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JMS Letters

Dear Sir,

Hb Sagami [β139(H17)Asn → Thr]: a new Hemoglobin Variant not Detected by Isoelectrofocusing and Propan-2-ol Test, was Detected by Electrospray Ionization Mass Spectrometry

The detection and identification of variant hemoglobins (Hbs) are clinically important and the information obtained from variant Hbs has furthered the study of diseases associated with various pathological proteins. More than 670 kinds of variant Hbs have been reported.[1] Most of these were detected by their mobilities on electrophoresis or isoelectrofocusing (IEF).[2] These variants mainly have substitutions between amino acids with different charges. In a group of variants with substitutions of amino acids of the same charge, the conformational difference may be responsible for the abnormal mobility.[3] Unstable Hb variants often have electrophoretically silent (non-separable) mutations, hence they cannot be identified by electrophoresis but can be detected by stability testing. Since Wada *et al.*[4] and Matsuo *et al.*[5] introduced the identification of variant Hbs using mass spectrometry (MS) in 1981, the procedure has been widely used.[6–8] We have studied Hb specimens for detection of hemoglobinopathy by IEF and electrospray ionization (ESI) MS.[9,10] Variant globin chains with more than a 10 Da difference from normal chains could be detected by the MS method that we used when mixtures of normal and abnormal globins were analyzed. Most of the variants examined so far were discernible by both IEF and ESI-MS. Some cases (substitution between amino acids of close molecular mass) were detected by IEF but not by ESI-MS. In the present study, we noted a new variant, which was detected solely by ESI-MS, i.e. an electrophoretically silent and propan-2-ol test-negative variant.

The hemoglobin from a 49-year-old healthy non-diabetic Japanese male was studied to clarify the cause of an unusual profile of ion-exchange high-performance liquid chromatography (HPLC) for routine HbA1c measurement (with an automated HbA1c analyzer, Hi-AUTO A1c type 8150, Kyoto Daiichi Kagaku, Kyoto, Japan). The HbA1c value was not accurate because the peak at the retention time of normal HbA1c overlapped with an unusual peak. Roughly, HbA1c measured five times during the past 5 years was 0.4–1.4% (normal range 4.3–5.8%). Other laboratory data were as follows: red blood cells, 5.50×10^{12} l^{-1} (normal); hemoglobin, 14.5 g dl^{-1} (normal); mean corpuscular volume, 78.1 fl (microcytic); mean corpuscular hemoglobin, 26.3 pg (hypochromic); platelets, 205×10^{9} l^{-1} (normal); and white blood cells, 7.50×10^{9} l^{-1} (normal). He had had no serious illness before this examination. The 17% propan-2-ol stability test of the patient's hemolyzate was negative. The variant Hb was not detected by isoelectric focusing (see inset of Fig. 1). The ESI-MS of the globin mixture showed doublet ion peaks of β-chain. The molecular mass of one ion peak corresponded to normal β-chain and the other was 12.7 Da smaller than normal β-chain (Fig. 1).

The sequence was determined by tandem MS coupled with HPLC with globin digests. A globin mixture of normal and abnormal components and α- and β-chains was cleaved with trypsin and lysyl endopeptidase.[10] Globin mixtures of both non-derivatized and oxidized globin were examined. The experiment with oxidized globin was performed to study the sequence of the core region as described previously.[10] The peptides were analyzed by HPLC/ESI-MS/MS. From the reconstructed selected ion chromatograms, the ions corresponding to all normal expected peptides from α- and β-chains and the ions 13 Da less than all normal counterparts were plotted. The search for oxidized peptides did not reveal any abnormal peptides, hence the mutation is not within core peptides (βT10 and 12). An abnormal peptide was found in digests of non-derivatized globin 13 Da smaller than βT14. The reconstructed selected ion chromatogram is shown in Fig. 2 and the abnormal peptide was designated β*T14. The collision-induced dissociation (CID) spectra (Fig. 3) of normal and abnormal peptides contained the common y series ions below m/z 538.9 (y_1–y_5), which coincided with the y ions of the sequence Ala–Leu–Ala–His–Lys of the β-chain, 140–144 (nomenclature for fragment ions according to Biemann[12]). The ion y_6 at m/z 653.2 in the CID spectrum of the normal βT14 [Fig. 3(b)] was not observed in the spectrum of β*T14 [Fig. 3(a)]. Instead, a prominent ion was observed at m/z 639.9 in the CID spectrum of the β*T14. The ions assigned from y_7 to y_{11} in the spectrum of β*T14 correlated with the values after subtracting 13 Da from the mass number of y_7–y_{11} ions in the normal βT14 spectrum. The difference between the two prominent ions at m/z 538.9 and 639.9 observed in the β*T14 spectrum was 101.0 Da, which corresponded to a threonine residue. Therefore, we concluded that the asparagine at position 139 of the normal β-chain was substituted by threonine in the variant β-chain, [β139(H17)Asn → Thr]. The sequence was confirmed by protein sequencer using a peptide, β*T14, isolated by reversed phase HPLC. We named this novel variant Hb Sagami, based on the district in which it was detected.

The peak height of the ion of the abnormal β-chain was about twice that of normal β-chain (Fig. 1). The ratio of the abnormal to normal β-chain estimated by the ESI-MS was comparable to the ratio of abnormal to normal hemoglobin measured by HPLC. Figure 4 shows a profile of hemoglobins of the proband obtained by ion-exchange HPLC with use of PolyCAT A (Poly LC, Columbia, MD, USA), in which chromatogram was developed by a shallow linear gradient of a high ionic strength and high pH buffer.[13] The fractions of each HPLC peak were analyzed by ESI-MS, and the observed mass values corresponded to the theoretical values of the molecular species as shown in Fig. 4. For the HPLC of HbA1c for routine analysis, elution was effected by a stepwise increase to a higher ionic strength buffer. In routine HPLC, Hb Sagami co-eluted with HbA. By HPLC with the same resin, in which buffer was changed not stepwise but as a slow gradient, normal and abnormal Hb were separated. The unusual peak that overlapped with HbA1c in routine HPLC was also separated from Hb A1c by HPLC and the observed molecular masses of the fraction measured by MS corresponded to normal α-chain and glycated mutant β-chain, indicating it to be glycated Hb Sagami. The values of HbA1c of the proband measured by routine HPLC were low because glycated Hb Sagami eluted earlier than normal A1c, and was not included for quantification. The content of glycated abnormal Hb was about twice that of normal HbA1c. HbA1c of normal plus glycated abnormal Hb was about 5%, which is a reasonable level for non-diabetic patients.

The ratio of ion peaks of abnormal and normal peptides (β*T14 and βT14) was about 2:1 (Fig. 2). An abnormally high ratio of variant to normal hemoglobin in propositus, as observed by HPLC of hemoglobins, ESI-MS of globins and also LC/MS of a mixture of tryptic peptides, all point to a compound heterozygosity for the β-globin variant and β-thalassemia on the normal β-chromosome. The low values of the mean corpuscular volume and mean corpuscular hemoglobin and the high level of HbA_2 determined by HPLC (Fig. 4) and MS (Fig. 1) support the existence of β-thalassemia.

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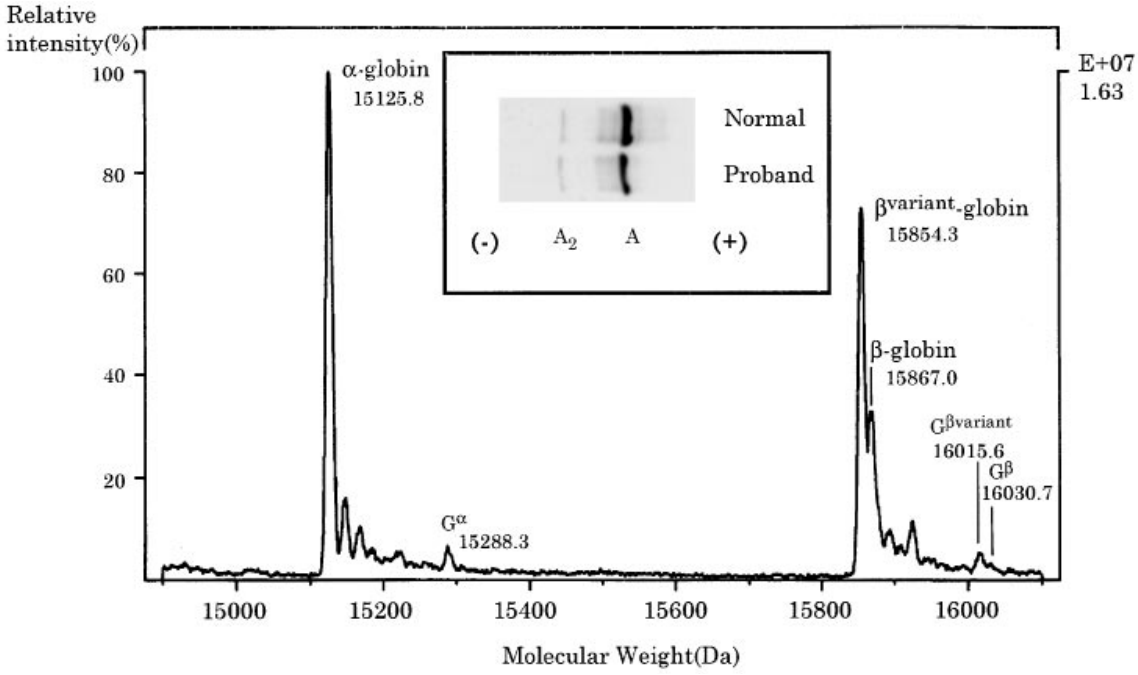

Figure 1. A transformed ESI mass spectrum of the globin mixture of the proband. A 5 μl volume of the globin solution (10 pmol μl^{-1}) was introduced via a sample loop at a flow rate of 5 μl min^{-1} into a TSQ-7000 triple-stage quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an ESI ion source. Full ES scanning was performed at *m/z* 900–1300 in 8 s and was summed over 10 scans to obtain the final spectra. α^A-Globin, β^A-globin and $\beta^{variant}$-globin indicate α-chain of normal, β-chain of normal and β-chain of variant Hb, respectively. G^α and G^β represent the glycated normal α- and β-chains, respectively, and $G^{\beta variant}$ the glycated variant β-chain. The peak annotation was based on the molecular mass of the species. The inset shows the IEF on a polyacryamide gel containing Pharmalyte (pH range 6–9) of the hemolysates from the proband and a normal control. IEF was carried out according to Basset *et al*.[11]

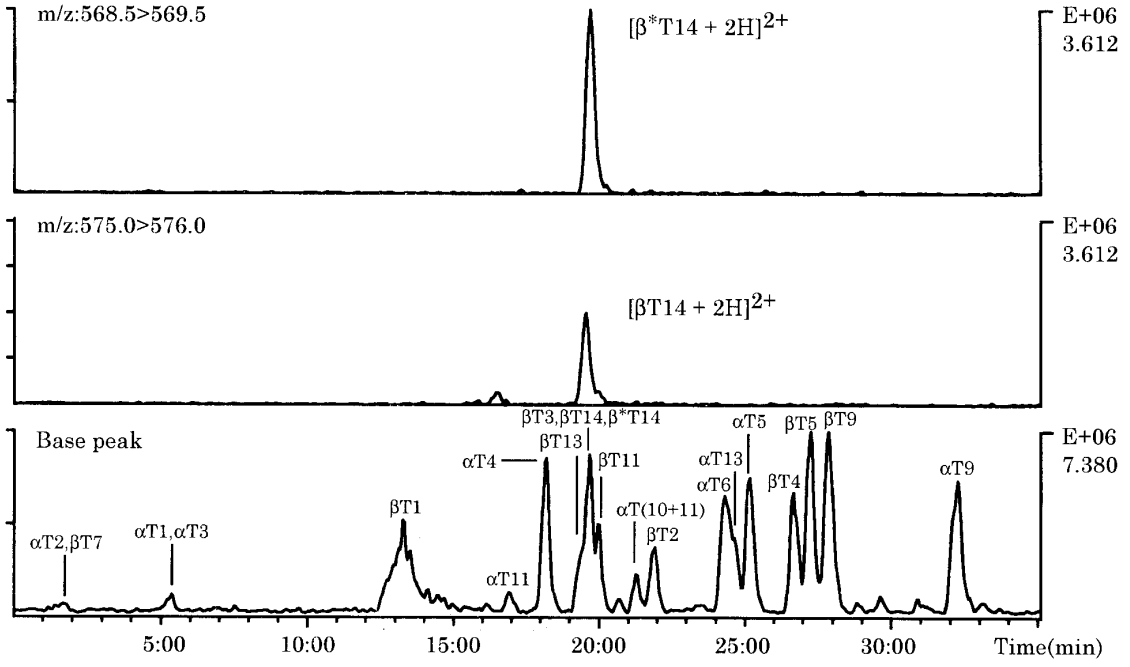

Figure 2. Reconstructed selected ion chromatograms and base ion chromatogram obtained by LC/ESI-MS. Intact globin mixture was digested with TPCK-trypsin (Worthigton Biochemical, Freehold, NJ, USA; Lot 33K996) and lysyl endopeptidase (Wako, Osaka Japan; Lot YLR9326) in 0.1 M ammonium hydrogencarbonate (pH 8.4) at 37 °C overnight.[9,10] The digests were dissolved in acetonitrile–water (2:98) that contained 0.1% trifluoroacetic acid, and 10 μl of the solution were injected into the HPLC system. '*m/z* 568.5 > 569.5' represents a doubly charged ion of a peptide, β*T14, 13 Da smaller than βT14, and '*m/z* 575.0 > 576.0' a doubly charged ion of βT14. Identities of the peptides labeled in the base ion chromatogram were confirmed by their CID spectra.

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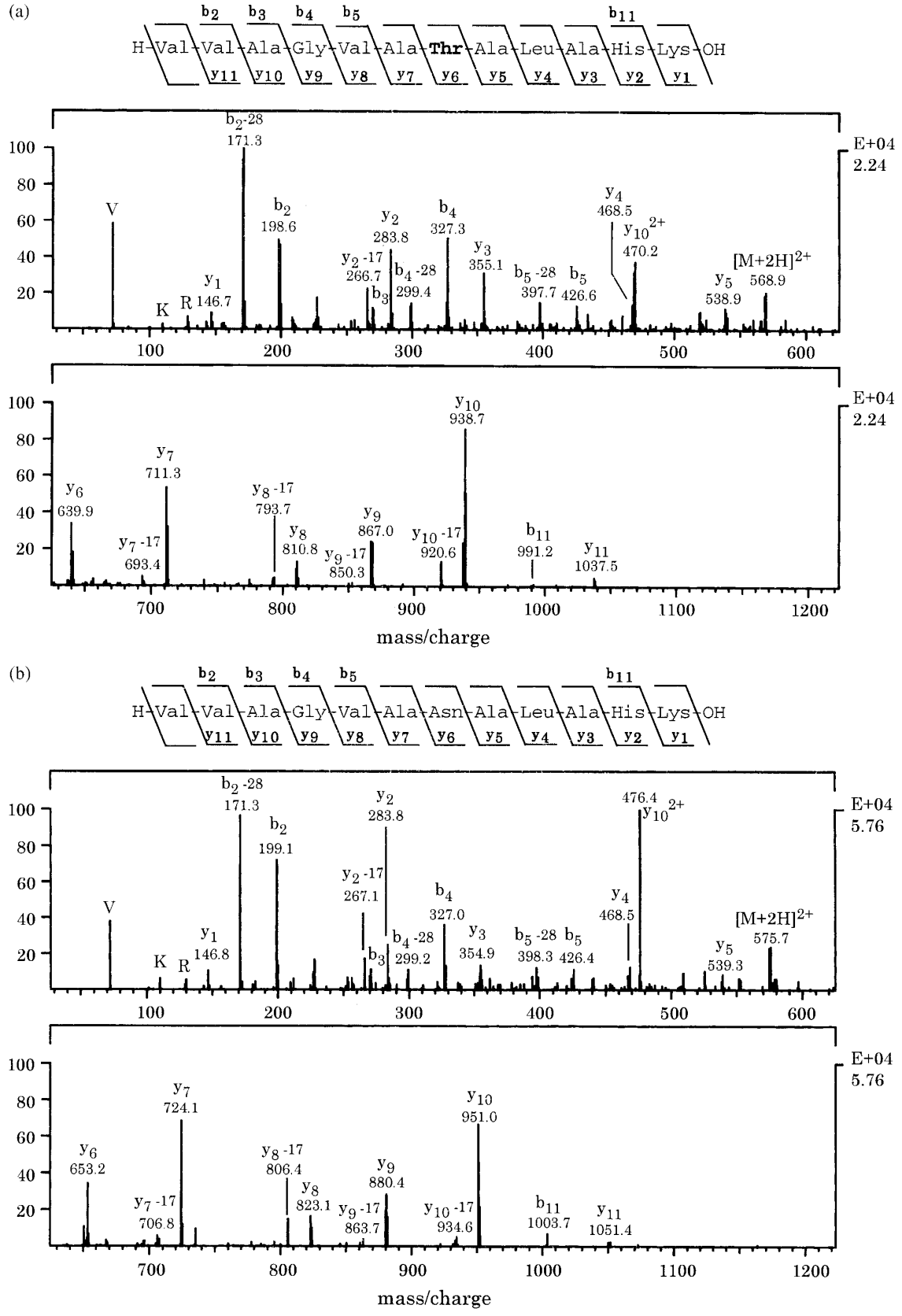

Figure 3. Comparison of the CID mass spectra of (a) a precursor ion of *m/z* 568.9, the doubly charged ion of abnormal *β**T14, and (b) that of *m/z* 575.3, the doubly charged ion of normal *β*T14. The interval between each adjacent y series ion gives the molecular mass of the proposed amino acid, which is shown at the top of each part of the figure.

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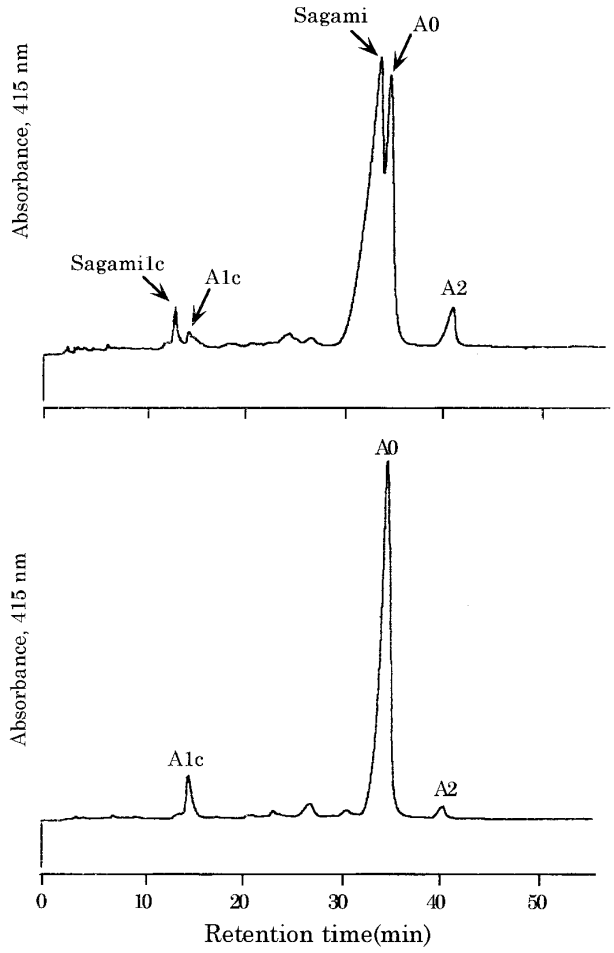

Figure 4. HPLC (PolyCAT A) separation of hemoglobin components in hemolyzates from the proband (top) and from a normal individual (bottom). HPLC was performed according to the reference.[13] The peaks were identified by comparison between profiles of the proband and a normal individual and by analysis by MS. Sagami1c represents glycated Hb Sagami, identified from the mass spectrum, which showed the ion peaks corresponding to non-glycated normal α-chain and glycated abnormal β-chain. Sagami represents Hb Sagami, identified by the ions corresponding to normal α-chain and non-glycated abnormal β-chain. Other peaks: A1c, HbA1c; A_0, Hb A_0; A_2, HbA_2.

The reported abnormal Hbs on β139 were Hb Hinsdale 139Asn → Lys, which showed a reduced affinity for oxygen and reduced cooperativity,[14] Hb Geelong 139Asn → Asp, which is unstable,[15] and HbAurora 139Asn → Tyr, which showed a high oxygen affinity.[16] Experiments on oxygen affinity are needed.

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TOYOFUMI NAKANISHI,[1] AYAKO MIYAZAKI,[1] MASAHIKO KISHIKAWA,[1] AKIRA SHIMIZU,[1]* YOSHIKAZU AOKI[2] and MIYAKO KIKUCHI[3]

* *Correspondence to*: A. Shimizu, Department of Clinical Pathology, Osaka Medical College, 2–7, Daigakucho, Takatsuki, Osaka 569, Japan

shimizu@poh.osaka-med.ac.jp

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[1] Department of Clinical Pathology, Osaka Medical College, 2–7, Daigakucho, Takatsuki, Osaka, 569, Japan

[2] Laboratory for Clinical Investigation, Kanagawa Health Association, 58, Nihon-ohdori, Naka-ku, Yokohama, 231, Japan

[3] Department of Comprehensive Health Check, Kanagawa Health Association, 58, Nihon-ohdori, Naka-ku, Yokohama, 231, Japan

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