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ORIGINAL ARTICLE

Hb J-CAPE TOWN [α92(FG4)Arg→Gln (α1), C*G*G→C*A*G] IN SOUTHERN ITALY FOUND IN A PATIENT WITH ERYTHROCYTOSIS

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□ *A high oxygen affinity hemoglobin (Hb) variant, Hb J-Cape Town [α92(FG4)Arg→Gln (α1), C*G*G→C*A*G] was identified in a 30-year-old woman patient from Cosenza (Southern Italy) who had previously been diagnosed with juvenile polycythemia in other hospitals. The occurrence of the variant Hb was assessed by both cation exchange chromatography and liquid chromatography-mass spectrometry (LC-MS) analyses. A detailed structural and functional characterization of the variant was performed at both the protein and DNA level. Structural investigation of the Hb variant by mass spectrometric methodologies and peptide sequencing identified the amino acid replacement as Arg→Gln at α92. The corresponding DNA mutation CGG→CAG was assigned to codon 92 of the α1 gene by DNA sequencing. These findings highlight the importance of investigating the hypothesis of a high affinity variant in the presence of a polycythemia so as to avoid unnecessary bone marrow examination or radioactive treatment. This report represents the first observation of the Hb J-Cape Town variant in Italy.*

Keywords Hb J-Cape Town, Erythrocytosis, Polycythemia vera, High oxygen hemoglobin (Hb), Mass spectrometry (MS)

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INTRODUCTION

Hb J-Cape Town is a rare hemoglobin (Hb) variant with a slightly high oxygen affinity characterized by the substitution Arg→Gln at α92(FG4). It was first described in a colored woman living in Cape Town, South Africa, found to carry a "fast moving" variant with the mobility of Hb J (1). The occurrence of Hb J-Cape Town in three other, apparently unrelated families, one from Johannesburg, one Caucasian family from Cape Town and a "colored" family from Kakamas in the northern Cape Province, was then reported (2–4). More recently, several patients carrying Hb J-Cape Town were also found in Japan during a systematic abnormal Hb survey (5–7). In all cases, mild erythrocytosis and reduced cooperativity were observed.

This paper reports the first observation of Hb J-Cape Town in Italy in a 30-year-old woman patient from Cosenza (Southern Italy), who had been previously diagnosed with juvenile polycythemia in other hospitals. The variant Hb was structurally characterized at both the protein and DNA level. Protein chemistry procedures in conjunction with mass spectrometric techniques confirmed the amino acid substitution Arg→Gln at position 92 of the α chain. The corresponding DNA mutation was determined as C*G*G→C*A*G at codon 92 of the α1 gene by DNA sequencing.

MATERIALS AND METHODS

Routine Examinations

Unless otherwise specified, blood was collected with EDTA as anticoagulant. Hematological data were obtained by standard procedures using a Cell-Dyn 3700 Hematology System (Abbott Laboratories, Abbott Park, IL, USA). Red cell lysates were analyzed by electrophoretic techniques at both alkaline pH (on cellulose acetate) and acidic pH (on agar citrate). The different Hb components were separated and measured by cation exchange high performance liquid chromatography (HPLC) using the VARIANT II™, β-Thalassemia Short Program (Bio-Rad Laboratories, Hercules, CA, USA). The isopropanol precipitation test was carried out as described by Huisman and Jonxis (8).

Structural Characterization of the Variant Hemoglobin

The hemolysate from the patient was directly analyzed by liquid chromatography-mass spectrometry (LC-MS) on a Quattro Micro LC-MS system (Waters, Milford, MA, USA) coupled to an HP 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) using the conditions described by Carbone *et al.* (9). Individual globin chains were purified by reversed phase

HPLC on an HP 1100 system (Agilent Technologies) essentially as described by Carbone *et al.* (9). The variant globin was digested with trypsin and the resulting peptide mixture was directly analyzed by matrix-assisted laser desorption ionization (MALDI) MS using a Voyager DE PRO Reflectron instrument (Applied BioSystems, Framingham, MA, USA) as reported before (10). The peptide mixture was fractionated by reversed phase HPLC on an HP 1100 system (Agilent Technologies) using a C_{18} narrow bore column (11). Automated Edman degradation was performed on individual purified peptide fractions using a PROCISE Mod 491 (Applied BioSystems).

DNA Analysis

DNA was obtained from peripheral blood leukocytes by the salting-out extraction procedure. The mutation of Hb J-Cape Town was determined by DNA sequencing. Amplifications of individual α1- and α2-globin genes were performed using specific primers for each gene (12). Sequence analysis of each amplified α-globin gene was accomplished by using two common internal primers: 5′-CTG AGC GAC CTG CAC GCG CAC-3′ (coordinates GeneBank NG_000006.1: 34145–34162 and 37949-37966) and ASeq4ASII: reverse 5′-AAG GCG CCA TCT CGC CCC TC-3′ (coordinates GeneBank NG_000006.1: 34242-34223 and 38046-38027). Sequence analysis of the amplified fragment was carried out on a gel purified sample (Qiagen Gel Purification Kit; Qiagen Inc., Valencia, CA, USA) on an Applied BioSystems 3100 automated sequencer using the PRISM™ Dye Terminator Cycle Sequencing Kit (Applied BioSystems, Norwalk, CT, USA).

RESULTS

Case Report

A 30-year-old woman from Cosenza (Southern Italy) was admitted to the Microcitemia Unit of the Cardarelli Hospital, Naples, Italy, in December 2003 because of a mild erythrocytosis, observed but not always present, since 1995. Her clinical history revealed that her mean Hb level was between 15 and 16 g/dL (with a maximum peak at 19 g/dL), while the red blood cells (RBCs) ranged between 5.0 and 5.5×10^{12}/L. The PCV value was at the upper limit of normal, sometimes close to 0.50 (L/L), and observed once to be as high as 0.56 (L/L). In contrast, the white blood cells and the platelets were always in the normal range. The patient had suffered from a thyroid cancer in 2000 for which she underwent surgery and radiometabolic therapy with ^{131}I. She also reported heavy menstrual cycles and oral iron therapy was necessary in 1997 and 1999. Before coming to our

Division, the patient consulted several hematological laboratories and underwent a bone marrow biopsy with negative cytogenetic results. In spite of this, in July 2003 she was diagnosed with polycythemia vera. The patient (MT) was then examined at our Division together with her husband (ADG).

Hematological data were collected from both subjects by routine laboratory procedures and are summarized in Table 1. A familial investigation could not be performed as the patient was adopted and her natural parents were unknown. The proband showed a mild erythrocytosis (RBC 5.4×10^{12}/L), increased Hb concentration (16.0 g/dL) with a normal Hb A_2 level (2.1%) and reticulocyte count (1.0%). The husband showed a clear heterozygous α-thalassemic phenotype with a $-\alpha^{3.7}/\alpha\alpha$ mutation.

Hemoglobin Analysis

Agar citrate (pH 6.0) electrophoresis of the blood samples from the two subjects showed only normal Hb components. Cellulose acetate electrophoresis of the Hb sample from MT displayed a "fast moving" band characterized by the electrophoretic mobility of a Hb J, whereas the same analysis carried out on the blood sample from ADG was normal. Accordingly, when the hemolysate sample from MT was analyzed by cation exchange HPLC on the VARIANT II™ instrument (Bio-Rad Laboratories), the occurrence of an abnormal Hb component between Hb F and Hb A was clearly detected. The abnormal Hb accounted for about 23% of the total Hb, clearly indicating the involvement of the α chain. The isopropanol stability test on the patient's red cells was negative.

TABLE 1 Hematological Parameters of the Subjects

Parameters	MT	Normal Values (females)	ADG	Normal Values (males)
Sex-Age	F-30		M-31	
RBC (10^{12}/L)	5.4	4.0–5.2	5.8	4.5–5.9
Hb (g/dL)	16.3	12.0–16.0	14.3	13.5–17.5
PCV (L/L)	0.48	0.36–0.46	0.43	0.41–0.53
MCV (fL)	89.0	80.0–100.0	75.0	80.0–100.0
MCH (pg)	29.3	26.0–34.0	24.7	26.0–34.0
Reticulocytes (%)	1.0		0.8	
Hb A_2 (%)	2.1	2.0–3.4	2.4	2.0–3.4
Hb F (%)	0.9	<1.0	0.9	<1.0
Hb X (%)	22.0		–	
α Genotype	αα/αα		$-\alpha^{3.7}/\alpha\alpha$	
Isopropanol test	Negative		Negative	
Serum iron (μg/dL)	27.0	40.0–145.0	65.0	60.0–175.0
Ferritin (ng/mL)	8.0	10.0–200.0	97.0	30.0–300.0

Abnormal Hemoglobin Analysis

Red cells from the proband (MT) were hemolyzed and the individual globins directly analyzed on an LC-MS (Waters) system. The total ion current (TIC) profile of the LC-MS analysis, *i.e.*, the current associated with the ionization of each individual protein component in the electrospray source, revealed the presence of an abnormal peak eluting after the normal α-globin with a molecular mass of about 28 Da lower than the normal α chain (experimental mass 15098.9 ± 0.6 Da, theoretical mass 15126.6 Da). On the basis of DNA single point mutations, this mass difference could only be accounted for by three amino acid substitutions, *i.e.*, Arg→Lys, Arg→Gln or Val→Ala (13).

The variant chain was purified by preparative HPLC, digested with trypsin and an aliquot of the resulting peptide mixture was directly analyzed by MALDI/MS, as previously described (10). An anomalous peak, absent in the normal α chain tryptic digest, was detected at m/z 1059.8 and tentatively assigned to the abnormal peptide 91–99 with a molecular mass decreased by 28 Da (αT-8,9, expected mass value 1087.7 Da), thus confining the structural variation within this region.

Unfortunately, on the basis of these data, several putative amino acid substitutions could be inferred (Arg92 to either lysine or glutamine, Val93 or Val96 to alanine). The mixture of tryptic peptides was then fractionated by reversed phase HPLC, as shown in Figure 1, and the individual fractions collected and analyzed by MALDI/MS. The abnormal peptide could easily be identified by its unique mass value (see insert of Figure 1) and the corresponding HPLC fraction was submitted to automated Edman degradation using a PROCISE protein sequencer (Applied BioSystems). The amino acid sequence of the abnormal peptide was determined as Leu-*GLN*-Pro-Val-Asn-Phe-Lys, corresponding to the 91–99 fragment containing a glutamine residue at position 92 instead of the normally occurring arginine. This amino acid replacement identified the variant as Hb J-Cape Town (1).

DNA Analysis

DNA of the proband and her husband was obtained from peripheral blood leucocytes by the salting-out method. The ADG sample was investigated for α-thalassemia (thal) mutations, while the DNA mutation corresponding to Hb J-Cape Town was determined in the sample of MT by direct sequencing. A heterozygous $-\alpha^{3.7}/\alpha\alpha$ thalassemic deletion was detected by the gap-polymerase chain reaction (gap-PCR) method following amplification of the DNA sample from ADG (12,14).

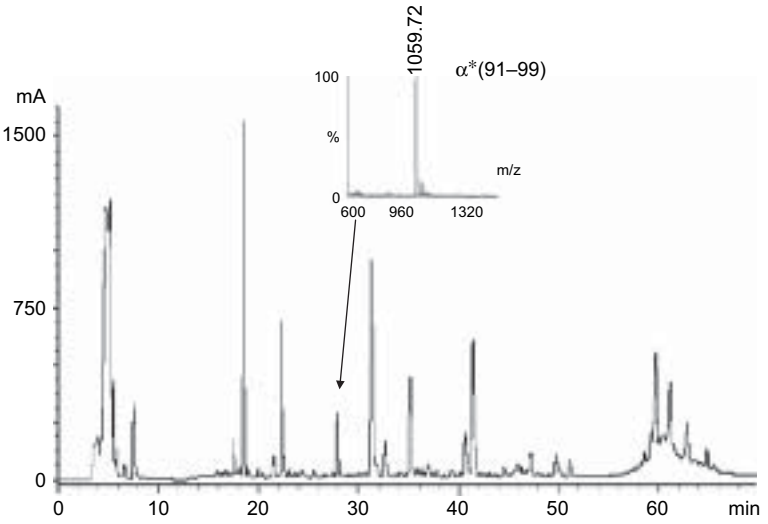

FIGURE 1 Reversed phase HPLC separation of tryptic peptides from the abnormal α chain. The abnormal peptide was identified by MALDI/MS analysis as depicted in the insert.

DNA sequencing was performed by PCR amplification of the α2 and α1 genes from the MT sample using two suitable internal primers, each specific for a single α gene. The two genes were sequenced by an Applied BioSystems 3100 automated sequencer. Figure 2 shows the DNA sequence at codon 92 of the α1 gene displaying the single point mutation at the second position, from C*G*G→C*A*G.

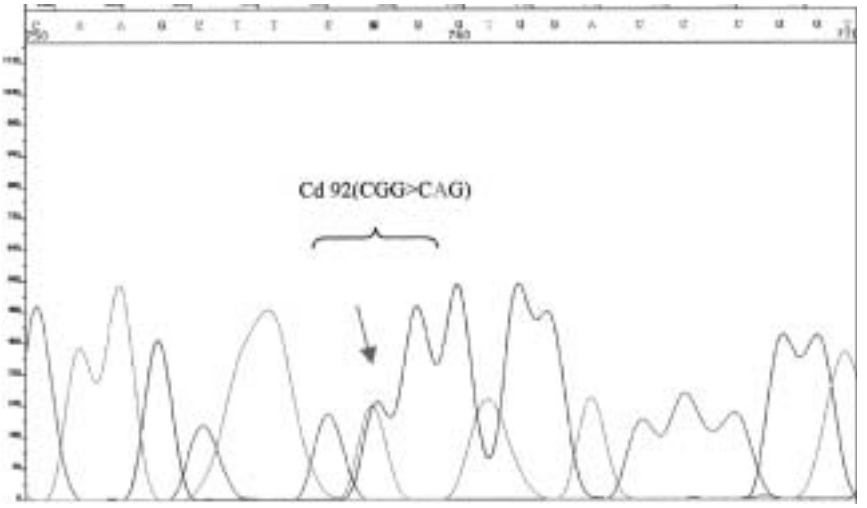

FIGURE 2 The DNA sequence at codon 92 of the α1 gene displaying the single point mutation at the second position of codon 92 (C*G*G→C*A*G).

DISCUSSION

This paper reports the first observation of the high oxygen affinity variant Hb J-Cape Town [α92(FG4)Arg→Gln (α1), C*G*G→C*A*G] in a 30-year-old woman from Cosenza (Southern Italy), who was diagnosed with juvenile polycythemia vera, despite the negative response of a bone marrow biopsy. However, when the hemolysate from the patient's red blood cells was examined by ion exchange HPLC, the occurrence of an abnormal Hb containing an abnormal α chain was easily detected. The variant α chain was then characterized by LC-MS analysis and the abnormal globin was purified by reversed phase HPLC. The amino acid replacement was determined by mass spectrometric methodologies and peptide sequencing as Arg→Gln at position 92 of the α chain, corresponding to Hb J-Cape Town. The corresponding DNA mutation was determined as G→A at the second position of codon 92 of the α1 gene by DNA sequencing.

Hb J-Cape Town was first described in a colored woman living in Cape Town, South Africa, as a "fast moving" variant with the mobility of Hb J (1). This variant was subsequently observed in three other apparently unrelated families (2–4) and, more recently, was found in several Japanese families (5–7). Hb J-Cape Town displayed only a moderate increase in oxygen affinity, and in heterozygotes, it was shown to be responsible for a slight degree of polycythemia (1,2,15). Arg92 in the α chain is located at the α1/β2 interface and its substitution with different residues impairs the normal rotational transition from the deoxygenated low-affinity state to the oxygenated high-affinity state, tending to lock the Hb into the high-affinity relaxed state (16). Interestingly, only three further mutations at position 92 of the α genes have been reported so far, *i.e.*, Hb Cemenelum (Arg→Trp) (17), Hb Chesapeake (Arg→Leu) (18) and Hb Monou (Arg→Pro) found in a Japanese male (HbVar database: http://globin.psu.edu/hbvar/menu.html). However, the α92 variants display rather different functional properties with both Hb J-Cape Town and Hb Cemenelum showing only a slight increase in oxygen affinity with normal hematological data. On the other hand, Hb Chesapeake is a very high oxygen affinity variant causing mild polycythemia (19). This demonstrates that, even for some key residues of the α1/β2 interface, the degree at which the functional properties are altered depends upon the specific residue occupying this position (17).

Results presented in this paper underline the importance of investigating the hypothesis of a high affinity variant in the presence of a diagnosis of polycythemia vera so as to avoid unnecessary bone marrow investigation or radioactive treatment. In fact, according to the guidelines of the American Society of Hematology, a number of investigations were proposed for those

with an absolute erythrocytosis to confirm the presence of a primary (PV) or secondary erythrocytosis that might be related to the occurrence of a high oxygen affinity Hb (20).

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