemia deletions (- 3.7, - 4.2). Analysis was performed using deletion-specific PCR amplification followed by agarose gel electrophoresis of the resulting PCR fragments.

Results-No - 4.2 deletion was detected, however, 18 (31.6%) out of 57 analyzed cases demonstrated the - 3.7 deletion, either in the homozygous or heterozygous state.

Conclusion-This study suggests that the - 3.7 deletion is a common cause of microcytic hypochromic anemia in Iran. The results are in accordance with previous studies, which report a remarkably high frequency of - 3.7 in the Middle East. Routine screening for this mutation will improve the molecular diagnosis of anemia in Iran.

Keywords • Alpha-thalassemia • Iran • deletion analysis

Introduction

Alpha-thalassemia is regarded as one of the most common hemoglobin disorders in the world and is caused by the absence or reduced synthesis of alpha-globin chains.¹ The genetic incidence for this disease varies between 1% and 98% throughout the tropics and subtropics.² There are two copies of alpha-globin genes on the short arm of chromosome 16.

The gene arrangement of the alpha-globin gene cluster is shown in Figure 1. The presence of two copies of alpha-globin genes per haploid genome makes the pathology of alpha-thalassemia far more complicated than that of beta-thalassemia.

The normal alpha-globin genotype is shown as $\alpha\alpha/\alpha\alpha$. There are two major types of alpha-thalassemia; α^0 and α^+ . Alpha⁺-thalassemia represents a condition in which one of the two linked alpha-globin genes on a chromosome is inactivated. This can be caused either by deletion (-) or mutation (T). The heterozygous states for these types can be represented as $-\alpha/\alpha\alpha$ or $\alpha^T\alpha/\alpha\alpha$,

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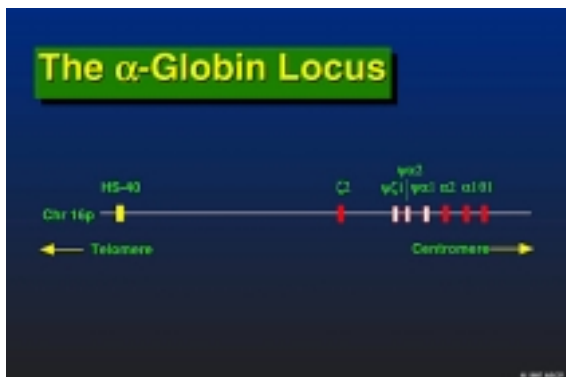


Figure 1. The alpha-like gene cluster is located on chromosome 16, p13.3. This cluster contains three functional genes ζ (embryonic) and $\alpha 1$ $\alpha 2$ (adult). θ seems to be functional and HS-40 is a regulatory element, which is essential for alpha-globin-gene function. Therefore, the alpha-globin gene locus provides two alpha-globin genes per haploid genome, four genes in all.^{2,3} (Adapted from Sabath et al, 2001)⁴

respectively. Alpha⁰-thalassemia represents a condition, in which both α -globin genes on a chromosome are inactivated. The heterozygous genotype in this case is shown as $--/\alpha\alpha$. In certain populations, specific α^0 deletions are more common; the heterozygous state of the Mediterranean or Southeast Asian deletions are represented as $--Med/\alpha\alpha$ and $--SEA/\alpha\alpha$, respectively.⁵

Heterozygous state of $\alpha+$ -thalassemia does not cause any significant hematological changes. However, α^0 -thalassemia is associated with moderate hypochromia and microcytosis similar to that of beta-thalassemia trait. The loss of two alpha-globin genes on one chromosome (the heterozygous state for α^0 -thalassemia: $--/++$) or the loss of one alpha-globin gene on each of the two homologous chromosomes (the homozygous state for $\alpha+$ -thalassemia: $-+/-+$) produces similar phenotypes. The absence of three alpha-globin genes resulting from the compound heterozygous state for α^0 and $\alpha+$ -thalassemia, a condition known

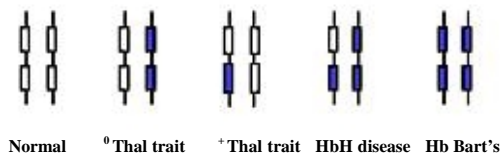


Figure 2. Graphic representation of alpha-globin gene arrangements in normal, α^0 , $\alpha+$, HbH and Hb Bart's.

as hemoglobin H disease, gives rise to a moderately severe anemia, with splenomegaly and a distinct shortening of the red cell survival. The most severe type of α -thalassemia is hydrops fetalis (Hb Bart's) resulting from the homozygous state for α^0 -thalassemia ($--/--$) and the absence of alpha chain production, which leads to death either *in utero* or immediately after birth.⁶ Figure 2 demonstrates a graphic representation of each condition.

Unlike beta-thalassemia mutations, which are mostly point mutations, the majority of α -thalassemia mutations are deletions of one or both alpha-globin genes. The most common single gene deletions of α -thalassemia are $-\alpha 3.7$ and $-\alpha 4.2$, while the $--/SEA$, Filipino type ($--/FIL$) and $--/Med$ are the most common double-gene deletions of α -thalassemia.⁷

Each alpha-globin gene is located within a homologous area of 4 kb length including two non-homologous sections. The homologous regions were most likely created by an ancient duplication event, which were then subdivided by subsequent insertions and deletions to give three homologous subsegments on each alpha-globin gene. These are called X, Y, and Z boxes. Duplicated Z regions are separated by 3.7 kb and the duplicated X regions are 4.2 kb apart.^{8,9}

The most common $-\alpha+$ -thalassemia single gene deletions $-\alpha 3.7$ and $-\alpha 4.2$ are the result of misalignment and crossover between the homologous boxes during meiosis. The mechanism of their creation is shown in Figure 3 and Figure 4 respectively.

The aim of this study was to determine the some common α -thalassemia mutations in Iran. A total of 57 patients were analyzed for -3.7α and -4.2α deletions using PCR and agarose gel electrophoresis. The results of this study demonstrate a relatively high frequency of -3.7 and no occurrence of -4.2 in the population under investigation.

Materials and Methods

After pertinent genetic counseling and recording of relevant hematological values, 10 mL of blood was drawn from 57 subjects with normal Hb A2 levels, low MCV, and normal or slightly reduced HCT and Hb levels. Genomic DNA was extracted according to standard protocols.¹² A method described by Baysal and Huisman¹³ was

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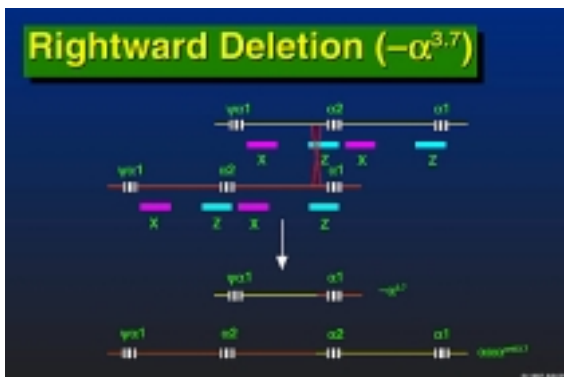


Figure 3. $-\alpha_{3.7}$ deletion can occur during meiosis where misalignment and reciprocal cross-over between homologous Z boxes leads to deletion of 3.7 kb DNA on one chromosome. As a result of this event, described as a rightward deletion, a chromosome is produced with either one α -globin gene ($-\alpha$) or triplicated alpha-globin genes ($\alpha\alpha\alpha$). (Depending on the site of cross-over there are three types of $-\alpha_{3.7}$ rearrangements designated 3.7 I, II and III).^{10,11} (Adapted from Sabath et al, 2001).⁴

used to detect the two most common α -thalassemia single gene deletion mutations, $-\alpha_{3.7}$ and $-\alpha_{4.2}$.

To detect $-\alpha_{3.7}$, two separate PCR reactions were performed simultaneously for each DNA sample. In one reaction, a pair of primers (A and B) was used to amplify the part of the chromosome

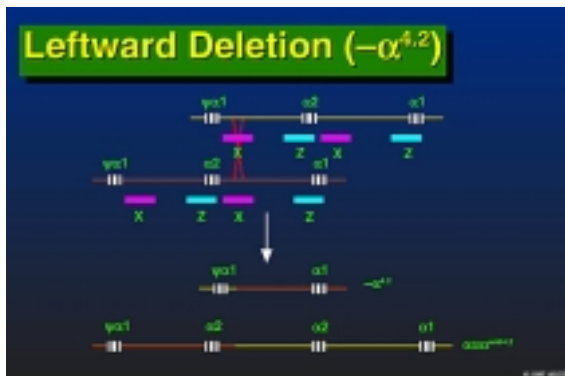


Figure 4. Similar to the mechanism of $-\alpha_{3.7}$ deletion, misalignment and reciprocal cross-over during meiosis between the homologous X boxes, a leftward deletion gives rise to deletion of 4.2 kb DNA, creating a chromosome with either one alpha-globin gene ($-\alpha_{4.2}$) or three alpha-globin genes ($\alpha\alpha\alpha$). (The genetic recombination involving the Z boxes occurs more frequently than those involving the X or Y boxes).^{10,11} (Adapted from Sabath, et al, 2001).⁴

with $-\alpha_{3.7}$ deletion. Another pair (A and C) was used to amplify the normal gene. For $-\alpha_{4.2}$, three primers (D, E and F) were used in a single PCR reaction to amplify both the deleted and the normal gene. In this multiplex PCR reaction, primers D and E amplify the part of chromosome with the $-\alpha_{4.2}$ deletion and primers D and F amplify the normal chromosome (Figure 5). Approximately 0.7 μ g DNA is used for each PCR reaction (25 μ L). The amplification buffer was composed of 67 mM tris-HCl, pH 8.8, 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 0.10 mg/mL BSA, 10 mM beta-mercaptoethanol, 4.0 mM MgCl_2 , 7.5% DMSO, and 200 μ M dNTPs. Also, 25 picomoles of each primer were used for detection of $-\alpha_{3.7}$ genotype. For detection of the $-\alpha_{4.2}$ deletion the concentration of the three primers (D, E and F) were 20, 15 and 5 picomoles, respectively. For each PCR reaction, 2 units of taq polymerase were used in a thermal cycler (Eppendorf, Germany). The PCR condition was 94 °C for one minute, 55 °C for one minute and 72 °C for one minute for D, E and F primers, respectively. The PCR products were run on a 1% agarose gel using TAE buffer and the bands were visualized on an UV transilluminator after staining with ethidium bromide (Figure 6).

Results

Regarding the $-\alpha_{3.7}$ deletion, detection of the fragment corresponding to the normal chromosome (primers A and C) and the fragment derived from the $-\alpha_{3.7}$ deletion (primers A and B) had the same size (1.8 kb). The heterozygous state for the $-\alpha_{3.7}$ deletion ($-\alpha_{3.7}/\alpha\alpha$) could be identified if 1.8 kb bands were present in both reactions, while in the homozygous state ($-\alpha_{3.7}/-\alpha_{3.7}$), bands are present only with primers A and B. The presence of a 1.8 kb band with primers A and C and the absence with primers A and B indicates the normal genotype for this deletion. Figure 6 shows gel electrophoresis of PCR products obtained from PCR reactions for $-\alpha_{3.7}$ deletion analysis. For each individual the first lane corresponds to the PCR products of the normal reaction and the second lane corresponds to the PCR product of the mutant reaction. Cases a (lanes 1, 2), b (lanes 3, 4) and f (lanes 11, 12) were normal, case c (lanes 5, 6) was homozygous, and case d (lanes 7, 8) was heterozygous for the $-\alpha_{3.7}$ deletion. PCR amplification failed for case e (lanes 9, 10).

As the fragments corresponding to the normal

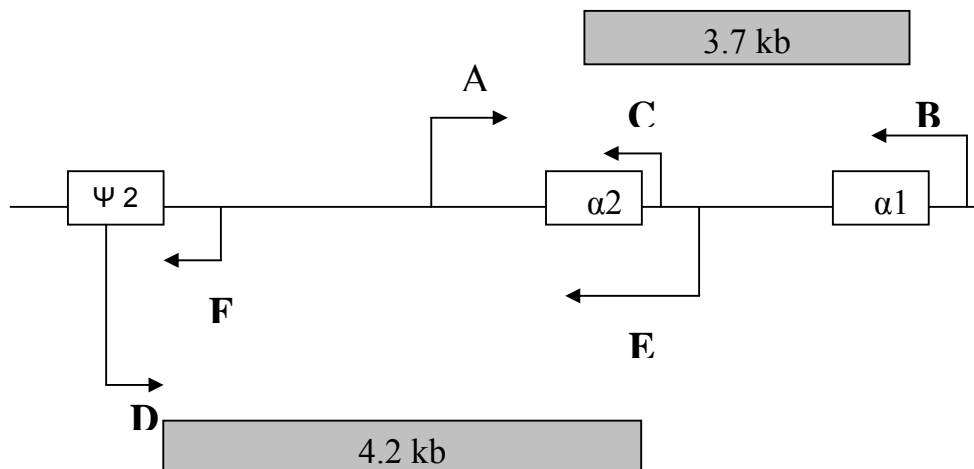


Figure 5. Location of primers A, B and C for detection of $-\alpha 3.7$ and primers D, E and F for detection of the $-\alpha 4.2$ single gene deletions.

chromosome (581 bp) and the chromosome carrying the $-\alpha 4.2$ deletion (2.1 kb) are of different sizes, to identify the $-\alpha 4.2$ deletion, two PCR reactions using primers D, E and F, were carried out in the same tube.

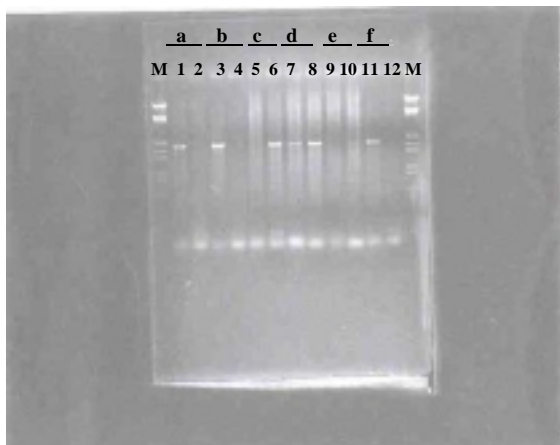


Figure 6. A 1% agarose gel electrophoresis of the amplification products obtained from the PCR reactions for $-\alpha 3.7$ deletion analysis. For each individual the first lane corresponds to the PCR product of the normal reaction (primers A and C) and the second corresponds to the PCR product of the mutant reaction (primers A and B). Cases a (lanes 1 and 2), b (lanes 3 and 4) and f (lanes 11 and 12) are normal for $-\alpha 3.7$ as there is a 1.8 kb band in the first reaction only. Case c (lanes 5, 6) is homozygous for $-\alpha 3.7$ as there is a 1.8 kb band in the second reaction only. Case d (lanes 7 and 8) is heterozygous for $-\alpha 3.7$ as there are bands in both reactions. PCR amplification failed in case e (lanes 9, 10).

Presence of both bands indicate the heterozygous state ($-4.2 \alpha/\alpha$), while presence of only one band, either 2.1 kb or 581 bp, indicates the homozygous or the normal state ($-4.2\alpha/-4.2\alpha$ or $\alpha\alpha/\alpha\alpha$) for this mutation respectively.

Out of 57 samples, 10 were homozygous for $-\alpha 3.7$ ($-\alpha 3.7/-\alpha 3.7$), 8 were heterozygous ($-\alpha 3.7/\alpha$) and the remaining 39 were normal ($\alpha\alpha/\alpha\alpha$) for this deletion. No $-\alpha 4.2$ deletion was identified. To summarize, 31.6% of the sample investigated in our study carried the $-\alpha 3.7$ deletion.

Discussion

The most common mutations of α -thalassemia are single or double alpha-globin gene deletions and the most common single gene deletions are $-\alpha 3.7$ and $-\alpha 4.2$. The $-\alpha 3.7$ deletion has a worldwide distribution among all racial groups^{14,15} while the $-\alpha 4.2$ deletion is less common and has been reported to be prevalent among Southeast Asians and Saudi Arabians.¹⁶ Alpha-thalassemia single gene deletion mutations in homozygous or heterozygous states do not have significant clinical implications. However, the compound heterozygous state for these mutations and α^0 -thalassemia can give rise to Hb H disease, which, has a high frequency in the Mediterranean and Southeast Asian regions ($--Med/-\alpha 3.7$ and $-SEA/-\alpha 3.7$). In addition, the principal pathophysiological mechanism that leads to anemia in beta-thalassemia is the destructive effects of the excess alpha chains produced during red cell maturation. Various combinations of alpha- and beta-

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thalassemia determinants lead to phenotypes that are significantly different to that of disease separately. Inheritance of one or more α -thalassemia determinants, together with beta-thalassemia, causes a milder clinical phenotype than beta-thalassemia patients with four intact alpha-genes.¹⁷ On the other hand, inheritance of triplicated alpha-globin genes together with the beta-thalassemia trait gives rise to phenotypic characteristics of beta-thalassemia intermedia.^{18,19}

Alpha-thalassemia is not as prevalent as beta-thalassemia in Iran but a large number of cases with reduced MCV and MCH, normal Hb A2 and F levels, and normal iron levels are being referred to the molecular biology clinics throughout the country as suspicious cases of α -thalassemia or silent beta-thalassemia carriers. It presents causes for concern and need to determine the common α -thalassemia mutations for the Iranian population. The $-\alpha 3.7$ deletion is relatively common in Iran. The condition of the other 39 patients, who could be of beta-thalassemia silent type or carry other types of α -thalassemia mutations, remained unidentified. These samples should be further investigated to determine the other types of α -thalassemia mutations in Iran. This will be valuable in carrier identification and prenatal diagnosis for prevention of the deleterious forms of α -thalassemia such as Hb H and Hb Bart's. In addition, in a country like Iran with a considerably high prevalence of beta-thalassemia, the likelihood of co-inheritance of alpha- and beta-thalassemia is high, resulting in occurrence of a large variety of different phenotypes. While there has been extensive research on finding the spectrum of beta-thalassemia mutations in Iran, there have been no comprehensive studies on α -thalassemia to date and this is the first report of a common α -thalassemia mutation in this country.

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