Delineation of the Molecular Basis of δ- and Normal HbA2 β-Thalassemia

By Paolo Moi, Elisabetta Paglietti, Adele Sanna, Carlo Brancati, Antonio Tagarelli, Renzo Galanello, Antonio Cao, and Mario Pirastu

In this study, we used cloning and sequence analysis to define the molecular defect in two δ-thalassemia genes, one associated with reduced output of δ-globin chains (δ-δ) from a Sardinian and the other with a complete suppression of δ-chain production from the affected locus (δ-δ) from a Southern Italian. Sequence analysis of the δ-δ gene showed a G→T substitution at the first nucleotide of codon 27 (δ-27) which produces an amino acid change (Ala→Ser) and presumably activates a cryptic splice site located at this position. Therefore, only a fraction of the transcript is processed from this site, as indicated by the clinical phenotype of δ-δ. DNA sequencing of the δ-δ gene revealed a T→C substitution at position 1 of IVS-1, which abolishes the splicing at this site and thus leads to complete deficiency of normal mRNA explaining the clinical phenotype of δ-δ. Oligonucleotide analysis was used to confirm the coinheritance of the δ-27 mutation in a group of Sardinians with thalassemia like phenotype and normal HbA2 level who, on the basis of genetic criteria, were supposed to be double heterozygous for δ-thalassemia and β-thalassemia. The definition of δ-thalassemia defects in each high-risk area facilitates identification of double heterozygotes for δ- and β-thalassemia by DNA analysis and may thus improve genetic counseling.

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MATERIALS AND METHODS
Gene cloning and sequence analysis. DNA was extracted from peripheral blood leukocytes as previously described. Total genomic DNA of one subject with δ-θ-thalassemia [II-3 of family A (Fig 1)] and one with δ-θ-thalassemia [II-1 of family B (Fig 1)] were digested with the restriction enzyme BglIII, which cuts outside the δ-globin gene. The 7- to 10-kilobase (kb) fragment of the digested DNA was collected by density sedimentation through a continuous sucrose gradient, ligated to a derivative of phage λ, Charon 27, in the compatible BamHI unique site and in-vitro packaged. The recombinant phages were propagated in Escherichia coli and phage clones with the 8-kb insert, which contains the δ-globin gene but not the β-globin gene, were isolated. The 2.3-kb PstI fragment, which includes the whole δ-globin gene, was subcloned into the plasmid pUC 9 (Pharmacia, Uppsala, Sweden). Sequence analysis was performed by the dideoxynucleotide chain termination method directly on supercoiled plasmid using several oligonucleotide primers distributed along the δ-globin gene. The IVS-2 of the δ-globin gene was subcloned in the M13 phage and sequenced with M13 universal primer.

Oligonucleotide analysis. Normal HbA1c δ-thalassemia heterozygotes were studied for the presence of the δ-27 mutation defined in this study (G→T substitution at the first nucleotide of codon 27 of the δ-globin gene) using two oligonucleotide probes, 19 nucleotides long, one (δ-19mer) complementary to the normal δ gene in the region surrounding codon 27 and the other (δ-19mer) complemen-

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MOLECULAR BASIS OF δ-thalassemia

531

A

I

II

F

A

0.3

0.6

1.6

2.7

12.0

2.0

2.0

11.4

δδ11

δδ

\( \delta^11\)

\( \delta^1\)

\( \delta^1\)

\( \delta^1\)

\( \delta^1\)

\( \delta^1\)

\( \delta^1\)

\( \delta^1\)

Fig 1. Pedigree and essential hematologic data of the two families studied.

RESULTS

Family studies. Figure 1 shows the pedigree and essential hematologic data from the two families studied, which have been previously reported.6,7 In family A (from Calabria, Southern Italy) the mother (I-2), with elevated HbF level (12%) is a carrier of a deletional form of \( \delta^\alpha\)-thalassemia;12 the father (I-1), who has low HbA2 level (1.6%) and normal MCH and MCV values, is considered to have \( \delta\)-thalassemia. Two children (II-1 and II-3) have thalassemia-like RBC indices and elevated HbF level (13.2% and 11.4%). Their HbA2 was not detectable. These hematologic findings indicate that both are double heterozygotes for \( \delta^\alpha\)- and \( \delta^\beta\)-thalassemia. Both parents in family B (from Sardinia) are normochromic and normocytic, with HbA2 level of 0.96% and 1.51% (mean of three determinations) respectively; \( \alpha\)-globin gene mapping showed that they are \( \alpha\)-thalassemia-2 carriers (\(-\alpha/\alpha\alpha\)). The son (II-1) has an extremely low HbA2 level of 0.44, as determined by microchromatography. Gene mapping showed that he was homozygous for \( \alpha\)-thalassemia-2 (\(-\alpha/\alpha\alpha\)). He has a mean corpuscular volume (MCV) of 71 fL and a ratio of \( \alpha\)/\( \beta\)-globin chain synthesis of 0.69. In this family, the \( \delta\)-globin gene was associated with haplotype IX (according to Orkin nomenclature).19 On the basis of the extremely low HbA2 level and the homozygosity for the haplotype IX the son (II-1) was considered homozygous for \( \delta^\alpha\)-thalassemia.

Sequence analysis of \( \delta^\alpha\) - and \( \delta^\beta\)-thalassemia genes. The \( \delta\)-thalassemia genes from individuals II-3 of family A and II-1 of family B were cloned and completely sequenced from nucleotide 130 5' to the Cap site to nucleotide 200 3' to the polyadenylation signal of the \( \delta\)-globin gene. DNA sequencing revealed a T→C substitution in IVS-1 position 1 of the \( \delta^\alpha\) gene and a G→T substitution at the first nucleotide of codon 27 of the \( \delta^\beta\) gene (\( \delta^\alpha\)27) (Fig 2). The \( \delta^\alpha\)27 mutation produces an amino acid change at codon 27 (Ala→Ser).

Screening of Sardinian normal HbA2 \( \beta\)-thalassemia heterozygotes by oligonucleotide analysis. Oligonucleotide probes complementary to the \( \delta^\alpha\)27 mutation (\( \delta^\alpha\)l9mer) and to the normal gene (\( \delta^\beta\)l9mer) at position around codon 27 were hybridized to genomic DNA from individual II-1 of family B. DNA of this subject hybridized only to the \( \delta^\alpha\) l9mer probe and not with \( \delta^\beta\)l9mer probe, confirming the homozygous state for the \( \delta^\alpha\)27 gene (lane 7 in Fig 3). The presence of the \( \delta^\alpha\)27 mutation was also investigated in seven obligate \( \beta\)-thalassemia carriers with normal HbA2 level considered to be heterozygous for \( \delta\)-thalassemia in trans with the \( \beta\)-thalassemia gene, on the basis of genetic criteria and previous haplotype analysis.20 Six of seven showed heterozy-
gosity for the δ²⁷ gene, which was associated with haplotype IX. The remaining carrier, with haplotype II/V, was negative for this mutation as well as for the δ²⁴ IVS-1 nt 1 defect, which was searched using the same procedure with a specific oligonucleotide (data not shown). The mother (I-2 of family C in ref 10) of one of the double heterozygotes for δ²⁷- and β-thalassemia was homozygous for haplotype IX. The remaining carrier, with haplotype Il/V, was negative for this mutation as well as for the δ²⁴ IVS-1 nt 1 defect, which was searched using the same procedure with a specific oligonucleotide (data not shown).

DISCUSSION

In this study, we characterized the molecular defect in two δ-thalassemia genes, one associated with reduced δ-chain synthesis (δ⁺thal) from a Sardinian and the other with a complete δ-chain suppression from the affected locus (δ⁻thal) from a Southern Italian.

The single nucleotide substitution (T→C) at position 1 of IVS-1 of the δ⁺thalassemia gene involves the invariant dinucleotide GT at the donor splice site. As in other thalassemic disorders, substitution at position 1 in IVS-1 of the δ gene may lead to complete loss of splicing at the altered junction and thus to absence of normal δ-globin mRNA, explaining the phenotype of δ⁺thalassemia.

The G→T substitution at the first nucleotide of codon 27 of the δ⁺thal gene occurs in a region of δ-globin gene that has complete homology to the corresponding region of the β-globin gene (Fig 4). An identical mutation at codon 27 in β-globin gene results in production of a hemoglobin variant, Hb Knossos²³ (δ²⁷Ala→Ser) and leads to activation of a cryptic donor splice site.²⁴ A minor fraction of RNA transcript is abnormally spliced from this site to the normal IVS-1 acceptor, producing a β⁺-thalassemia phenotype. A similar mechanism may occur in the δ⁺²⁷ gene described in this study.

Oligonucleotide analysis allowed us to identify two homozygotes for the δ⁺²⁷ mutation. Their HbA₂ levels were 0.4% (II-1 of family B) and 0.8, respectively (I-2 of family C in ref 10). This discrepancy may be explained by the interaction of α-thalassemia (−α/−α) in subject II-1 of family B, which further decreases the HbA₂ level.

In 6 of 7 individuals who, on the basis of genetic criteria, were believed to be double heterozygotes for δ- and β-thalassemia, we confirmed the presence of the δ⁺²⁷ mutation by oligonucleotide analysis. In all these individuals, the δ⁺²⁷ gene was associated with haplotype IX. The remaining normal HbA₂ β-thalassemia carrier may have another δ-thalassemia mutation, as indicated by the presence of different chromosomal haplotypes (Ⅱ/Ⅴ). From these data, we may conclude that at the molecular level δ-thalassemia in Sardinia is heterogeneous. The δ⁺²⁷ mutation, however, appears to be the most prevalent defect. These findings have practical relevance for definition of the type of thalassemia in individuals with thalassemia-like RBC indices and normal HbA₂. This phenotype, indeed, may result either from α-thalassemia or double heterozygous state for δ- and β-thalassemia or δβ-thalassemia. Globin-chain synthesis may allow us to define the genotype. However, in populations in which α- and β-thalassemia are frequent, double heterozygotes for α- and β-thalassemia, with almost balanced globin-chain synthesis, are quite frequently detected.²⁵ In this instance, oligonucleotide analysis may allow direct detection of the δ⁺²⁷ mutation.
of the coinheritance of a δthal gene, explaining the normal HbA2 level in a β-thalassemia heterozygote.

By analogy to the β-thalassemia mutations, we can reasonably assume that in each population a limited number of δ-thalassemia lesions may be prevalent. Characterization of these mutations at the molecular level may be useful for identification of nontypical β-thalassemia heterozygotes and will thus improve genetic counseling.

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REFERENCES

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