

Homozygous β^+ thalassaemia owing to a mutation in the cleavage-polyadenylation sequence of the human β globin gene

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Abstract

A mild, non-transfusion dependent, β thalassaemia phenotype is described in a Dutch patient homozygous for a mutation in the cleavage-polyadenylation sequence of the β globin gene. The molecular basis of the mutation, AATAAA>AATGAA, was determined using denaturing gradient gel electrophoresis (DGGE) and direct sequencing of genomic DNA amplified by the polymerase chain reaction (PCR). Different fragments of the β globin gene were amplified and analysed on DGGE for the presence of mutations. The fragment with an abnormal melting behaviour was reamplified and the base substitution in the polyadenylation sequence was identified by direct sequencing.

In the past 20 years it has become clear that both the phenotype and the molecular basis of β thalassaemia show great heterogeneity. More than 90 mutations involving single base substitutions, small deletions, and insertions within the β globin gene or its immediately flanking sequences have been characterised to date. These mutations either affect transcription, RNA splicing, RNA modification, or translation, or produce unstable β globin chains.¹

Mutations at the RNA cleavage and polyadenylation sequence AATAAA, located 110 nucleotides 3' from the termination codon of the β globin gene, result in the formation of elongated and unstable mRNA molecules.² Four of these mutations have been described and they all appear to result in a β^+ thalassaemia phenotype.¹⁻³

The analysis of the phenotype expressed by a

particular mutation can be difficult in compound heterozygotes for two different molecular lesions, and therefore the description of homozygous patients can be of value. In this article we report the haematological and molecular analysis of a Dutch patient homozygous for a mutation at the cleavage-polyadenylation sequence of the β globin gene (AATAAA>AATGAA), who displayed a very mild β^+ thalassaemia phenotype.

Materials and methods

HAEMATOLOGICAL ANALYSIS

Haematological parameters were measured according to standard methods. Globin biosynthetic ratios in reticulocytes were measured by ³H-leucine labelling and isoelectric focusing. Identification of haemoglobins in haemolysates was carried out by starch gel electrophoresis.⁴

MOLECULAR ANALYSIS

DNA extraction and haplotype analysis were performed following standard procedures.⁵ Amplification using the polymerase chain reaction and direct sequencing of amplified material was performed as previously described.⁶ The template for the direct sequencing experiment was amplified with amplimers C and E, while a-D was used as a sequencing primer.

The gel apparatus and electrophoresis conditions for the denaturing gradient gel electrophoresis (DGGE) were as described in Myers *et al.*⁷ In short, one fifth of the amplification product was electrophoresed on a 6% polyacrylamide gel with a linearly increasing gradient of denaturant (100% denaturant = 7 mol/l urea/40% formamide (v/v)). For primers GC.A and pCO4, a-pCO4 and GC.B, and GC.a-B and IVS-2 a gradient from 45 to 75% denaturant was used; for primers C and D, and a-D and E a gradient from 25 to 55% denaturant was applied. The gels were run at 50 V, 30 mA for 16 hours. After electrophoresis the gel was stained with ethidium bromide (0.5 μ g/ml) for 30 minutes and examined under UV transillumination.⁸ The nucleotide sequence of the amplimers is given in the legend to fig 1.

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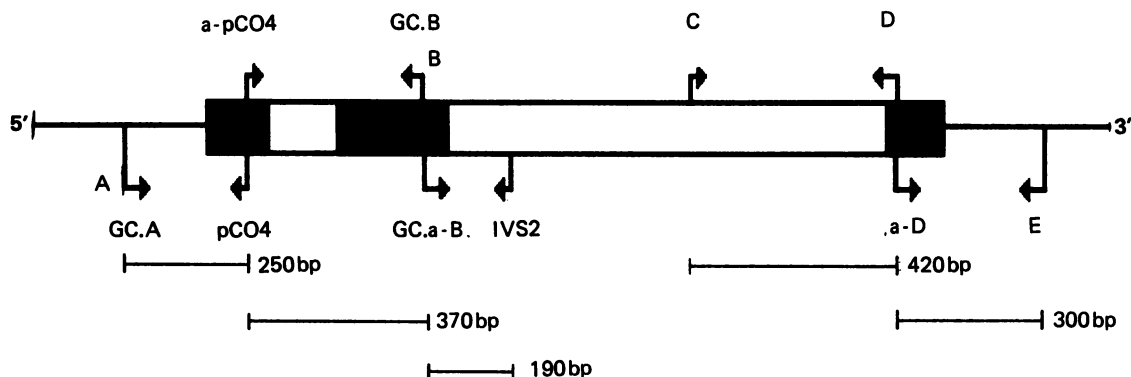


Figure 1 Schematic representation of the location and orientation of the amplimers in the β globin gene. Filled boxes represent coding regions, open boxes are introns. The following amplimers were used: (A) 5'-GTACGGCTGTTCATCACTTAG-ACCTCA-3'; pCO4: 5'-CAACTTCATCCACGTTCCACC-3'; (B) 5'-TGCAGCTTGTACAGTGCAGTCACT-3'; IVS-2: 5'-AAACGATCCTGAGACTTCCA-3'; (C) 5'-GTGTACACATATTGACCAAA-3'; (D) 5'-AGCACACAGAC-CAGCACGTT-3'; (E) 5'-AAATGCACTGACCTCCACA-3'. As a GC clamp the following sequence was attached to the 5' end of the amplimer: 5'-CGCCC GCCCGCCCGTGCCCCCGCGCCCGCCCCGCC-3'. The primers marked a- were synthesised in the opposite orientation.

Results

HAEMATOLOGICAL ANALYSIS

The proband is a Caucasian female with no apparent evidence of foreign ancestry. At 13 years she was admitted to hospital for a non-haematological condition and on that occasion an iron resistant hypochromic anaemia was discovered. Other laboratory findings included increased levels of bilirubin, very low haptoglobin, and reduced survival of red cells (15 days). A bone marrow biopsy showed a marked erythroid hyperplasia. No definite diagnosis was formulated at that time and despite various attempts at therapy, including a splenectomy carried out seven years later, the anaemia did not improve significantly.

When brought to our attention the patient was 26 years old. Physical examination showed pallor and slight jaundice in a tall young woman who expressed only moderate complaints of fatigue and dizziness. No other abnormalities, particularly no hepatomegaly, were found.

The haematological data of the patient are given in

Haematological data.

	Proband	Mother
Sex/age	F26	F49
Hb (g/dl)	9.8	14.1
RBC ($10^{12}/l$)	3.77	5.30
Ret (%)	280	11
SeFe (mg/l)	1.3	—
Bilirubin (mg/l)	1.9	—
PCV (l/l)	0.31	0.44
MCV (fl)	82	83
MCH (pg)	26.0	26.6
MCHC (g%)	31.6	32.04
Osmotic fragility	Very decreased	Slight decreased
Hb A ₂ (%)	4.6	3.7
Hb F (%)	5.6	0.4
β/α ratio	0.26	0.55

the table. The proband showed a hypochromic anaemia with a strong haemolytic component, normal MCV, low MCH, decreased osmotic fragility, unbalanced β/α globin synthesis ratio, and increased levels of Hb A₂ and Hb F. A peripheral blood smear showed anisopoikilocytosis, hypochromia, basophile stippling, target cells, and the presence of schistocytes and erythroblasts (~50% of all nucleated cells). The patient became pregnant at 30 and 32 years of age. The first pregnancy ended with the premature birth of an immature infant who died of sepsis shortly after birth. During the second pregnancy, which resulted in the delivery at term of a healthy baby, the patient was repeatedly transfused in order to keep the Hb levels between 12 and 13 g/dl.

The mother of the proband is an apparently healthy woman whose heterozygosity for β thalassaemia is indicated only by a moderate increase of Hb A₂ and an unbalanced β/α globin synthesis ratio. The same abnormality, an increase of Hb A₂ and slightly decreased osmotic fragility, was found in an aunt and two brothers of the mother. The father and other members of the family were not available for examination; the only important information we could obtain was the existence of consanguinity between the proband's parents.

On the basis of the clinical, haematological, and biochemical data, we concluded that the patient had a very mild form of β thalassaemia caused (considering the consanguinity of the parents) by homozygosity for the same kind of mutation.

MOLECULAR ANALYSIS

Various regions of the β globin genes of the proband were amplified with different primer combinations

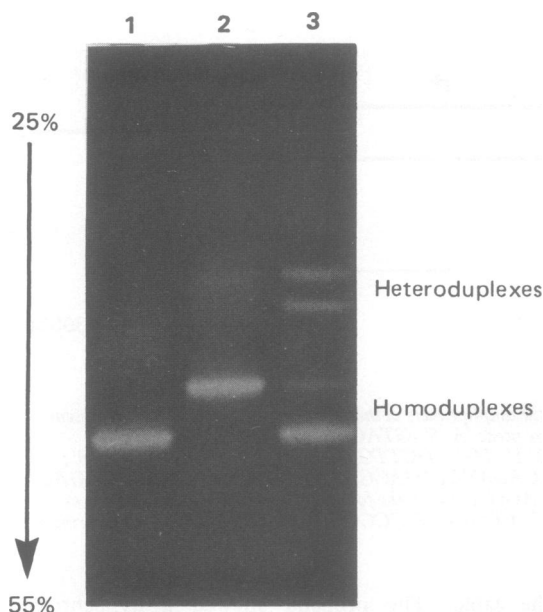


Figure 2 Ethidium bromide stained polyacrylamide gel containing a denaturing gradient from 25 to 55% denaturant. β globin fragments amplified with amplimers a-D and E from the following subjects were loaded: lane 1, the proband; lane 2, a control sample; lane 3, an artificial heterozygote consisting of DNA from the proband mixed with DNA from a normal control sample. The wild type homoduplex in the artificial heterozygote has a lower intensity than the mutant homoduplex, probably owing to a lower concentration of the wild type DNA in the sample. The percentage of denaturant is indicated on the left hand side of the picture. The position of homo- and heteroduplexes in the gel is indicated on the right hand side of the picture.

(fig 1) and the amplified DNA was analysed by denaturing gradient gel electrophoresis (DGGE) to predetermine the region of the gene where the mutation is located.⁸

The PCR products obtained with amplimers a-D and E (fig 2, lane 1) showed a homoduplex fragment with a faster electrophoretic mobility compared to the homoduplex from the normal control (fig 2, lane 2). The artificial heterozygote, a mixture of DNA from the proband and the normal control, showed four different bands (fig 2, lane 3). The two lower bands correspond to the normal wild type and mutant homoduplexes, while the two upper bands correspond to the heteroduplexes formed by the annealing of normal and mutant alleles.

Direct sequence analysis of the same region, using a nested primer, showed the presence of the AATAAA>AATGAA mutation in the cleavage-polyadenylation signal on both alleles. RFLP analysis indicated that the proband is homozygous for haplotype II, according to the nomenclature of Orkin *et al.*⁹

Discussion

The highly conserved sequence AATAAA, found 10 to 30 nucleotides upstream of most polyadenylation sites in eukaryotic mRNA, forms a part of the recognition site for endonucleolytic cleavage of primary transcripts.¹⁰

In a cloned β thalassaemia gene containing an AATAAA>AACAAA mutation, a fraction of the β globin mRNA extends at least 900 bp 3' of the normal polyadenylation site. The cleavage and polyadenylation at the normal polyadenylation site is markedly inefficient but not completely extinguished.² The presence of normally processed RNA probably accounts for the mild phenotype in carriers of a mutation in the cleavage-polyadenylation sequence.²

Elongated RNA transcripts have also been observed in a human α thalassaemia gene containing an AATAAA>AATAAG mutation in the cleavage polyadenylation sequence of the $\alpha 2$ globin gene.^{10 11} When this gene is transfected into HeLa cells it not only fails to process the 3' end, but also fails to terminate transcription.¹⁰ The amount of normal $\alpha 2$ mRNA present in the reticulocytes of the homozygous α thalassaemia patient is about 10 to 20% of that found in a normal subject.¹¹

Jankovic *et al.*³ described the AATAAA>AATGAA mutation in the β globin gene of Yugoslavian, Greek, and Bulgarian subjects. Haematological data from these subjects, all heterozygotes or compound heterozygotes, displayed a rather mild β^+ thalassaemia phenotype comparable to that of the mother of the proband described in this paper. Since no information was provided by Jankovic *et al.*³ on the haplotype on which the mutation occurs in these subjects, we cannot speculate on the origin of the mutation in the Dutch patient.

Until now four different mutations in the AATAAA sequence of the β globin gene (AACAAA, AATGAA, AATAGA, AATAAG) have been described, causing a mild form of β thalassaemia.¹⁻³ A combination with other more severe types of β thalassaemia might result in a transfusion dependent β thalassaemia major syndrome, while in combination with milder mutations, an intermediate type of thalassaemia, like in the proband, is usually observed.³

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