

Molecular characteristics of three hemoglobin variants observed in a Chinese population: Hb Ube-1 [β 98 (FG5) Val \rightarrow Met], Hb Ube-2 [α 68 (E17) Asn \rightarrow Asp] and Hb Ube-4 [α 116 (GH4) Glu \rightarrow Ala]

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Abstract. In this study we report three cases of Hb Ube-1, one case of Hb Ube-2 and one case of Hb Ube-4 in mainland China. One case of Hb Ube-1 had the clinical manifestation of hemolytic anemia. Hb Ube-2 and Hb Ube-4 did not appear to be associated with clinical or hematological abnormalities. The variants were detected by Hb electrophoresis during a thalassemia screening. Genomic DNA was extracted from the peripheral blood leukocytes of Hb specimens. α 1, α 2 and β -globin genes were amplified by polymerase chain reaction (PCR). All variants were confirmed by DNA sequence analysis and the PCR-restriction fragment length polymorphism assay.

Introduction

A recent query of the Human Hemoglobin Variant database (HbVar) (<http://globin.cse.psu.edu>) identified 652 variants of the α -globin chain and 775 variants of the β -globin chain. The majority of these hemoglobin variants do not have any hematological or clinical effects and are often found incidentally, for example during newborn screening or health examination, and are finally identified by DNA sequencing.

Hb Ube-1 [β 98 (FG5) Val \rightarrow Met], also known as Hb Koln, was first observed in Cologne, Germany, by Pribilla in 1965. It is often found in Europe and America, and has occasionally been reported in Japan, South Korea and Taiwan (1-3). Hb Ube-1, in which the β 98 (FG5) valine residue is replaced by methionine (G \rightarrow A), is the most frequent unstable hemoglobin. However, it presents very few features that allow its distinc-

tion from other unstable hemoglobin variants. Mild anemia, jaundice and persistent excretion of dark urine characterize the clinical manifestations of Hb Ube-1. The relationship between the amino acid substitution (Val \rightarrow Met) and the instability of this hemoglobin suggests that the valine residue is in contact with the heme in the normal β -chain. The substitution of valine for methionine changes the steric configuration of the β -globin, which either alters the enzymatic reduction of iron or results in an increased conversion of meta-hemoglobin into denatured hemoglobin. When Hb Ube-1 is in the oxygenated conformation, the molecule is saturated with heme groups and is stable. The transition to the deoxygenated state results in the loss of heme groups, the instability of Hb and the precipitation and formation of Heinz bodies, which are cytoplasmic degradation products attached to the cell membrane (4). Heinz bodies induce hemolysis and splenomegaly (5).

Hb Ube-2 [α 68 (E17) Asn \rightarrow Asp] was originally observed in a Japanese individual in 1967 (6). To our knowledge, it has only ever been observed in the Japanese and Taiwanese population (7). This Hb variant resulted from an AAC \rightarrow GAC mutation at codon 68 of the α -globin gene. Hb Ube-2 carriers do not have clinical symptoms or hematological changes, and their oxygen affinity is normal.

Hb Ube-4 [α 116 (GH4) Glu \rightarrow Ala (α 1)] is an abnormal hemoglobin, that was first observed in a Korean family residing in Japan (8). To the best of our knowledge, all Hb Ube-4 carriers have been found in Korea or Japan (9). This hemoglobin variant results from a GAG \rightarrow GCG mutation at codon 116 of the α 1-globin gene (10). Hb Ube-4 carriers also have no clinical symptoms or hematological changes.

According to previous reports, Hb Ube-1, Hb Ube-2 and Hb Ube-4 have not been reported in mainland China. In this study, we report the molecular and clinical features of these three rare variants in mainland China.

Materials and methods

Hematological analysis. Specimens were analyzed by cellulose acetate electrophoresis (pH 8.6) or agar gel electrophoresis (pH 8.6), and the percentage of Hb variants was measured by densitometry on alkaline electrophoresis or automated high-performance liquid chromatography (HPLC) (11,12). Tests for

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Table I. Primers and length of globin amplification.

Primers	5'-3' sequences	Length of PCR products (bp)
α 1-B	CCATGCCTGGCACGCTTTGCTGAG	880
α 1-L	TCCCCACAGACTCAGAGAGAACC	
α 2-D	AACACCTCCATTGTTGGCACATTCC	880
α 2-L	TCCCCACAGACTCAGAGAGAACC	
β -3F	GTGTACACATATTGACCAAA	442
β -4R	AGCACACAGACCAGCACGTT	

Table II. Summary of the hematological findings of the three Hb Koln carriers.

Parameter	Proband	Mother	Father	Brother
Gender-age (years)	M-21	F-43	M-45	M-1.5
RBC ($10^{12}/l$)	4.61	3.32	4.84	3.93
Hb (g/l)	132.0	96.0	143.0	90.0
MCV (fl)	95.0	97.4	85.2	76.2
MCH (pg)	28.6	28.9	29.5	22.9
RDW-CV (%)	15.8	17.2	12.4	20.4
Ret (%)	4.9	4.1	1.1	5.3
Hb A (%)	89.9	89.8	96.6	87.7
Hb F (%)	1.0	0.9	0.5	3.7
Hb A2 (%)	2.7	2.8	2.9	2.5
Hb Koln (%)	6.4	6.5	-	6.1
Basophilic stippling of erythrocytes	+	+	-	+

RBC, red blood cell count; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red blood cell distribution width; Ret, reticulocytes.

Hb stability were carried out using the isopropanol and heat methods (13,14). Hb variants were dissociated by parachloro-mercuribenzoate (PCMB), which differentiates α - or β -globin mutation (15).

DNA analysis. Genomic DNA was extracted from the peripheral blood leukocytes of these specimens with the genomic DNA mini-preparation kit (Decipher Bioscience Shenzhen Ltd.) as previously described (16). α - and β -globin genes were amplified by polymerase chain reaction (PCR) in a MJ Mini thermal cycler (Bio-Rad Co.). The primer and product lengths were described previously (17-19) (Table I). The β -globin gene was sequenced using primers that flank the three exons of the β -globin gene, and DNA was amplified using Takara LA *Taq*. After initial denaturation at 97°C for 7 min, 35 cycles of PCR (94°C for 30 sec, 46°C for 30 sec and 72°C for 2 min) were carried out.

The amplification process of the α -globin gene was similar to that of the β -globin gene. The α 1-globin gene was amplified in 50- μ l PCR reaction mixtures containing 0.1 μ g DNA, 15 pmol of primers, 200 μ mol dNTPs and 2.5 units *Taq*DNA polymerase (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., China) in 10 mmol/l Tris-HCl (pH 8.0), 50 mmol/l KCl and 3 mmol/l MgCl₂. PCR

conditions were as follows: 95°C for 3 min to activate the DNA polymerase, followed by 35 cycles at 98°C for 40 sec, 60°C for 5 sec and 72°C for 1 min. All amplified products were separated on 2% agar gel and detected with UV light after staining with ethidium bromide. The PCR products were analyzed by DNA sequencing with the ABI 3700 automated sequencer.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The β -globin gene was amplified with the sense primer 5'-ACTCTTGGGTTTCTGATAGGCACT-3' and antisense primer 5'-AAAAGAAGGGGAAAGAAAACATC-3'. The length of the PCR products was 228 bp. Primer Premier 5.0 software showed that this PCR product of the β -globin gene created two *Hin*II DNA restriction sites in Hb Ube-1, and only one restriction site in the normal control. Hb Ube-1 could be digested into 59-, 105- and 164-bp bands, while the normal control could only be cut into two 164-bp bands.

The α 1-globin gene was amplified using the primers shown in Table I. The PCR products of the α 1-globin gene created three *Hin*II DNA restriction sites in Hb Ube-2 and two restriction sites in the normal control. Therefore, Hb Ube-2 could be cut by *Hin*II into 51-, 350-, 401- and 488-bp bands, while the normal control could only be cut into 401- and 488-bp bands.

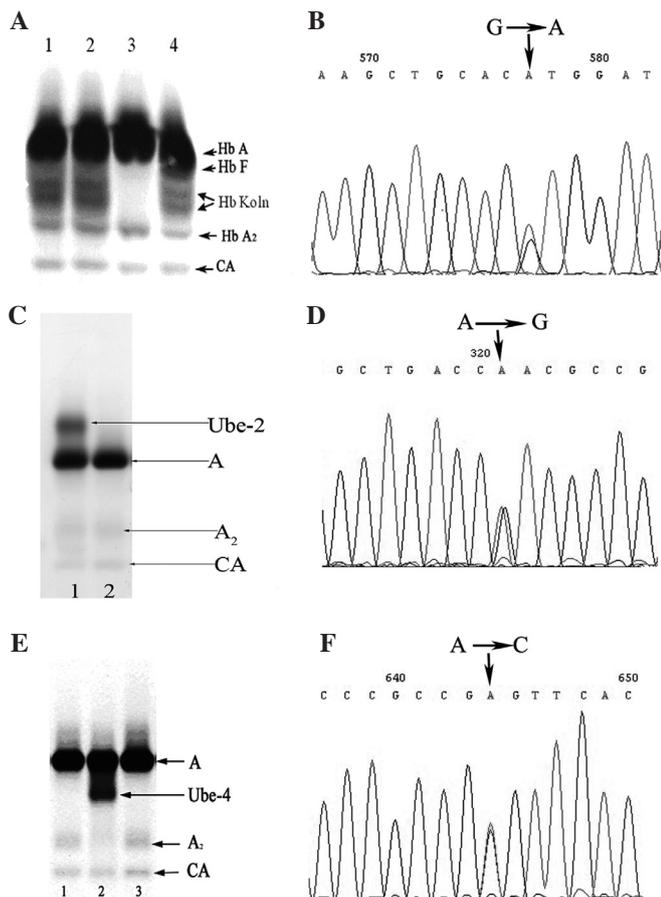


Figure 1. Hemoglobin electrophoresis and DNA sequencing analysis. (A) Hemoglobin analysis of Hb Koln carriers with cellulose acetate electrophoresis at pH 8.6. Lane 1, proband; 2, mother; 3, father; 4, brother. (B) β -globin gene sequences of Hb Koln carrier. DNA sequencing revealed a G→A substitution at codon 98 of the β -globin gene. (C) Hemoglobin analysis of the Hb Ube-2 carrier by agarose gel electrophoresis. Lane 1, Hb Ube-2; 2, normal control. (D) Sequence analysis of the amplified α -globin gene. The downward arrow indicates the A→G substitution at codon 68 of the α -globin gene (Hb Ube-2). (E) Hemoglobin analysis of the Hb Ube-4 carrier with agarose gel electrophoresis. Lane 1, Hb Ube-4; 2, normal control. (F) Sequence analysis of the amplified α -globin-1 gene. The downward arrow indicates the A→C substitution at codon 116 of the α -globin-1 gene (Hb Ube-4).

Exon 3 of the $\alpha 1$ -globin was amplified with the sense primer 5'-CGGCCCTGACCCTCT-3' and antisense primer 5'-GTACGGGTGCAGGAAGGG-3'. The length of the PCR product was 232 bp. The PCR product was digested by *Bsh1236I*. Hb Ube-4 could be cut into 72- and 160-bp bands, while the normal control could not be digested, and maintained the 232-bp band.

The digestive products were separated with DNA markers on 2% agar gels containing ethidium bromide. The bands were visualized under UV light.

Results

Hb Ube-1 [p98 (FG5) Val→Met]. The proband was a 21-year-old Chinese male from Hunan, China. He was a medical outpatient seen for an abnormal hemoglobin on electrophoresis at Chaozhou Central Hospital, Guangdong, China. Physical examination revealed that the sclera was mildly jaundiced, the spleen was touchable below the left rib edge, and the liver

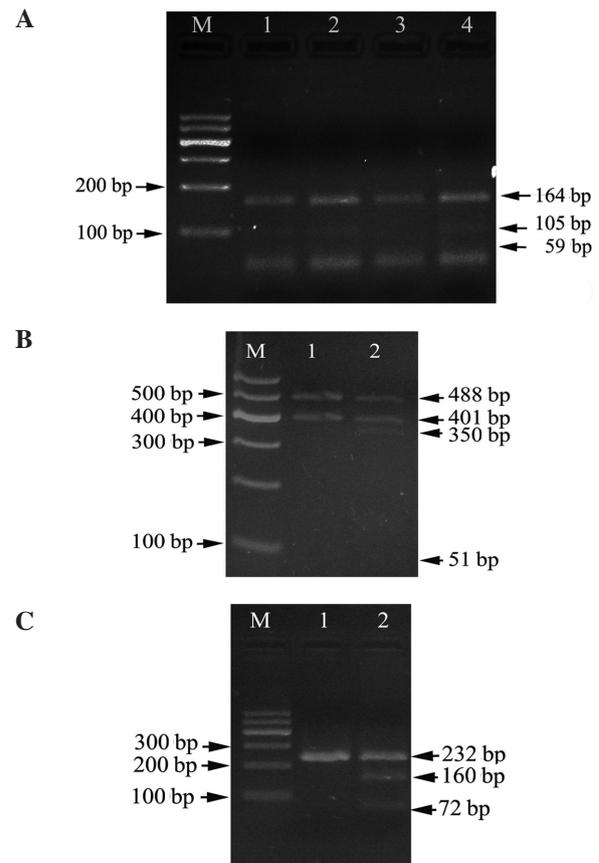


Figure 2. Results of PCR products digested by restriction enzymes. (A) Results of PCR product digested by *HinIII* to identify Hb Ube-1. Lane 1, proband; 2, mother; 3, father; 4, brother; M, marker. Lanes 1, 2 and 4 represent heterozygotes of Hb Ube-1, and lane 3 a normal case. (B) Results of PCR product digested by *HinII* to identify Hb Ube-2. Lane 1, normal control; 2, proband; M, marker. The proband was a heterozygote of Hb Ube-2. (C) The results of PCR product digested by *Bsh1236I* to identify Hb Ube-4. Lane 1, normal control; 2, proband; M, marker. The proband was a heterozygote of Hb Ube-4, the normal control was undigested amplified DNA.

was not touchable. Liver function tests revealed total bilirubin levels of 39.3 $\mu\text{mol/l}$ (2-20 $\mu\text{mol/l}$) and indirect bilirubin of 35.6 $\mu\text{mol/l}$ (2-14 $\mu\text{mol/l}$). The hematological data of the family is summarized in Table II. This variant migrated as a multiple Hb component between Hb A2 and Hb A on cellulose acetate electrophoresis at pH 8.6 (Fig. 1A). Its percentage was 6-7% of the total Hb, as measured by HPLC. Thermic instability and isopropanol precipitation tests were positive, revealing that this abnormal hemoglobin was unstable. Heinz bodies were observed in the peripheral blood (data not shown).

Sequencing of the three exons of the β -globin gene indicated a transition from G→A in the first position of codon 98 (Fig. 1B). The results of the PCR-RFLP are shown in Fig. 2A. These results revealed that the proband, his mother and his brother were Hb Ube-1 carriers.

Hb Ube-2 [a68 (E17) Asn→Asp]. This abnormal hemoglobin was found in a 31-year-old female who was a medical outpatient for premarital thalassemia screening in Chaozhou Central Hospital. A complete blood count at the time of Hb analysis revealed the following: RBC $3.16 \times 10^{12}/\text{l}$, Hb 113 g/l, MCV 99 fl, RDW 12.9%; WBCs and platelets were normal.

The Hb variant patterns were revealed by agar gel electrophoresis at pH 8.6 (Fig. 1C). This variant moved towards the node faster than Hb A, but slower than Hb H. The Hb variant, Hb A and Hb A2 were measured at 26.16, 71.3 and 2.54%, respectively. The Hb stability tests were normal.

This abnormal hemoglobin was dissociated by PCMB, and it was found that the hemoglobin variant was due to an α -globin mutation. The PCR products of the α -globin gene were sequenced and an AAC→GAC mutation at codon 68 of α 1-globin was observed (Fig. 1D). PCR-RFLP identified Hb Ube-2 (Fig. 2B). These results were all suggestive of Hb Ube-2.

Hb Ube-4 [α116 (GH4) Glu→Ala (α1)]. The proband was a 30-year-old asymptomatic Chinese male medical outpatient from the Chaozhou area who was admitted for premarital thalassemia screening. The abnormal hemoglobin was found by Hb electrophoresis and the blood sample was sent to our laboratory for further identification. A complete blood count at the time of Hb analysis showed RBC $5.16 \times 10^{12}/l$, Hb 155 g/l, MCV 88 fl, MCH 30.2 pg, RDW 12.9%; WBCs and platelets were normal, as was his red blood cell morphology. The Hb variant patterns were revealed by agar gel electrophoresis at pH 8.6 (Fig. 1E). This variant moved slightly faster than Hb S at an alkaline pH. The sample was analyzed by automated HPLC, and the percentages of the Hb variant, Hb A2 and Hb A were 20.61, 1.66 and 77.73%, respectively.

The abnormal hemoglobin was dissociated by PCMB and found to be an α -globin mutation. The whole α 1-globin gene was amplified by PCR, and sequence analysis showed a GAG→GCG substitution at codon 116, which resulted in a transition from glutamine (Glu) to alanine (Ala) (Fig. 1F). The results of PCR-PFLP are shown in Fig. 2C.

Discussion

The majority of hemoglobin variants do not have any hematological or clinical effects; they are often found incidentally during procedures such as thalassemia screening. To our knowledge, Hb Koln is the most common unstable hemoglobin variant, and causes hemolytic anemia. Mild anemia, jaundice, persistent excretion of dark urine and splenomegaly characterize the clinical manifestations of Hb Ube-1. The significant reduction of certain globin chains produces the effects of thalassemia, such as hemolytic anemia and microcytes (4). However, in our study, the proband had no anemia or microcytes, since Hb Koln increased the oxygen affinity of hemoglobin, stimulating the secretion of erythropoietin and resulting in megaloblastic erythropoiesis. The brother of the proband had microcytes as well as anemia, possibly due to iron deficiency ($5.3 \mu\text{mol}/l$). In accordance with previous reports, the content of Hb Koln in these three Hb Koln carriers was 6–7%, less than that of Hb variants which resulted from the common β -chain mutation (20).

Most Hb variants present very few features that allow distinction from other Hb variants. Differential diagnosis of these relevant abnormal hemoglobins mainly depends on DNA sequence analysis of amplified α - and β -globin genes. To date, there have been no reports on the use of PCR-RFLP for the identification of Hb Ube-1, Hb Ube-2 and Hb Ube-4. In our study, using Primer Premier 5.0 software, it was found that the

G→A substitution at Hb Ube-1, the A→G mutation at Hb Ube-2 and the A→C mutation at Hb Ube-4 created a *HinIII*, *HinII* and *BshI236I* DNA restriction site, respectively. A dependable method was devised by selective amplification of a DNA fragment of the α - and β -globin genes with specific primers, followed by digestion of the amplified product by restriction enzymes that recognize artificially created or naturally occurring restriction sites.

Diagnosis of these relevant abnormal hemoglobins is problematic in routine screening, and the development of new methods is expected to aid in providing an accurate diagnosis. Nowadays, the confirmation of Hb variants is accomplished by DNA sequence analysis and PCR-RFLP, which provide a rapid, simple and accurate procedure for identifying all Hb variants. Multiplex allele specific PCR, high-performance liquid chromatography or high-resolution melting may also be applied to the diagnosis of abnormal hemoglobins.

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References

- Carrell RW, Lehmann H and Hutchison HE: Haemoglobin Koln (beta-98 valine-methionine): an unstable protein causing inclusion-body anaemia. *Nature* 210: 915-916, 1966.
- Ricco G, Ravazzolo R, Capaldi A, Trento M, Lecchi M, Sartori ML, Furlani C and Rietto GB: Koln haemoglobinopathy in Italy. *Panminerva Med* 23: 227-233, 1981.
- Chang YH, Hur M, Lee DS, Park SS, Kim BK, Park S, Ohba Y, Hattori Y and Cho HI: The first case of Hb Koln [beta98 (FG5) Val→Met] in Korea. *Hemoglobin* 23: 287-289, 1999.
- Miranda RP, Fonseca F and Figueiredo S: Hb Koln [α 2 β 298 (FG5) Val→Met] identified by DNA analysis in a Brazilian family. *Br J Genet* 20: 126-128, 1997.
- Furia FG and Miller DR: Oxygen affinity in hemoglobin Koln disease. *Blood* 39: 398-406, 1972.
- Iuchi I, Shimasaki S, Hidaka K, Ueda S, Shibata S, Mizushima J and Harada H: Hb Ube-2 [alpha68 (E17) Asn replaced by Asp]: the second instance in Japan. *Hemoglobin* 5: 599-603, 1981.
- Chen CM, Liu SC, Huang CH, Lee TP, Chan WL and Chang JG: Hb Ube-2 in a Taiwanese subject: an A→G substitution at codon 68 of alpha2-globin gene. *Hemoglobin* 26: 99-101, 2002.
- Ohba Y, Miyaji T, Matsuoka M, Morito M and Iuchi I: Characterization of Hb Ube-4: alpha116 (GH4) Glu yield Ala. *Hemoglobin* 2: 181-186, 1978.
- Iuchi I, Hidaka K, Ueda S, Shibata S, Hitomi F and Takesue A: Hb Ube-4 [alpha116 Glu leads to Ala]: a second independent instance found in a Korean family of Japan. *Hemoglobin* 2: 561-563, 1978.
- Lee ST, Weykamp CW, Lee YW, Kim JW and Ki CS: Effects of 7 hemoglobin variants on the measurement of glycohemoglobin by 14 analytical methods. *Clin Chem* 53: 2202-2205, 2007.
- Schneider RG: Differentiation of electrophoretically similar hemoglobins – such as S, D, G and P; or A2, C, E and O – by electrophoresis of the globin chains. *Clin Chem* 20: 1111-1115, 1974.
- Gu YC, Wilson JB and Webber BB: Hb Ottawa [alpha15 (A13) GLY→ARG] found in Hubei Province, People's Republic of China. *J Tonqi Med Univ* 6: 185-187, 1986.
- Carrell RW and Kay R: A simple method for the detection of unstable haemoglobins. *Br J Haematol* 23: 615-619, 1972.
- Ockelford PA, Liang AY, Wells RM, Vissers M, Brennan SO, Williamson D and Carrell RW: Hemoglobin Volga, beta 27 (B9) Ala replaced by Asp: functional and clinical correlations of an unstable hemoglobin. *Hemoglobin* 4: 295-306, 1980.
- Ohba Y, Hattori Y, Yoshinaka H, Matsuoka M, Miyaji T, Nakatsuji N and Hirano M: Urea polyacrylamide gel electrophoresis of PCMB precipitate as a sensitive test for the detection of the unstable hemoglobin subunit. *Clin Chim Acta* 119: 179-188, 1982.

16. Lin M, Wu JR, Yang LY, Zou HY, Wang Q and Zheng L: Hb Q-H disease: two cases in a Cantonese family. *Blood Cells Mol Dis* 41: 259-260, 2008.
17. Robert JC, Ronald B and Kenneth AP: Multiplexed genotyping of β -globin variants from PCR-amplified newborn blood spot DNA by hybridization with allele-specific oligodeoxynucleotides coupled to an array of fluorescent microspheres. *Clin Chem* 46: 996-998, 2000.
18. Huang Y, Lin M and Yang LY: Hb CS-H disease combined with β -thalassemia. *Blood Cells Mol Dis* 44: 215-216, 2010.
19. Lin M, Wu JR, Yang LY, Chen H, Wang PP, Wang Q and Zheng L: Hb G-Waimanalo: occurrence in combination with alpha-thalassemia-1 Southeast Asian deletion. *Blood Cells Mol Dis* 42: 36-37, 2009.
20. Ideguchi H, Ishikawa K, Katsuno M, *et al*: A case of unstable hemoglobin (Hb Koln). *Rinsho Ketsueki* 27: 1645-1649, 1986.