A Family With Segregating Triplicated Alpha Globin Loci and Beta Thalassemia

By Renzo Galanello, Rita Ruggeri, Elisabetta Paglietti, Maria Addis, Maria A. Melis, and Antonio Cao

In this article we report a Sardinian family, in which a β-thalassemia gene and a triple α-globin loci, counterpart of the rightward deletion type α-thalassemia-2, were segregating. The analysis of the genotype–phenotype correlations in the different family members allowed us to give an outline of the manifestations associated with different genotype combinations. The heterozygote for the triple α-loci showed no consistent abnormal clinical or hematologic characteristics and presented balanced α/β-globin chain synthesis. In the homozygous state for this lesion, the only phenotypic expression was a slightly imbalanced globin chain synthesis. The combination of heterozygous β-thalassemia with the heterozygous state for the triple α-globin loci produced no clinical manifestations and showed a hematologic phenotype indistinguishable from that of heterozygous β-thalassemia. On the other hand, the combination of the homozygous state for the triple α-globin gene loci and the heterozygous state for β-thalassemia produced a clinical picture of thalassemia intermedia with a very mild clinical course, minor increase of fetal hemoglobin (HbF) levels, and a pronounced imbalance of globin chain synthesis.

Between the severe transfusion-dependent homozygous form of β-thalassemia (thalassemia major) and the very mild or symptom-free heterozygous carrier state, there are forms of thalassemia of intermediate severity that do not require transfusion therapy (thalassemia intermedia). This clinical syndrome may result from many different disorders of hemoglobin synthesis, producing a lesser globin chain imbalance and thus a milder condition than that seen in thalassemia major.

We report a Sardinian family in which a β-thalassemia gene and a triple α-globin loci were segregating. The propositus, showing the phenotypic manifestations of thalassemia intermedia, was found to have a combination of the homozygous state for α-thalassemia and the homozygous state for the triple α-globin loci. This new genetic interaction should be added to the list of disorders producing thalassemia intermedia.

Materials and Methods

Red cell indices were measured with a Coulter Counter model S, and other hematologic data were obtained according to standard procedures. Hemoglobin electrophoresis was carried out on cellulose acetate plates (Tian III, Helena Lab., Beaumont, TX), using a Tris-EDTA borate buffer, pH 8.6. Hemoglobin A2 was determined by column chromatography and fetal hemoglobin (HbF) by alkali denaturation. Supravital staining of the erythrocytes with methyl violet and brilliant cresyl blue for intracellular inclusion was performed by standard methods. Serum ferritin was determined using a radioimmunoassay (Ramco Laboratories, Houston, TX). Globin chain synthesis analysis in peripheral blood reticulocytes was carried out by methods already described.

DNA was prepared from white blood cells by phenol-chloroform isoaamyl alcohol extraction and ethanol precipitation. Ten micrograms of DNA was digested with restriction endonucleases Eco RI, Bam HI, Bgl II, and Hind III at 37°C for 24 hr using the conditions recommended by the manufacturers from whom they were purchased. The restricted DNA was then electrophoresed in 0.8% agarose for 16–18 hr and transferred to nitrocellulose filters. In order to establish the α-globin genotype, the filters were hybridized with specific 32P-labeled α-globin gene probe prepared by nick-translation, as described by Maniatis et al. from α-globin genes cloned in plasmid JW-101. Restriction enzyme-digested DNA was also hybridized with γ, y, ψ, β, and β probes in order to analyze the arrangement of the β-like globin gene cluster. Polymorphic restriction sites were used to determine the haplotype around the β-globin cluster. The sites studied were Hind II, 5' to the ε-globin gene, Hind III in the second intervening sequence (IVS) of the γ-globin, and Aγ-globin genes, Hind II within and 3' to the ψβ gene, Ava II in the second IVS of β-globin, and Bam HI, 3' to the β gene.

Results

Clinical Findings

A 28-yr-old Sardinian man (II-9) (Fig. 1) was referred at our outpatient service for slight anemia and mild jaundice, which was first noted at between 1 and 2 yr of age. The parents were first cousins once removed. He had never received a blood transfusion, but was repeatedly treated with different hematincs and iron. Physical examination showed normal growth and development, no skeletal deformity, and a slight hepatosplenomegaly. Leg ulcers and gallstones were not observed. Cardiac evaluation, including echocardiography, revealed no abnormality.

Hematologic Findings (Table 1)

The propositus had hemoglobin values in the range of 8–10 g/dl at repeated determinations and a typical
thalassemic blood picture. His hemoglobin pattern showed 7.56% HbF and 4.85% HbA₂, the rest being HbA. The red blood cells were microcytic with mean cell volume (MCV) values in the range of 59–67 fl and hypochromic with MCH values around 20–21 pg. The reticulocyte count was 3.3%, and there were rare (2/100 white blood cells) nucleated red blood cells in peripheral blood. Supravital staining of peripheral blood with methyl violet showed very few red blood cells with inclusion bodies. Bilirubin levels were slightly increased (total 2.7 mg/dl, conjugated 0.43 mg/dl). Serum ferritin was 275 ng/ml (normal values 25–280 ng/ml). There was a pronounced imbalance of globin chain synthesis, with an α/non-α ratio of 4.56. This clinical and hematologic phenotype can be classified within the broad definition of thalassemia intermedia.¹

**Restriction Endonuclease Analysis**

α-Globin gene mapping. DNA from the propositus digested with Eco RI, which cleaves the human DNA outside the two α-globin gene loci,¹³ produced a new band 27.0 kb (not shown) in length, which is 3.7 kb longer than the normal 23.0 kb band¹⁴ (Fig. 2). This elongated band has already been shown to contain a triplicated α-globin loci, the counterpart of the rightward deletion α-thalassemia-2 genotype.¹⁴ Since the DNA from our propositus produced no normal fragments, both of his no. 16 chromosomes should contain the triplicated α-globin loci.

Digestion of the DNA of the propositus with Bam HI, which also cleaves the human DNA outside the two α-globin gene loci, produced a new fragment 18.0 kb in length, which is 3.7 kb longer than the 14.5 kb

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**Table 1. Hematologic Data of the Propositus (II-9) and His Family According to Their Globin Genotypes**

<table>
<thead>
<tr>
<th>α-Globin Genotype:</th>
<th>aαa/aαa</th>
<th>aαa/aαa</th>
<th>aαa/aαa</th>
<th>aαa/aαa</th>
<th>aαa/aαa</th>
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<tbody>
<tr>
<td>β-Globin Genotype:</td>
<td>a/β²</td>
<td>a/β²</td>
<td>a/β²</td>
<td>a/β²</td>
<td>a/β²</td>
</tr>
<tr>
<td>Subjects:</td>
<td>II-4</td>
<td>II-9</td>
<td>II-1</td>
<td>II-6</td>
<td>I-2</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>35</td>
<td>28</td>
<td>42</td>
<td>34</td>
<td>66</td>
</tr>
<tr>
<td>RBC (x 10¹²/liter)</td>
<td>4.48</td>
<td>4.46</td>
<td>4.19</td>
<td>5.30</td>
<td>4.30</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>9.8</td>
<td>10.1</td>
<td>13.3</td>
<td>16.1</td>
<td>13.2</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>30.0</td>
<td>30.9</td>
<td>38.3</td>
<td>46.2</td>
<td>37.2</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>67</td>
<td>67</td>
<td>91</td>
<td>88</td>
<td>86</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>21.9</td>
<td>21.8</td>
<td>31.9</td>
<td>30.4</td>
<td>30.7</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>31.9</td>
<td>31.8</td>
<td>33.9</td>
<td>35.1</td>
<td>34.5</td>
</tr>
<tr>
<td>HbA₂ (%)</td>
<td>4.73</td>
<td>4.85</td>
<td>2.70</td>
<td>2.83</td>
<td>2.41</td>
</tr>
<tr>
<td>HbF (%)</td>
<td>2.8</td>
<td>7.6</td>
<td>0.9</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Globin chain synthesis α/β ratio</td>
<td>3.57</td>
<td>4.56</td>
<td>1.83</td>
<td>1.47</td>
<td>1.07</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>2.1</td>
<td>3.3</td>
<td>2.3</td>
<td>—</td>
<td>1.2</td>
</tr>
</tbody>
</table>
normal one\(^{14}\) (Figs. 2 and 3). This pattern, with no normal Bam HI bands but only an elongated band, again indicates the homozygous state for the triple \(\alpha\)-globin loci.

This interpretation was further substantiated by the analysis of the Bgl II map. Digestion of DNA from our propositus with Bgl II, which cuts human DNA outside and between the two \(\alpha\)-globin loci,\(^{15}\) produced, besides the two normal fragments 12.5 and 7.2 kb in length containing the \(\alpha_2\) and \(\alpha_1\) loci, respectively, an additional fragment 3.7 kb in length, which was found to contain the third \(\alpha\)-globin locus (Figs. 2 and 4). This Bgl II map is very similar to that already observed in heterozygotes for the triple \(\alpha\)-globin loci.\(^{14,16-18}\) The 3.7-kb band present in our propositus was, however, much more intense as compared to that of heterozygotes (II-2, II-7, III-1) (Fig. 4) for the triple \(\alpha\)-globin loci, indicating a higher amount of hybridizing DNA sequences than expected in the heterozygous state for this lesion.

Digestion of DNA from our propositus, with Hind III, which cleaves the coding sequence of the \(\alpha\)-globin structural gene at the position corresponding to amino acids 90 and 91,\(^{19}\) produced a normal pattern with 16.0, 3.7 and 4.5 kb fragments (Figs. 2 and 5). The 3.7-kb band was, however, more intense than that produced either from normal DNA or from DNA of heterozygotes for the triple \(\alpha\)-globin loci, which in turn produce a band with higher intensity as compared to normal DNA.

From these results we may conclude that our propositus is a homozygote for the triple \(\alpha\)-globin loci (\(\alpha\alpha\alpha/\alpha\alpha\alpha\)). Moreover, because of the high HbA2 levels, he is...
Mutation (nonsense mutation at codon 39) in this population. Thus, the $\beta$-thalassemia gene segregating in our family is very likely the common $\beta^S$ Sardinian amber termination mutant.

Family Studies (Tables 1 and 2 and Fig. 1)

The mother of the propositus (I-2) was hematologically normal. The $\alpha/\beta$-globin chain synthesis ratio fell within the normal range, and $\alpha$-globin gene mapping showed a pattern compatible with the heterozygous state for the triple $\alpha$-globin loci. In fact, Bam HI and Eco RI produced both the normal and the elongated bands, and the 3.7-kb bands obtained after digestion with both Bgl II and Hind III were less intense than in the propositus (Figs. 3–5). Her genotype should be $(\alpha aa/\alpha aa; \beta^A/\beta^A)$. His wife (II-10) was hematologically normal and showed a normal $\alpha$-globin gene map $(\alpha aa/\alpha aa; \beta^A/\beta^A)$ (Figs. 3–5).

A sister (II-1) showed neither clinical nor hematologic thalassemia-like manifestations. In particular, HbA2 levels were repeatedly normal. Her $\alpha/\beta$-globin chain synthesis ratio was 1.66, which is within the range already found in Sardinian $\beta^S$-thalassemia carriers (1.51 ± 0.26). The restriction enzyme pattern was identical to that of the propositus. Her genotype can be written: $(\alpha aa/\alpha aa; \beta^A/\beta^A)$.

A brother (II-3) had normal hematologic features and balanced $\alpha/\beta$-globin chain synthesis. DNA analysis showed a normal complement of $\alpha$-globin gene loci.

A sister (II-4) showed a slight microcytic anemia, with a Hb level below 10 g/dl and, apart from a slight spleen enlargement, no clinical manifestations. Her hemoglobin pattern was characterized by increased HbA2 (4.73%) and HbF (2.80%) levels. There was a minor increase of bilirubin. Reticulocyte count was 2.1%, and there were few nucleated red blood cells in peripheral blood. Supravital staining of peripheral blood with methyl violet showed very few red blood cells with inclusion bodies. The $\alpha/\beta$-globin chain synthesis ratio was 3.57. The presence of spleen enlargement, moderate anemia, nucleated red blood cells and red blood cells with inclusion bodies in peripheral blood, associated with a remarkable imbalance of

<table>
<thead>
<tr>
<th>Family M.</th>
<th>Hinc II $5^\prime$</th>
<th>Hind III $\gamma$</th>
<th>Hinc II $\gamma$</th>
<th>Ave II $3^\prime$</th>
<th>Bam HI $3^\prime$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-2 Normal/normal</td>
<td>+/−</td>
<td>−/+</td>
<td>−/+</td>
<td>−−/+</td>
<td>−/+</td>
</tr>
<tr>
<td>II-9 $\beta^s$/normal</td>
<td>+/+</td>
<td>−/−</td>
<td>−/−</td>
<td>−/+</td>
<td>−/+</td>
</tr>
<tr>
<td>II-4 Normal/normal</td>
<td>−/−</td>
<td>+/+</td>
<td>−+</td>
<td>+/−</td>
<td>+/+</td>
</tr>
<tr>
<td>III-1 $\beta^s$/normal</td>
<td>+/−</td>
<td>−/+</td>
<td>−/+</td>
<td>−/+</td>
<td>+/+</td>
</tr>
</tbody>
</table>

*Bold symbols indicate haplotypes of the $\beta$-thalassemia chromosome.
TRIPLE α-GLOBIN LOCI AND β-TALASSEMIA

1039

globin chain synthesis, led us to consider this patient as a milder form of thalassemia intermedia.1 The restriction enzyme pattern was identical to that of the propositus (Figs. 3–5). Her genotype is therefore (αααα/αααα; βthal/β+).

A brother (II-6) had normal hematologic characteristics associated with a slight imbalanced α/β-globin chain synthesis (1.47). DNA analysis of the α-globin gene cluster indicates the homozygous state for triple α-globin gene loci.

A sister (II-7) had normal red cell indices and Hb pattern, and α/β-globin chain synthesis was slightly unbalanced (1.19). The restriction enzyme pattern was identical to that of the mother. Her genotype is therefore (αααα/αααα; β+β+).

One of the two daughters (III-1) showed a slight microcytic anemia, with Hb levels around 10 g/dl and thalassemia-like red blood cell modifications. Her hemoglobin pattern showed 4.53% HbA2 and 5.20% HbF. This hematologic phenotype is similar to that found in the β-thalassemia carrier state in childhood.22 α-Globin gene analysis showed the restriction enzyme pattern of the heterozygous state for the triple α-loci (Figs. 3–5). Her genotype should be (αααα/αααα; βthal/β+). The other daughter had the same hematologic characteristics. Material for DNA analysis was not available.

DISCUSSION

The restriction endonuclease mapping of the DNA from the propositus clearly shows a pattern indicative of the homozygous state for triplicated α-globin loci. This triple α-globin loci is the result of an unequal crossing over after misalignment of the α-globin genes on one chromosome with the α-globin genes on the other chromosome, producing, on one side, a DNA strand with one α-globin gene deleted and hence 3.7 kb shorter than normal (the rightward deletion α-thal-2 genotype) and on the other side, a DNA with an additional α-globin gene, 3.7 kb longer than normal.14,16

The propositus was the product of a consanguineous marriage and received one chromosome with the triplicated α-globin loci from his mother. The other chromosome, bearing the same lesion, was likely inherited from his father who, because of the high degree of consanguinity, probably shared the same genetic defect with his wife. He definitely is also a carrier of a high HbA2 β-thalassemia gene, since the presence of six α-globin genes cannot explain his high HbA2 levels. His sister, who is homozygous for the triple α-globin loci has, in fact, normal HbA2 levels.

According to the haplotype analysis produced in this study, the β-thalassemia gene segregating in this family is probably the nonsense β^19 mutation, which is the most common and very likely the unique β-thalassemia determinant in Sardinians (Pirastu M and Kan YW, unpublished data).

The analysis of the genotype–phenotype correlations in the different members of this family allows us to develop an outline of the clinical manifestations associated with different genotype associations.

The heterozygote (I-2) for the triple α-loci, with five α-globin genes per diploid cell (αααα/ααααα), shows no consistent abnormal clinical and hematologic manifestations according to previous findings.14,16 Globin chain synthesis is either normal or slightly imbalanced.

The homozygous state for this lesion, with six α-globin genes per diploid cell (ααααα/αααααα), expresses no hematologic manifestations, but shows a slightly imbalanced α/β-globin chain synthesis, with an α/β ratio in the order of the values found in the β-thalassemia carrier state.21

The combination of heterozygous β-thalassemia with the heterozygous state for the triple α-globin loci produces no clinical manifestations and is associated with a hematologic phenotype similar to that of heterozygous β-thalassemia, even though the HbF levels were at the higher extreme of the variable spectrum seen in the β-thalassemia carrier state. In fact, the moderate anemia observed in the subject (III-1) with this gene complex is of a similar degree to that commonly occurring in β-thalassemia heterozygotes in infancy and childhood.22

The combination of the homozygous state for the triple α-globin loci with six α-globin genes per diploid cell and the heterozygous state for β-thalassemia produces a clinical picture of thalassemia intermedia, with an albeit variable, but always mild, clinical course, minor increase of HbF levels, and a pronounced imbalance of globin chain synthesis.

Our results are not surprising, because a normal cell with four copies of α-globin genes and two copies of β-globin genes produces a slight excess of α-chains, which is quickly removed to achieve balanced α/β-globin chain synthesis.1 On the other hand, the loss of function of one β-globin gene is associated with the hematologic phenotype of heterozygous β-thalassemia, while the deletion of one α-thalassemia gene is usually associated with no clinical manifestations. Accordingly, the extra α-globin gene, which was found by mRNA studies to be functional,14,23 produces no or very slight globin chain imbalance. This imbalance became evident at globin chain synthesis analysis when there are two extra α-globin genes per diploid cell. When one extra α-globin gene is associated with the loss of function of one β-globin gene, the imbalance should be the sum of the effects produced by each of
these lesions separately. However, our case with this genetic combination shows a phenotype indistinguishable from that of the β-thalassemia carrier state. Finally, a more consistent imbalance of globin chains, and hence a clinical phenotype of thalassemia intermedia, is produced in subjects with the loss of the function of one β-globin gene associated with two extra α-globin genes.

The genetic combination we have described should be a very rare cause of thalassemia intermedia either in American blacks or in Sardinians, where the frequency of the triple α-globin loci is very low, being 0.0036 in the former and 0.004 in the latter. On the other hand, this combination should be more common in the Greek Cypriots, in whom the frequency of the triple α-globin loci is 0.05.

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