Identification of a Rare Hemoglobin Variant, Hb Nagasaki [β17(A14)Lys→Glu], Found in a Japanese by the Techniques of Protein Chemistry and the DNA Analysis

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ABSTRACT. This paper describes a rare hemoglobin variant discovered in an apparently healthy Japanese male during an assay of his Hb A_{IC} level using a cation exchange HPLC, HLC-723GHb. The abnormal β globin was isolated by CM-52 cellulose column chromatography, aminoethylated and digested with TPCK-trypsin. Amino acid analysis of the abnormal peptide observed on a reverse phase HPLC showed a replacement of Glu for Lys at β17, identifying it as Hb Nagasaki [β17 (A14)Lys→Glu]. This conclusion was further confirmed by DNA sequencing of the amplified-cloned β globin gene, namely the nucleotide sequence of the β17 codon was changed from AAG (Lys) to GAG (Glu). Globin biosynthesis (non-α/α=0.98) was normal.

Key words: abnormal hemoglobin — Hb Nagasaki [β17(A14)Lys→Glu] — protein chemistry — DNA analysis — biosynthesis

Recently many abnormal hemoglobins (Hbs) have been observed in an unusual chromatographic elution pattern during an assay of the Hb A_{IC} level using the cation exchange high performance liquid chromatography (HPLC) system, HLC-723GHb.[1-3] We encountered a Japanese male, who visited a hospital for his regular physical check-up, with an unusual chromatographic elution pattern of HLC-723GHb (Fig 1). Isoelectric focusing (IEF) and HPLC with a DEAE column of his hemolysate indicated the presence of an abnormal Hb. This paper describes the results of the structural analysis of this Hb using protein chemistry and DNA analysis techniques.

MATERIALS AND METHODS

The periperal blood used here was collected with heparin or EDTA-2Na as an anticoagulant. The Hb A_{IC} level was determined by use of HLC-723GHb system (Tosoh Ltd Co, Tokyo, Japan).[4] Hematological and biochemical examinations were carried out using ordinary methods. The preparation of his hemolysate was done by a usual method.[5] IEF of the hemolysate was carried out on a polyacrylamide gel plate containing carrier ampholytes [Ampholine pH 3.5-10.0 and pH 7-9; LKB AB, Uppsala, Sweden, Pharmalyte pH 6.7-7.7; Pharmacia Biochemicals, Uppsala, Sweden].[6] Analysis of Hbs was done by HPLC with a DEAE column (DEAE-5PW, 7.5×75 mm, Tosoh Ltd Co,

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Tokyo, Japan) in which a linear gradient buffer containing a mixture of 20 mM Tris-HCl (pH 8.0) with 0.5 M NaCl-20 mM Tris-HCl (pH 8.0) was used as an eluent. Quantitative analysis of Hbs (Hb A_2, HbA, Hb F and abnormal Hb) was done by the relative ratio of the square of the Hb peak revealed on DEAE-HPLC. Hb instability was determined by the isopropanol precipitation test.[7] The globin obtained by treatment of the hemolysate with HCl-acetone was chromatographed on a CM-cellulose column (CM-52, Whatman International Ltd Co, Kent, UK) to separate it into normal α and β chains, and the abnormal chain.[8] The aminoethylated (AE) abnormal β chain was digested with TPCK-trypsin (Trypsin Type XIII, Sigma Chemical Co, St. Louis, MO, USA).[9] The resulting peptides were analyzed on a reverse phase HPLC using a TSKgel ODS80Ts (4.6×250 mm, Tosoh Ltd Co, Tokyo, Japan) to detect the abnormal peptide.[1] The amino acid composition of the peptide was determined with an automatic amino acid analyzer (Irica 5500, Irica Science Co, Tokyo, Japan), after it was hydrolyzed with 6N-HCl. Amino acid sequence was done by a Protein Sequencer (Model 120A/477A, Japan-Applied Biochemical Inc, Tokyo, Japan). DNA was obtained from white cells of his peripheral blood by the method of Poncz et al.[10] Amplification of DNA by the polymerase chain reaction (PCR) was done using a thermostable enzyme, Taq polymerase (Perkin-Elmer-Cetus Inc, Norwalk, CT, USA), as described in a previous paper.[11] The amplified DNA was double-digested with Pst I and Sph I, and ligated into the vector M13mp18 which had been digested with the same enzymes. The recombinant DNA was transfected into the bacteria JM109 by an E. Coli Pulser (Nippon Bio-Rad Laboratories, Tokyo, Japan). From the bacterial preparation, mixed single strand DNA (mix-ssDNA) and isolated cloned ssDNA (iso-ssDNA) were prepared as previously described.[11] Nucleotide sequencing was done by the dideoxy method[12] using a DNA sequencing kit [Sequenase Version 2.0, United State Biochemicals (USB), Cleavland, OH, USA]. Synthesized oligonucleotides were used as the sequencing primers.

Globin biosynthesis was performed by incubation of reticulocytes in an amino acid culture medium containing ^{3}H-leucine at 37℃ for 2 hours.[13] Globin was separated into α and β chains by CM-cellulose column chromatography[8] and the β to α chain ratio was calculated from the radioisotope counts incorporated into the chains.

RESULTS AND DISCUSSION

The hematological and biochemical findings of his peripheral blood showed no abnormalities [RBC $430\times10^4/\mu$l, WBC $3.7\times10^3/\mu$l, Hb 14.0 g/dl, Ht 42.9%, MCV 99.8 fl, MCH 32.6 pg, MCHC 32.6%, serum glucose 72 mg/dl, total bilirubin 0.7 mg/dl, serum Fe 88 μg/dl, total iron binding capacity (TIBC) 349 μg/dl] . The chromatographic elution pattern of HLC-723GHb of his blood suggested the presence of abnormal Hb from a broad Hb F peak, lowered Hb A_{IC} level, and broad Hb A peak, as shown in Fig 1. The IEF of his hemolysate showed the presence of a Hb variant that was migrated faster than Hb A. The mobility of the Hb was quite a bit larger than that of Hb Takamatsu with an amino acid substitution of Lys→Gln at β 120 (Fig 2).[14] The DEAE-HPL chromatogram of his hemolysate showed the elution pattern

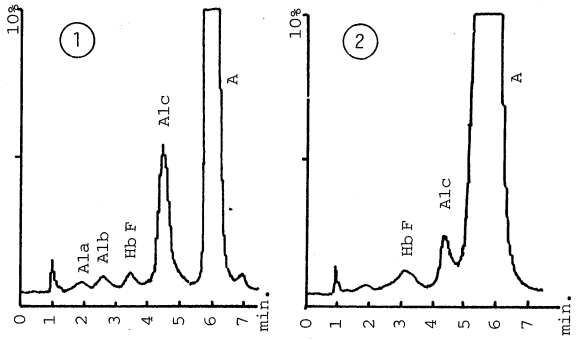

Fig 1. Comparison of the HLC-723GHb elution pattern of normal control with that of the patient. Chromatogram of the patient showed the broad peak of Hb F, the lower Hb A_{1C} level, and the broad Hb A peak. 1. Normal control 2. Proband

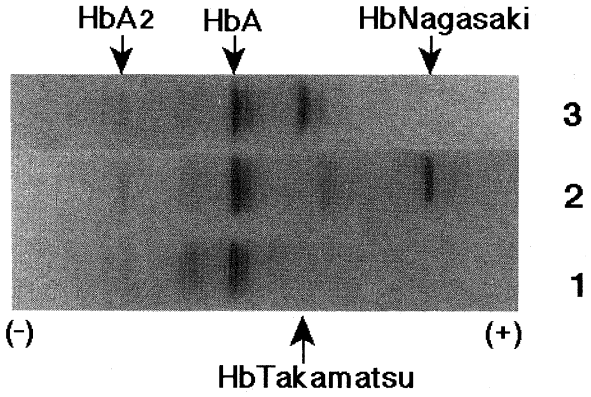

Fig 2. IEF of the hemolysates (pH range: 6-9). The patient's hemolysate showed the abnormal Hb band that was migrated faster than Hb A and Hb Takamatsu. 1. Normal control 2. Proband 3. Carrier with Hb Takamatsu

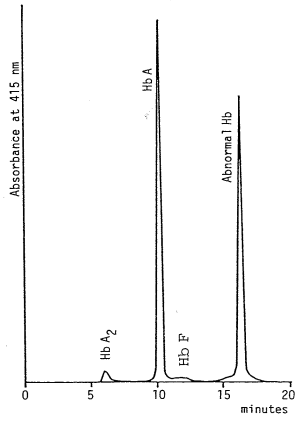

Fig 3. Hb analysis of the patient's hemolysate on DEAE-HPLC. The abnormal Hb peak was observed after the elution of the Hb A peak.

as shown in Fig 3, and the Hb composition of the total Hb was determined by the relative ratio of the square of these Hb peaks. The percentages of Hb A_2, Hb F, Hb A and the abnormal Hb were 2.6%, $<1\%$, 54.8% and 41.6% of the

hemoglobin, respectively. The instability test was negative. The abnormal globin chain, which eluted faster than the normal β-chain by CM-cellulose column chromatography, was collected, aminoethylated, and digested with TPCK-trypsin. Comparison of a reverse phased HPL-chromatogram of the tryptic peptides obtained from the normal globin chain with that obtained from the abnormal globin chain demonstrated that the βT-3 peptide peak disappeared and the βT-2 peptide peak was made smaller, but a new peptide peak was not distinctly observed in a routine analytic region, as shown in Fig 4. Although the peptide appeared at the position of the βT-2 peptide was

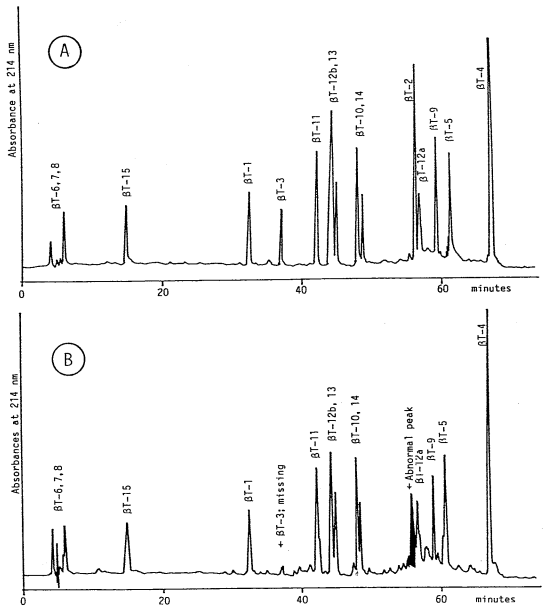

Fig 4. Separation of the peptides of the digest of the AE-abnormal β chain by reverse phase HPLC with a linear gradient of acetonitrile (from 0 to 50%) in a 9 mM trimethylamine-acetic acid buffer (pH 6.0). The chromatogram of the abnormal chain showed an absence of the βT-3 peptide peak. From the amino acid analysis of all peptides, the darkened peak was an abnormal peptide. A: AE-normal β chain. B: AE-abnormal β chain

expected to be abnormal, all peptides were collected to determine the amino acid composition. From the results, the amino acid composition of the peptide eluted at the position of the normal βT-2 peptide was expected to have a glutamic acid or glutamine residue for a lysine residue at β17(A14) and to combine the βT-2 peptide with the βT-3 peptide (Table 1). Variants possessing an amino acid substitution of Lys→Gln and Lys→Glu at β17 have identified to Hb Nikoshia[15] and Hb Nagasaki,[16] respectively. The electrophoretic behavior of the Hb described here leads to its being identified as Hb Nagasaki. The amino acid sequence of this abnormal peptide was analyzed by a Protein Sequencer, and the result demonstrated a Lys→Glu substitution at β17 as follows.

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          9                    15      17
Normal : Ser-Ala-Val-Thr-Ala-Leu-Trp-Gly-Lys-Val-Asn
Mutant : Ser-Ala-Val-Thr-Ala-Leu-Trp-Gly-Glu-Val
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TABLE 1. Amino acid composition of the abnormal peptide and the expected ratios of the βT-2 and βT-3 peptides

Amino Acid Residues	Found (mol/mol)	Expected Ratio βT-2	βT-3
Aspartic acid	2.44	0	2
Threonine	1.00	1	0
Serine	1.28	1	0
Glutamic acid	3.11	0	2
Glycine	3.56	1	3
Alanine	2.82	2	1
Valine	3.60	1	3
Leucine	1.98	1	1
Lysine	0.02	1	0
Arginine	0.82	0	1
Tryptophan	-	1	0

This conclusion was further confirmed by analysis of the nucleotide sequence of the mix-ssDNA and the iso-ssDNA by the dideoxy method. Although the result from mix-ssDNA suggested the presence of a nucleotide change in codon β17(AAG), it was clarified by sequencing of the iso-ssDNA. Nucleotide sequence coding around β17, as shown in Fig 5, revealed the alteration of A to G at the first base of the codon β17 AAG, which corresponded to Hb Nagasaki[16] with an amino acid substitution Lys (AAG)→ Glu (GAG).

Biosynthesis study of globin in reticulocytes was carried out, and the ratio of the β^{Total}(non-α) chain to the α chain was 0.98. No unbalanced biosythesis was observed between the non-α and α chains.

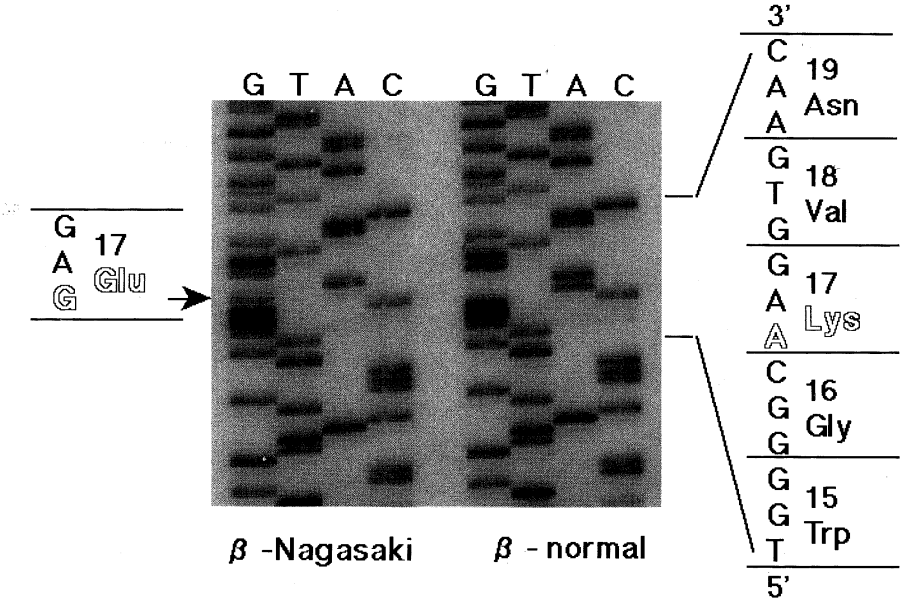

Fig 5. Autoradiograms of the sequencing gel of the cloned ssDNA (iso-ssDNA) of the β-globin genes. Analysis of the nucleotide sequence of the iso-ssDNA of the normal and abnormal (β-Nagasaki) genes showed the alteration of A to G at the first base of the codon β17(AAG).

The present case of Hb Nagasaki is only the second case since the first case was found in a Japanese family in Nagasaki prefecture in 1970. It has not yet been found in persons of different ethnic origins. Replacement of the amino acid at β17(A14) is generally considered to cause no hematological or clinical abnormalities, and the patient sutdied here showed no clinical or hematological abnormalities. In addition, a Japanese carrier of Hb J-Amiens with Lys→Asn substitution at β17 was also normal.[17]

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