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Casuïstiek

Hb-OSU Christiansborg: a rare abnormal hemoglobin observed in two independent families in The Netherlands

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The colonial heritage and the recent immigration from many different countries has generated in The Netherlands a large and very heterogeneous allochtonous population originating from areas which are often associated with elevated frequencies for a multitude of different hemoglobin abnormalities. We describe two families with Hb Osu-Christiansborg (β52 Asp–>Asn), a rare (2 cases earlier described in litterature), non-pathological β-gene mutant which migrate like HbS on Hb-electrophoresis at pH 8.6 and which could therefore be confused with this pathological mutant frequently occurring in the allochthonous population in The Netherlands. The two families were of African origin, one from Ghana, the other from the Dominican Republic, both families displayed the same mutation on the same haplotypes. This seems to indicate a single mutation event on a common ancestral framework. We describe the hematological and molecular analysis and compare the analytical results using HPLC separation at different conditions.

At least 10% of the Dutch population consists of recent immigrants and a group of about 1-2 million "autochthonous" inhabitants has early Asian, South-European or Black ancestors. A recent molecular study on a group of thalassemia carriers revealed the presence of 48 different β-thalassemia determinants. The common structural defects such as HbS, HbC, HbE, Hb Lepore, Hb Constant-Spring and HbD-Punjab were also largely represented and 30 different rare abnormal hemoglobins were found, 7 of which were described for the first time (Giordano et al. Community Genetics in press). In addition, 139 independent chromosomes with deletional and non-deletional α-thalassemia defects were characterized showing the presence of at least 14 different α-thalassemia determinants in The Netherlands (1). It has been calculated that at least 100.000 people of recent allochtonous origin can be expected to be carrier of some form of hemoglobinopathies in The Netherlands and that at least 30.000 of them are HbS carriers (2). Abnormal hemoglobins which behave similarly to HbS or HbE on electrophoresis or chromatography may generate a possible identification problem especially if confirmation by sickling test give a false negative result because of a HbS/α-thalassemia combination. The families described in this study are examples of potential misdiagnosis when standard hematological methods are used to detect hemoglobinopathies in a poly-ethnic population that have a large variety of hemoglobin abnormalities.

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PATIENTS and METHODS

Patients, who have been preselected for hematological abnormalities, are routinely referred to our laboratory for hemoglobinopathies analysis. Blood samples are vacuum collected in Na-EDTA or Li-Heparine and analyzed following a specific and extended hematological flow-chart (3). The hematological parameters are obtained from a semiautomatic counter Sysmex F300 (Sysmex-Toa Medical Electronics Co Ltd, Kobe, J.) determining the Haematocrit by capillary centrifugation. Red cell lysates are examined on starch gel electrophoresis at pH 8.6 (4). The HbA_2 and HbF fraction are usually estimated by automatic HPLC analysis (Variant, Biorad). The routine chromatographic analysis, performed at the Groene Hart Hospital, applies an ion exchange column (Mono-S HR 5/5, Pharmacia/LKB, Upsala, Sweden) and an HPLC set up based on Pharmacia elements (pumps type 2150-020; controller type 2152-020, Uvicord type 2510-010). Two gradients of different pH are used. The gradient at pH 5.7 consists of 10mM malonic acid in phase A and B, with the addition of 35 g/l NaCl in phase B. The gradient formation (percentage of phase B in A) is as follow: 1 ml isocratic run at 20%, up to 40% until 4 ml, up to 50% until 7 ml and up to 100% until 12 ml, followed by an isocratic flow until 15ml. The flow-rate is 2 ml/min. The gradient at

Table 1. Hematological data of family I

Individuals Gender/age	I-1 F34*	I-2 M ?	II-1 M13
Hb (mmol/l)	7.5		7.9
Haematocrit (l/l)	0.34		0.39
RBC (10^{12}/l)	4.3		4.5
MCV (fl)	78		86
MCH (amol)	1717		1742
Hb A_2 (%)	3.0		3.0
Hb F (%)	<1		<1
Hb-Osu (%)	46		45
Hp (g/l)	1.75		1.47
Osmotic frag.	↓↓		↓
S.Iron (µmol/l)	9.8		??
Ferritin (µg/l)	24		??
Erythr. Morph.~)	+		+
Incl. Bodies	absent		absent
β/α ratio	1.1		1.04
β abn %	44		45
α-genotype	αα/αα		αα/αα
HSmegaly	absent		absent
Haplotype @	11/2	4/? #	11/4

~): Erythrocyte Morphology: + = slightly, ++ = moderately, +++ = strongly aberrant; @: Haplotype definition according to ref. 14; #: deducted; *): propositus

Table 2. Hematological Data of Family II

Individuals Gender/age	I-1 M41	I-2 F36	II-1 F4*
Hb (mmol/l)	8.5	8.0	7.8
Haematocrit (l/l)	0.42	0.37	0.38
RBC (10^{12}/l)	4.76	4.05	5.06
MCV (fl)	88	91	74
MCH (amol)	1792	1972	1543
HbA_2 (%)	2.7	3.0	2.8
HbF (%)	0.3	1.0	1.6
HbOsu (%)	--	42	45
HbC (%)	30.7	--	--
Hp (g/l)	1.25	1.54	0.94
Osmotic frag.	↓↓↓	↓	↓
Iron (µmol/l)	--	--	18.2
Ferritin (µg/l)	--	--	32
Erythr. Morph.~)	++	+	++
Incl. Bodies	absent	absent	absent
β/α ratio	β^{A+C}/α=1.15	β^{A+O}/α=1.1	n.d.
β abn %	47	50	45
α-genotype	-αα/α	αα/αα	αα/αα
HSmegaly	absent	absent	absent
Haplotype @	5/1	11/4b	11/1

~): Erythrocyte Morphology: + = slightly, ++ = moderately, +++ = strongly aberrant; @: Haplotype definition according to ref. 14; *): propositus

pH 6.7 consists of 10mM Tris in phase A and B, with the addition of 11,7 g/l NaCl to phase B. The gradient formation as a percentage of phase B in phase A is developed at different flow-rates. At a flow-rate of 1.5 ml/min, 1.5 ml are eluted at 15% isocratically. Phase B is then increased to 35% up to 10.5 ml elution volume, when the flow-rate is increased to 2 ml/min and the percentage phase B is increased to 100% up to 20 ml, followed by isocratic elution until 23 ml with 100% B. The synthetic ratio of the α- and non-α-globin chains was determined using a modified method (Giordano et al. submitted for publication), based on standard procedures (5,6). Genomic DNA is isolated by selective lysis (7) and high salt extraction (8). The molecular analysis of the β-gene is performed as previously described by selective amplification from genomic DNA of overlapping β-gene fragments (Fig. 1), followed by mutation detection on denaturing gel gradient electrophoresis (DGGE) (9). The mutations are confirmed by direct, solid-phase sequencing of the PCR products (10) using magnetic beads as solid support (11) and FITC-labelled M13-I sequencing primer on an Automated Laser Fluorescent DNA Sequencing apparatus (A.L.F. Pharmacia). The PCR reactions are carried out in a forced water circulation thermocycler (12). The haplotype of the β-gene clusters of the family is established using 7 polymorphic sites distributed over a 50 Kb sequence ranging from 1Kb 5' to the Î gene until 5 Kb 3' to the β-gene (13,14). The a-gene deletion was identified by standard Southern blot procedure, using Eco-RI and Bgl-II endonucleases and hybridization with a 32P labelled a- and z-gene probe (1).

Case presentation

Family I

The propositus was a 34-years-old woman of Black/Asiatic ancestry originary from the Dominican republic who was hospitalized with abdominal complains and a moderate microcytic hypochromic anemia. No hemolysis, hepatosplenomegaly, icterus or cyanosis were present. Amylases were slightly elevated and the direct Coombs test was positive. On Hb-electrophoresis an anomalous pattern compatible with the HbS mutant was observed but the sickle test was negative. After appendicitis was diagnosed, a laparoscopic appendectomy was performed and two units of packed red cells were delivered. The patient was released with a cure of ferro-fumarate and because of the "HbS like" abnormal hemoglobin a blood sample of her son was collected. No blood from the father was available.

The 13-year-old son presented with the same "HbS like" electrophoretic pattern and negative sickle test and a mild microcytic hypochromic anemia as in the mother. The data of the biochemical/hematological analysis are summarized in Table 1.

Family II

The propositus, a 4-years-old Ghanese child, presented with a mild microcytic hypochromic anemia and an heterozygous electrophoretic pattern identical to the HbS heterozygous and an HPLC profile suggesting the presence of HbE. The sickle test was negative. The mother was carrier of the same mutant but without microcytic anemia. The father was heterozygous for the HbC mutant which is very frequent

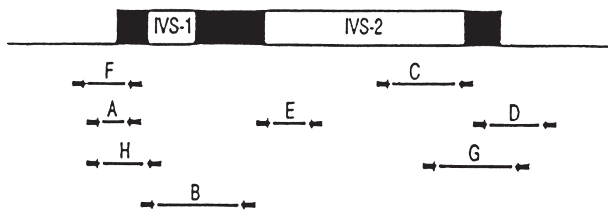

Figure 1. Amplification chart of the β-gene fragments used for point mutation pre-screening on DGGE

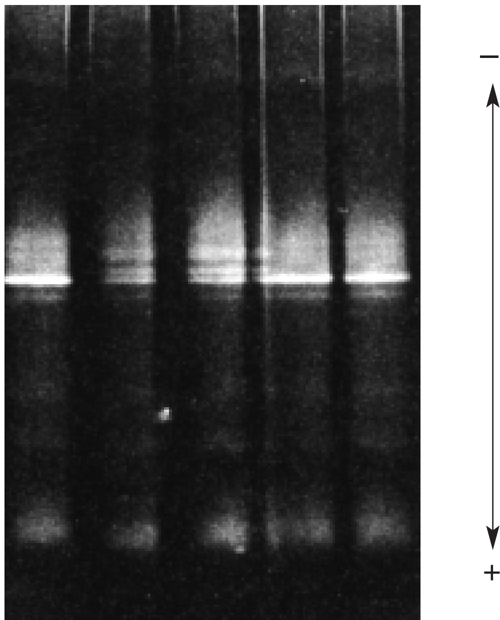

Figure 2. The PCR product of fragment B (as shown in fig. 1) presents on DGGE with an anomalous heterozygous pattern in the carriers of Hb-Osu Christianborg of family II, revealing the presence of the mutation. From left to right: Normal control, child carrier, mother carrier, father carrier of HbC with a normal fragment B, normal control.

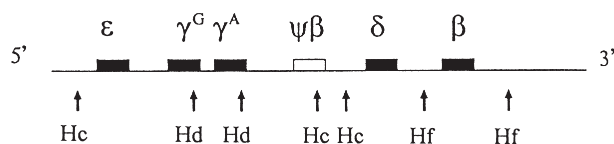

Figure 3. Scheme indicating the position of 7 polymorphic sites with their specific restriction enzymes (Hc: Hinc-II or Hind-II; Hd: Hind-III; Hf: Hinf-I)

in the West-African population but the percentage HbC was low suggesting the presence of α-thalassemia. The data of the biochemical/hematological analysis are summarized in Table 2.

Results

The hematological data of the two families summarized in table 1 and 2 confirm the non pathological character of the Hb-Osu Christianborg trait which is expressed at the expected rate without hemolysis, cellular inclusions, globin chain synthesis unbalance or hepatosplenomegaly.

Molecular analysis: 8 fragments, covering the complete β-gene (figure 1), were amplified and analyzed

Table 3a. Results of the β-Cluster haplotype analysis of family I

Fragm.	position	enzyme	I-1	II-1
1	5'-ε	*Hinc* II	+/–	+/–
2	Gγ	*Hind* III	–/+	–/–
3	Aγ	*Hind* III	–/+	–/–
4	ψβ	*Hinc* II	–/–	–/–
5	3'-ψβ	*Hinc* II	+/+	+/+
6	5'-β	*Hinf* I	+/+	+/+
7	3'-β	*Hinf* I	+/+	+/+
Haplotype			11/2	11/4

Table 3b. Results of the β-Cluster haplotype analysis of family II

			I-1	I-2	II-1
1	5'-ε	*Hinc* II	–/+	+/–	+/+
2	Gγ	*Hind* III	+/–	–/–	–/–
3	Aγ	*Hind* III	–/–	–/–	–/–
4	ψβ	*Hinc* II	–/–	–/–	–/–
5	3'-ψβ	*Hinc* II	+/–	+/+	–/+
6	5'-β	*Hinf* I	–/–	+/–	–/+
7	3'-β	*Hinf* I	+/–	+/–	–/+
Haplotype			5/1	11/4b	1/11

on DGGE. Fragment two, was the only one who revealed an anomalous DGGE pattern, indicating the presence of the mutation (figure 2). Direct sequencing of the same fragment revealed the GAT—>AAT single base substitution which induces the Asp—>Asn substitution at cd52, previously described as Hb-Osu Christiansborg. In family II the father was also carrier of the HbC mutant. The decreased level of expression was induced by the coexistence of an α-thalassemia defect which was confirmed by the slightly umbalanced synthetic β/α ratio and by standard southernblot analysis revealing an heterozygosity for the -$\alpha^{3.7}$ deletion (data not shown).

Haplotype analysis of the β-globin gene cluster, according to (14) (figure 3) revealed the association of Hb-Osu Christianborg with haplotype 11 in all carriers of both families (table 3a and b).

Discussion

750 structural hemoglobin defects have been described up to now involving the various globin chains (15). The majority of these mutants is rare, often virtually non- or moderately pathological in the heterozygous and even in the homozygous state. However, combination of rare mutants with β-thalassemia or HbS showing intermediate or severe pathologies have been reported (5,6) as have defects induced by unstable or crucially modified globin chains with a more or less dominant phenotype (16). Among the many hemoglobin mutants, only a limited number of prevalent mutations, selected by malaria, is frequently found, and often selectively in different populations. In the case of immigration countries with a very heterogeneous allochtonous populations and with individuals of mixed ethnic origin, the number of expected mutations may become very large indeed.

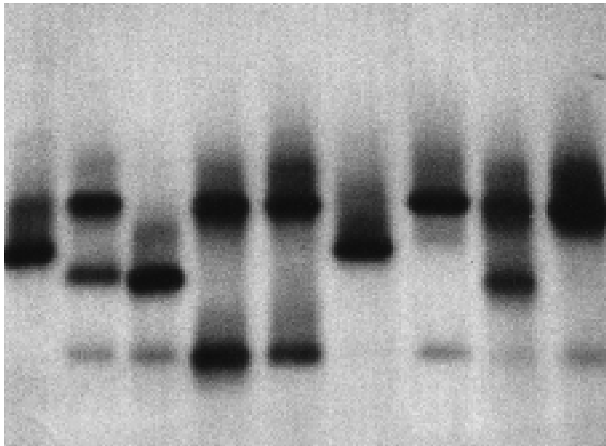

Figure 4a. Starch gel at pH 8.6 showing the electrophoretic patterns of several red cell lysates. From the left to the right: 1) the cordblood of an heterozygous α^+-thalassemia newborn; 2) an adult heterozygous HbS; 3) an adult with sicklecell disease (HbS/S or HbS/β-thal); 4) an adult heterozygous for the HbC mutant; 5) an adult heterozygous for the HbE mutant; 6) a 1 year old baby with β-thalassemia major; 7) an adult heterozygous β-thalassemia; 8) an adult heterozygous for the Hb-Osu Christianborg mutant; 9) an adult with a normal pattern.

In these cases a specific detection method is needed for the screening of a large scale of mutations (9).
Hb-Osu Christiansborg [β52(D3)Asp–>Asn], is classified as non pathological in the heterozygous form (15). Pathology associated with this mutant should not be expected neither in the heterozygous nor in the homozygous form because aspartic acid at position β52 is an external residue and is not involved in crucial contacts between the subunits, heme stability, DPG binding or Bohr effect. Two different mutants are described at the same codon, Hb-Ocho Rios (Asp–>Ala) and Hb-Summer Hill (Asp–>His), presenting both with normal heterozygous phenotypes.
Hb-Osu Christiansborg was originally observed in a Ghanese patient in combination with HbS (17) and later in an Iranian family (18). Although these two cases were not analysed at the haplotype level it could be assumed that the mutant originated independently in the African and in the Iranian population. Conversely Hb-Osu in our two families, one from Ghana and the other from the Dominican Republic but both of African ancestry, was associated with haplotype 11 which was shared by all carriers, suggesting a single mutation event. Haplotype 11 is not very common in West-Africans (2.9%) and we assume therefore that the mutation found in our two families probably originated from the same ancestral ethnical group, who generated the actual Ghanese population.
Figure 4a and b show the electrophoretic mobility at pH 8.6 of Hb-Osu Christianborg and the separation on HPLC at two different pH compared with a standard HbE sample. It is quite clear that Hb-Osu Christiansborg presents with the same electrophoretic mobility as HbS when separated on electrophoresis and with a chromatographic pattern comparable to the HbE mutant when separated by standard HPLC. Without further analysis Hb-Osu Christianborg could easily be mistaken for one of those two mutants.

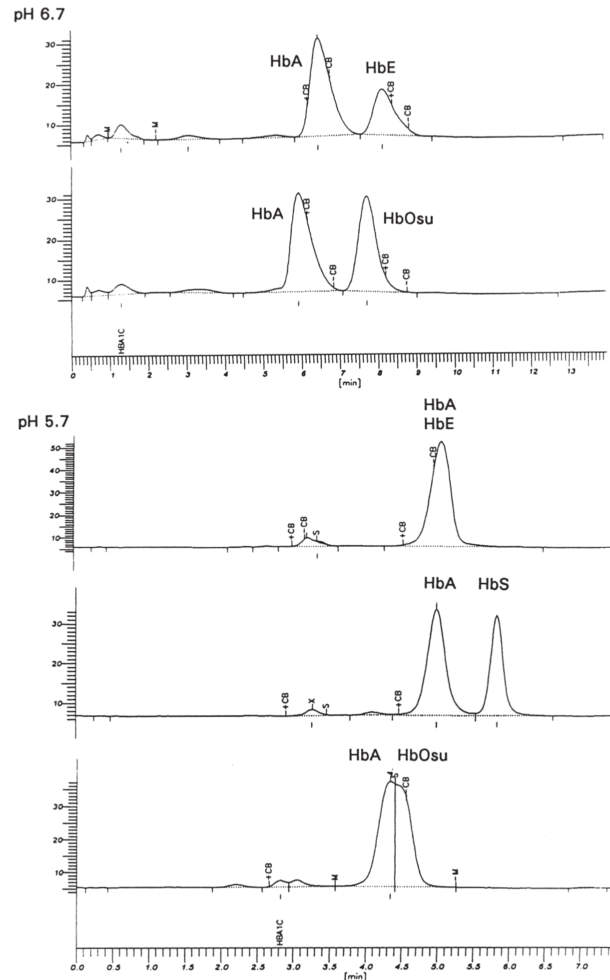

Figure 4b. From top to bottom, HPLC diagrams at pH 6.7 of HbA/E and HbA/Osu heterozygotes; HPLC diagrams at pH 5.7 of HbA/E, HbA/S and HbA/Osu heterozygotes.

Evidently, Hb-Osu Christianborg is considered a rare mutant. However, 2 cases in the relatively small African subpopulation in The Netherlands in a short time could mean, that this mutant is perhaps more frequently found than one could presume. In this case one should consider the possibility of a recurring pitfall in the diagnostic of HbS or HbE in The Netherlands.

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