The molecular basis of β thalassaemia in Bulgaria

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SUMMARY Bulgaria is in a geographical area where β thalassaemia is relatively common. The frequency of carriers is 2 to 3% of the population. Data on the molecular characteristics of the disorder were obtained from the study of 33 homozygous patients and 57 β thalassaemia carriers. As in other Mediterranean ethnic groups, haplotype I and the splicing mutation in IVS-1 nt 110 are the most common. Haplotype V is second in frequency and is associated with three different mutations. The second most common mutation, $\beta^{\circ}39$, is found in association with haplotype II in 80% of cases. A rare haplotype, possibly resulting from a crossover between a haplotype II and a haplotype V chromosome, was found in two thalassaemia carriers in association with frameshift 6.

Altogether four mutations (IVS-1 nt 110, $\beta^{\circ}39$, frameshift 6, and IVS-1 nt 6) account for 67% of the thalassaemia chromosomes. Their detection would permit direct fetal DNA analysis in 84% of the families studied (45% fully informative). RFLP analysis (haplotype plus *Ava*II $\psi\beta$) is 100% informative in 79% of the high risk families.

β thalassaemia is a severe hereditary anaemia, whose molecular basis is a well known example of genetic heterogeneity. Prenatal diagnosis of the disorder by fetal blood sampling and globin chain synthesis studies is being gradually replaced by direct or indirect DNA analysis. The latter approach reduces the medical and psychological problems of second trimester prenatal diagnosis. Its feasibility, however, depends on the genetic basis of the disease in the target population. Therefore, choosing the approach to the prevention of β thalassaemia in a particular geographical area requires preliminary knowledge about the underlying molecular defects.

Bulgaria is on the periphery of the Mediterranean region where β thalassaemia is a common genetic disorder. The average frequency of the β thalassaemia trait is 2 to 3% of the population.¹ This figure seems high enough to justify a programme for the primary prevention of the disease. Meanwhile a strategy should be devised for preventing the birth of further affected children in known high risk families by means of fetal globin chain analysis or DNA studies. The molecular basis of β thalassaemia in the Bulgarian population has not been studied in detail so far. In the present paper we report data on

the chromosomal background and prevalent thalassaemia mutations and on the feasibility of RFLP and direct DNA analysis for the prevention of β thalassaemia in Bulgaria.

Materials and methods

The study involved 33 homozygous, transfusion dependent patients and 57 β thalassaemia carriers. The patients and families were referred by paediatric departments and units from various regions of the country. DNA extraction from heparinised venous blood and restriction endonuclease analysis were performed essentially as described.² The polymorphic restriction sites³⁻⁶ studied are shown in table 1. Haplotypes were numbered according to Orkin *et al.*⁷ In addition, the *Ava*II site 3' to the β globin gene⁸ was included in the RFLP analysis feasibility study. Detection of the most common molecular defects relied on the previously reported association between haplotypes and β thalassaemia mutations in Mediterranean populations.⁷⁻¹¹ For the identification of frameshift 6,¹² IVS-2 nt 1,¹³ and IVS-2 nt 745⁷ mutations, restriction enzymes *Mst*II, *Hph*I, and *Rsa*I were used. Detection of the other common mutations was undertaken with ³²P end labelled synthetic oligonucleotides which were hybridised to dried gels with *Bam*HI digested

TABLE 1 β globin cluster haplotypes in normal and thalassaemia chromosomes.

5'→3'									Chromosomes	
	ϵ	G γ	A γ	$\psi\beta I$	δ	β			β^A (%) (n=53)	β^h (%) (n=60)
	HincII	HindIII	HincII	HincII		AvaII	BamHI			
I	+	-	-	-	-	+	+	40	50	
II	-	+	+	-	+	+	+	6	13	
III	-	+	-	+	+	+	-	6	-	
IV	-	+	-	+	+	-	+	8	-	
V	+	-	-	-	-	+	-	13	15	
VI	-	+	+	-	-	-	+	4	8	
VII	+	-	-	-	-	-	+	9	7	
VIII	-	+	-	+	-	+	-	-	-	
IX	-	+	-	+	+	+	+	12	2	
11	-	+	+	-	+	+	-	2	3	
13	-	+	+	-	-	+	+	-	2	

genomic DNA or, alternatively, to dot blots with enzymatically amplified DNA (polymerase chain reaction, PCR¹⁴) of the β globin gene region including exons 1 and 2. Amplification by the PCR was performed in a 50 μ l reaction volume containing 1 μ g genomic DNA, 50 pmol each of primers SR24 (5'CTTACCAAGCTGTGATTCCA 3') and SR10 (5'CTCCCTTCCTATGACATGAAC 3'), 1.2 units of heat stable DNA polymerase from *Thermus aquaticus* (Cetus, Emeryville, CA), and 0.2 mmol/l each of dATP, dCTP, dGTP, dTTP in 1 $\times$ reaction buffer (50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3, 1.5 mmol/l MgCl₂, 0.01% gelatin). The reaction mix was placed into a glass capillary tube and then sealed at both ends. The capillary tubes were placed into the common reaction vessel of a computer assisted home made machine consisting of three water baths, with their operating temperatures indicated. The DNA was initially denatured for 120 seconds at 94°C, and then underwent 30 cycles consisting of denaturation at 94°C for 90 seconds, annealing at 55°C for 120 seconds, and DNA synthesis at 70°C for 180 seconds.

For spot blots, after addition of 400 μ l 0.4 mol/l

NaOH, 0.25 mol/l EDTA solution, 2 μ l of the amplified DNA samples diluted in 200 μ l 1 $\times$ TE buffer were applied to a Pall Biodyne nylon membrane (Pall BioSupport Division, Glen Cove, NY) using a spotting apparatus Micro-Sample Filtration Manifold (Schleicher and Schuell). Application was in duplicate; one of the spots was used for hybridisation to the normal probe and the other to the mutant probe. The probes were 5' end labelled with ³²P- γ -ATP. DNA primers and probes were synthesised using a Gene Assembler (Pharmacia LKB). The synthetic oligonucleotides with the normal (β^A) and the mutated (β^h) sequences and the hybridisation temperatures used here are shown in table 2. Hybridisation of dried gels was performed at the temperatures indicated in 6 $\times$ NET (0.9 mol/l NaCl, 0.09 mol/l Tris-HCl, pH 8, 0.006 mol/l EDTA), 0.1% SDS, 5 $\times$ Denhardt's 10% dextran sulphate, 100 μ g/ml denatured salmon sperm DNA, and 2 $\times$ 10⁶ cpm of 5' end labelled oligonucleotide. The gels were washed in 6 $\times$ SSC for 30 minutes at room temperature, and then for one to three minutes at the hybridisation temperature.

TABLE 2 DNA sequences of the synthetic oligonucleotides for the detection of specific mutations and the hybridisation temperatures.

IVS-1 nt 1	(G→A) ¹⁵	β^h β^A	5'CCTGGGCAGATTGGTATCA 3' 5'TGATACCAACCTGCCAGG 3'	53°C 55°C
IVS-1 nt 5	(G→T)	β^h β^A	5'GGCAGGTTGTTATCAAGGT 3' 5'ACCTTGATACCAACCTGCC 3'	51°C 53°C
IVS-1 nt 6	(T→C) ¹⁵	β^h β^A	5'GCAGGTTGGCATCAAGGTT 3' 5'AACCTTGATACCAACCTTC 3'	53°C 51°C
IVS-1 nt 110	(G→A) ¹⁶	β^h β^A	5'GCCTATTAGTCTATTTTCC 3' 5'AATAGACCAATAGGCAGAG 3'	47°C 49°C
β^{39}	(C→T) ¹⁰	β^h β^A	5'AGAACCTCTAGGTCCAAGG 3' 5'CCTTGGACCAAGAGTTCT 3'	53°C 55°C

TABLE 3 Feasibility of prenatal RFLP analysis in 24 high risk families.

	100%	50%	0%
Haplotype RFLPs	12* (50%)	9 (38%)	3 (12%)
Haplotype + <i>Ava</i> II $\psi\beta$	19† (79%)	5 (21%)	—

*7/12—two restriction sites.

†12/19—two restriction sites.

Results

HAPLOTYPES IN THE β GLOBIN GENE CLUSTER
Haplotype analysis was performed on 57 normal and 66 thalassaemic chromosomes. The haplotype could be unequivocally established in 53 normal and 60 mutant chromosomes (table 1). Although significant heterogeneity was observed, haplotype I was found to be the most common in both normal and mutant chromosomes, as is the case in other Mediterranean populations.^{7 17-19} Haplotype V came second, followed by haplotype II, which is in agreement with the findings of Kollia *et al*²⁰ in the Greek population, and differs from the data on other Mediterranean ethnic groups. Haplotypes III and IV were occasionally seen in normal chromosomes, while haplotype VIII was not found in the group studied. Two unusual haplotypes were established in one normal and three mutant chromosomes, as can be seen in table 1. These have been observed

previously in a study of German families with haemoglobinopathies²¹ and designated 11 and 13.

FEASIBILITY OF PRENATAL DIAGNOSIS BY RFLP ANALYSIS

Out of the 57 thalassaemia carriers studied, 41 (72%) were heterozygous for the haplotypes. From this figure the expected frequency in 100% informative families (with both parents heterozygous for the haplotypes) can be estimated at 54%. The actual data on the informativeness of RFLP analysis were obtained from the study of 24 nuclear families with at least one affected thalassaemic child (table 3). As can be seen from the figures in this table, only half of the high risk families are 100% informative for the haplotype RFLPs. In our group of β thalassaemia carriers, 86% of the homozygotes carried haplotype I on both chromosomes. In an attempt to increase the proportion of informative families, we added the *Ava*II $\psi\beta$ polymorphic site to the RFLPs studied.

This polymorphic marker has been reported previously to increase dramatically the number of informative families in the Cypriot population,⁸ while it had an insignificant effect on the feasibility of indirect prenatal DNA analysis in northern Italy.²² In our series the analysis of the *Ava*II $\psi\beta$ polymorphism increased the proportion of fully informative families to 79% (table 3), thus having a considerable effect on the feasibility of fetal RFLP analysis.

TABLE 4 β thalassaemia mutations and haplotypes in chromosomes from Bulgarian patients with thalassaemia major.

Haplotype									Total
	Frameshift 6	IVS-1 nt 1	IVS-1 nt 6	IVS-1 nt 110	$\beta 39$	IVS-2 nt 1	54 nt 7-8 nt	Uncharacterised	
I				21	1			8	30
II					8				8
V	3	4				2			9
VI			5						5
VII				1			2	1	4
IX								1	1
11	2								2
13			1						1
Uncharacterised	1				1		1	3	6
Total	6	4	6	22	10	2	3	13	66

LINKAGE DISEQUILIBRIUM OF THE *Ava*II $\psi\beta$ POLYMORPHISM

The 3.9 kb allele (absence of the *Ava*II 3' $\psi\beta$ restriction site) was observed in 57% of haplotype I thalassaemia chromosomes but not in normal haplotype I chromosomes or in normal or mutant chromosomes of any other haplotype. The absence of this polymorphic site therefore showed a marked association with mutant haplotype I chromosomes, all of which were subsequently shown to have the common Mediterranean IVS-1 nt 110 mutation.²³

THE PREVALENT MUTATIONS

The mutation was identified in 53 (80%) of the thalassaemia chromosomes (table 4). The splicing defect in IVS-1 nt 110 was found in 22 chromosomes and was almost invariably associated with haplotype I. The next most common mutations were $\beta^{\circ}39$ (present in 10 chromosomes), which was associated with haplotype II in 80% of cases, IVS-1 nt 6 (six chromosomes), correlating mainly with haplotype VI, and frameshift 6. In our study frameshift 6, which is often associated with haplotype V, was also found in two chromosomes carrying haplotype 11, possibly resulting from a recombination between a mutant haplotype V chromosome and a normal haplotype II chromosome. Haplotype V, which was second in frequency in the group studied, was associated with three different mutations, namely IVS-1 nt 1, frameshift 6, and IVS-2 nt 1. So far in our study we have been unable to identify the mutation in 13 out of 66 thalassaemic chromosomes. Eight of these are haplotype I, *Ava*II $\psi\beta$ (+) chromosomes, thus suggesting that in the Bulgarian population haplotype I may be associated with some less common mutations.

Discussion

As a result of the genetic heterogeneity of β thalassaemia in the population studied, only five out of 33 patients have been shown to be homozygous for the same mutation (four for IVS-1 nt 110 and one for $\beta^{\circ}39$). Since four mutations (IVS-1 nt 1, $\beta^{\circ}39$, IVS-1 nt 6, and frameshift 6) account for 67% of the thalassaemic chromosomes, we tested the feasibility of direct fetal DNA analysis using three oligoprobes and direct restriction analysis (*Rsa*I). Testing for these four defects would permit the identification of the mutation in both fetal chromosomes in 45% of the families and exclude compound heterozygosity in another 39%. The higher proportion of fully informative families in Cypriots and northern Italians reported by Thein *et al*¹⁶ emphasises further the significant molecular heterogeneity of β thalassaemia in Bulgaria. Nevertheless, our study

indicated that direct prenatal DNA analysis would be feasible in 84% of the high risk families investigated, backed up by RFLP or globin chain synthesis analysis in the 50% informative couples.

Conclusion

In this study we have analysed the chromosomal background and the most common mutations leading to β thalassaemia in a peripheral Mediterranean population which so far has not been investigated in detail. Although a variety of haplotypes and mutations has been observed, the Bulgarian population is similar to other Balkan ethnic groups in terms of the prevalence of haplotype I and the splicing mutation in IVS-1 nt 110 (associated with the 3.9 kb fragment of the *Ava*II $\psi\beta$ polymorphic site). Four molecular defects, namely IVS-1 nt 110, $\beta^{\circ}39$, IVS-1 nt 6, and frameshift 6, account for 67% of the mutations in Bulgarian thalassaemia patients. Our findings suggest that in the population studied haplotype I may be associated with a mutation which is less common in other Mediterranean areas. The prenatal detection of β thalassaemia by means of indirect DNA analysis (haplotype RFLPs and *Ava*II $\psi\beta$) would be 100% feasible in 79% of the families in this study. Direct identification of the mutation with the aid of oligonucleotide probes specific for the mutations in IVS-1 nt 110, $\beta^{\circ}39$, and IVS-1 nt 6, and direct restriction analysis for frameshift 6 is possible in 84% of the families with 100% informativeness in 45%. RFLP haplotype analysis will still be helpful in choosing the correct approach to fetal diagnosis and as a back up method, unless a very large battery of oligonucleotide probes and extensive amplification of the β globin gene are used for direct DNA analysis.

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References

- 1 Tasheva E, Toshkov S, Dobrova A. Hemoglobinopathies in Bulgaria. *Hemoglobin* 1987;11:523-9.
- 2 Old J. Fetal DNA analysis. In: Davies KE, ed. *Human genetic diseases: a practical approach*. Oxford: IRL Press, 1986.
- 3 Antonarakis SE, Boehm CD, Giardina PJV, Kazazian HH. Nonrandom association of polymorphic restriction sites in the β -globin gene cluster. *Proc Natl Acad Sci USA* 1982;79:137-41.
- 4 Jeffreys A. DNA sequence variants in G γ , A γ and β -globin genes in man. *Cell* 1979;18:1-10.
- 5 Driscoll MC, Baird M, Bank A, Rachmilewitz A. A new polymorphism in the human β -globin useful in antenatal diagnosis. *J Clin Invest* 1981;68:915-9.

- ⁶ Kan YW, Lee KY, Furbetta M, Angius A, Cao A. Polymorphism in DNA sequence in the β -globin region: application to prenatal diagnosis of β^0 -thalassaemia in Sardinia. *N Engl J Med* 1980;**302**:185-8.
- ⁷ Orkin SH, Kazazian HH, Antonarakis SE, Goff SC, Boehm CD. Linkage of β -thalassaemia mutations with DNA polymorphisms in the human β -globin gene cluster. *Nature* 1982;**296**: 627-31.
- ⁸ Wainscoat JS, Thein SL, Old JM, Weatherall DJ. A new DNA polymorphism for prenatal diagnosis of β -thalassaemia in Mediterranean populations. *Lancet* 1984;ii:1299-301.
- ⁹ Orkin SH, Marham AF, Kazazian HH. Direct detection of the common Mediterranean β -thalassaemia gene with synthetic DNA probes. An alternative approach for prenatal diagnosis. *J Clin Invest* 1983;**71**:775-9.
- ¹⁰ Pirastu M, Kan YW, Cao A, Conner BJ, Teplitz RL, Wallace RB. Prenatal diagnosis of β -thalassaemia: detection of a single nucleotide mutation in DNA. *N Engl J Med* 1983;**309**:284-7.
- ¹¹ Thein SL, Wallace RB. The use of synthetic oligonucleotides as specific hybridization probes in the diagnosis of genetic disorders. In: Davies KE, ed. *Human genetic diseases: a practical approach*. Oxford: IRL Press, 1986.
- ¹² Chang JC, Alberti A, Kan YW. β -thalassaemia lesion abolishes the same Mst II site as the sickle cell mutation. *Nucleic Acids Res* 1983;**11**:7789-94.
- ¹³ Baird M, Driscoll C, Schreiner H, et al. A nucleotide change at a splice junction in the human β -globin gene is associated with β^0 -thalassaemia. *Proc Natl Acad Sci USA* 1981;**78**:4218-21.
- ¹⁴ Saiki RK, Scharf S, Faloona F, et al. Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 1985;**230**:1350-4.
- ¹⁵ Rosatelli C, Leoni GB, Tuveri T, Scalas MT, Di Tucci A, Cao A. β thalassaemia mutations in Sardinians: implications for pre-natal diagnosis. *J Med Genet* 1987;**24**:97-100.
- ¹⁶ Thein SL, Wainscoat JS, Old JM, et al. Feasibility of prenatal diagnosis of β -thalassaemia with synthetic DNA probes in two Mediterranean populations. *Lancet* 1985;ii:345-7.
- ¹⁷ Camaschella C, Saglio G, Serra A, et al. DNA polymorphism analysis in the Italian population and prenatal diagnosis of thalassaemia. In: Fraccaro M, Simoni G, Brambati B, eds. *First trimester fetal diagnosis*. Berlin: Springer Verlag, 1985.
- ¹⁸ Maggio A, Acuto S, LoGioco P, et al. β^A and β^{thal} DNA haplotypes in Sicily. *Hum Genet* 1986;**72**:229-30.
- ¹⁹ Chibani J, Vidaud M, Duquesnoy P, et al. The peculiar spectrum of β -thalassaemia genes in Tunisia. *Hum Genet* 1988;**78**:190-2.
- ²⁰ Kolia P, Karababa P, Sinopoulou K, Boussiou M, Loukopoulos D. Distribution of the β -gene cluster haplotypes in Greece. Feasibility of prenatal diagnosis of thalassaemia. In: *VII International Congress of Human Genetics, Berlin*. Berlin: Springer Verlag: 1986:4-6.
- ²¹ Oehme R, Kohne E, Horst J. DNA-polymorphic patterns linked to the β -globin genes in German families affected with hemoglobinopathies and thalassemias: a comparison to other ethnic groups. *Hum Genet* 1985;**71**:219-22.
- ²² Camaschella C, Saglio G, Serra A, Cremonesi L, Travi M, Ferrari M. Fetal diagnosis of β -thalassaemia by DNA analysis in Italy. *Lancet* 1986;i:390.
- ²³ Thein SL, Wainscoat JS, Old JM, Wallace RB, Weatherall DJ. The AvaII $\psi\beta$ polymorphism is linked to the common Mediterranean β -thalassaemia mutation. *Br J Haematol* 1985;**61**:747.

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