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ARTICLE

A complex haemoglobinopathy diagnosis in a family with both β^o- and $\alpha^{o/+}$-thalassaemia homozygosity

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The occurrence of point mutation α-thalassaemia and of complex combinations of haemoglobin defects is underestimated. Haemoglobinopathies, the most frequent monogenic recessive autosomal disorder in man, occur predominantly in Mediterranean, African and Asiatic populations. However, countries of immigration with a low incidence in the indigenous population, are now confronted with a highly heterogeneous array of imported defects. Furthermore, the occurrence of severe phenotypes is bound to increase in the near future because of the endogamous growth of the ethnical minorities and the lack of prevention. We describe an Afghan family in which both partners of a consanguineous relationship are carriers of a β- as well as an α-thalassaemia determinant. The combination of defects was revealed by the *in vitro* measurement of the β/α biosynthetic ratio and was characterised at the DNA level. The molecular defects involved are the Cd5(-CT), a Mediterranean β^o-thalassaemia mutation, and the $\alpha_2^{o/+}$-thalassaemia AATA(-AA) polyadenylation defect. The α-thalassemia defect is a rare RNA-processing mutant described only twice before in heterozygous form in Asian-Indian patients. The mutation suppresses the expression of a α_2 gene and reduces the expression of the less efficient, 3' located α_1 gene as well, inducing a near α^o-thalassaemia phenotype. This defect is now described for the first time in the homozygous condition in one of the children who, in addition to being homozygous for the α-thalassaemia point mutation, is also a carrier of the β^o-thalassaemia defect. A previously described homozygous case of the $\alpha^{o/+}$-thalassaemia condition, caused by a similar polyadenylation defect, was characterised by a severe HbH disease. However, the patient described here present at 7 years of age with severe caries, like his β-thalassaemia homozygous brother but without hepatosplenomegaly, haemolysis or severe anaemia. The haematological analysis revealed 9.5 g/dl Hb; 5.4×10^{12}/l RBC; 0.33 l/l PCV; 61 fl MCV; 17.6 pg MCH and 6.2% of HbA_2. The biosynthetic ratio β:α was 1.6 and no HbH fraction was detectable either on electrophoresis or as inclusion bodies. The

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parents reported no complications during pregnancy, at birth, or in the neonatal period in rural Afghanistan. We presume therefore that the counterbalancing effect induced by the co-existing β-thalassaemia defect could have modified a potentially severe perinatal HbH disease into a strongly hypochromic but well compensated 'α°-like heterozygous' thalassaemia phenotype. The risk of a severe HbH disease, could have been easily missed in this family which was referred because of a child affected with β-thalassaemia major.

Keywords: β-thalassaemia; α-thalassaemia; globin chains synthesis

Introduction

Haemoglobinopathies, the most frequent monogenic recessive autosomal disorder in man, occur predominantly in Mediterranean, African and Asiatic populations. The selection mechanism induced by the protection against *malaria tropica*, which is an advantage to the carrier, has given rise to a high level of these traits. Carriers of haemoglobinopathy are healthy persons with mild haematological symptoms but the children of couples at risk have a 25% chance of becoming severely affected. Prevention of major haemoglobinopathies is technically feasible and is achieved by carrier and partner diagnostics, by giving information and genetic counselling and by eventually offering prenatal diagnosis. In countries of immigration, where the carrier frequency was originally very low, a highly heterogeneous array of various defects has arisen, mostly concentrated in the population of the ethnic minorities. Therefore the occurrence of severe phenotypes is bound to increase in the countries of immigration if no prevention is offered to the populations at risk. On the other hand, the large variety of defects, often present in polyethnic populations, makes prevention at the molecular level an intricate matter. As an example of a complex prevention case we present a large Afghan family, living in The Netherlands, which was examined for the presence of the β-thalassaemia trait and which already had a child affected with β-thalassaemia major. The three-generation family consisted of 23 individuals comprising two grandparents, four married couples and 13 children in the third generation. Ten individuals were found to be carriers but only the nuclear family who had already a β-thalassaemia homozygous child was found at risk of β-thalassaemia major. During routine analysis of the nuclear family, all carriers presented with elevated HbA_2 levels and parameters compatible with their state but with an unexpectedly balanced globin chain biosynthesis. Having excluded the possibility of an artefact, the presence of a β–α defect combination was investigated at the molecular level.

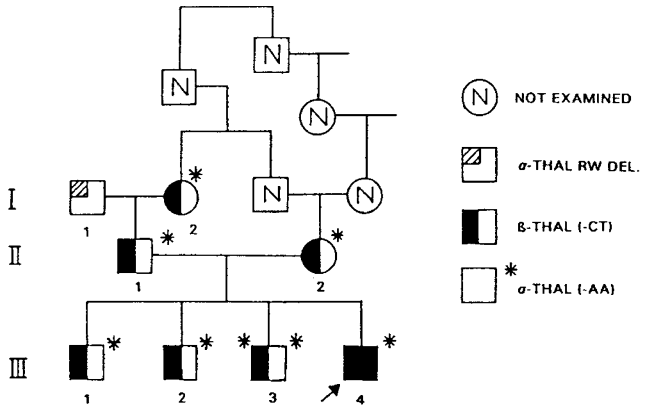

Figure 1 *Pedigree of the family*

Materials and Methods

Blood samples were vacuum-collected in Li-Heparin and Na-EDTA. The haematological parameters were obtained from a semiautomatic counter Sysmex F300 (Sysmex-Toa Medical Electronics Co Ltd, Kobe, Japan). Red cell lysates were examined on starch gel electrophoresis at pH 8.6,[1] The HbA_2 fraction was estimated by ion exchange column chromatography.[2] The HbF concentration was established by alkaline denaturation.[3] The synthetic ratio of the α and non-α globin chains was determined using a modified method based on standard techniques. The method consists of the measurement of the ^{3}H-labelled leucine which is incorporated in the α and β globin chains, separated from the newly synthesised protein obtained from *in vitro* incubated reticulocytes. This method is described in detail elsewhere.[4] Genomic DNA was isolated by selected lysis[5] and high salt extraction.[6] The α-gene deletion was identified by standard Southern blot procedure, using *Eco*-RI and *Bgl*-II endonucleases and hybridisation with a ^{32}P-labelled α and ζ gene probe.[7] The α gene point mutation was characterised by DGGE and sequence analysis as previously described.[8] The molecular analysis of the β gene was done by selective amplification from genomic DNA of overlapping β gene fragments followed by denaturing gradient gel electrophoresis (DGGE) as previously described.[9] Direct solid-phase sequencing of the PCR product[10] was achieved using magnetic beads as solid support[11] and fluorescein-labelled Universal sequencing primer on an Automated Laser Fluorescent DNA Sequencing apparatus (ALF Pharmacia, Sweden) or using a BigDye^{tm} Terminator mix on an ABI 377 automated sequencer (Applied Biosystems, Perkin Elmer Corporation, Foster City,

Table 1 Haematological data of the family members

Individuals	*I*-1	*I*-2	*II*-1	*II*-2	*III*-1	*III*-2	*III*-3	*III*-4
Sex/age	*M64*	*F67*	*M37*	*F29*	*M11*	*M8*	*M7*	*M3*[a]
Hb (g/dl)	13.4	12.8	13.12	10.5	11.3	11.8	9.5	10.6
PCV (L/L)	0.43	0.42	0.43	0.38	0.36	0.38	0.33	0.28
RBC (10^{12}/L)	5.07	4.80	5.68	4.86	5.14	5.27	5.4	3.68
MCV (fl)	85.0	88.0	76.0	78.0	70.0	72	61	76
MCH (pg)	26.4	26.6	23	21.4	21.9	22.4	17.5	28.8
MCHC (g/dl)	31.1	30.3	30.3	27.6	31.3	30.9	28.8	37.9
Hb A_2 (%)	2.70	5.2	6.4	5.8	6.0	5.8	6.2	3.4
Hb F (%)	<1	<1	<1	<1	<1	<1	<1	8.0
Hp (mg/dl)	143	181	177	194	112	76	87	85
Osmotic frag.	□	□□	□□	□□	□□	□□	□□	□
RBC morph.	+	+++	++	++	++	++	+++	+
Incl. bodies	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
□□/□ ratio	–	–	0.9	1.02	1.02	0.85	1.6	0.12[b]
□ genotype	–□/□□	±/□□	±/□□	±/□□	±/□□	±/□□	□□□	□□□□
□ defect	RW	(–AA)	(–AA)	(–AA)	(–AA)	(–AA)	(–AA)	(–AA)
□ genotype	□/□	□°/□	□°/□	□°/□	□°/□	□°/□	□°/□	□°/□°
□ defect	–	(–CT)	(–CT)	(–CT)	(–CT)	(–CT)	(–CT)	(–CT)
Transfusion	no	no	no	no	no	no	no	yes

[a]=propositus; [b]=□/□ only; □ or □ = decreased or increased; n.d. = not detected; +, ++, +++ = increase in abnormality; □ genotype ± = □$_2$ POLY A mutation.

CA, USA). The PCR reactions were carried out in a forced water circulation thermocycler.[12]

Results

Haematological Data and Clinical Report

Eight members of the three-generation family were examined (Figure 1). All β-thalassaemia carriers presented with moderate haematological abnormalities and elevated HbA_2 levels. The propositus, a 3-year-old boy (III-4), affected with β-thalassaemia major was kept above 10 g of Hb by a transfusion regime at 5-week intervals. His length and weight were normal, and although severe and extended caries were observed, no significant hepatosplenomegaly was present.

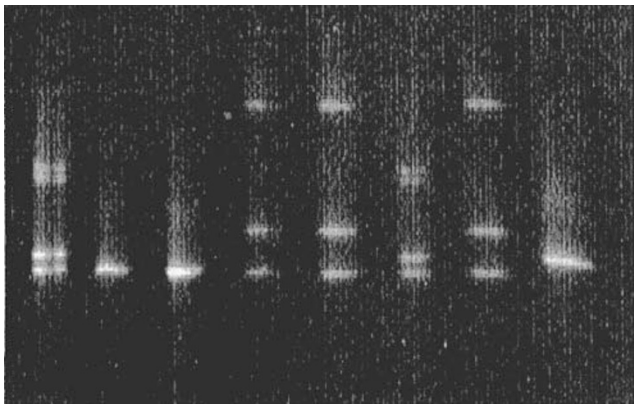

Figure 2 *DGGE patterns of fragment A in the 6 members of the family who are all carriers of the cd5(-CT) mutation: Lane 1, III-3 (heterozygous); lanes 2 and 3, III-4 (homozygous); lanes 4,5,6 and 7, III-1, III-2, II-2 and II-1 respectively (all heterozygous, but note that lanes 4,5 and 7 are anomalous because of the cd2 polymorphism). In lane 8 the normal w.t. pattern without the cd2 polymorphism*

The child III-3 presented with the pallor of a strongly microcytic hypochromic intermediate anaemia but without physical complaints. No hepatosplenomegaly or other evident anomalies were observed. Like his brother (III-4), the child had extensive caries but no history of severe perinatal anaemia was reported by the parents. The haematological data of the family are summarised in Table 1.

Molecular Analysis of the β Genes

The β gene was analysed in all family members by DNA amplification of 8 gene fragments, for DGGE and sequencing. The PCR fragment which covers part of the β gene promoter and of the first exon, analysed by DGGE produced an anomalous denaturing pattern and revealed upon DNA sequencing the dinucleotide deletion (-CT) at cd5 (Figure 2).

The cd5(-CT) mutation induces a β^o-thalassaemia phenotype, and is quite common in the Mediterranean area, especially in the Moroccan population. Although uncommon in Asians the mutation was found in homozygous form in the propositus (III-4) and in

heterozygous form in the consanguineous parents, in the three older children, and in the grandmother.

Molecular Analysis of the α Genes

Restriction fragment analysis of the α gene cluster revealed the presence of an $-\alpha^{3.7}$ thalassaemia deletion heterozygosity in the grandfather but this deletion was not found in the rest of the family (data not shown). The α2 gene fragment amplified with the S3–S6 primers as indicated in Figure 3a produced an anomalous pattern on DGGE (data not shown).

Sequencing analysis of the same fragment revealed the presence of a dinucleotide deletion AATA(-AA) disrupting the poly-adenylation site of the α2 gene (Figure 3b). This defect was present in heterozygous form in the grandmother, in both parents, and in three children including the propositus affected with β-thalassaemia major. The child who carries the α-thalassaemia point mutation in homozygous form (III-3) was also heterozygous for the β-thalassaemia mutation.

Discussion

At least 140000 haemoglobinopathy carriers are present in the autochthonous and allochthonous Dutch population. Due to the high heterogeneity of this population a large variety of different defects can be expected and unusual combinations of defects may arise which can only be recognised with specialised analyses.[13] Without globin chain synthesis determination and point mutation analysis of the β and the α gene, the present family would have been diagnosed only at risk for a classic high HbA_2 β-thalassaemia. Although α-thalassaemia phenotypes are mostly induced by deletions, 36 different point mutation

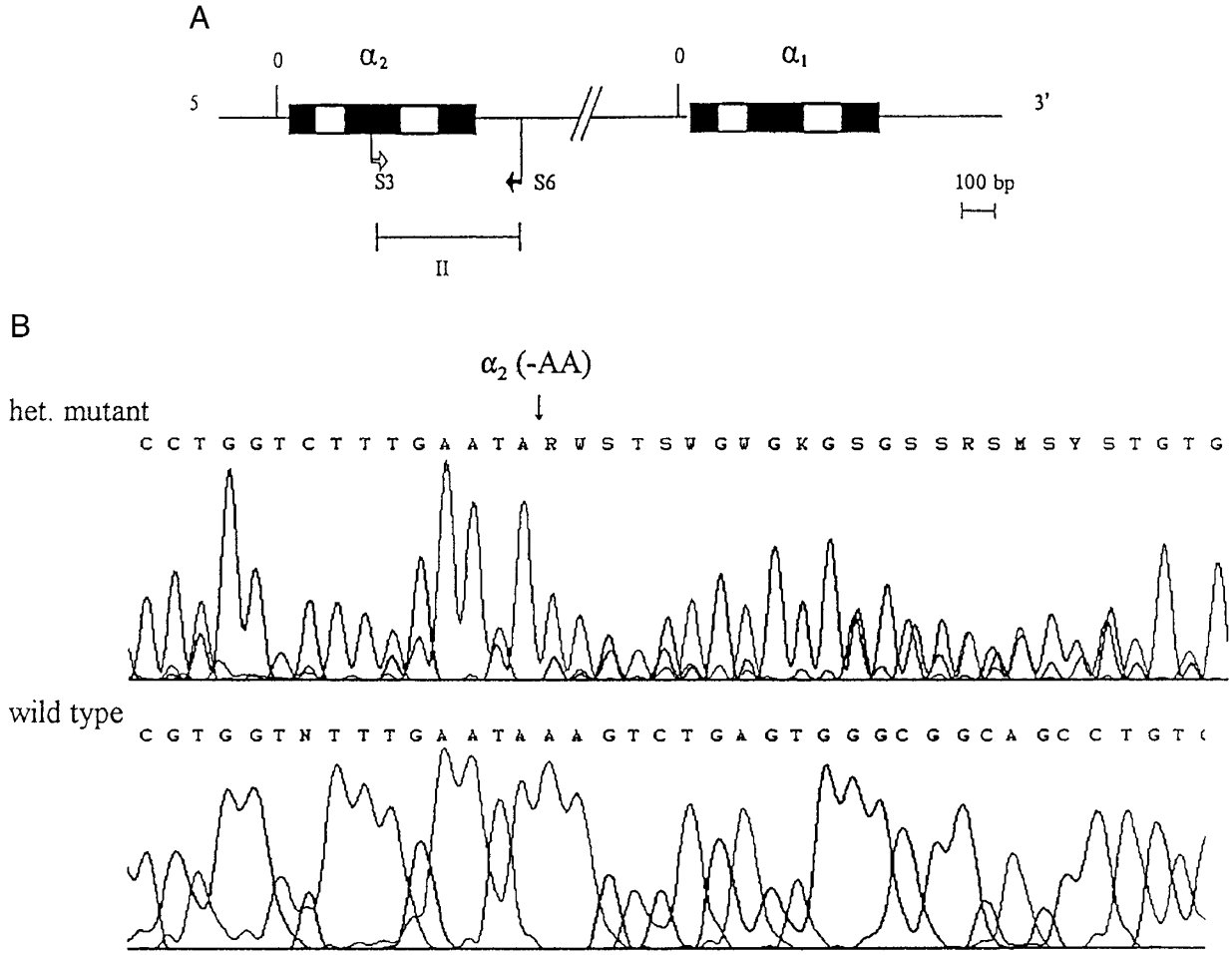

Figure 3 (a) and (b) *Schematic representation of the duplicated α globin genes. Fragment II has been used for direct sequencing using BigDye[tm] terminator mix on an ABI 377 automated sequencer.* **(b)** *Sequence section of fragment II indicating the (-AA) deletion at the poly-adenylation site of the α2 gene*

determinants have also been described to date.[14] Among these, four different polyadenylation site mutations of the α_2 gene with a near α^o-thalassaemia phenotype have been characterised. These defects affect not only the function of the mutated α_2 gene but also downregulate the 3' located α_1 gene by transcriptional interference.[15] The cases of HbH disease in combination with β-thalassaemia which have been previously described[16,17] concern deletion defects easily detectable by standard Southern blotting procedures. The polyadenylation defect described in this family has been reported only once before, causing a near α^o-thalassaemia phenotype ($\alpha^{o/+}$-thalassaemia) in a heterozygote[8] and is reported for the first time in the homozygous form in the present family. A similar $\alpha^{o/+}$-thalassaemia polyadenylation defect previously described in an homozygous case[18] was characterised by a severe HbH disease. Our patient, however, presented at 7 years of age with an intermediate anaemia but without hepatosplenomegaly or haemolysis and no HbH fraction was detectable either on electrophoresis or as cellular inclusions.

Because no major complications were reported during pregnancy, or in the perinatal period, we assume that the co-existing β^o-thalassaemia heterozygosity reduced the biosynthetic unbalance to a moderate $\beta:\alpha = 1.6$, modifying a potentially severe perinatal HbH disease into a strongly hypochromic but well compensated 'α^o-like heterozygous' thalassaemia phenotype.

HbH disease presents, per definition, with a degree of variability[19,20] and can be influenced by non α gene related factors as happens in all cases where non-functional protein products are present in the mature erythrocytes.[21,22] However, in view of the combination of defects, the progeny of this family must be considered at risk not only for the homozygous β^o-thalassaemia condition but also for a possibly severe HbH disease that could result from the homozygous state of the $\alpha^{o/+}$-thalassaemia polyadenylation defect, in the absence of a compensating β-thalassaemia heterozygosity. On the other hand, the homozygous β^o-thalassaemia child, is maintained at a good Hb level (10–14 g in pre- and post-transfusion conditions, respectively) by a relatively modest 5-week transfusion regime, and still has 8% endogenous HbF and no measurable haemolysis. We propose that the absence of haemolysis in this patient is partially due to the coexisting α-thalassaemia heterozygosity and to the presence of residual γ-chain expression, a combination of factors which reduces the amount of unbound α chains to cause ineffective erythropoiesis and haemolysis.

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