Molecular Analysis of $\beta^\circ$-Thalassemia Intermedia in Sardinia


In this study we have carried out $\alpha$- and $\beta$-globin gene analysis and defined the $\beta$-globin gene polymorphisms in a group of patients with thalassemia intermedia of Sardinian descent. A group of patients (109) with thalassemia major of the same origin served as control. Characterization of the $\beta$-thalassemia mutation showed either a frameshift mutation at codon 6 or a codon 39 nonsense mutation. We found that homozygotes for the frameshift mutation at codon 6 or compound heterozygotes for this mutation and for the codon 39 nonsense mutation develop thalassemia intermedia more frequently than thalassemia major. The frameshift mutation at codon 6 was associated with haplotype IX that contains the C-T change at position −158 5′ to the Gγ globin gene implicated in high $\gamma$ chain production and thus the mild phenotype. In patients' homozygotes for codon 39 nonsense mutation, those with thalassemia intermedia more frequently had the two-gene deletion form of $\alpha$-thalassemia, or functional loss of the $\alpha_2$ gene as compared with those with thalassemia major. In a few siblings with thalassemia major and intermedia, the thalassemia intermedia syndrome correlated with the presence of the $\alpha1/\alpha2$ genotype. No cause for the mild phenotype was detected in the majority of patients who had not inherited either haplotype IX or $\alpha$-thalassemia.

IN THE SARDINIAN population, the predominant type of $\beta$-thalassemia is the $\beta^\circ$ variety. Molecular characterization has shown that the most common defect is codon 39 nonsense mutation ($\beta^\circ$−39), which accounts for 95% of the $\beta$-thalassemia chromosomes followed by a frameshift at codon 6 ($\beta^\circ6^\circ$ = 2%). The great majority of homozygotes or compound heterozygotes for these mutations develop the transfusion-dependent form of $\beta$-thalassemia (thalassemia major). However, some manifest the milder nontransfusion-dependent condition referred to as thalassemia intermedia. Genetic factors thought to be capable of reducing the severity of homozygous $\beta$-thalassemia are mild $\beta$-thalassemia mutations, coinherited $\alpha$-thalassemia, and nondeletion hereditary persistence of fetal hemoglobin (HPFH). In a previous study in the Sardinian population using a limited number of patients, coinherited deletion $\alpha$-thalassemia ($\alpha1$−$\alpha$) has been found more frequently in patients with thalassemia intermedia than in those with thalassemia major.

In this study we have attempted to define the genetic factors that may be responsible for the mild forms of $\beta^\circ$-thalassemia in Sardinians by the analysis of $\alpha$- and $\beta$-globin genes and $\beta$-globin gene polymorphisms.

We found that patients' homozygotes for a frameshift at codon 6 or compound heterozygotes for this mutation and codon 39 nonsense mutation more frequently develop thalassemia intermedia than thalassemia major. A frameshift at codon 6 was always associated with haplotype IX, which contains the C→T change at position −158 5′ to the Gγ gene and may be responsible for high $\gamma$ chain production and mild phenotype. Coinherited $\alpha$-thalassemia may have contributed to the development of the mild phenotype. In patients' homozygotes for codon 39 nonsense mutation, those with thalassemia intermedia more often had the two-gene deletion form of $\alpha$-thalassemia and functional loss of the $\alpha_2$ gene than those with thalassemia major, indicating that these forms of $\alpha$-thalassemia, by reducing the overall globin chain imbalance, may have contributed to the development of the mild phenotype.

PATIENTS AND METHODS

Sixty-one Sardinian patients with the clinical phenotype of $\beta^\circ$-thalassemia intermedia were investigated. Their hemoglobin levels ranged from 6.8 to 12.4 g/dL. None of these patients was transfusion dependent. A total of 109 Sardinian patients with thalassemia major served as controls.

Hematologic measurements were performed with a Coulter Counter model S (Coulter Electronics, Hialeah, FL). Hemoglobin electrophoresis was carried out on Titan III cellulose acetate plates, pH 8.6 (Helena Lab, Beaumont, TX), Hb A2 was determined by DE-52 microchromatography, and Hb F by alkaline denaturation. Globin chain synthesis analysis was carried out according to Kan et al. DNA restriction endonuclease analysis was performed according to previously described methods. The $\alpha$-globin genotype was determined by digestion of the DNA with the restriction endonucleases BamHI or BglII and hybridization with $\alpha$- and $\beta$-globin specific probes, respectively, as previously described.

Nei I mapping was carried out to screen for the presence of the most common nondeletion form of $\alpha$-thalassemia occurring in Sar- dinians, namely the initiation codon mutation of the $\alpha_2$ gene, which abolishes an $\alpha$ Nei I site.

The $\gamma$-globin gene arrangement was studied by digesting the DNA with the restriction enzymes BglII, EcoRI, HindIII, BamHI, and BclI and hybridizing with a $\gamma$-specific probe. The $\gamma$-probe used was the $\gamma$-globin cDNA plasmid JW 151.11 Polymorphic restriction-site analysis was used to determine the haplotypes at the $\beta$-globin gene cluster. The sites studied were HindIII 5′ to the $\epsilon$-globin gene, HindIII in the second intervening sequence (IVS) of the Gγ, and Aγ.
globin genes, HincII within and 3' to the ψβ gene, AwaII in the second IVS of the β-globin gene, and BamHI 3' to the β-gene.

We also investigated our patients for the presence of an XmnI site resulting from a C → T substitution at position -158 5' to the G, gene because this mutation has been associated with high G, expression in sickle cell anemia as well as β-thalassemia.23,25 To define the β-thalassemia mutations, since in Sardinians the most common defects are the β6–39 and a frameshift at codon 6, we screened DNA from our patients first with an oligonucleotide probe capable of detecting the β6–39 mutation25 and then with MstII restriction endonuclease, which directly detects the frameshift at codon 6, because this mutation abolishes the MstII recognition site at this position.3

RESULTS

Clinical aspects. Patients with thalassemia intermedia had mean hemoglobin (Hb) values of 9.7 ± 1.8 g/dL and never received transfusions. No differences in Hb levels in relation to the β-globin haplotype, α-globin gene arrangement, and type of β-thalassemia mutation were detected.

As far as the other hematologic parameters evaluated (red cell indices, HbA2%, and α/γ-globin chain synthesis ratio) the HbA2% and the α/γ ratio showed differences in relation to the α-globin genotype, while no differences were detected in relation to the different β-globin haplotypes. HbA2% was 2.2 ± 0.8 in patients with the αα/αα genotype, 3.1 ± 0.7 in those with the -α/αα genotype, and 3.9 ± 0.6 in those with the -α/-α genotype. The difference between patients with the αα/αα genotype and those with the -α/-α and -α/αα genotype were statistically significant (P < .001).

The values of the α/γ-globin synthesis ratio were 2.31 ± 0.5 in patients with the αα/αα genotype and 1.98 ± 0.5 and 1.8 ± 0.4 in subjects with -α/αα and -α/-α genotypes respectively. Differences between the subjects with the αα/αα genotype and those with the -α/-α genotypes were statistically significant (P < .05). In the control group Hb levels at presentation were 6.4 ± 0.63 g/dL.

Characterization of β-thalassemia mutations. A representative oligonucleotide analysis is shown in Fig 1. Forty-three of 61 patients with thalassemia intermedia investigated were homozygotes for codon 39 nonsense mutation (70.5%), one was a homozygote for a frameshift at codon 6 (1.6%), and 17 (27.9%) were compound heterozygotes for these mutations (Table 1). In the control group with thalassemia major, 95.2% had the β6–39/β6–39 genotype, and 4.8% were β6–39/β6–6 genetic compounds. As compared with those with thalassemia major, patients with thalassemia intermedia had a higher frequency of a frameshift at codon 6 (P < .001; Table 2).

α-Globin gene analysis. A representative Southern blot

Table 1. β-Globin Haplotype, α-Globin Gene Arrangement XmnI Site and Hb Levels in Patients With Thalassemia Intermedia Homozygotes for the β6–39 Mutation (A) or β6–6/β6–39 Genetic Compounds (B) and in a Homozygote for β6–6 Mutation (C)

<table>
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<tr>
<th>No. Patients</th>
<th>Haplotype</th>
<th>α-Globin Genes</th>
<th>XmnI Site</th>
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used for characterization of the α-globin gene arrangement is shown in Fig 2. In homozygotes for the codon 39 nonsense mutation, the frequency of the (−α/−α) globin genotype (−3.7 kb deletion) and nondeletion α-thalassemia affecting the α2 gene (ATG → ACG mutation of the α2 gene) was significantly higher (P < .005) in patients with thalassemia intermedia than those with thalassemia major. However, no difference was observed between the two groups in the frequency of the single α-globin gene deletion (−α/αα).

α-Globin gene analysis was also carried out in three families in which one sibling had thalassemia intermedia and the other one thalassemia major. In one family the patient with thalassemia major had a single α-globin gene deletion (−α/αα), while the other with thalassemia intermedia had the deletion of two α-globin genes (−α/−α). In the other two families both siblings had the −α/−α globin genotype. However, in both patients with thalassemia major and the (−α/−α) α-globin genotype, the disease was unusually mild, as indicated by late presentation (2 and 3 years, respectively) and onset of transfusion dependence (4 and 6 years, respectively).

In the group of thalassemia intermedia patients compound heterozygotes for a frameshift at codon 6 and codon 39 nonsense mutation or homozygote for a frameshift at codon 6, four had the −α/αα globin genotype and one the −α/ααNcoI α genotype, while in patients with thalassemia major four had the αα/αα and one the −α/αα globin genotype.

Polymorphism haplotype analysis. Both thalassemia intermedia and major contain the β6-39 mutation in four different chromosomal haplotypes, haplotype II being the most frequent according to Orkin et al. The β6 frameshift mutation was linked in all cases to haplotype IX. The only difference in the distribution of haplotypes between the two groups of patients was the higher frequency of haplotype IX, both in the homozygous (1.6%) and in the heterozygous state (32.8%) in thalassemia intermedia as compared with thalassemia major (P < .001).

The C → T substitution at position −158 to the Gs gene, which results in the presence of a novel XmnI site, was found in all chromosomes with haplotype IX and never in other haplotypes. The percent of Gs was 60.6 ± 3.5 in XmnI negative patients (−/−) and 66.5 ± 2.9 in XmnI positive subjects (−/+)(P < .01). The only subject homozygote for the XmnI positive site had % Gs = 71.7.

Gene analysis. In one patient with thalassemia intermedia and one with thalassemia major, we found a DNA pattern indicative for a triple γ-gene arrangement in one chromosome (Gs − Gs − As). Both these patients showed no distinctive clinical or hematologic features.

Family studies. We attempted to detect the coinheritance of nondeletion HPFH by the analysis of HbF levels in all parents of our patients. Only two of the parents of patients...
with thalassemia intermedia had HbF levels higher than 4% (9% and 22% respectively). Excluding these two subjects, mean HbF level % were 1.01 ± 0.64 in parents of patients with thalassemia major, and 1.19 ± 0.78 in parents of patients with thalassemia intermedia.

**DISCUSSION**

This study shows that Sardinian patients homozygous for a frameshift mutation at codon 6 or compound heterozygotes for this mutation and codon 39 nonsense mutation develop thalassemia intermedia more frequently than thalassemia major. A frameshift at codon 6 was associated without exception with haplotype IX, which contains the C → T change at position −158 5′ to the G, globin gene and creates a novel Xmn1 site. This nucleotide substitution has been previously associated with increased expression of the Gγ gene and/or increased HbF production in patients with sickle cell anemia or thalassemia. In our patients the C → T substitution at −158 may indeed be responsible for high γ chain expression and in this way may have reduced the overall globin-chain imbalance, thus ameliorating the clinical picture of homozygous β-thalassemia. As previously suggested, this mutation becomes activated solely in the presence of erythropoietic stress and therefore is expressed only in patients with either thalassemia or sickle cell anemia, while it is silent in both normal individuals and in heterozygotes for β-thalassemia or sickle mutation. The presence of the two-gene deletion or nondeletion α-thalassemia affecting the α2 gene may have contributed to the development of the mild clinical picture.

In patients homozygous for the codon 39 nonsense mutation, we have confirmed in a large series the results of a previous investigation indicating that the −α/−α genotype is positively associated with thalassemia intermedia; but we have excluded the existence of any association between thalassemia intermedia and the −α/αα genotype. In this study we have also shown that the thalassemia intermedia syndrome correlates with the −α/−α genotype in thalassemia intermedia and major siblings. Lastly this paper has also demonstrated that nondeletion α-thalassemia affecting the α2-globin gene occurs more frequently in thalassemia intermedia than in thalassemia major.

These results indicate that the deletion of two α structural genes or functional loss of the α2-globin gene by nondeletion mechanism, by reducing the overall globin-chain imbalance, may result in less ineffective erythropoiesis and thus milder phenotype.

The discrepancy between the effect of a single α-globin gene deletion (−α/αα) and nondeletion mutation of the α2 gene, presumably depends on the more severe defect of α-chain production resulting from the nondeletion α-thalassemia chromosome, as compared with the deletion α-thalassemia (−α) chromosome in which there is a compensatory increase of α-globin chain output. A more consistent effect of α-thalassemia was seen in homozygous βγ-thalassemia, in which even the coinheritance of single α-globin gene deletion is associated with thalassemia intermedia. Because the clinical phenotype of thalassemia depends on the degree of globin chain imbalance, it seems obvious that a minor reduction of the α-globin output, as in the −α/αα genotype, is sufficient to reduce the overall globin chain imbalance in βγ-thalassemia but not in βα-thalassemia in which the globin chain imbalance is more marked.

In this study there were, however, a limited number of patients either with the −158 mutation or with the −α/−α genotype who developed thalassemia major. Because we used very strict criteria for the definition of thalassemia major (i.e., Hb levels <7 g/dL for at least three consecutive months), we can reasonably exclude the chance to have erroneously considered this group of patients as affected by thalassemia major. While we have no explanation for this finding, we can postulate the presence of genetic factors negatively influencing the γ-chain expression or the proliferation potential of the stem cells in these patients. Further studies in the γ-globin gene region may define the reasons for this discrepancy and characterize the molecular mechanism for the mild clinical picture in those patients who did not inherit either the −158 mutation or α-thalassemia.

**ACKNOWLEDGMENT**

We thank Cecilia Bachis for her editorial assistance.

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