Bull Yamaguchi Med Sch 31 : 47-54, 1984

Identification of Abnormal Hemoglobins

VI. Separation of Peptides by Reversed Phase High Performance Liquid Chromatography. Application to a New Case of Hb M Iwate

*Yuzo Ohba, Takaoki Miyaji, Miyoko Matsuoka and Nobuyuki Kitoh**

Department of Clinical Laboratory Science, Yamaguchi University School of Medicine, and *Department of Pediatric Cardiology, Shimonoseki Saiseikai General Hospital

(Received June 28, 1984)

Abstract Reversed phase high performance liquid chromatography on a C_{18} column (μBondapak,Waters) was applied to the identification of Hb M Iwate found in a newborn girl. The abnormal α chain was retained in the column even longer than the β chain, and well separated peaks for the normal α, β, abnormal α, $^{G}\gamma$ and $^{A}\gamma^{I}$ chains were eluted in sequence by the A/B solvent system of Huisman et al. It was co-eluted with the $^{G}\gamma$ chain by C/D solvent system of Shelton et al. The normal and abnormal αT9s were easily purified by a single peptide mapping of the tryptic hydrolysate of the total α chain mixture. Each of them was hydrolyzed with chymotrypsin, and the products were purified by peptide mapping on the same column.

The father and grandfather of the baby carried the same abnormal hemoglobin. The abnormal α chain comprised about 24% of the total α chain in the three carriers.

Key Words: High performance liquid chromatography, Abnormal hemoglobin

Introduction

Abnormal hemoglobins occur infrequently among Japanese[1]. They are extremely heterogenous, however, and virtually every abnormal hemoglobin found in a Japanese deserves identification by precise delineation of an amino acid sequence abnormality in either the α or non-α (β, γ or δ) chain. Through two decades of experience, we have established our methods for chemical identification of abnormal hemoglobins[2-6], where we used filterpaper electrophoresis and chromatography (fingerprinting) as the most important part of the whole procedure. Recent progress in high performance liquid chromatography (HPLC) has revolutionized the methods for separation of globin chains and the fragments therefrom[7]. We describe here the application of this latest technique to the identification of Hb M Iwate found in a newborn baby in Shimonoseki.

Materials and Methods

Blood samples were studied by a set of routine screening tests for hemoglobinopathy ("Hb Study") as was described in a previous paper[6]. Absorption

spectrum of acid methemoglobin was recorded by means of Hitachi 624 Spectrophotometer on an approximately 40 μmol/1 hemoglobin solution in 1/15 mol/1 phosphate buffer (pH 6.6) containing 1 mmol/1 potassium ferricyanide. Isoelectric focussing (IEF) was performed by the polyacrylamide gel thin layer method of Basset et al[8]. The procedures of Huisman et al (A/B developer system)[9] and of Shelton et al (C/D developer system)[10] were followed in the separation of globin chains by reversed phase HPLC, where we used a μBondapak C_{18} columm (3.9 mm ID×25 cm L), U6K Universal Injecter, 6000A Chromatographic Pump, 660 Solvent Programmer and 441 UV-Detector with a fixed wave length of 214 nm (Waters Associates), S-310A UV Detector with variable wave length (Soma) and Pantos U-228 Recorder (Nihondenshi). The absorbance of the effluent was continuously recorded at 214 and 280 nm.

Hemolysate was prepared and heme was removed by conventional methods. The α chains were prepared by the urea CM-cellulose column chromatography[11]. Appropriate fractions were pooled and globins were recovered by lyophilization after being transferred into 0.2 mol/1 acetic acid by Sephadex G-25 gel filtration. The α chains were dissolved 1 g/dl in 0.02 mol/1 acetic acid and hydrolyzed by 0.01 g/dl trypsin (TPCK-treated, Worthington) at pH 8-9 (pH adjusted with trimethylamine) for 4 h at 37°C. Essentially the same conditions were applied to chymotryptic (α chymotrypsin, Worthington) hydrolysis of αT9. The tryptic, as well as chymotryptic, hydrolysate was mapped by the reversed phase HPLC on another μBondapak C_{18} column using a linear gradient of acetonitrile from 0 to 32%, either in 9 mmol/1 trimethylamine-10 mmol/1 acetic acid (pH 5.6)[12] or in 0.05% (v/v) trifluoroacetic acid. The effluent containing relevant peptides was collected manually. The peptides were hydrolyzed in 20% (w/w) HCl at 105°C for 24 h, and their amino acid compositions were determined by Hitachi 835 Amino Acid Analyzer. The results were expressed by molar ratios. Ornithine was used as an internal standard for quantitation of αT9.

Results

The propositus, a Japanese newborn girl, was transferred to the Department of Pediatric Cardiology of Shimonoseki Saiseikai General Hospital because of cyanosis. However, a deep cyanosis under a normal or even higher than normal arterial pO_2 suggested a hemoglobinopathy, e.g. Hb M disease, rather than a congenital heart disease, and a blood sample was referred to Central Laboratory of Yamaguchi University Hospital. The baby was born otherwise uneventfully at a full term. Her birth weight was 3,600 g. The results of blood counting 3 days after birth were as follows: RBC 5.50×10^{12}/1, VPRC 0.605 1/1, Hb 20.5 g/dl, MCV 110.0 fl, MCH 37.3 pg, MCHC 33.9 g/dl, reticulocyte 4.2%, nucleated erythrocyte 1/100 WBC, platelet 217/10 OIF and WBC 8.3×10^{9}/1.

The fully oxygenated blood was chocolate-colored, as was the red cell lysate. Absorption spectrum of acid methemoglobin indicated the presence of Hb M in her hemolysate because of flattening of the indentation at 600 nm[13]. A hemoglobin band of abnormally dark color focussed in the cathodic side of Hb F in the IEF (Fig. 1), while the abnormal band migrated in the anodic side of Hb F in cellulose acetate membrane electrophoresis at pH 7.2. Better separation was achieved after converting the hemolysate into methemoglobin form. The abnormal and normal methemoglobin bands were extracted from the cellulose acetate membrane at pH 7.2. Absorption spectrum of the abnormal hemoglobin was characteristic of Hb M, i.e. protrusion rather than indentation of the absorbance arround 600 nm[13].

The father and patternal grandfather of the baby were also cyanotic. Examination of their blood confirmed the diagnosis of Hb M disease (Fig. 1). IEF and electrophoresis of the hemolysates from the adults revealed a minor abnormal band representing an abnormal Hb A_2. The demonstration of this minor abnormal band in addition to the major abnormality disclosed that the α chain was abnormal in this M-type hemoglobin. Heat denaturation (50°C) and isopropanol tests were negative in the adults. These

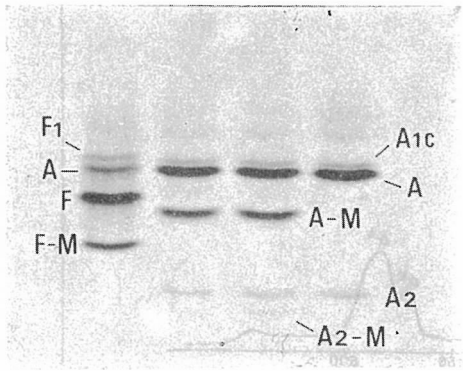

Fig. 1 Isoelectric focussing of hemolysate. The anode is to the top. 1) The propositus (a newborn), 2) Father, 3) Grandfather and 4) Normal adult. F-M, A-M and A_2-M signify molecules composed of the M-type α subunits associated with the γ, β, and δ subunits, respectively.

The abnormal α chain emerged much slower than normal in the reversed phase HPLC using the A/B solvent system (Fig. 2), whose peak area comprised 28.2, 28.0 and 28.1% of the total α chain areas in the baby, her father and grandfather, respectively. These values overestimated the relative amounts of the abnormal hemoglobin bacause of the difference in the specific absorptivity between the two α chains (introduction of a tyrosyl residue in the abnormal α chain: see below), but they did show that the abnormal hemoglobin comprised about the same proportion in the three carriers. The abnormal α chain co-emerged with the $^{G}\gamma$ chain when the column was developed by the C/D solvent system.

The urea CM-cellulose column chromatography of the total globin from the baby separated four major peaks, the γ, β, abnormal and normal α chains in order (Fig. 3). The two α chains, which partially overlapped, were pooled together and subjected to

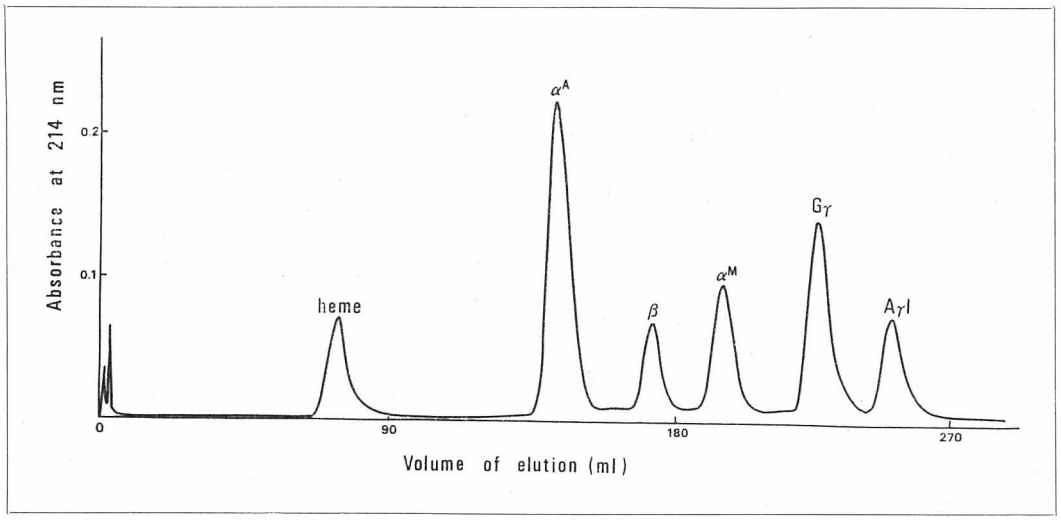

Fig. 2 Separation of globin chains by reversed phase HPLC. 20 μl of hemolysate from the propositus containing about 0.5 mg of hemoglobin was directly applied to a 3.9 mm×25 cm column of μBondapak C_{18}, which was developed by the A/B solvent system. α^M designates the abnormal α chain emerging even later than the β chain.

tests were spuriously positive in the baby because her hemoglobin consisted largely of normal and abnormal fetal hemoglobin (Hb F).

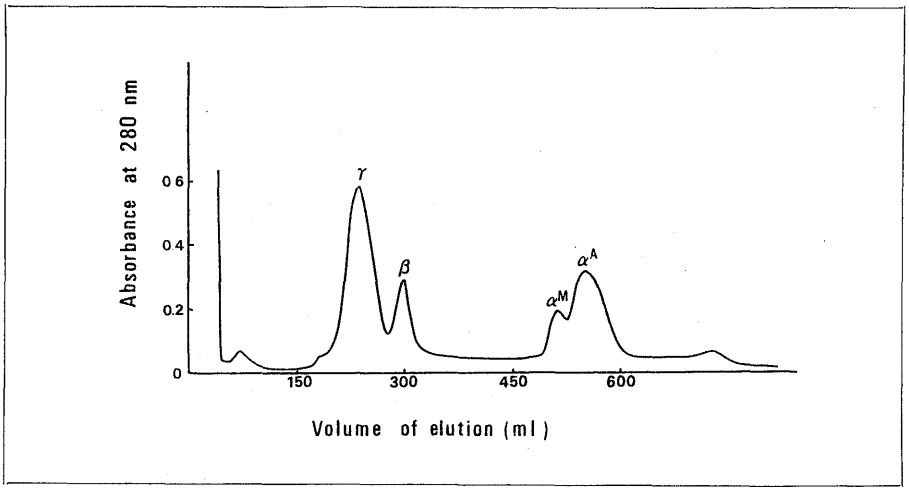

Fig. 3 Preparation of globin chains by urea CM-cellulose (CM-52) column chromatography.

tryptic hydrolysis. In the reversed phase HPLC of the tryptic hydrolysate, an extra peak with absorbance at 280 nm in addition to 214 nm emerged after normal αT9 (Fig. 4). Amino acid composition of the abnormal tryptic peptide agreed with that expected for an αT9 in which one of three histidyl residues was substituted by a tyrosyl (Table 1). The yield of amino acid from the abnormal αT9 was 23.8% of the sum of those from normal and abnormal αT9. Peptide mapping of the chymotryptic hydrolysate of αT9 (Fig. 5) and subsequent amino acid analysis of each fragment (Table 1) disclosed that histidyl residue at position 87 was replaced by tyrosine, while those at 72 and 89 were intact. Although the carboxyl site of leucyl residue at position 86 was hydrolysed by chymotrypsin in the normal αT9, hydrolysis at the carboxyl site of the newly introduced tyrosyl at position 87 was preferred in the abnormal αT9 (Fig. 6). These data identified the abnormal hemoglobin as Hb M Iwate or α87 (F8) His→Tyr[13].

Discussion

The reversed phase HPLC separates peptides according to their size and polarity[7]. Mutual separation of the $^{G}\gamma$ and $^{A}\gamma^{I}$ chains exemplifies the high resolving power of the column (Fig. 2). The difference between the two γ chains is whether there is glycine or alanine at position 136[14], i.e. the $^{A}\gamma^{I}$ chain has a molecular mass only 14 daltons larger than that of $^{G}\gamma$ with a molecular mass of 15,997. The amino acid substitution in Hb M Iwate is from a hydrophilic histidine to a hydrophobic, more bulky tyrosine, resulting in loss of one positive charge at the acidic pH and net increase of 26 daltons to the original molecular mass of the normal α chain of 15,125. The abnormal α chain is therefore retained in the C_{18} column much longer than normal.

The same effects were realized in the reversed phase HPLC of tryptic peptides, where the abnormal αT9 emerged later than normal. We routinely use the volatile buffer system of pH 5.6. We also use the more acidic trifluoroacetic acid system as an

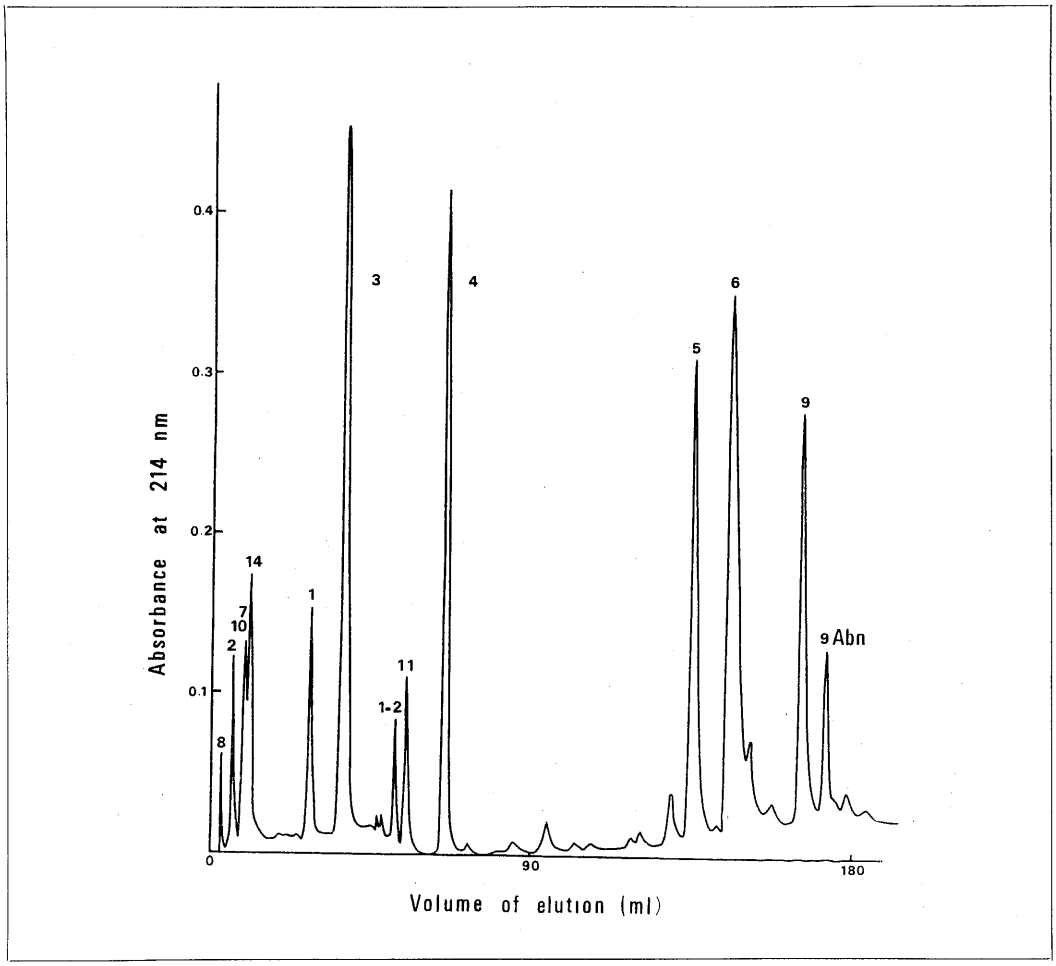

Fig. 4 Peptide mapping of tryptic hydrolysate by reversed phase HPLC. 30 μl of 2 g/dl solution was applied to a μBondapak C_{18} column, which was developed by the trimethylamine-acetic acid/acetonitrile system at a flow rate of 1.5 ml/min. Only the 214 nm tracing is shown. Numbers designate the tryptic peptide numbers of the α chain. Those peaks which absorb light at 280 nm are αT3 (trp), 4, 6, 14 and the abnormal αT9 (9Abn) (tyr).

alternative because the electric property and polarity of individual peptide may drastically change according to pH. Monitoring of the effluent at 280 nm in addition to 214 nm is useful for locating the peptides containing tryptophanyl or tyrosyl residue. This has been particularly effective in the study of Hb M Iwate; the presence of a tyrosyl residue in the abnormal tryptic peptide was evident in the HPLC mapping (Fig. 4), and also the most important chymotryptic fragment containing the tyrosyl could readily located (Fig. 5).

We deliverately mixed up the abnormal and normal α chains after their partial separation by the urea CM-cellulose column

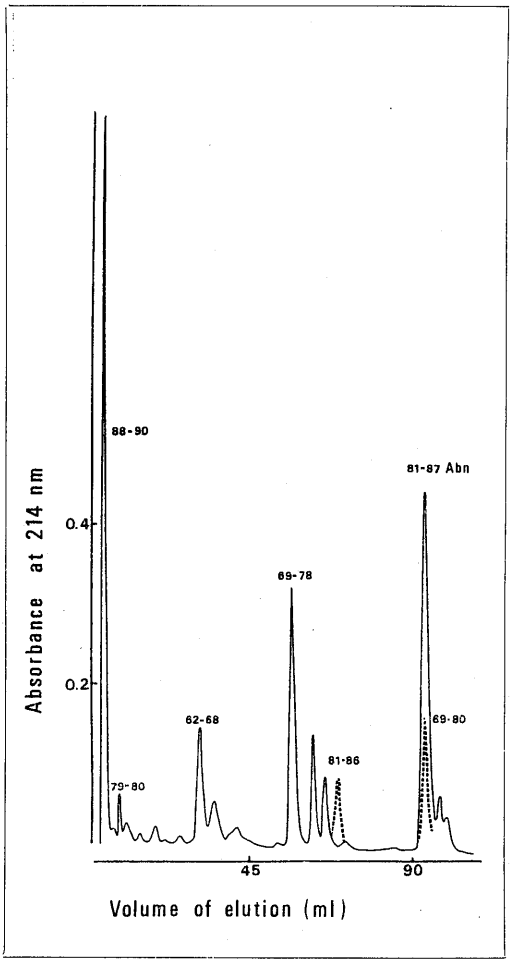

Fig. 5 Peptide mapping of chymotryptic hydrolysate from the abnormal αT9 on the same column as in Fig. 4, but it was developed by the trifluoroacetic acid/acetonitrile system. Only the 214 nm tracing is shown. Numbers indicate the residue numbers of the α chain which are included in each fragment. An abnormal peptide 81–87, the only peptide with absorbance at 280 nm, emerges together with peptide 69–80. Normal αT9 lacks the peptide 81–87 and has 81–86 (broken lines). Amino acid composition of the peptide 81–87 in Table 1 has actually been calculated by subtracting that for 69–80 from the result on the mixture.

chromatography. Complete recovery of the normal and abnormal αT9 from a single reversed phase HPLC of the tryptic digest of the α chain mixture and subsequent amino acid analysis of each, with an internal standard, enabled for the first time an accurate determination of the amount of M Iwate α chain relative to the total α chains. Conventional methods for the determination of relative amount of an abnormal hemoglobin have not been applicable to Hb M Iwate because its visible and ultraviolet absorption spectra are abnormal even after dilution with a cyanmethemoglobin reagent. Hb M Iwate is a unique ferrihemoglobin which is stabilized in a deoxy conformation[15] and does not bind cyanide to transform into cyanmethemoglobin.

In a previous paper dealing with identification of Hb Matsue-Oki or α75 Asp→Asn[4], we solved the problems in purification of αT9 by introducing Sephadex G-25 gel filtration of tryptic digest. Although this technique was subsequently applied successfully to another case of Hb M Iwate (unpublished), the reversed phase HPLC is much simpler, speedy and versatile. The resolving power of the reversed phase HPLC for tryptic peptides from globin is comparable or even superior to the traditional filter-paper fingerprinting. It offers far better reproducibility, nearly complete recovery rate, speed and extreme flexibility. Free amino acids and very small peptides, e.g. di- or tri-peptides, are difficult to handle with this column, however, because they may emerge all together without interacting with the column. Ion exchange column chromatography and the traditional fingerprinting compensate for this minor defect of the reversed phase chromatography.

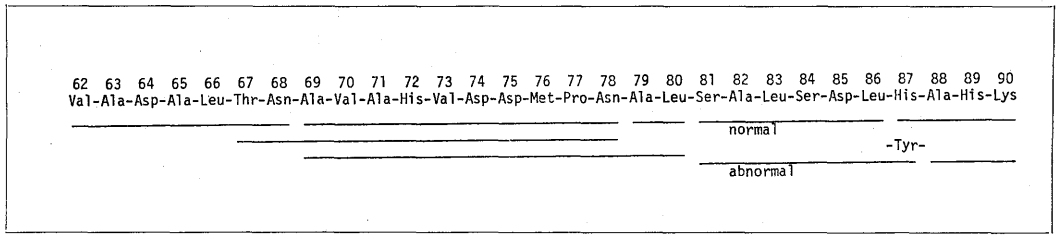

Fig. 6 Amino acid sequence of αT9. Numbers are the residue numbers of the α chain. Bars span the fragments released by chymotryptic hydrolysis which have been analysed in this study.

Table 1 Results of Amino Acid Analysis on α T9 and the Chymotryptic Peptides Therefrom

	Tryptic peptides		Chymotryptic peptides from abnormal αT9					Chymotryptic peptides from normal αT9		
	Abnormal αT9	Normal αT9	62–68	69–78	69–80	81–87	88–90	69–80	79–80	81–86
Asp*	5.89	5.95	1.75	2.85	2.81	0.94	tr	2.97	tr	1.06
Thr	0.98	0.95	0.96	–	–	–	tr	–	tr	–
ser	1.67	1.70	–	–	–	1.92	tr	–	tr	1.81
Glu	–	–	–	–	–	–	tr	–	–	–
Pro**	1.56	1.26	–	1.27	1.27	–	–	1.20	–	–
Gly	–	–	tr	–	tr	tr	tr	–	–	–
Ala	6.89	6.97	2.05	1.96	2.79	0.93	1.05	2.89	0.98	1.06
Val	3.24	3.31	1.16	2.09	2.14	–	–	2.03	–	–
Cys	–	–	–	–	–	–	–	–	–	–
Met	0.82	0.83	–	0.78	1.11	–	–	0.85	–	–
Ile	–	–	–	–	–	–	–	–	–	–
Leu	4.01	4.12	1.07	–	1.04	2.08	–	1.11	1.02	2.07
Tyr	0.87	–	–	–	–	1.13	–	–	–	–
Phe	–	–	–	–	–	–	–	–	–	–
Trp	–	–	–	–	–	–	–	–	–	–
Lys	1.11	1.07	–	–	–	–	1.01	–	–	–
His	1.99	2.84	–	1.05	0.83	–	0.94	0.96	–	–
Arg	–	–	–	–	–	–	–	–	–	–
yield#	0.83	1.26	1.65	0.98	0.63	1.86	2.49	0.99	3.22	4.00

* Aspartic acid plus asparagine.

** Values for proline are semiquantitative at most when the sample size is below 1 nmol.

\# The amounts actually loaded to the amino acid column (nmol).

References

1) Ohba, Y. and Miyaji, T.: Abnormal hemoglobins and thalassemia syndrome in Japan (in Japanese). *Byotaiseiri*, 1 : 382–388, 1982.

2) Ohba, Y., Matsuoka, M. and Miyaji, T.: Identification of abnormal hemoglobins. I. Fingerprinting, technique and limitation (in Japanese). *Ann. Rep. Protein Chem. Yamaguchi Univ. Sch. Med.*, 7 : 71–78, 1978.

3) Ohba, Y., Matsuoka, M. and Miyaji, T.: Identification of abnormal hemoglobins. II. Sephadex G-25 column chromatography of the tryptic peptides (in Japanese). *Ann. Rep. Protein Chem. Yamaguchi Univ. Sch. Med.*, 7 : 79-84, 1978.

4) Ohba, Y. Matsuoka, M. and Miyaji, T.: Identification of abnormal hemoglobins. Ⅲ. Analysis of αTpIX: identification of abnormal Hb Matsue-Oki (in Japanese). *Ann. Rep. Protein Chem. Yamaguchi Univ. Sch. Med.*, 7 : 85-90, 1978.

5) Ohba, Y., Matsuoka, M. and Fujisawa, K.: Determination of structure of abnormal hemoglobins (in Japanese). *Jpn. J. Clin. Pathol.*, special issue 33 : 29-45, 1978.

6) Ohba, Y., Hattori, Y., Yamamoto, K., Matsuoka, M. and Miyaji, T.: Identification of abnormal hemoglobins. V. Detection and identification of unstable hemoglobins (in Japanese). *Yamaguchi Med. J.*, 32 : 87-104, 1983.

7) Schroeder, W.A., Shelton, J.B. and Shelton, J. R.: High performance liquid chromatography in the identification of human hemoglobin variants. *In* S.M. Hanash and G.J. Brewer (eds), *Advances in Hemoglobin Analysis*, Liss, New York, 1981, p1-22.

8) Basset, P., Beuzard, Y., Garel, M.C. and Rosa, J.: Isoelectric focussing of human hemoglobin: its application to screening, to the characterization of 70 variants, and to study of modified fractions of normal hemoglobins. *Blood*, 51 : 971-982, 1978.

9) Huisman, T.H.J., Webber, B., Okonjo, K., Reese, A.L. and Wilson, J.B.: The separation of human hemoglobin chains by high pressure liquid chromatography. *In* S.M. Hanash and G.J. Brewer (eds), *Advances in Hemoglobin Analysis*, Liss, New York, 1981, p23-38.

10) Shelton, J.B., Shelton, J.R., Schroeder, W.A. and DeSimone, J.: Detection of Hb-Papio B, a silent mutation of the baboon β chain, by high performance liquid chromatography. Improved procedures for the separation of globin chains by HPLC. *Hemoglobin*, 6 : 451-464, 1982.

11) Clegg, J.B., Naughton, M.A. and Weatherall, D.J.: Separation of the α and β chains of human hemoglobins. *Nature*, 219 : 69-70, 1968.

12) Ishiguro, K., Ohba, Y., Hattori, Y., Miyaji, T., Oshida, Y., Tachinami, T., Takabatake, S. and Nakaizumi, K.: Hemoglobin Kansas in a Japanese family. *Hemoglobin*, 7 : 573-580, 1983.

13) Shibata, S., Miyaji, T., Iuchi, I., Ohba, Y. and Yamamoto, K.: Hemoglobin M's of the Japanese. *Bull. Yamaguchi Med. Sch.*, 14 : 141-179, 1967.

14) Shroeder, W.A., Huisman, T.H.J., Shelton, J. R., Shelton, J.B., Kleihauer, E.F., Dozy, A.M. and Robberson, B.: Evidence for multiple structural genes for the γ chain of human fetal hemoglobin. *Proc. Natl. Acad. Sci. U.S.A.*, 60 : 537-544, 1968.

15) Greer, J.: Three-dimensional structure of abnormal human hemoglobins M Hyde Park and M Iwate. *J. Mol. Biol.*, 59 : 107-126, 1971.