β thalassaemia mutations in Sardinians: implications for prenatal diagnosis

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SUMMARY In this study we have characterised by oligonucleotide hybridisation and direct restriction endonuclease analysis the β thalassaemia mutation in 494 Sardinian β thalassaemia heterozygotes. The most prevalent mutation, accounting for 95.4% of the cases, was the nonsense mutation at codon 39. The remainder, in decreasing order of frequency, were a frameshift at codon 6 (2.2%), β^+ IVS-1, nt 110 (0.4%), and β^+ IVS-2, nt 745 (0.4%). This information allows prenatal diagnosis by DNA analysis to be made in the great majority of Sardinian couples at risk for β thalassaemia.

In the Sardinian population, one β thalassaemia mutation, namely a C-T substitution at the codon corresponding to amino acid 39 (β^{39}), is widely prevalent accounting for the majority (95%) of cases of thalassaemia major and intermedia.^{1,2} Prenatal diagnosis in this population is therefore carried out at present in the great majority of cases by the analysis of amniocyte or chorionic villus DNA with an oligonucleotide probe able to detect the β^{39} mutation.^{3,4} The remaining cases, in whom the molecular defect has not yet been characterised, are monitored by fetal blood analysis.⁵

In order to extend prenatal diagnosis by DNA analysis to carriers of the less frequent mutations, in the present study we investigated the molecular basis of β thalassaemia in a group of β thalassaemia chromosomes from Sardinians in whom we excluded the presence of the β^{39} mutant. We found that in the non- β^{39} thalassaemia chromosomes, the most frequent mutation was the single nucleotide deletion at the GAG codon of the β -6 position (frameshift 6) of the β globin gene, resulting in a frameshift and thus in the genotype of β^0 thalassaemia.^{6,7} Because this mutation abolishes an *Mst*II restriction site, it can be detected directly by restriction endonuclease analysis. This information has led us to screen β thalassaemia carriers, in whom we excluded the presence of the β^{39} mutant, by *Mst*II analysis and allows prenatal diagnosis by DNA analysis in an additional group of Sardinian couples.

Patients

By screening 247 Sardinian couples, in whom both members were heterozygotes for β thalassaemia, with an oligonucleotide probe complementary to the β^{39} mutation, we detected the presence of this mutant in 471 subjects and excluded its presence in 23. In the non- β^{39} thalassaemia chromosomes, we investigated the nature of the molecular defect by haplotype analysis and oligonucleotide hybridisation or direct restriction endonuclease analysis. Oligonucleotide hybridisation was carried out to detect the following β thalassaemia mutations: the G-A substitution at position 110 of the first intervening sequence (IVS-1) (β^+ IVS-1, nt 110), the C-T substitution at codon 39 (β^{39}), the T-C substitution at position 6 (β^+ IVS-1, nt 6), and the G-A substitution at position 1 (β^0 IVS-1, nt 1).

Direct restriction endonuclease analysis was used to detect a frameshift at codon 6, which is recognised by *Mst*II,^{6,7} a C-G substitution at position 745 of IVS-2 of the β globin gene (β^+ IVS-2, nt 745), which is detected by *Rsa*I,⁸ and a G-A substitution at position 1 of IVS-2 (β^0 IVS-2, nt 1), which is detected by *Hph*I.^{9,10}

For each haplotype, the oligoprobe or the restriction enzyme to be used was selected on the basis of the known association between haplotype and specific β thalassaemia mutations.^{8,11} Those cases in whom the mutation was not characterised by this approach were tested with all four oligoprobes available, as well as with *Mst*II, *Hph*I, and *Rsa*I restriction endonuclease analysis (see below).

Methods

HAPLOTYPE ANALYSIS

DNA restriction endonuclease analysis was performed according to Goossens and Kan.¹²

The polymorphic restriction enzyme sites studied were: *HincII* 3' to the ϵ globin gene,¹³ *HindIII* within the G γ and A γ globin genes,¹⁴ *HincII* within and 5' to the $\psi\beta$ globin gene,¹³ *AvaII* within the second intervening sequence of the β globin gene,¹⁵ and *BamHI* 3' to the β globin gene.¹⁶

The presence of a polymorphic site is indicated as (+) and its absence as (-). The haplotypes are numbered according to Orkin *et al.*⁸ The probes used were genomic fragments corresponding to the ϵ , γ , $\psi\beta$, and β globin genes.

DIRECT ANALYSIS OF THE MUTATIONS

Oligonucleotide analysis was carried out as previously described.^{3, 17-19} Each mutation was analysed by means of two oligonucleotide (19-mers) probes, one (β^{th}) complementary to the β globin gene sequence around the mutation and one (β^{A}) homologous to the normal β globin gene sequences at the same position. They differ from each other by a single nucleotide placed in the middle of the sequence. The sequences of the oligonucleotides used were as follows: for the $\beta^{\circ}39$ mutation= β^{A} 5'-CCTTGGACCCAGAGGTTCT-3', $\beta^{\text{th}39}$ 5'-AGAACCTCTAGGTCCAAGG-3'; for the IVS-1 nt 110 mutation= β^{A} 5'-AATAGACCAATAGGCAGAG-3', $\beta^{\text{th}110}$ 5'-CTCTGCCTATTAGTCTATT-3'; for the IVS-1 nt 6 mutation= β^{A} 5'-AACCTTGATACCAACCTGC-3', $\beta^{\text{th}6}$ 5'-GCAGGTTGGCATCAAGGTT-3'; and for the IVS-1 nt 1 mutation= β^{A} 5'-TGATACCAACCTGCCAGG-3', $\beta^{\text{th}1}$ 5'-CCTGGGCAGATTGGTATCA-3'.

DNA (10 μ g) was digested with the restriction endonuclease *BamHI* according to the recommendations of the manufacturer (Bethesda Research Laboratories) and DNA fragments were separated by agarose gel electrophoresis. All β thalassaemia mutations (β^+ IVS-1, nt 110, $\beta^{\circ}39$, β^+ IVS-1, nt 6, β^+ IVS-1, nt 1) investigated occur in the 1.8 kb *BamHI* fragment which contains the 5' part of the β globin gene. Two identical gels were usually run, one hybridised to the β^{th} and the other to the β^{A} probe.

Results

Fig 1 shows representative autoradiograms of gels hybridised either with the $\beta^{\circ}39$ or the β^+ IVS-1, nt 110 probe, and fig 2 illustrates the detection of the frameshift at codon 6 by *MstII* analysis. This frameshift 6 is directly identified by *MstII* analysis

FIG 1 Autoradiograms of leucocyte DNA analysed with the oligomer method. (a) Hybridisation with the $\beta^{\circ}39$ probe. (b) hybridisation with the β^+ IVS-1, nt 110 probe. Lane 1: normal control; lanes 2, 3, 4, 5 and 6: heterozygotes for the $\beta^{\circ}39$ mutation; lane 7: heterozygote for the β^+ IVS-1, nt 110 mutation; lanes 8 and 9: homozygotes for the β^+ IVS-1, nt 110 and $\beta^{\circ}39$ mutation respectively.

because this mutation abolishes a *MstII* recognition site involving codon 6 of the β globin gene. DNA from normal subjects shows a 1.15 kb fragment. DNA from homozygotes for the frameshift at codon 6 have a longer 1.35 kb fragment, and heterozygotes for this mutation show both the 1.15 and 1.35 kb fragments.

The overall results are summarised in tables 1 and 2. The $\beta^{\circ}39$ mutation was found in 471 out of 494 (95.4%) β thalassaemia carriers investigated (table 1). In 11 subjects (2.2% of the total investigated and 47.8% of those in whom the $\beta^{\circ}39$ mutant was excluded), *MstII* analysis led to the identification of the frameshift at codon 6. This mutation was associated in 10 cases (90.9%) with haplotype IX and in one (9.11%) with haplotype I.

In two subjects (8.7% of those in whom the $\beta^{\circ}39$ mutation was excluded), oligonucleotide hybridisation showed the presence of the β^+ IVS-1, nt 110 mutation, which was associated in both with

Origin

TABLE 2 β thalassaemia mutations in Sardinians.

	No	%
β thalassaemia heterozygotes investigated	494	
$\beta^{\circ}39$	471	95.4
Frameshift at codon 6	11	2.2
β^+ IVS-1, nt 110	2	0.4
β^+ IVS-2, nt 745	2	0.4
Unknown	8	1.6

FIG 2 Southern blot analysis of the β^6 (-1 bp) mutation. Lanes 1, 2, and 3: leucocyte DNA from normal subjects (lane 1) and from β thalassaemia heterozygotes not carrying the β^6 (-1 bp) mutation (lane 2); lanes 4 and 5: leucocyte DNA from heterozygotes for the β^6 (-1 bp) mutation.

haplotype I. In two subjects (8.7%), *RsaI* restriction endonuclease analysis detected the presence of the β^+ IVS-2, nt 745 mutation, which was associated in both cases, with haplotype VII. In the remaining eight cases, we excluded by oligonucleotide hybridisation and restriction endonuclease analysis the presence of those mutations (β^+ IVS-1, nt 110, β^+ IVS-1, nt 6, β^+ IVS-1, nt 1, β° IVS-2, nt 1, β^+ IVS-2, nt 745, and β^6 (-1 bp)) which commonly occur in Mediterranean populations. In these cases the β thalassaemia mutation was associated in six with haplotype I, in one with haplotype III, and in one with haplotype VIII.

All the spouses of β thalassaemia heterozygotes with the frameshift at codon 6 had the $\beta^{\circ}39$ mutant. Prenatal diagnosis was undertaken in these cases, therefore, with the combination of oligonucleotide

hybridisation to detect the $\beta^{\circ}39$ mutant and *MstII* analysis for detecting the frameshift at codon 6.

Discussion

The results of this study confirm that the most prevalent β thalassaemia mutation in Sardinians is the $\beta^{\circ}39$ mutant,^{3 4} which accounts for 95% of β thalassaemia chromosomes. In the remainder, we detected the frameshift at codon 6 in 2.2%, the β^+ IVS-1, nt 110 in 0.4%, and the β^+ IVS-2, nt 745 in 0.4%. The relative frequencies of the different mutations found in this study contrast with the distribution observed in an American population of mixed Mediterranean background²⁰ in which the β^+ IVS-1, nt 110 was the most frequent mutation followed by the $\beta^{\circ}39$. This difference may be explained by the relative isolation of the Sardinian population since these mutations arose.

A frameshift at codon 6 was found to be associated more commonly with haplotype IX and rarely with haplotype I. Previous studies in Mediterranean people have shown this mutation associated with haplotypes I, IX, or V.^{8 20} Our results confirm, therefore, the existence of a close association between specific β thalassaemia mutations and haplotypes.

Oligonucleotide hybridisation and direct restriction endonuclease analysis allowed us to characterise the β thalassaemia mutation in 65% of the non- $\beta^{\circ}39$ thalassaemia chromosomes in Sardinians, confirming the validity of this approach for the delineation of specific β thalassaemia defects in a population. Cloning and sequence analysis of the

TABLE 1 Association of specific β thalassaemia mutations and chromosomal haplotypes in Sardinians.

Haplotype	Mutations			
	IVS-1, nt 110	IVS-2, nt 745	Frameshift 6	Uncharacterised
I+-----+	2	—	1	6
III+-----	—	—	—	1
VII+-----	—	2	—	—
VIII+-----	—	—	—	1
IX+-----	—	—	10	—

more rare mutations associated with haplotypes I and III are in progress.

The results of this study were immediately applied to screening prospective parents and for prenatal diagnosis in the Sardinian population. Every couple in whom both members are β thalassaemia heterozygotes is now screened for the presence of the β^{39} mutant by oligonucleotide hybridisation. Those in whom the presence of the β^{39} mutant is excluded are then screened for the frameshift at codon 6 by *MstII* analysis.

Through this approach we are at present able to define the β thalassaemia mutations in the great majority of couples presenting at the antenatal clinic and to accomplish prenatal diagnosis by DNA analysis using oligonucleotide hybridisation or direct restriction analysis in almost all the cases.

The results from this study are also relevant to the planning of prenatal diagnosis in a multiracial population. According to our studies a β thalassaemia heterozygote of Sardinian origin requesting prenatal diagnosis should be investigated first for the presence of the β^{39} mutant and second for a frameshift at codon 6, both of which may be easily detected by DNA analysis.

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