British Journal of Haematology, 1999, 105, 68–74

Unexpected pattern of β-globin mutations in β-thalassaemia patients from northern Portugal

José M. Cabeda,[1,2] Cristina Correia,[1] Alexandra Estevinho,[1] Carla Simões,[1] Maria Luis Amorim,[1] Luciana Pinho[1] and Benvindo Justiça[1] [1]*Clinical Haematology, Santo Antonio General Hospital, Porto, and* [2]*Abel Salazar Institute for the Biomedical Sciences, Porto, Portugal*

Received 21 October 1998; accepted for publication 29 December 1998

Summary. We characterized the genetic nature of β-thalassaemia in northern Portugal. Of the 164 patients studied three were β-thalassaemia major cases (one IVS-1-6/β°39 and two homozygous IVS-1-110). The analysis of the frequency of each mutation in the families revealed that the codon 6(−A) mutation was unexpectedly frequent (40%) and associated with the β-globin haplotype E, and not with the usual European and North African CD6(−A) haplotypes. In contrast, the frequency of IVS-1-6 (8%) and β°39 (19%) was found to be lower than in the rest of the country. The frequency of all other mutations was similar to previous reports for central/southern Portugal.

Six families carried none of the most frequent mutations in the Mediterranean area. These families were studied by gene sequencing, revealing that three families carried a previously described mutation (CD16 G→A). The remaining families carried previously unidentified mutations: one showed an 86 bp insertion in exon 2 (named HGSA) and two showed a deletion of a cytidine in codon 11 (CD11(−C)).

The results, showing a high frequency (82%) of β° mutations, strongly indicates that genetic counselling should be intensified as a means of preventing the spread of the severe mutations found.

Keywords: β-thalassaemia, β-globin, mutations, Portugal, codon 6(−A).

The β-thalassaemias have been reported in virtually all ethnic groups and geographical locations. Geographically, the β-thalassaemias seem to be more frequent in Mediterranean populations and regions of Africa, the Arabian peninsula, Turkey, Iran, India and South-East Asia (Amselem *et al*, 1988). However, the β-globin mutations found in each of these regions vary (Kazazian & Boehm, 1988), indicating that different mutations may have arisen as independent mutational events in different geographical areas. These mutations were then dispersed through differential migration patterns, giving rise to the mutational pattern seen today.

The frequency of β-thalassaemia in Portugal seems to be around 0·45% in total, varying from north to south (Martins *et al*, 1993). The genetic characterization of β-globin mutations in Portuguese thalassaemic individuals has in the past focused mainly in the areas with the highest incidence of the disease, i.e. the central and southern areas of the country (Coutinho-Gomes *et al*, 1988; Lavinha & Baiget, 1992; Faustino *et al*, 1992; Tamagnini *et al*, 1993). This has resulted in the assumption regarding the homogeneity of the mutations throughout the country, as well as of a low or even null incidence of the disease in the north (Martins *et al*, 1993). In contrast to this, a large number of families carrying β-thalassaemia mutations have been diagnosed and followed in our hospital. Although the small number of β-thalassaemia major cases found is in accordance with a lower frequency of β-thalassaemia in the north than in central/southern Portugal, the number of affected families identified does argue against the reported null incidence of this disease in this area. Since some β-globin mutations are known to induce mild phenotypes (Molina *et al*, 1994), it is possible that the reported null frequency in the north of the country derives from a different pattern of β-globin mutations. The present report aims at closing the gap between the characterization of β-thalassaemia in the northern and central/southern areas of the country. The screening studies were performed at a local level, and included family studies starting from previously characterized carriers not included in the National Haemoglobinopathies screening programmes. The identified carriers were then subjected to genetic studies to characterize the nature of the β-globin mutations found in the β-thalassaemia families identified.

Correspondence: Dr José Manuel Cabeda, Clinical Haematology, Hospital Geral de Sto. António, R. D. Manuel II (ex cicap), 4050 Porto, Portugal. e-mail: jcabeda@mail.telepac.pt.

MATERIALS AND METHODS

Individuals studied. The study included 382 individuals belonging to 111 β-thalassaemia minor families. No selection criteria were applied apart from the individual's willingness to cooperate in the study. Each individual studied was investigated for personal and family history of anaemia and splenomegaly. Blood samples were collected and haemoglobin level, red blood cell indices, red cell morphology, reticulocyte count and serum iron stores were determined. Whenever MCV <78 fl and MCH <28 pg were found, HbA2 and HbF were determined, and haemoglobin electrophoresis (isoelectric focusing) was performed. All individuals showing MCV <79 fl, MCH <27 pg and HbA2 >3·5% were considered carriers, and were requested to be included in the genetic study.

For the screening tests, a control group of 48 individuals consisting of newly registered blood donors was also studied. This group consisted of 28 males and 20 females with ages ranging from 17 and 55 years (mean age 33 years in the female group and 27 in the male group) who had never been phlebotomized.

Of the 382 individuals screened, a total of 164 affected individuals (representing 88 families) agreed to cooperate in the genetic study and were genetically characterized for the presence of mutations in the β-globin gene. An additional group of 95 regular blood donors was used as controls in the genetic analyses.

Screening tests performed. Blood cell counts and red cell indices were determined on an automatic cell counter (MAXM-Coulter) calibrated with reference standards from Coulter Diagnostics Inc.

Haemoglobin A2 was quantified by microcolumn chromatography with glycine-potassium cyanide developers (Helena Laboratories). Estimation of haemoglobin F was performed using the modified Betke alkaline denaturation method (Betke *et al* 1959; Huisman & Jonxis, 1977). More recently, an automated procedure (Hb Gold Analyser, Menarini Diagnostics) for the quantification of HbA2 and HbF was implemented.

Haemoglobin isoelectric focusing was performed using precast pH 5·5–8·5 agarose gels (FMC-Bioproducts, U.S.A.), according to the supplier's recommendations.

Detection of β-globin mutations. DNA was prepared from 200 μl of peripheral blood with the Qiamp Blood Kit (Quiagen, U.S.A.) according to the supplier's recommendations. One tenth of the prepared DNA was subsequently used to amplify the β-globin gene. β-globin gene amplification was performed by PCR using previously described specific β-globin primers (Table I). The reactions were performed in a total volume of 100 μl containing 1·5 mM $MgCl_2$, 200 μM each dNTP (dATP, dTTP, dGTP and dCTP), 0·2 μM of each primer, 2 units of AmpliTaq DNA Polymerase (Apligene-Oncor, U.S.A.) in the buffer supplied by the enzyme manufacturer. Two different PCR reactions were performed: one using the primers CD7 and SR10 (Table I) and the other using CD7 and IVS1-110R (Table I). One tenth of the PCR products of each reaction was digested overnight at the appropriate temperature with 20 units of restriction enzyme (Table I) in the buffer supplied by the enzyme manufacturer (New England Biolabs, Beverly, U.S.A.). Restriction fragments were subsequently analysed by gel electrophoresis on ethidium bromide containing precast 4% 3:1 Nusieve gels (FMC-Bioproducts). The sizes of the restriction fragments observed are shown in Table I. Subsequently, all results were confirmed by a microwell hybridization based technique, the MDX-Betha Gene 1 Kit (Bio-Rad, U.S.A.), according to the supplier's recommendations.

Haplotype analysis. Determination of the β-globin haplotypes associated with the β-thalassaemia mutations was done by PCR-RFLP. DNA preparation and PCR reactions were performed as above, using the primers listed in Table II. Four-fifths of the PCR products were digested overnight at the appropriate temperature with 20 units of the appropriate restriction enzyme (Table II) in the buffer supplied by the enzyme manufacturer (New England Biolabs, Beverly, U.S.A.). Restriction fragments were subsequently analysed by gel electrophoresis on ethidium bromide containing precast 4% 3:1 Nusieve gels (FMC-Bioproducts). In the text, polymorphic markers will be described by their natural genetic order (A–F).

Table I. Size of the restriction fragments observed in the presence or absence of mutations.

PCR primers	PCR start*	PCR end*	PCR product (bp)	Enzyme used	Mutation detected	Restriction fragments (bp)	
						Wild type	Mutated
CD7† + SR10†	61976	62705	730	*BSA BI*	IVS-1-1	730	426/304
				BSU 36I	Codon 6(−A)	228/213/201/88	429/213/88
				Sfa NI	IVS-1-6	488/242	317/242/171
				Mae I	β°39	540/114/76	272/268/114/76
				Ava II	β°37	454/214/62	668/62
						454/276	730
CD7† + IVS-1-110R‡	61976	62417	442	*Mbo I*	IVS-1-110	413/21/8	434/8

*Nucleotide numbers as in HUMHBB locus sequence (GenBank accession number U01317).
† Primers CD7 and SR10 were described by Spielberg *et al* (1989).
‡ Primer IVS-1-110R was described by Lindeman *et al* (1991).

Table II. β-globin haplotype analysis. PCR primers and restriction reaction details.

Marker	Gene	Primer*	Restricted enzyme	PCR start†	PCR end†	RE cut sites†	Expected bands (bp)
A	5′ϵ	F=AATACTCACAAAGTAGCCAGT R=ATGATATCCATCTCTCCCATT	*Hinc II*	18770	19220	19097‡	451+327+124
B	Gγ	F=TTGAAAGTCAGCTCTGTGTGT R=ACATAAAACCCTTTGTGGCTC	*Hind III*	35525	36135	35768‡	611+368+243
C	Aγ	F=TTGAAAGTCAGCTCTGTGTGT R=ATAAATGAGGAGCATGCACAC	*Hind III*	40461	41120	40684‡	660+437+223
D	$\psi_{\beta 1}$	F=ACCCTTAGATATTTGCACTAT R=CAATCAATATCACGTTGCCTA	*Hinc II*	46178	47021	46790‡	844+612+232
E	3'$\psi_{\beta 1}$	F=AGGGAAGTTTGTTTGAGACCT R=TCCTACAGCAGACAGTCTTTT	*Hinc II*	49874	50428	49993‡	555+436+119
F	β	F=GAGGGTTTGAAGTCCAACTC R=CCCTTCCTATGACATGAACTTAA	*Ava II*	61958	62702	62430/ 62644‡	472+273+214+59
D+E		F=ACCCTTAGATATTTGCACTAT R=TCCTACAGCAGACAGTCTTTT	*Hinc II*	46178	50428	46790‡ 49993‡	−/− → 4251 +/− → 612+3639 −/+ → 3815+436 +/+ → 612+3203+436

*F=Forward primer; R=Reverse primer; primer sequence is indicated in the direction 5′ → 3′.
†Nucleotide numbers as in HUMHBB locus sequence (GenBank accession number U01317).
‡Indicated restriction site is the polymorphic site.

Co-segregation of polymorphisms D and E was analysed by a long-PCR-RFLP approach. The long-PCR was performed in a 50 μl reaction, containing 360 μM of each dNTP (dATP, dTTP, dCTP, dGTP), 2 mM $MgCl_2$, 0·3 μM of each primer (Table I, reaction D + E), 50 mM Tris-HCl (pH 9·0), 15 mM $(NH_4)_2SO_4$, 0·1% Triton X-100 and 2 units of Dynazyme EXT DNA polymerase (Finnzymes Oy, Finland). The reaction mixture was subject to an initial denaturing step at 94°C for 2 min, followed by 40 cycles of amplification. The amplification cycles consisted of a denaturation step at 94°C for 30 s, followed by an annealing step at 57°C for 30 s and an extension step at 68°C for 1 min. A final 5 min extension step at 68°C was also performed. Restriction enzyme digestions were performed as described above, using the *Hinc II* enzyme. Restriction fragments were visualized on ethidium bromide containing precast 1% agarose gels (FMC-Bioproducts, U.S.A.).

Gene sequencing. β-globin gene sequence was done by direct PCR sequencing. Briefly, PCR reactions were performed on a total volume of 50 μl containing 200 μM each dNTP (dATP, dTTP, dCTP, dGTP), 0·2 μM of each primer, 2 mM $MgCl_2$, 50 mM Tris-HCl (pH 9·0), 15 mM $(NH_4)_2SO_4$, 0·1% Triton X-100 and 2 units of Dynazyme EXT DNA polymerase (Finnzymes Oy, Finland). The reaction mixture was subject to an initial denaturing step at 94°C for 5 min, followed by 40 cycles of amplification. The amplification cycles consisted of a denaturation step at 94°C for 30 s, followed by an annealing step at 57°C for 30 s, and an extension step at 68°C for 30 s. A final 5 min extension step at 68°C was also performed. The PCR products were checked on a 4% Nusieve (3:1) agarose gel by gel electrophoresis, and purified from primers and nucleotides by ethanol precipitation on $MgCl_2$ salt. PCR products were then mailed to Cambridge Bioscience (U.K.) for direct sequencing by dye terminator chemistry on an automated Perkin Elmer ABI Prism sequencer.

RESULTS

Gene mutation frequencies

Of the 164 patients studied only three were found to have β-thalassaemia major (2%). Of these, one turned out to be a compound heterozygote (β°39 and IVS-1-6); the two others belonged to the same family and were found to be homozygous for IVS-1-110.

The analysis of the frequency of each mutation in the patients studied (Table III) revealed that the codon 6(−A) mutation was unexpectedly frequent (40%). In contrast, the frequency of IVS-1-6 (8%) and β°39 (19%) was found to be lower than anticipated (Table III). All other mutations studied (IVS-1-1, β°37, IVS-1-110 and IVS-2-1) were found not to differ significantly from the previously described frequencies in Portugal (Table III).

Haplotype analysis

In order to establish whether the unexpectedly high frequency of the CD6(−A) mutation was due to a newly occurring mutation event, or the result of specific population migration patterns, we studied the β-globin haplotype associated with the CD6(−A) mutation. The results showed that all CD6(−A) affected families studied had compatible haplotypes segregating with the CD6(−A) mutation. The haplotype found to be associated with this mutation was −−−+++. This haplotype had been previously identified and named haplotype E (Rouhabi *et al*, 1988). However, to the best of our knowledge, this is the first report of this haplotype associated with the CD6(−A) mutation.

Table III. Geographical distribution of some β-globin mutations.

Region*	Mutation frequency									
	CD6 (−A)	β°39	IVS-1-1	IVS-1-6	IVS-1-110	β°37	IVS-2-1	CD16 TGG→TGA	−90 (C→T)	IVS-1-130 (G→C)
Portugal†,‡,§ (this study)	40% (35/88)	19% (17/88)	16% (14/88)	8% (7/88)	10% (9/88)	0% (0/88)	1% (1/88)	3% (3/88)	—	—
Portugal[1]	—	36%	21%	23%	17%	—	—	—	—	—
Portugal[2]	—	39%	26%	15%	17%	—	—	—	—	—
Portugal[3]	—	56%	33%	—	11%	—	—	—	—	—
Portugal[4]	—	33%	16%	26%	8%	—	—	15%	2%	1%
Spain[1]	—	63%	4%	17%	10%	—	—	—	—	—
Spain[5]	—	31%	22%	9%	27%	—	0%	—	—	—
Spai6[2]	5%	64%	4%	16%	9%	—	—	—	—	—
Greece/Italy[7]	2%	28%	9%	—	34%	—	—	—	—	—
Mediterranean[8]	1%	17%	13%	13%	39%	0%	3%	—	—	—
Bulgaria[9]	6%	20%	4%	10%	28%	—	—	—	—	—
Egypt[10]	4%	2%	10%	19%	27%	2%	4%	—	—	—
Algeria[11]	19%	—	6%	3%	25%	—	—	—	—	—
Tunisia[12]	16%	—	1%	10%	7%	—	—	—	—	—

*Superscript numbers denote references as follows: (1) Lavinha & Baiget, 1992; (2) Faustino *et al*, 1992; (3) Coutinho-Gomes *et al*, 1988; (4) Tamagnini *et al*, 1993; (5) Molina *et al*, 1994; (6) Amselem *et al*, 1988; (7) Kazazian *et al*, 1984b; (8) Beris *et al*, 1995; (9) Kalaydjiveva *et al*, 1989; (10) Noveletto *et al*, 1990; (11) Rouabhi *et al*, 1988; (12) Chibani *et al*, 1988.

† Percentage of families affected (number observed/number studied).

‡ Six families showed none of the tested mutations, and were studied by gene sequencing. Three of these families were found to carry the CD16 TGG→TGA mutation, and three others were found to carry two new mutations (see text for details on the new mutations).

§ One family showed a double heterozygosity (β°39/IVS-1-6).

β-globin gene sequencing

We then sequenced the β-globin gene in the six families that showed none of the studied mutations.

The first patient to be sequenced showed a PCR product heterogenous in size, and thus was anticipated to carry an insertion. The sequence data (Fig 1; GenBank accession number AF059180) obtained from two affected relatives confirmed this prediction, showing an 86 bp insertion. This insertion in exon 2 of the β-globin gene consists of a duplication of the immediately downstream sequence, causing a frameshift and a premature stop codon (Fig 1). The predicted protein sequence carries the first 70 amino-acids of the normal β-globin, followed by 19 mutated aminoacids (Fig 1B). The resulting polypeptide is thus 58

Table IV. Geographical distribution of the β-globin haplotypes associated with the CD6(−A) mutation.

	Haplotype*							Reference
Northern Portugal	−	−	−	+	+	+	(E)	Present report†
Mediterranean basin	+	−	−	−	−	+	(I)	Cao, 1989; Kazazian *et al*, 1984b
	−	+	−	+	+	+	(IX)	Cao, 1989; Kazazian *et al*, 1984b
	+	−	−	−	−	+	(Va)	Cao, 1989; Kazazian *et al*, 1984b
Bulgaria	+	−	−	−	−	+	(V)	Kalaydjieva *et al*, 1989
	−	+	+	−	+	+	(11)	Kalaydjieva *et al*, 1989
Egypt	+	−	−	−	−	+	(Va)	Noveletto *et al*, 1990
Tunisia	+	−	−	−	−	+	(Va)	Chibani *et al*, 1988
	−	+	−	+	+	+	(IX)	Chibani *et al*, 1988
	−	−	−	−	−	+	(A)	Chibani *et al* 1988
Algeria	+	−	−	−	−	+	(I)	Rouhabhi *et al*, 1988; Bennani *et al*, 1994
	−	+	−	+	+	+	(IX)	Rouhabhi *et al*, 1988; Bennani *et al*, 1994
	−	−	−	−	−	+	(A)	Rouhabhi *et al*, 1988; Bennani *et al*, 1994

*Haplotype as previously described (Orkin *et al*, 1982; Rouhabhi *et al*, 1988; Chibani *et al*, 1988; Bennani *et al*, 1994).

† Haplotype E was previously described in Algeria by Rouhabhi *et al* (1988), but not associated with the CD6(−A) mutation.

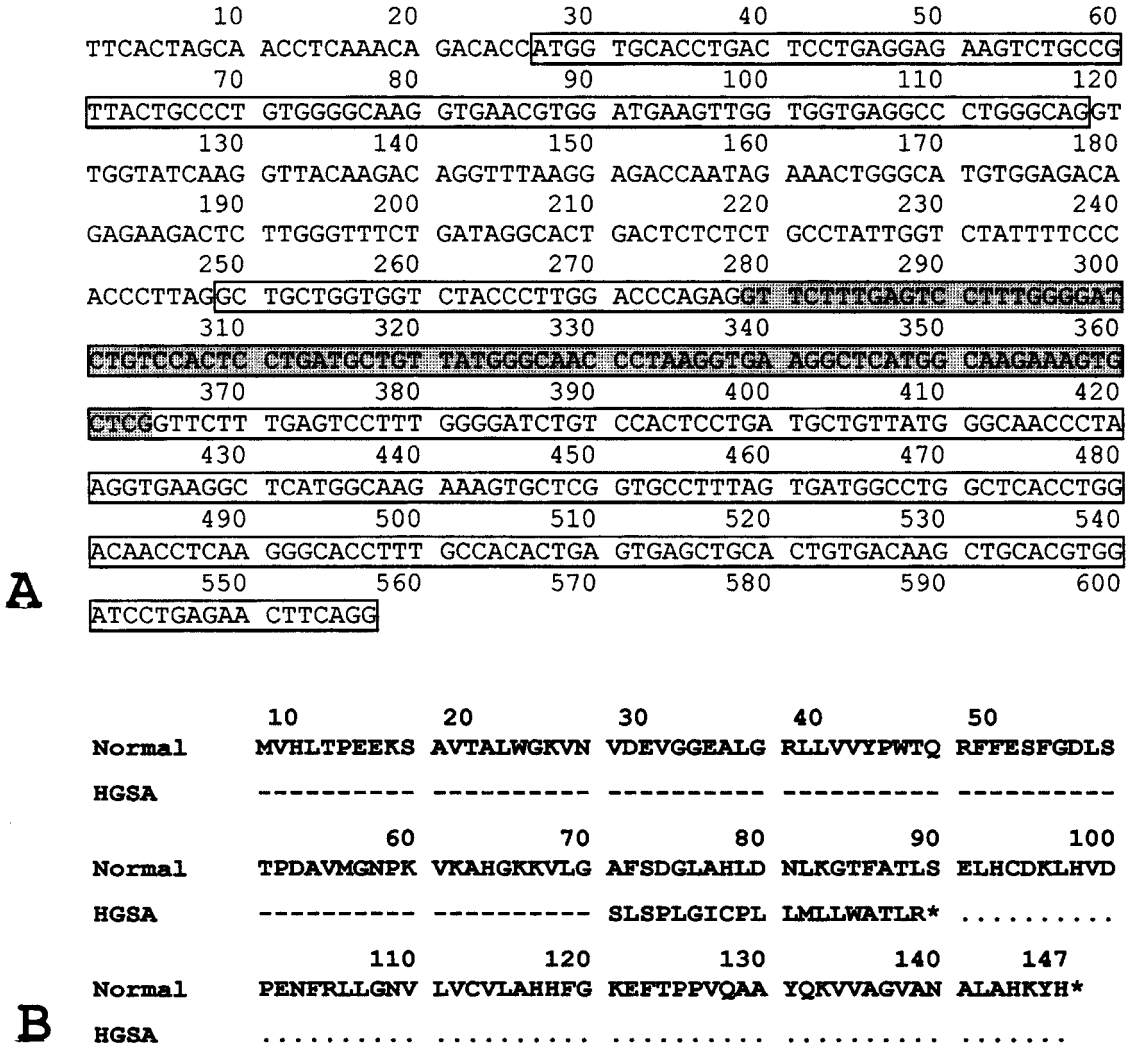

Fig 1. Nucleotide (A) and deduced protein sequence (B) obtained from the β-globin gene of two β-thalassaemia-affected family relatives carrying the HGSA 86 bp insertion. ATG at nucleotides 27–29 is the initiation codon. Exonic sequences are boxed. The mutation was found to consist of an 86 bp insertion (greyed sequence). This insertion is a duplication of nucleotides 365–450 of this sequence. Insertion originates a frameshift, resulting in 19 new aminoacids followed by a stop codon. The resulting protein consists of 89 aminoacids. The first 70 aminoacids are identical to the wild-type beta-globin; the remaining 19 are the result of the frameshift. Protein is prematurely ended by a frameshift originated stop codon.

aminoacids shorter than the wild type. The boundaries of the insertion were found to carry an imperfect splicing sequence (AG|GT...CG|GT; see Fig 1).

Two other, non-related, patients showed a deletion of a cytidine at codon 11 (GenBank accession number AF083884). Codon 11 in wild type is GCC. The deletion of the second C changes this codon to GCG. This change does not produce an amino acid change in this codon due to genetic code redundancy, but introduces a nonsense frameshift. The first 11 amino acids of the resulting polypeptide are similar to the wild type. Deletion originates a frameshift that changes amino acids 12 to 18 and then a stop codon occurs. The resulting deduced polypeptide has thus only 18 amino acids, and is therefore probably non-functional.

Three other non-related individuals showed the presence of the previously described (Kazazian *et al* 1984a) G→A substitution at codon 16, making this mutation more frequent (3·4%) among our patients than IVS-2-1.

DISCUSSION

Screening studies performed in Portugal have concluded that the frequency of β-thalassaemia in the north of the country is nearly nil (Martins *et al*, 1993); this has resulted in the absence of extended genetic studies in this region. Most authors have therefore assumed that the genetic nature of β-thalassaemia in Portugal is similar throughout the whole country. The present report is, to the best of our knowledge, the first large genetic study of β-thalassaemia-affected families in northern Portugal. The results argue strongly against the homogeneity of β-thalassaemia mutations in Portugal, since CD6(−A), an almost absent mutation in the

central and southern areas of Portugal, represents almost 40% of the affected families. Interestingly the highest frequency of this mutation occurs in North African countries (Algeria and Tunisia, see Table IV). Therefore, if migration patterns were the reason for its high prevalence in Portugal, one would expect it to be at least as frequent in the south of the country. Accordingly, it was not surprising that we found a different haplotype associated with the CD6(−A) mutation than the one observed in Algeria, Tunisia and the rest of Europe (Table IV). These results could be explained by two mechanisms: (1) a recombination event between $^{G}\gamma$ and $^{A}\gamma$ loci of an haplotype IX chromosome carrying the CD6(−A) mutation (frequent in the Mediterranean basin), and another chromosome negative for the $5'\epsilon$ and the $^{G}\gamma$ polymorphisms; (2) an independent origin of CD6(−A) mutation in Portugal, Africa and Europe. Although no valid data exists to exclude either possibility, the fact that no haplotype IX CD6(−A) families were identified seems to favour the second possibility.

The results reported showed that by studying the six most frequent mutations in the Mediterranean basin we could identify the mutation in 94% of the studied families. The remaining 6% were the subject of sequencing studies that have identified two new mutations, and the discovery that CD16 (T→C) should be included in the first genetic screenings, since it was present in 3% of the studied families.

The two newly described mutations were found to be nonsense mutations. One of these (named HGSA after our hospital name) is, to the best of our knowledge, the first report of a large insertion causing a β-thalassaemia phenotype. Interestingly, the insertion seems to result from an intragenic duplication. The sequencing data showed that the mutation originates a frameshift, causing a premature stop codon which would predict the mutation to exhibit a β° phenotype, since it creates a truncated protein. However, the haematological parameters observed in the two carriers studied did not confirm this prediction (Hb 12·2 g/dl, MCV 63·8 fl, MCH 20·3 pg, HbA2 5·82%, HbF 3·36%). The explanation of this fact is at present elusive and deserves further investigation. The origin of this mutation is also not clear, although one possibility is that this duplication is reminiscent of a retrotransposon or retroviral sequence insertion. However, the duplications resulting from the activity of these genetic elements are usually much shorter (Lewin, 1997).

The other newly described nonsense mutation is a frameshift mutation occurring at codon 11 as the result of a cytidine deletion. In this case the resulting protein is far too small to be functional.

In conclusion, the low frequency of mutated alleles in the population in general must not detract our attention from the fact that β° mutations were present in 82% of the affected families. Thus, as β-thalassaemia is not a major health concern in northern Portugal it must be due to the low genetic penetrance of the mutations. However, if, due to lack of genetic counselling, the frequency of these mutations increases with time, they could produce a major public health problem, as homozygous β° thalassaemia cases would become frequent. Consequently, β-thalassaemia genetic counselling in northern Portugal should not be regarded as a low medical priority.

REFERENCES

Amselem, S., Nunes, V., Vidaud, M., Estivill, X., Wong, C., d'Auriol, L., Vidaud, D., Galibert, F., Baiget, M. & Goossens, M. (1988) Determinations of the spectrum of β-thalassemia genes in Spain by use of dot-blot analysis of amplified β-globin DNA. *American Journal of Human Genetics*, 43, 95–100.

Bennani, C., Bouhass, R., Perrin-Pecontal, P., Tamouza, R., Malou, M., Elion, J., Trabuchet, G., Beldjord, C., Benabadji, M. & Labie, D. (1994) Anthropological approach to the heterogeneity of β-thalassemia mutations in Northern Africa. *Human Biology*, 66, 369–382.

Beris, P., Darbellay, R. & Exterman, P. (1995) Prevention of β-thalassemia major and Hb Bart's hydrops foetalis syndrome. *Seminars in Hematology*, 32, 244–261.

Betke, K., Marti, H.R. & Schlict, I. (1959) Estimation of small percentages of fetal haemoglobin. *Nature*, 184, 1877.

Cao, A. (1989) β-Thalassaemia mutations in Mediterranean population. *British Journal of Haematology*, 71, 309–312.

Chibani, J., Vidaud, M., Duquesnoy, P., Bergé-Lefranc, J.L., Pirastu, M., Ellouze, F., Rosa, J. & Goossens, M. (1988) The peculiar spectrum of β-thalassemia genes in Tunisia. *Human Genetics*, 78, 190–192.

Coutinho-Gomes, M.P., Gomes-da-Costa, M.G., Braga, L.B., Cordeiro-Ferreira, N.T., Loi, A., Pirastu, M. & Cao, A. (1988) β-Thalassemia mutations in the Portuguese population. *Human Genetics*, 78, 13–15.

Faustino, P., Osório-Almeida, L., Barbot, J., Espírito-Santo, D., Gonçalves, J., Romão, L., Martins, M.C., Marques, M.M. & Lavinha, J. (1992) Novel promoter and splice junctional defects add to the genetic, clinical or geographical heterogeneity of β-thalassemia in the Portuguese population. *Human Genetics*, 89, 573–576.

Huisman, T.H.J. & Jonxis, J.H.P. (1977) *The Haemoglobinopathies: Techniques of Identification, Clinical and Biochemical Analysis*, Vol. 6. Dekker, New York.

Kalaydjieva, L., Eigel, A. & Horst, J. (1989) The molecular basis of β-thalassemia in Bulgaria. *Journal of Medical Genetics*, 26, 614–618.

Kazazian, H.H. & Boehm, C.D. (1988) Molecular basis and prenatal diagnosis of β-thalassemia. *Blood*, 72, 1107–1116.

Kazazian, H.H., Jr, Orkin, S.H., Antonarakis, S.E., Sexton, J.P., Boehm, C.D., Goff, S.C. & Waber, P.G. (1984a) Molecular characterization of seven beta-thalassemia mutations in Asian Indians. *EMBO Journal*, 3, 593–596.

Kazazian, H.H., Jr, Orkin, S.H., Markham, A.F., Chapman, C.R., Youssoufian, H. & Waber, P.G. (1984b) Quantification of the close association between DNA haplotypes and specific β-thalassaemia mutations in Mediterranians. *Nature*, 310, 152–154.

Lavinha, J. & Baiget, M. (1992) Beta thalassemia in Spain and Portugal: epidemiology and molecular pathology. *Hematology Reviews*, 6, 113–116.

Lewin, B. (1997) *Genes VI*. Oxford University Press.

Lindeman, R., Hu, S.P., Volpato, F. & Trent, R.J. (1991) Polymerase chain reaction (PCR) mutagenesis enabling rapid non-radioactive detection of common β-thalassemia mutations in Mediterraneans. *British Journal of Haematology*, 78, 100–104.

Martins, M.C., Olim, G., Melo, J., Magalhães, H.Á. & Rodrigues, M.O. (1993) Hereditary anaemias in Portugal: epidemiology, public health significance and control. *Journal of Medical Genetics*, 30, 235–239.

Molina, M.A., Romero, M.J., Abril, E., Delgado, I., Cano, R.M., Garrido, F., Pablos, J.M. & Garrido, M.L. (1994) Frequencia de las alteraciones moleculares de la β-thalassemia heterocigota en el sur de espana y su relacion con el fenótipo hematológico. *Sangre*, 39, 253–256.

Noveletto, A., Hafez, M., Deidda, G. Di-Rienzo, A., Felicetti, L., El-Tahan, H., El-Morsi, Z., El-Ziny, M., Al-Tonbary, Y., Sittien, A. & Terrenato, L. (1990) Molecular characterization of β-thalassemia mutations in Egypt. *Human Genetics*, 85, 272–274.

Orkin, S.H., Kazazian, H.H., Antonarakis, S.E., Goff, S.C., Boehm, C.D., Sexton, J.P., Waber, P.C. & Giardina, P.J.V. (1982) Linkage of β-thalassaemia mutations and β-globin gene polymorphisms with DNA polymorphisms in human β-globin gene cluster. *Nature*, 296, 627–631.

Rouabhi, L., Lapoumeroulie, C., Amselem, S., Krishnamoorthy, R., Adjrad, L., Girot, R., Chardin, P., Benabdji, M., Labie, D. & Beldjord, C. (1988) DNA haplotype distribution in Algerian β-thalassemia patients: an extended evaluation by family studies and representative molecular characterization. *Human Genetics*, 79, 373–376.

Spielberg, R., Aulehla-Scholz, C., Erlich, H. & Horst, J. (1989) A β-thalassemia gene caused by a 290 base pair deletion: analysis by direct sequencing of enzymatically amplified DNA. *Blood*, 73, 1695–1698.

Tamagnini, G.P., Gonçalves, P., Ribeiro, M.L.S., Kaeda, J., Kutlar, F., Baysal, E. & Huisman, T.H.J. (1993) Beta-thalassemia mutations in the Portuguese: high frequencies of two alleles in restricted populations. *Hemoglobin*, 17, 31–40.