Delineation of Specific β-Thalassemia Mutations in High-Risk Areas of Italy: A Prerequisite for Prenatal Diagnosis

By Mario Pirastu, Giuseppe Saglio, Clara Camaschella, Angela Loi, Anna Serra, Tiziana Bertero, Wilma Gabutti, and Antonio Cao

In this study, we defined by haplotype characterization combined with oligonucleotide hybridization or direct restriction endonuclease analysis the specific β-thalassemia mutations in a representative sample of β-thalassemia chromosomes from patients with homozygous β-thalassemia originating from different parts of Italy. We characterized the mutations in 90% of the thalassemia chromosomes and found that three mutations, namely the β^IVS 1-110, β^-39 and β^'IVS 1-6 are prevalent in the Italian population. Most of the patients investigated were compound heterozygotes for two β-thalassemia mutations, and only a few were homozygotes for one mutant. On the basis of these findings, we predict that prenatal diagnosis in this population would be feasible in most cases by fetal DNA analysis with the oligonucleotide method using a limited number of oligonucleotide probes selected after screening parents for the most common β-thalassemia mutations. We have also devised a method based on hybridization with a mixture of two oligonucleotides that allows rapid and simultaneous screening of prospective parents for the two most frequent mutations in Italians, the β^IVS 1-110 and β^-39 mutants. This method may be applicable to prenatal diagnosis in cases at risk for the genetic compound of these mutations.

© 1988 by Grune & Stratton, Inc.

Prenatal diagnosis of β-thalassemia can now be accomplished by the analysis of choriocarcinoma or amniocytic DNA. Most β-thalassemias are caused by single nucleotide substitution, addition, or deletion of a few nucleotides, and may be directly detected by using complementary oligonucleotide probes. Only a few β-thalassemia mutations are due to a gross rearrangement of the DNA or affect a restriction recognition site and thus may be identified directly by Southern blot analysis. Alternatively, fetal diagnosis may be carried out indirectly by linkage analysis with polymorphic restriction sites at the β-globin gene cluster. Large-scale prevention of β-thalassemia requires the extension of prenatal testing to prospective couples without previous children. In these cases when no children are available only methods that directly detect the mutation can be used since linkage analysis entails a cumbersome and sometimes futile family study. The critical prerequisite for large-scale application of the direct method of DNA analysis is definition of the molecular basis of β-thalassemia in the target population. According to the few studies carried out thus far, a limited number of β-thalassemia mutations are prevalent in each population. Thus, in each population, prenatal diagnosis could be accomplished in most cases by the use of a few oligonucleotide probes and restriction enzymes able to detect the most frequent β-thalassemia mutations.

Recent studies have shown that in general, in every population, each specific β-thalassemia mutation is associated with one or a few patterns of polymorphic restriction sites at the β-globin gene cluster (haplotype). Oligonucleotide hybridization, associated with cloning and sequence analysis of a group of chromosomes from each haplotype, may lead to the characterization of the molecular basis for β-thalassemia in each population. Because information on the distribution and frequency of β-thalassemia mutations in the various at-risk areas of Italy is scarce, we have now characterized the haplotypes associated with β-thalassemia chromosomes from patients with homozygous β-thalassemia originating from different parts of Italy and defined the β-thalassemia mutation contained in each haplotype by oligonucleotide hybridization or restriction endonuclease analysis.

The results show that three mutations, the G-A substitution at position 110 of the first intervening sequence (β^IVS 1-110), the C-T substitution at codon 39 (β^-39), and the T-C substitution at position 6 (β^'IVS 1-6) are the most prevalent (80%). Thus, prenatal diagnosis in this population can be accomplished in most cases by using three oligonucleotide probes complementary to these mutations. Because more than one mutation predominates, the thalassemia major phenotype is often caused by double heterozygosity for two mutations, commonly the β^IVS 1-110 and the β^-39. We therefore devised a method based on hybridization with a mixture of two oligonucleotide probes that allows a rapid and simultaneous screening of prospective parents for the two most frequently occurring β-thalassemia mutations found in Italians.

PATIENTS AND METHODS

Peripheral blood samples were collected from 110 Italian patients affected by thalassemia major (87 cases) or intermedia (23 cases) followed at the Departments of Pediatrics and Internal Medicine in Torino University where patients from different high-risk areas of...
Italy are admitted. All parents of these patients were typical high Hb A1 β-thalassemia carriers. To assess the frequency of specific β-thalassemia mutations in each area, only patients whose parents originated from the same Italian region were included. Patients of Sardinian descent were excluded because the molecular bases of origin came from the same Italian region were included. Patients of fl-thalassemia mutations in each area, only patients whose parents from each haplotype were studied either by the chromosomes fl-thalassemia mutations as defined by previous studies were hybridized to the fl-globin gene,23,24 and DNA fragments at the same sequences at the same polymorphism were assigned the polymorphism studies were carried out by means of two oligonucleotides probes, and one () mutation, the 1-globin gene,23,24 were hybridized together to the 1-globin gene,23,24 with haplotype V (9) haplotype VI (19) with the oligonucleotide probe complementary to the I1IVS 1-6 mutation, and chromosomes with haplotype V (9) with the fl-globin gene,23,24 BamHI 3' to the β-globin gene. The haplotypes are numbered according to Orkin et al.14

The probes used were genomic fragments corresponding to the ε, γ, Ψ, and β-globin genes. In case of ambiguous combination, family studies were carried out to assign the polymorphism pattern correctly.

Direct analysis of the mutation. A group of β-thalassemia chromosomes from each haplotype was studied either by the oligonucleotide technique or by restriction endonuclease analysis. The oligonucleotide probe or restriction enzyme used was selected on the basis of the pattern of association between haplotypes and specific β-thalassemia mutations as defined by previous studies in the Mediterranean populations.14,17

Oligonucleotide analysis was carried out as previously described12,26 to detect the following β-thalassemia mutations: β1IVS 1-110, βo39, β1IVS 1-6, G-A substitution at position 1 of IVS-1 of the β-globin gene (β1IVS 1-1), and T-G substitution at position 116 of IVS 1 (βoIVS 1-116). The analysis of each mutation was carried out by means of two oligonucleotides (19-mers) probes, one (βo) complementary to the β-globin gene sequence around the mutation, and one (β1) homologous to the normal β-globin gene sequences at the same position. The probes differed from each other by a single nucleotide position in the middle of the sequence. The oligonucleotides were purified by gel electrophoresis and 5' end-labeled with 32P by T4 polynucleotide kinase.

Chromosomes with haplotype I (42) were analyzed with the oligonucleotide probe complementary to the β1IVS 1-110 mutation, chromosomes with haplotype II (23) and III (8) with the oligonucleotide probe homologous to the βo39 gene, chromosomes with haplotype VI (19) with the oligonucleotide probe complementary to the β1IVS 1-6 mutation, and chromosomes with haplotype V (9) with an oligonucleotide probe complementary to the βoIVS 1-1 mutation.

Ten micrograms of DNA was digested with the restriction endonucleases BamHI or AccI, according to the recommendations of the manufacturer (Amersham, Buckinghamshire, UK), and DNA fragments were separated by agarose gel electrophoresis. All β-thalassemia mutations examined (β1IVS 1-110, βo39, β1IVS 1-6, βoIVS 1-1) reside in the 1.8-kilobase (kb) BamHI fragment that contains the 5' part of the β-globin gene. With AccI digestion, the βo39 mutation resides in a 1.6-kb β-globin-specific fragment, the other three mutations occur together in a 0.7-kb fragment.

Usually two identical gels were run, one hybridized to the βo probe and the other to the β1 probe. Gels containing AccI fragments were hybridized to a mixture of the β1IVS 1-110 and βo39 probes. Washing and hybridization were carried out at 54°C for the βo39, β1IVS 1-6, βoIVS 1-1 probes, and at 42°C for the β1IVS 1-110. Experiments with the mixture of the β1IVS 1-110 and βo39 probe were performed at 50°C.

Mutations contained in haplotype VI and III were investigated by direct restriction endonuclease analysis. Chromosomes with haplotype VII (8) were studied with the restriction enzyme Rsal, which recognizes a new site created by the C-G substitution at position 745 of the IVS 2 of the β-globin gene (βoIVS 2-745).14 Chromosomes with haplotype III (3) were investigated with the restriction enzyme Hphi, which detects the G-A substitution at position 1 of the IVS 2 (βoIVS 2-1) of the β-globin gene.27

RESULTS

Haplotypes and distribution. We defined the haplotypes of 220 β-thalassemia chromosomes from Italian patients and found, as expected from previous studies,28 an extreme heterogeneity (Table 1). A notable exception occurred in the Delta Po area, where only three haplotypes were detected and haplotype VI was completely lacking.

The frequency and distribution of haplotypes in the different Italian regions are summarized in Table 1. In all regions, the most frequent was haplotype I, which accounts for 41% of the total. Excluding the populations from the Delta Po area and Campania, the second and third in order of decreasing frequency were haplotypes II and VI, which represent 23% and 14% of the total, respectively.

Only a minority of the patients (31%) were homozygous for one haplotype, most frequently haplotype I (16%), II (10%), or VI (6%), and very rarely haplotype V (1.8%) and VII (0.9%). The majority (69%) showed the genetic combination of two haplotypes, most frequently, as expected, haplotype I-III.

Detection of specific mutation. Figure 1 shows representative autoradiograms of leukocyte DNA from thalassemia patients hybridized with different oligonucleotide probes, and Table 2 summarizes the results. Screening of β-thalassemia chromosomes with an oligonucleotide probe or restriction enzyme selected on the basis of the pattern of association between haplotypes and specific β-thalassemia mutations showed that 34 of 42 (80%) of the chromosomes with haplotype I had the β1IVS 1-110 mutation, 28 of 31 (91%) of those with haplotype II-IX showed the βo39 mutation, and 7 of 8 (87%) of those with haplotype VII had the β1IVS 2-745 mutation. All chromosomes with haplotypes V, VI, and III contained the βoIVS 1-1, the β1IVS 1-6, and βoIVS 2-1 mutations, respectively.

Chromosomes (13) in which the mutation was not defined

<p>| Table 1. Haplotypes Associated with β-Thalassemia Chromosomes in Different Italian Regions |
|-------------------------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Region</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>IX</th>
<th>Others</th>
<th>No. of Chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Po Delta</td>
<td>18</td>
<td>11</td>
<td>—</td>
<td>—</td>
<td>8</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>37</td>
</tr>
<tr>
<td>Puglia</td>
<td>17</td>
<td>17</td>
<td>2</td>
<td>5</td>
<td>13</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>62</td>
</tr>
<tr>
<td>Basilicata</td>
<td>6</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>12</td>
</tr>
<tr>
<td>Calabria</td>
<td>27</td>
<td>5</td>
<td>5</td>
<td>9</td>
<td>3</td>
<td>3</td>
<td>—</td>
<td>—</td>
<td>52</td>
</tr>
<tr>
<td>Campania</td>
<td>6</td>
<td>—</td>
<td>2</td>
<td>—</td>
<td>3</td>
<td>—</td>
<td>—</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Sicilia</td>
<td>16</td>
<td>14</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>1</td>
<td>4</td>
<td>—</td>
<td>46</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>51</td>
<td>14</td>
<td>31</td>
<td>11</td>
<td>17</td>
<td>2</td>
<td>220</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>17.7%</td>
<td>29.6%</td>
<td>17.8%</td>
<td>15.1%</td>
<td>17.4%</td>
<td>2.9%</td>
<td>20.2%</td>
<td></td>
</tr>
</tbody>
</table>

From www.bloodjournal.org by guest on October 3, 2017. For personal use only.
Fig 1. Detection of four $\beta$-thalassemia mutations by oligonucleotide hybridization. The mutations examined reside in the 5' part of the $\beta$-globin gene, which is included in a BamHI 1.8-kb fragment (arrow). N, normal chromosomes; I, II, III, V, VI, and VII are thalassemia chromosome haplotypes. Probe is specific for the $\beta^+39$ mutation (a) and hybridization is evident in lanes 2 and 3 (homozygote and heterozygote for the $\beta^-39$ mutation, respectively); probe is specific for the $\beta^+IVS1-110$ mutation (b) and positive hybridization is evident in lanes 1 and 3 (homozygote and heterozygote for the $\beta^-IVS1-110$ mutation, respectively); probe is complementary to the IVS 1-6 mutation (c) and hybridization is evident in lanes 2 and 3 (homozygote and heterozygote for the $\beta^+IVS1-6$ mutation, respectively); probe is specific for the $\beta^-IVS1-1$ mutation (d) and hybridization is evident in lanes 2 and 3 (homozygote and heterozygote for the $\beta^+IVS1-1$ mutation, respectively). The sequences of the oligonucleotide probes are: $\beta^+39$ GGAACCTGGATCTCCAAGA, $\beta^-IVS1-110$ CTCTGCCTATTAGTCTATT, $\beta^-IVS1-6$ GCAGGTTGGCATCAAGGTT, and $\beta^+IVS1-1$ CCTGGCGAGATTGGAATCA.

by this approach were screened by all oligonucleotide probes available and HphI, MstII, and RsaI restriction endonuclease analysis. The $\beta^-39$ mutant was detected in 3 of 42 chromosomes with haplotype I, and the $\beta^+IVS1-110$ was detected in 1 of 23 chromosomes with haplotype II.

In the remaining nine $\beta$-thalassemia chromosomes, we were not able to identify the mutation. Hb Knossos, which in the Mediterranean area is associated in the heterozygous state with normal Hb A2 levels, was reasonably excluded in the undefined nine $\beta$-thalassemia chromosomes because all the patient’s parents had elevated Hb A2 levels.

The distribution of the $\beta$-thalassemia mutations in Italy is depicted in Fig 2. Three mutations, namely the $\beta^+IVS1-110$, $\beta^+39$, and $\beta^+IVS1-6$ accounted for 80% of the $\beta$-thalassemia lesions. Most of the patients were compound heterozygotes for two different $\beta$-thalassemia alleles. The most frequent combination was between the $\beta^+IVS1-110$ and $\beta^-39$ mutations.

Simultaneous detection of two different mutations. A mixture of two oligonucleotides ($\beta^+IVS1-110$ and $\beta^-39$) was used to screen prospective parents for these two mutations simultaneously and to detect homozygous $\beta$-thalassemia in patients doubly heterozygous for these mutations. As shown in Fig 3, AccI digestion produces two $\beta$-globin-specific fragments, one 0.7 kb long containing the $\beta^+IVS1-110$ mutation and one 1.6 kb long in which the $\beta^-39$ mutation resides. DNA from a carrier of the $\beta^+IVS1-110$ mutation shows hybridization of the 0.7-kb fragment (lane 1); DNA from a normal subject shows no hybridization either of the 0.7-kb or the 1.6-kb fragments (lane 2). DNA from a carrier of the $\beta^-39$ mutation shows hybridization of the 1.6-kb fragment (lane 3); and DNA from a thalassemic patient, genetic compound for these mutations, shows hybridization of both fragments (lane 4). This method

Table 2. Association Between Haplotypes and Specific $\beta$-Thalassemia Mutations in Italians

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Haplotypes</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>IX</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Chromosomes</td>
<td></td>
<td>42</td>
<td>23</td>
<td>9</td>
<td>19</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>$\beta^+IVS1-110$</td>
<td></td>
<td>34</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta^-39$</td>
<td></td>
<td>3</td>
<td>22</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta^+IVS1-1$</td>
<td></td>
<td></td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta^+IVS1-6$</td>
<td></td>
<td></td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta^+IVS2-745$</td>
<td></td>
<td></td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta^+IVS2-1$</td>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown*</td>
<td></td>
<td>5</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*In these chromosomes, we excluded the presence of the mutations listed in Table 2 as well as frameshift at codon 6.
DISCUSSION

This study confirms that β-thalassemia in the Italian population is very heterogeneous. Six different mutations were identified by the combination of oligonucleotide hybridization and restriction endonuclease analysis. Several other mutations would certainly have been identified by cloning and sequencing β-globin genes that escaped definition by this approach. The frequencies of specific mutations found in this study are in close agreement with those previously established in North Americans of Italian origin.\(^{14,29}\) We confirmed that each specific β-thalassemia mutation is generally associated with a specific chromosome haplotype.\(^7\) However, a specific mutation was also found associated with two or three different haplotypes. For instance, the \(\beta^+\) IVS 1-110 mutation, which is most frequently associated with haplotype I,\(^{14,17}\) was also detected in haplotype II, and conversely the \(\beta^a-39\) mutation, which is usually contained either in haplotype II or IX,\(^{14,17}\) was found also to be associated with haplotype I.

Notwithstanding the large heterogeneity of the molecular defects identified and the differences in their relative frequencies among populations originating from different Italian regions, on the whole, three mutations, namely the \(\beta^+\) IVS 1-110, the \(\beta^a-39\), and the \(\beta^+\) IVS 1-6 accounted for 80% of the β-thalassemia lesions. Apart from Sardinia, where the \(\beta^a-39\) nonsense mutation is the predominant lesion,\(^{18,20}\) less

---

**Fig 2.** Distribution of the common β-thalassemia mutations in different Italian regions. The number of chromosomes studied were 16 in Po Delta (1), 30 in Puglie (2), 12 in Basilicata and Campania (3), 30 in Calabria (4), and 24 in Sicilia (5).

Data for the Sardinian population (6) were published in a previous article.\(^6\) The symbols \(\beta^+110\) and \(\beta^+6\) correspond to the \(\beta^+\) IVS 1-110 and \(\beta^+\) IVS 1-6 mutations, respectively.

---

**Fig 3.** Acc I map within the β-globin gene region (left) and autoradiograms of gels hybridized with a mixture of two oligonucleotides complementary to the \(\beta^a-39\) and the \(\beta^+\) IVS 1-110 mutations, respectively (right). The \(\beta^+\) IVS 1-110 and the \(\beta^a-39\) mutation reside in the 0.7-kb and 1.6 kb fragment, respectively. Hybridization of the 0.7-kb fragment from heterozygote for the \(\beta^+\) IVS 1-110 mutation DNA is apparent, hybridization of the 1.6-kb fragment from DNA of a compound heterozygous state for these mutations.

\(\beta th^{39}/\beta th^+IVS1-nt110\)
heterogeneity was found in the Delta Po area, where only two mutations, \( \beta^* \text{IVS} 1-100 \) and \( \beta^0-39 \), were detected.

The results of the distribution and relative frequency of specific \( \beta \)-thalassemia mutations in the various at-risk areas of Italy produced in this study will be very useful for planning antenatal diagnosis in the Italian population. The most appropriate oligonucleotide probes or restriction enzymes for each high-risk area can be used to screen both parents. The application of this approach led us to make a successful antenatal diagnosis by oligonucleotide hybridization of chorionic villus DNA in a case at risk for the genetic combination of the \( \beta^0-39 \) and the \( \beta^* \text{IVS} 1-110 \) mutations.\(^{31}\)

The \( \beta^* \text{IVS} 1-6 \) mutation requires additional information on genetic counseling. This mutation is reported to be associated with mild phenotype.\(^{22,23}\) Clinical information was available for 20 patients who carry haplotype VI, which is invariably associated with this mutation in Italy (Table 2). Five patients homozygous for this mutation have thalassemia intermedia and do not require blood transfusion. Of the 15 patients doubly heterozygous for this mutation and another \( \beta \)-thalassemic lesion, 6 have mild thalassemia and 9 require blood transfusion from an early age. The types of mutation interacting with the \( \beta^* \text{IVS} 1-6 \) are not different for the two groups. Thus, in families at risk for this mutation, this information should be provided at genetic counseling.

The method we have devised for screening for two specific mutations simultaneously allow us either to identify or exclude the presence of the two most frequent mutations in Mediterraneans. This method can also be applied to antenatal diagnosis for those cases at risk for the most frequent genetic compound in Italians, the \( \beta^* \text{IVS} 1-110 \) and \( \beta^0-39 \) combination. Double heterozygotes for other combinations can similarly be determined using different restriction enzymes to screen for several different mutations at the same time.

For couples at risk for more rare \( \beta \)-thalassemia mutations prenatal diagnosis can be carried out either by fetal blood analysis\(^ {24} \) or, for those who have children, by linkage analysis with polymorphic restriction sites.\(^ {1,5} \)

Our results would also be relevant for developing antenatal diagnosis programs in North American couples of Italian origin. The knowledge of the Italian region from which prospective parents originate may indeed indicate with a high degree of probability the types of molecular defects that should be investigated with synthetic oligonucleotide probes or direct restriction endonuclease analysis.

ACKNOWLEDGMENT

We thank Dr. Y. W. Kan for his continuing advice and Rita Loi for editorial assistance.

REFERENCES

20. Pirastu M, Galanello R, Doherty M, Tuveri T, Cao A, Kan YW: The same \( \beta \)-globin gene mutation is present on nine different \( \beta \)-thalassemia chromosomes in a Sardinian population. Proc Natl Acad Sci USA 84:2882, 1987
Delineation of specific beta-thalassemia mutations in high-risk areas of Italy: a prerequisite for prenatal diagnosis

M Pirastu, G Saglio, C Camaschella, A Loi, A Serra, T Bertero, W Gabutti and A Cao