Molecular Characterization of β-Thalassemia Intermedia in Patients of Italian Descent and Identification of Three Novel β-Thalassemia Mutations

By S. Murru, G. Loudianos, M. Deiana, C. Camaschella, G.V. Sciarratta, S. Agosti, M.I. Parodi, P. Cerruti, A. Cao, and M. Pirastu

In this study, we have defined by dot-blot analysis with allelic specific oligonucleotide probes or direct sequencing on amplified DNA the β-thalassemia mutations in a large group of patients (23) of Italian descent with thalassemia intermedia. These patients had one parent with either the silent β-thalassemia carrier phenotype or borderline-normal hemoglobin A0 (HbA0) levels (2.5% to 3.5%). Nearly all were genetic compounds for a severe β-thalassemia mutation and a β-thalassemia mutation associated with high residual output of β-globin chains (β+ intervening sequence [IVS]-I-nt6, β-87, β-101), indicating that inheritance of a mild β-thalassemia allele, even in a single dose, is the most common molecular mechanism producing thalassemia intermedia in the Italian population. In three cases, in whom we failed to define by dot-blot analysis the mutations, we sequenced the β-globin gene and found three novel β-thalassemia mutations, which are certainly very rare because they have been hitherto detected solely in a single patient. These mutations consist of: (1) a T-A substitution at position 2 of IVS-I, in a patient compound heterozygote for this mutation and the −87 promoter mutation; (2) a G-C substitution at position 844 of IVS-II, in a patient heterozygous for this mutation who showed normal sequences at the in trans β-globin gene (The reason for the presence of clinical manifestations in a β-thalassemia heterozygote has not been defined.); and (3) a deletion of one nucleotide (−T) at codon 126, resulting in a frameshift and readthrough of the 5′ untranslated region and most likely producing an elongated Hb molecule of 156 amino acid residues, in a patient heterozygous for this mutation with normal β-globin gene sequences at the other locus.

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MATERIALS AND METHODS

Subjects

This study includes a group of 23 patients affected by thalassemia intermedia (Table 1), the large majority of Southern Italian descent, who were followed either at “Clinica Medica A,” Torino, or at “Centro Microcitemia Ospedale Galliera,” Genova. These patients had one parent with either the silent β-thalassemia carrier phenotype or borderline-normal HbA0 level (2.5% to 3.5%).

Hematologic Analysis

The red blood cell (RBC) indices were obtained with an automated cell counter (Technicon H6000). HbA0 was quantified by microchromatography,1 Fetal Hb was determined by alkali denaturation;2 Hb chain biosynthesis was performed according to Kan et al; and Hb electrophoresis was performed on cellulose acetate by standard methods.
Table 1. Hematologic Features in 23 Thalassemia Intermedia Patients of Italian Descent

<table>
<thead>
<tr>
<th>No.</th>
<th>Hb (g/dL)</th>
<th>HbA₂ (%)</th>
<th>HbF (%)</th>
<th>Genotype</th>
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<td>1</td>
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<td>3.7</td>
<td>63</td>
<td>β⁺-87/β⁺-IVS-I-nt110</td>
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<td>11</td>
<td>4.5</td>
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<tr>
<td>4</td>
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<td>4.5</td>
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DNA Analysis

DNA was isolated from peripheral blood leukocytes by phenol/chloroform extraction using standard techniques. Polymorphism haplotype analysis at the β-globin gene cluster was performed according to Orkin et al. Amplification of the β-globin gene was obtained from position −158 5' to the CAP site to position 60 3' to the polyadenylation signal by PCR, as previously described. In addition, we amplified: (1) a region 5' to the β-globin gene spanning from position −670 to position −60 5' to the CAP site using a pair of oligonucleotides with the following sequences: (A) 5' TGCAACAGACACATTGATT 3' and (B) 5' TGCCTCTGGAGTAGATTGGTGG 3'; (2) the second intervening sequence starting from codon 100 to position 705 in the IVS-II with the following oligonucleotides: (C) 5' CCTGAGACTTACGGTGA 3' and (D) 5' TCAGTACACATATGCA 3'.

Screening for known β-thalassemia mutations was performed by dot-blot analysis on amplified DNA with a series of oligonucleotide probes complementary to the most common β-thalassemia defects in the Mediterranean area.

DNA sequencing analysis was performed by the dideoxy chain termination method of Sanger et al on amplified single-strand DNA, using the enzyme T7 DNA polymerase (Sequenase US Biomedical Corporation). Sequencing gel and autoradiography were performed by standard techniques. To confirm the presence of the mutations defined by sequencing analysis we performed dot-blot analysis with ASO probes complementary to these mutations. Their sequences are as follows: family A, 5' CCTGAGAGACTTACGGTGA 3'; family B, 5' TTATCCTCCTGACCACAGC 3'; family C, 5' CCACCGAGCGGCTG 3'.

RESULTS

Detection of Known Mutations

The patients' DNA was screened for the presence of the most common β-thalassemia mutations in Mediterranean populations by dot-blot analysis on amplified DNA with ASO probes. The results are summarized in Table 1. The largest group includes 10 patients in whom we detected the β⁺-IVS-I-nt6 (T-C) mutation either in the homozygous state (two cases) or in the compound heterozygous state with other β⁺ or β⁺ thalassemia defects (β⁺-IVS-I-nt1, β⁺-IVSII-nt1, β⁺-39, β⁺-IVS-I-nt110) (eight cases). In seven patients we found the compound heterozygous state for the β⁺101 (C-T) promoter mutation and other β⁺ or β⁺ mutations (β⁺-39, β⁺-IVS-1nt1, β⁺-6, β⁺-IVS-I-nt110). In one of these families with the β⁺101 (C-T) mutation, one sibling, affected by a milder form of thalassemia intermedia, had the −101 defect in combination with a β⁺ globin gene with normal sequences but a complex rearrangement (−T+ATA) at position −530 5' to the CAP site, while the other two siblings, more severely affected, showed in addition the presence of the triple α-globin gene.

Four patients had the β⁺87 (C-G) promoter mutation; in three of them it was in the double heterozygous state with the β⁺-110 defect, while in the last one (no. 4 in Table 1) we were unable to define the other β-thalassemia defect. In two other patients (nos. 22 and 23 in Table 1) we failed to detect the mutation in either β⁺-globin genes. In these three cases we performed direct sequencing on amplified β⁺-globin genes.

Sequencing of Unknown Mutations

Family A. The propositus of family A, a 33-year-old woman, (no. 4 in Table 1) presented at 2 years of age with a thalassemia-like clinical picture. She received a regular transfusional program from 2 to 4 years, when, because of progressive splenomegaly, she was splenectomized. Following splenectomy, transfusions became necessary only sporadically. Hematologic analysis performed before starting transfusions showed: Hb 7.7 g/dL, mean corpuscular volume (MCV) 70 fl, mean corpuscular hemoglobin (MCH) 21 pg, HbA₂ 4.5%, and HbF 70%. A large number of nucleated RBCs were present in the peripheral blood smear. Both parents were heterozygous for typical high HbA₂, β-thalassemia.

Haplotype analysis showed the homozygosity of haplotype I. The α-globin gene arrangement was normal.

Direct sequencing of the amplified β⁺-globin gene showed in one chromosome the C-G transversion at position −87, which is a relatively common mild β-thalassemia mutation in Mediterranean populations, and in the other chromosome a novel mutation, i.e., a T-A transversion at position 2 of IVS-1 (β⁺-IVS-I-nt2, T-A), as shown in Fig. 1. This mutation was confirmed by oligonucleotide analysis with oligonucleotide probes complementary to the mutation or to the normal β⁺-globin gene sequence at the same position.

Family B. The proband of this family (no. 22 in Table 1), originating from Southern Italy (Puglia), is a 45-year-old woman affected by mild microcytic anemia, moderate splenomegaly, and gallstones. No thalassemia-like skeletal changes were detected. She received sporadic transfusions during the pregnancies. Hematologic analysis showed: Hb 8.2 g/dL, MCV 67 fl, MCH 18 pg, HbF 5%, and HbA₂ 5.7%. In conclusion, this patient has a mild thalassemia-like
intermedia phenotype, while her hematologic features are typical of heterozygous β-thalassemia.

She married a hematologically normal man. Of her four children, one showed a typical β-thalassemia carrier phenotype, the others being normal.

Haplotype analysis showed homozygosity for haplotype 1.

The α-globin gene arrangement was normal.

Direct sequencing of amplified β-globin gene showed a C-G transversion at position 844 of IVS-II (Fig 2). Normal DNA sequences were detected in the other β-globin gene. The presence of the IVS-II-nt844 (C-G) mutation was confirmed by oligonucleotide analysis using an ASO probe.

Family C. The proband of this family, originating from Northern Italy (Picmonte), is a 23-year-old woman with mild anemia and moderate spleen enlargement (II-1 in Fig 3A). She has never required transfusions. Pertinent hematologic data are reported in Fig 3A and in Table 1 (no. 23). Brilliant cresyl blue staining showed a limited number of erythrocytes with inclusion bodies in the peripheral blood. Inclusion bodies with the morphologic aspect of Fessas bodies were observed in the bone marrow-nucleated RBCs. No abnormal Hb was detected either by agar gel and cellulose acetate electrophoresis or isoelectrofocusing. Similarly, no abnormal globin chains were seen by globin chain synthesis analysis on CM-52 chromatography or isoelectrofocusing performed using standard incubation times. Heat and isopropanol stability tests gave normal results.

The father is hematologically normal (MCV = 83 fl; HbA2 = 3.4%; a/α ratio = 1.1). The mother shows normal RBC indices (MCV = 79 fl) and Hb pattern (HbA2 = 2.5%), but has an increased α/α-globin chain synthesis ratio (1.5), consistent with a diagnosis of the silent β-thalassemia carrier state.

Direct sequencing of the amplified β-globin gene in the proposita (Fig 3B) showed a deletion of a single nucleotide (−T) at codon 126 (normal sequence = GTG). This deletion results in a frameshift and readthrough of untranslated 3' β-globin gene sequences until a new stop codon (TAA) at position 156 is encountered. This mutation most likely results in the production of an elongated β-globin chain with additional 10 amino acids.

The presence of this molecular defect was confirmed by dot-blot analysis using an ASO probe.

Sequence analysis of the β-globin gene of the proband's father (I-1 in Fig 3A) was entirely normal, while that of the mother (I-2 in Fig 3A) showed a complex rearrangement at position −530 5' to the CAP site of the β-globin gene consisting of the insertion of an ATATA sequence and a deletion of a T (Fig 4). This sequence variation shows an additional TA insertion (ATATA v ATA) as compared with the rearrangement previously detected in a silent carrier of Albanian descent.10 The (−T +ATA) rearrangement has been seen in a large number of individuals who are either normal or affected by β-thalassemia or have different hemoglobinopathies (HbE or HbS).11,12 However, the normal persons so far studied have not been investigated by globin chain synthesis analysis, which is the only ACG T

Fig 1. Direct sequencing of amplified DNA from the propositus of family A. On the left, the autoradiogram shows the sequence at the splice junction between exon I and intron I. The donor site GT is mutated in GA (framed in the normal [N] and in the mutated [M] sequences). At the bottom, a graphic representation of the β-globin gene. An arrow marks the mutation point.

Fig 2. Sequencing gel of amplified β-globin gene from the propositus of family B, which shows the C-G substitution (circled) at position 844 in the polypyrimidine stretch at the 3' end of the large intervening sequence (IVS-II), five bases 5' to the consensus AG dinucleotide sequence (boxed). At the top, the β-globin gene is schematically shown with the mutation point indicated by an arrow.
MOLECULAR BASES OF β-THALASSEMIA INTERMEDIA

DISCUSSION

In this study we have characterized the β-thalassemia mutations in a large group of patients of Italian descent with thalassemia intermedia, who have one parent with either the silent β-thalassemia carrier phenotype or borderline-normal HbA, levels (2.5% to 3.5%).

Nearly all were compound heterozygotes for a severe β-thalassemia defect and a β-thalassemia mutation associated with a high residual output of β-globin chains (β'-IVS-1-nt6, β-101, β-87), indicating that the inheritance of a mild β-thalassemia allele, even in single dose, is the most common molecular mechanism producing thalassemia intermedia in the Italian population.

In those patients in whom we failed to define the mutations using this approach, we sequenced the entire β-globin gene on amplified DNA and found three novel β-thalassemia defects. These mutations are certainly very rare or even unique because they have been detected so far only in a single patient.

The T-A substitution at position 2 of IVS-1 alters the splice donor site. In analogy to the effect of other mutations at this position, normal splicing is most likely completely suppressed and the resultant phenotype is that of β⁺ thalassemia. At position 2 of IVS-1, T-G and T-C substitutions have already been reported in a Tunisian and in a Black patient, respectively. In both cases the clinical phenotype was that of β⁺ thalassemia. Our patient with the phenotype of thalassemia intermedia was a compound heterozygote for the –87 promoter mutation and the IVS-1-nt2 (T-A) mutation. Because it is most likely that the IVS-1-nt2 (T-A) mutation is associated with absent β-chain production, the –87 promoter mutation, which results in a
consistent output of β-globin chains, is responsible for the mild phenotype observed in our patient.

The second case described here is a patient with a very mild form of thalassemia intermedia in whom gene sequencing showed a single nucleotide substitution at position 844 of IVS-II while the other β-globin gene was normal. This substitution occurred seven nucleotides 5’ to the AG dinucleotide sequence of the splice acceptor site within the polypyrimidine stretch, which is mutated from CCTCCCACGC to CCTGCCACAG. A number of mutations have been detected in proximity of the invariant dinucleotide AG of the acceptor junction of the IVS-II, including the IVS-II position 849 (A-G),\textsuperscript{15} the IVS-II position 849 (A-C),\textsuperscript{16} the IVS-II position 848 (C-A),\textsuperscript{17,18} and the IVS-II position 843 (T-G).\textsuperscript{19} In analogy with the 843 (T-G) mutation, the IVS-II-nt 844 (C-G) described here most likely reduces the efficiency of splicing at the normal splice acceptor site of IVS-II and results in the production of β⁺ thalassemia. Our patient was heterozygous for the IVS-II-nt844 (C-G) mutation, while the other β-globin gene, from position -670 5' to the CAP site to position 60 3' to the polyadenilation site, had entirely normal sequences. These molecular findings are consistent with the hematologic features of heterozygous β-thalassemia presented by this patient. Clinically, however, we observed mild clinical thalassemia-like manifestations for which we have at the present time no explanations.

The third case had a thalassemia intermedia-like clinical condition associated with a limited number of inclusion bodies in peripheral erythrocytes and bone marrow-nucleated RBCs. Gene sequencing showed in one of the β-globin genes a nucleotide deletion (−T) at codon 126, resulting in a frameshift and readthrough of the 3' untranslated region until a new stop codon (TAA) is encountered at position 156 (Fig 3B). The other β-globin gene showed normal sequences. This mutation most likely results in an elongated Hb with an additional number of normal sequences. The resulting abnormal Hb molecule seems to be very unstable because it escaped detection by agar gel or cellulose acetate electrophoresis or isoelectrofocusing. Inclusions in the peripheral erythrocytes are most likely composed of precipitated Hb variants and amino acids at the carboxy terminus. The resulting abnormal Hb molecule seems to be very unstable because it escaped detection by agar gel or cellulose acetate electrophoresis or isoelectrofocusing. Accordingly, no abnormal globin chain was seen on CM-52 chromatography or isoelectrofocusing. Inclusions in the peripheral erythrocytes are most likely composed of precipitated Hb variants and α chains in excess.

According to the place of birth of the proband, this new Hb variant will be indicated as Hb Vercelli. This variant was not detected in the parents, indicating that it arose as a spontaneous mutation. It is interesting to note that the patient with this abnormal Hb is of Northern Italian extraction, a region where β-thalassemia occurs very rarely.

Hb Vercelli should be added to the growing list of mutations that result in the production of an elongated Hb molecule 156-amino-acids long, as in our case.\textsuperscript{20,21} These mutations manifest in the heterozygous state with a thalassemia-like intermedia phenotype frequently associated with inclusion bodies in peripheral erythrocytes. They occur sporadically or show a dominant transmission pattern. In the majority of them, protein analysis fails to detect the abnormal Hb because of its high instability. From the practical point of view, these mutations should be suspected in sporadic patients with a thalassemia-like intermedia phenotype and normal parents or in those families with a thalassemic disorder transmitted as a dominant character.

The mother of our patient with Hb Vercelli (I-2 in Fig 3A) shows normal hematologic features and normal Hb pattern, but has an unbalanced globin chain synthesis and, thus, may be classified as a silent β-thalassemia carrier. β-globin gene sequencing showed at position −530 5' to the CAP site a complex rearrangement consisting of deletion of a T nucleotide and insertion of the sequence ATATA after DNAse I protection studies, are indicated by a bracket and an arrow (when no endpoint could be determined); FAM.C = sequences found in the subject I-2 of this family: at position −590 5' to the CAP site, an ATATA insertion and a T deletion occurs; ALB.FAM. = sequences found in the original patient with the silent β-thalassemia carrier phenotype of Albanian descent.
possibility that the silent β-thalassemia carrier status, characterized by decreased expression of the β-globin gene, may result from a tighter binding of this repressor to the mutated −T+ATA or to the closely related −T+ATATA-rearranged sequence. Further studies are needed to define the role, if any, of the DNA region around position −530 in the function of the β-globin gene.

Definition of rare β-thalassemia mutations such as those characterized in this study may expand our knowledge on the functional anatomy of the β-globin gene and extend our diagnostic capabilities for the prevention of β-thalassemia.

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REFERENCES

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