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First case of a single heterozygote of an abnormal hemoglobin, Hb Stanmore, [β111(G13)Val→Ala]

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Abstract

We describe a hemoglobin β-chain mutant detected incidentally in an unusual profile of glycated hemoglobin (HbA1c) measured by ion-exchange HPLC. Analysis of intact globin by electrospray ionization mass spectrometry (ESI-MS) and peptide analysis by on-line HPLC-ESI-MS-MS revealed the substitution, [β111(G13)Val→Ala], which was confirmed by DNA analysis. This was the second case of Hb Stanmore. As the first case combined β^0-thalassemia, and the family study in that case showed no case of Hb Stanmore without combined thalassemia, the case presented here is the first case of single heterozygote, and the first Japanese case. Hb Stanmore is isoelectrophoretically silent with only mild clinical symptoms, although stability by isopropanol test was positive.
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1. Introduction

Several hemoglobin (Hb) variants have been found in analysis of glycohemoglobin (HbA1c) by ion-exchange high-performance liquid chromatography (HPLC), which is used to monitor glycemic control in diabetic patients [1–5]. The detection and identification of abnormal Hbs is clinically essential to diagnosis in patients suffering from abnormal Hb related symptoms, as well as to examine unusual profiles of HPLC for HbA1c measurement. Wada et al. used mass spectrometry for abnormal Hb determination in 1981 [6], using field desorption ionization, and this has been improved by the use of modern soft ionization mass spectrometry. We have used electrospray ionization mass spectrometry (ESI-MS) for this purpose. In addition, critical devices have been applied to prepare peptides most suitable for ESI-MS analysis. Here we report an abnormal Hb which was difficult to detect without ESI-MS, as it was isoelectrophoretically silent with only mild clinical symptoms.

2. Materials and methods

2.1. Case report

A hemoglobin specimen from a 41-year-old Japanese female was subjected to structural study because

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of an accidentally-discovered unusual profile on ion-exchange HPLC for routine HbA1c measurement using an ADAMS™ A1c HA-8160 analyzer (Arkray, Kyoto, Japan). A notch before the HbA1c peak was observed as shown in Fig. 1 in an initial analysis of the patient's Hb. The origin of the notch was unclear as this profile was not reproducible, it may have been a peak of denatured abnormal Hb component. Nonetheless, we analyzed the Hb by ESI-MS, and two clear β-chain peaks were found. Therefore, the detection of this variant was accidental. HbA1c was 4.8% by HPLC (HA8150, Arkray, Kyoto, Japan), and 5.0% by immunoassay (DCA2000, Bayer Medical, Chicago, USA). Plasma glucose was also within normal range (79 mg dl^{-1}, 87 mg dl^{-1}).

Hematological data were as follows: First examination at 40-years-old: RBC 3.58×10^{12} l^{-1}, Hb 9.1 g dl^{-1}, MCV 81.3 fl, MCH 25.4 pg, MCHC 31.3 g dl^{-1}, Platelet 240×10^{9} l^{-1}, WBC 35.7×10^{9} l^{-1}; Fe 23 mg dl^{-1} (40–162), Ferritin 5 ng ml^{-1} (10–80), Haptoglobin 160 mg dl^{-1} (19–170), LDH 226 IU l^{-1} (200–400). At 41-years-old: RBC 3.33×10^{12} l^{-1}, Hb 9.7 g dl^{-1}, MCV 87.7 fl, MCH 29.1 pg, MCHC 33.2 g dl^{-1}, WBC 31.0×10^{9} l^{-1}; Reticulocyte 1.4%. By supplement of iron tablet (sodium ferrous citrate), the anemia and iron level recovered to almost normal level, iron 77 mg dl^{-1}, ferritin 24 ng ml^{-1} and Hb 11.6 g dl^{-1}. She had been suffering from a mild type of Behçet's disease since she was 38-years-old (recurrent aphtha on oral mucosa, folliculitis and polyarthralgia). She had also complained of genital bleeding due to adenomyomatosis of the uterus for a couple of years, which may have caused the mild chronic iron deficient anemia. The 17% isopropanol stability test of the patient's hemolysate was positive, suggesting the presence of an unstable Hb. She had no episode of hemolysis. The degree of hemolytic anemia caused by the unstable nature of variant Hb in this patient was very small or none.

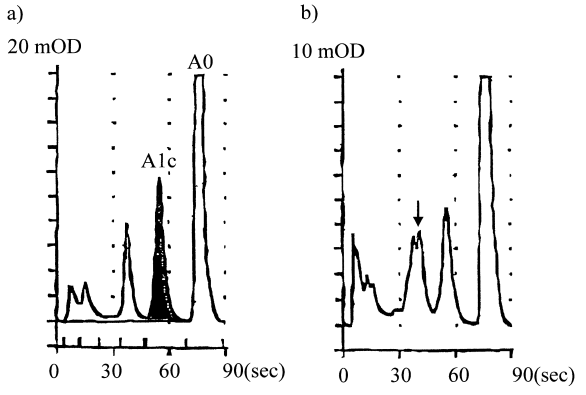

Fig. 1. A profile of ion-exchange HPLC for routine HbA1c measurement using an analyzer, ADAMS™ A1c HA-8160 analyzer, Arkray, Kyoto, Japan. (a) Normal control; (b) The case presented here.

2.2. MS analysis of globins and peptides

Two hundred μl of hemolysate (Hb ca. 10 mg dl^{-1}) was added into 10 ml of cold acid acetone (0.02 N HCl in acetone). The precipitate was washed with cold acetone three times and then lyophilized with nitrogen gas. Globins were dissolved in 50% methanol containing 1% acetic acid. This solution (50 pmol μl^{-1}) was introduced into a TSQ-7000 triple-stage quadrupole mass spectrometer equipped with an electrospray ion source (Finnigan MAT, San Jose, CA) at a flow-rate of 5 μl min^{-1}. Scan range was m/z 800–1300 over 3 s in the positive ion mode and data were summed over 10 scans to obtain the final spectra. Calibration was done using the peptide Met–Arg–Phe–Ala and horse apo-myoglobin.

Variant β-chain was prepared by HPLC using reversed-phase HPLC (Aquapore RP-300 7 μ, 250× 4.6 mm, Perkin-Elmer, Norwalk, CT). Solvent A was 20% isopropanol and 80% solution of 0.2% trifluoroacetic acid (TFA) in water, and solvent B was 55% isopropanol and 45% of the same TFA solution. The gradient was as follows: 0–20% B from 0–2 min, 20–40% B from 2–35 min, 40–60% B from 35–45 min, then isocratic at 60% B for 10 min. The flow-rate was 0.8 ml min^{-1} and elution of the chromatography was monitored at 280 nm [7]. Each fraction was collected and dried under vacuum (VEC-310, Iwaki Glass Co. Ltd., Tokyo, Japan).

The isolated abnormal β-chain (ca. 0.3 mg) was dissolved in 50 m*M* ammonium bicarbonate (pH 8.0) and separated into two tubes. One was cleaved with *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone(TPCK) treated trypsin (1:50 weight ratio), the other was cleaved with endoproteinase Glu-C (1:25 weight ratio, Roche Diagnostics GmbH, Mannheim, Germany) and Asp-N (1:125 weight ratio, Roche Diagnostics GmbH, Mannheim, Germany) 3 h at 37 °C. Trypsin peptides were analyzed by a HPLC

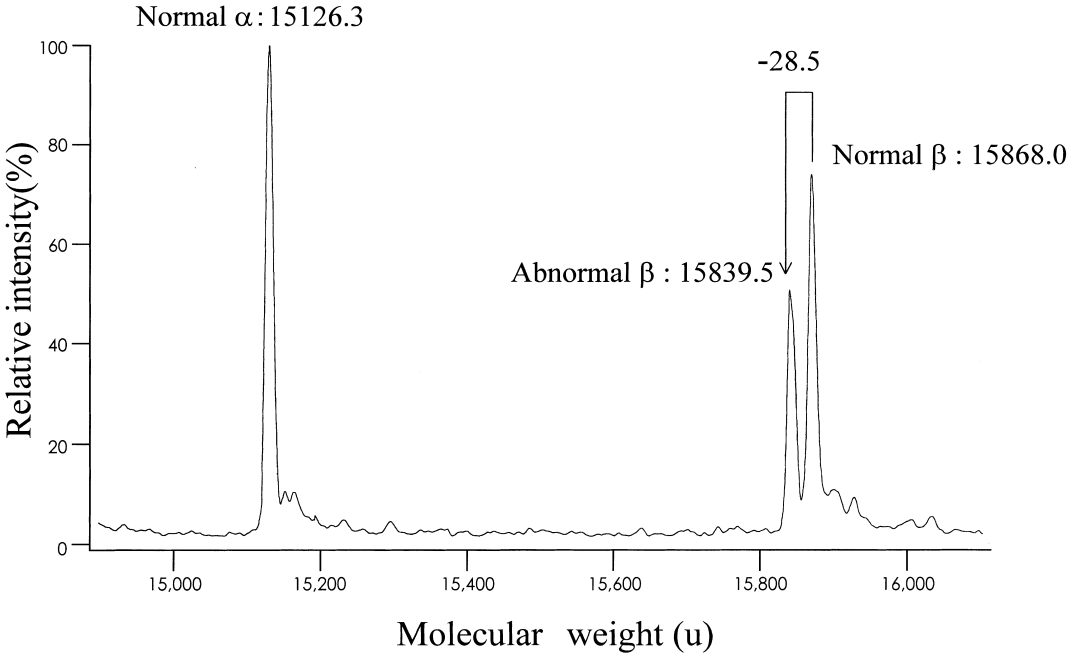

Fig. 2. A transformed ESI-MS spectrum of intact globin chain prepared from the case.

column (XTerra™ MS C_{18}, 2.1×150 mm, 5 μm, Waters Corporation, Milford, MA) connected to a quadrupole ion trap mass spectrometer, LCQ, equipped with an ES ion source (Finnigan MAT, San Jose, CA). Peptides in 50 m*M* ammonium bicarbonate were injected and eluted with a gradient of 2–60% acetonitrile containing 0.02% TFA and 0.1% acetic acid over 60 min at a flow-rate of 200 μl min^{-1}. For each LC–ESI-MS analysis, 10 μl of ca. 10 pmol was loaded.

ESI (LCQ) spectra were recorded by scanning the quadrupole from m/z 400–2000 u. Data was analyzed using data analysis system Xcalibur™ version 1.1 (Finnigan Corp. 1999). Calibration was performed using the peptide Met–Arg–Phe–Ala, caffeine and Ultramark K162. The structure of the peptides was determined according to the principles established by Biemann et al. [8].

2.3. Isoelectricfocusing (IEF)

Isoelectricfocusing (IEF) of the hemolysate was carried out according to the report of Basset et al. [9] on a polyacrylamide gel containing Pharmalyte (pH range 6–9).

2.4. DNA sequence analysis

For the DNA analyses, total genomic DNA was extracted from the leukocytes. Approximately 0.1 ng of the DNA was amplified by polymerase chain reaction (PCR) and 30 base primers flanking Hb gene region containing exon 3, according to the sequence described by Hutt et al. [10]. Amplification of the DNA was performed according to Hutt et al. [10]. DNA sequencing was performed with an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems Inc., Foster City, CA). The primers used for sequencing were the same as those used for amplification.

3. Results

3.1. Globin analysis

The variant was not detected by isoelectric focusing or ion-exchange HPLC but was detected by electrospray ionization mass spectrometry (ESI-MS). Fig. 2 shows the transformed spectrum of a globin

derived from the patient. A peak representing normal α-subunit was seen at mass, 15 126.3±0.5 u and the ions of β-subunit separated into two components; one was 15 868.0±0.9 u and the other was 15 839.5±0.5 u. The former corresponded to the molecular mass of the normal β-chain, and the latter was 28.5 u smaller than the normal β-chain.

3.2. Peptides analysis

Variant β-chain was separated by HPLC using reversed-phase resin. Fig. 3 shows the chromatography, and the β-chain peaks split, designated β1 and β2 fractions as shown in the figure. Suspected abnormal β-chain was eluted slightly before normal β-chain. Both fractions were pooled and dried. The isolated β-chains were cleaved with trypsin, endoproteinase Glu-C and Asp-N, and applied to on-line LC–ESI-MS. Fig. 4 shows base ion chromatograms by HPLC–ESI-MS. Identities of the peptides annotated in the base ion chromatogram were confirmed by their CID spectra. From reconstructed selected ion chromatograms of β1 fraction, we searched for the ions corresponding to all normal expected peptides from β-chain (Fig. 5a). All peaks except small tryptic peptides (βT-6, 7, 8, 15) were detected, but the intensity of the ion peak of βT-12 was weak (see intensity number noted at on the upper right of each chromatogram). The ion 28 u less than normal βT-12 were plotted (Fig. 5b). An abnormal peptide, 28 u smaller than βT-12, was found and the abnormal peptide was designated βT*-12. From these analyses, the peak of βT-12 was found at 35.51 min as seen in the chromatogram, Fig. 4 bottom, and the peak of βT*-12 was seen at 34.52 min as shown in Fig. 4 top. Fig. 6 shows the CID spectra of normal and abnormal βT-12. Doubly-charged ions of the normal βT-12, m/z 861.0 and the abnormal βT*-12, m/z 847.1 were used for analyses of CID spectrometry. The CID spectra of normal and abnormal peptides included the common y series ions (y4–y9) which coincided with the y ions of the sequence, 116-112 of β-chain, that is, His–Ala–Leu–Val–Cys. The ions y10–y14 in the CID spectrum of the normal βT-12 were not observed in the spectrum of the abnormal βT*-12. Instead of this, a prominent series of y ions was observed in the βT*-12. The mass difference of ions y10–y14 between βT-12 and βT*-12 was 28 u. Therefore, we concluded that the valine at the position 111 of the normal β-chain is substituted by alanine. This is the same structure formarly reported as Hb Stanmore [11].

The sequence was also confirmed as G*T*C→G*C*C at the codon of 111 by nucleotide sequencing.

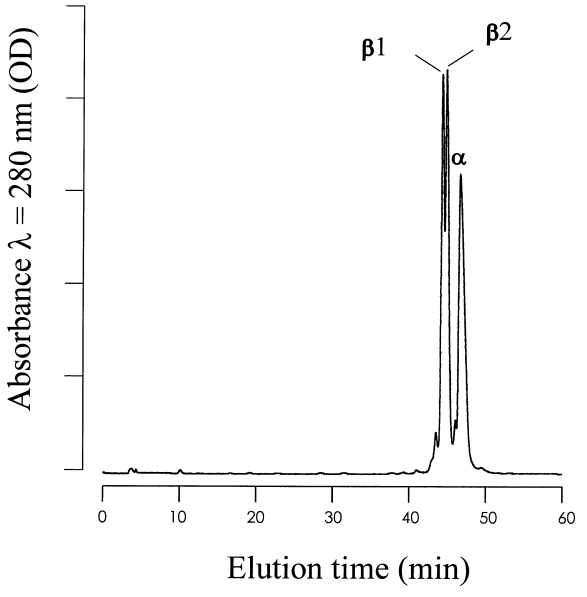

Fig. 3. A chromatogram by reversed-phase HPLC (Aquapore RP-300 7 μ, 250×4.6 mm). Globin (1.4 mg) was applied.

4. Discussion

The abnormal structure of the hemoglobin reported in the present paper was determined to be [β111(G13)Val→Ala]. This was the second case of Hb Stanmore. The propositus of the first report of Hb Stanmore was a double-heterozygote for the variant Hb and β^0-thalassemia, and Italian extraction. The variant was proven to be an unstable Hb by isopropanol test. Family studies of the first report revealed two siblings and parents to have thalassemia, but none to have the unstable Hb [11]. Therefore, the case presented here is the first case of single heterozygote, that is, heterozygote of Hb Stanmore and normal Hb genes. This is also the first

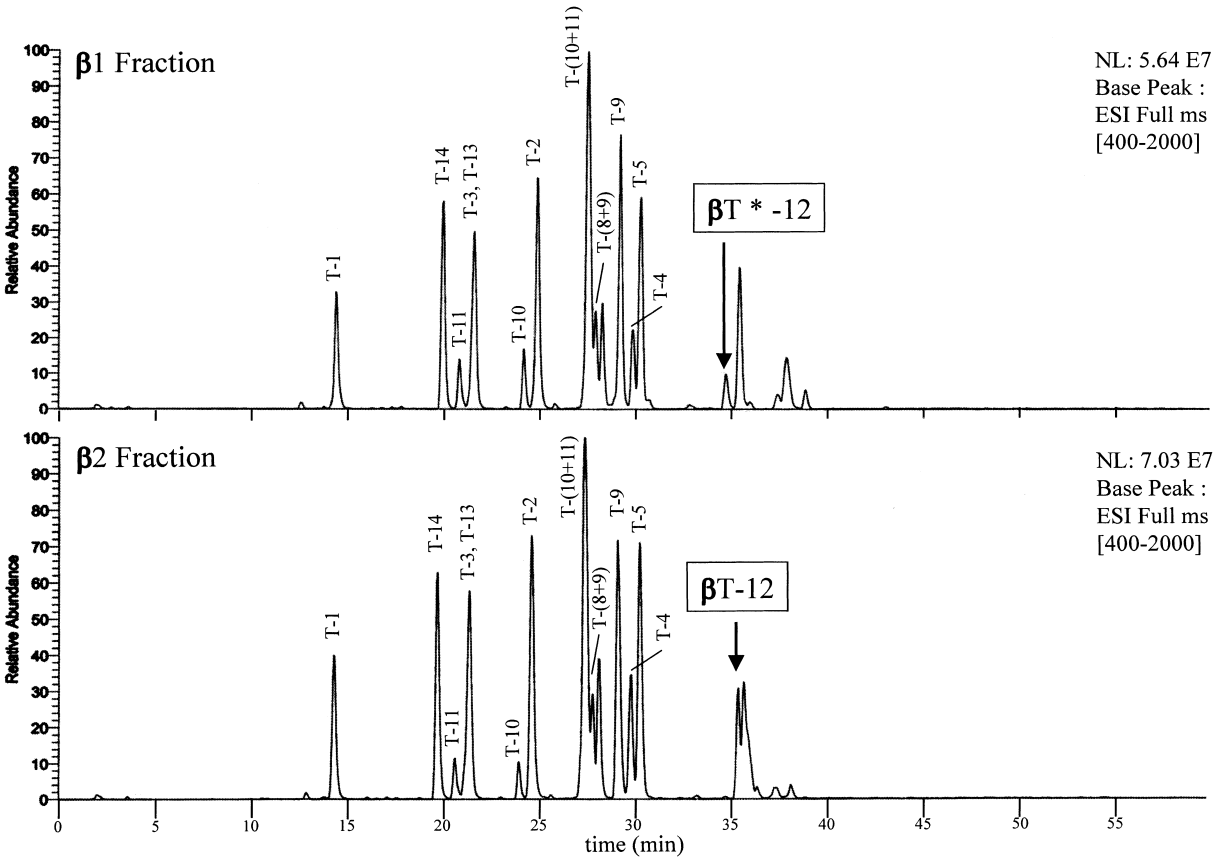

Fig. 4. Base ion chromatogram by HPLC–ESI-MS derived from peptides obtained by digestion of β-chain with trypsin. βT: tryptic peptide from β-chain, numbers are from N-terminal. Upper: analysis of fraction of β1 (abnormal β-chain), Lower: analysis of fraction of β2 (normal β-chain). βT*-12: an abnormal peptide.

Japanese case. Hb Stanmore is isoelectrophoretically silent, clinical symptoms of the trait without thalassemia were very mild, although stability by isopropanol test was positive. The case in the first report of Hb Stanmore might have been subjected to Hb analysis because of combined β^0-thalassemia. Our case was incidentally subjected to Hb analysis. This variant may easily be overlooked and conceivably more new clinically and isoelectrophoretically silent variants will be found by analysis with ESI-MS.

More than 840 kinds of abnormal Hb's have been found and new variants have been added just recently [12]. Modern soft ionization mass spectrometry, e.g., ESI-MS, has contributed to these discoveries. We have devised methods for preparing peptides suitable for ESI-MS analysis: i.e. (1) enzyme digestion in a short time (less than 3 h) and avoiding lyophilization of digests to protect against formation of insoluble peptides (core); (2) use of endoproteinases Glu-C and Asp-N in addition to trypsin to cover the sequence regions of small tryptic peptides; (3) Automatic figuring of mass chromatograms of each anticipated mass of peptides using the LCQ Deca and analysis soft wear integrated into the instrument. By this system, we determined many Hb variants quickly and reliably, which are essential properties for clinical application. We reported a variant determined by our updated protocol in the present paper.

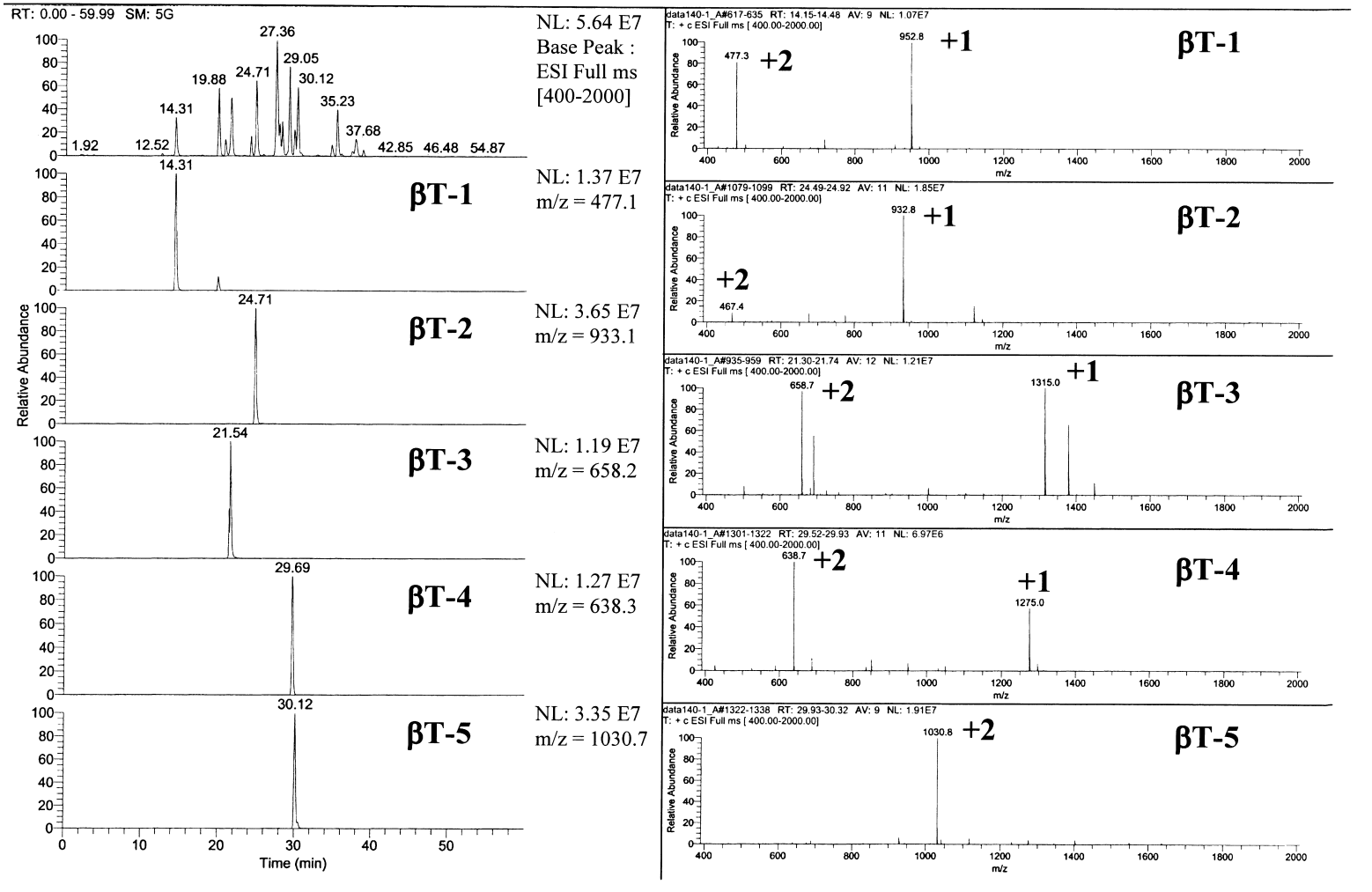

Fig. 5. Reconstructed selected ion chromatograms. (a) The ions corresponding to normal expected peptides from β-chain were investigated. All peaks except small tryptic peptides (βT-6, 7, 8, 15) were detected, but the intensity of the ion peak of βT-12 was weak (see intensity number noted on the right upper of each chromatogram). (b) The ions 28 u less than normal βT-12 were plotted. An abnormal peptide, 28 u smaller than βT-12, was found and the abnormal peptide was designated βT*-12.

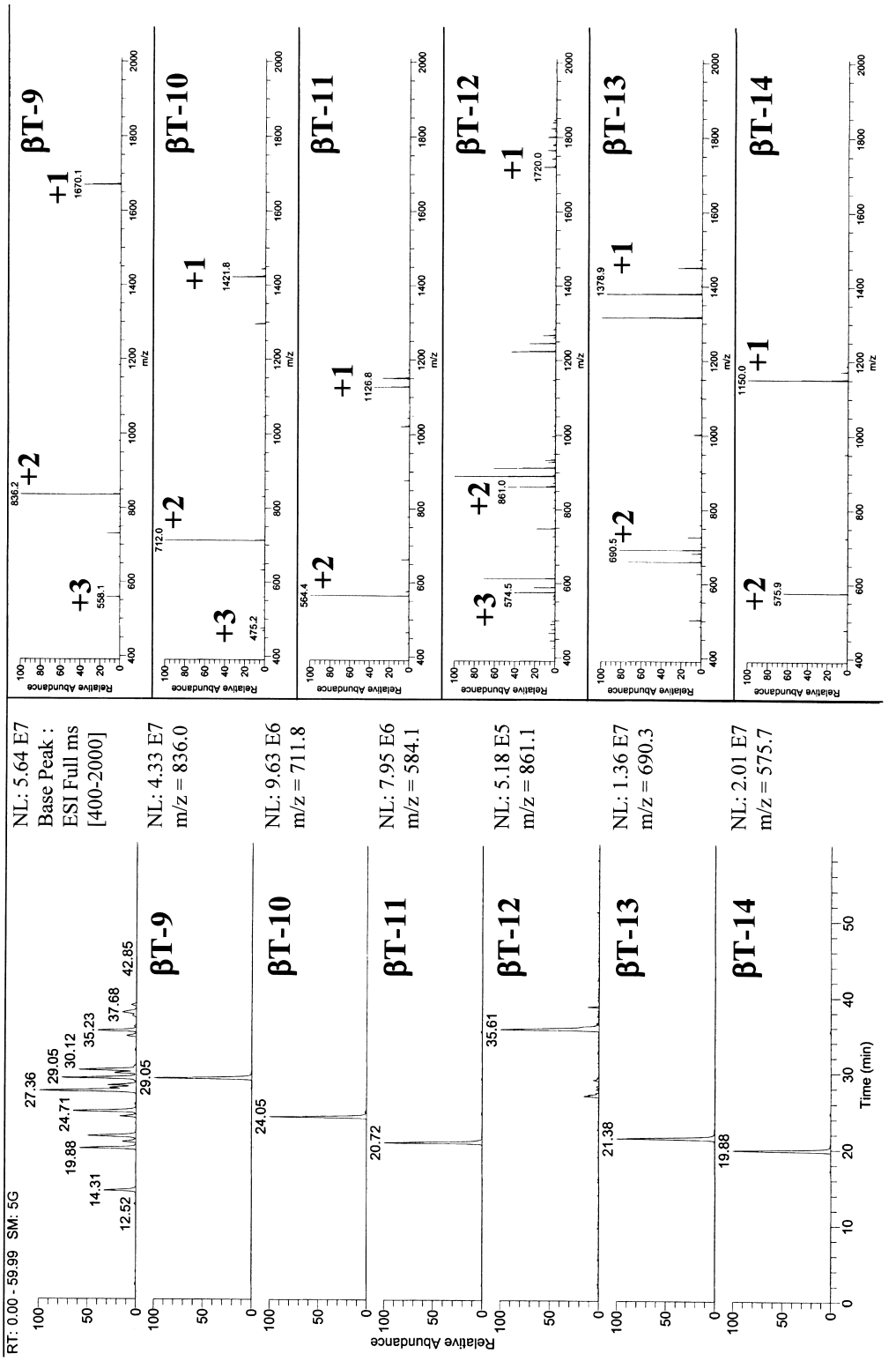

Fig. 5. (*continued*)

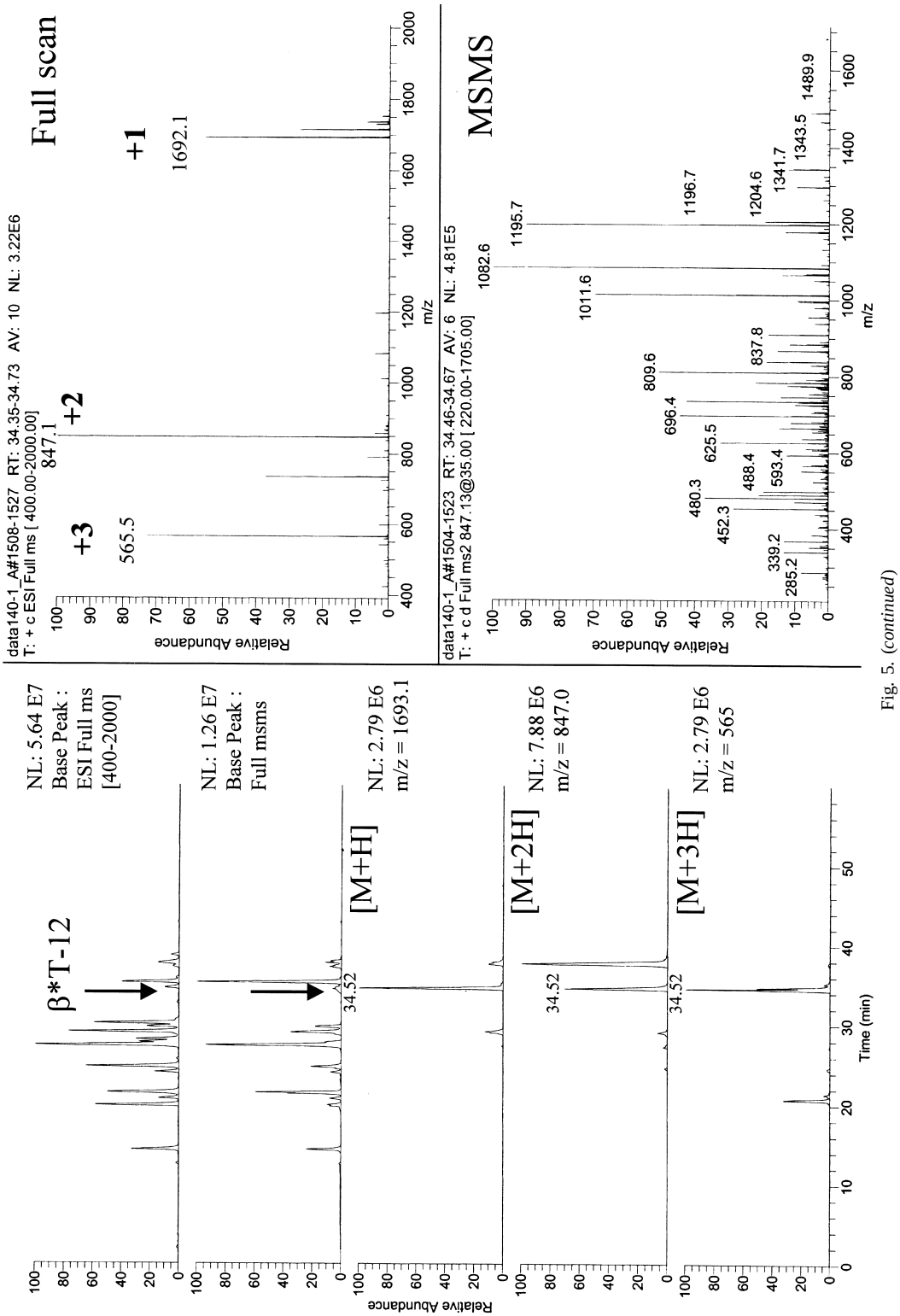

Fig. 5. (*continued*)

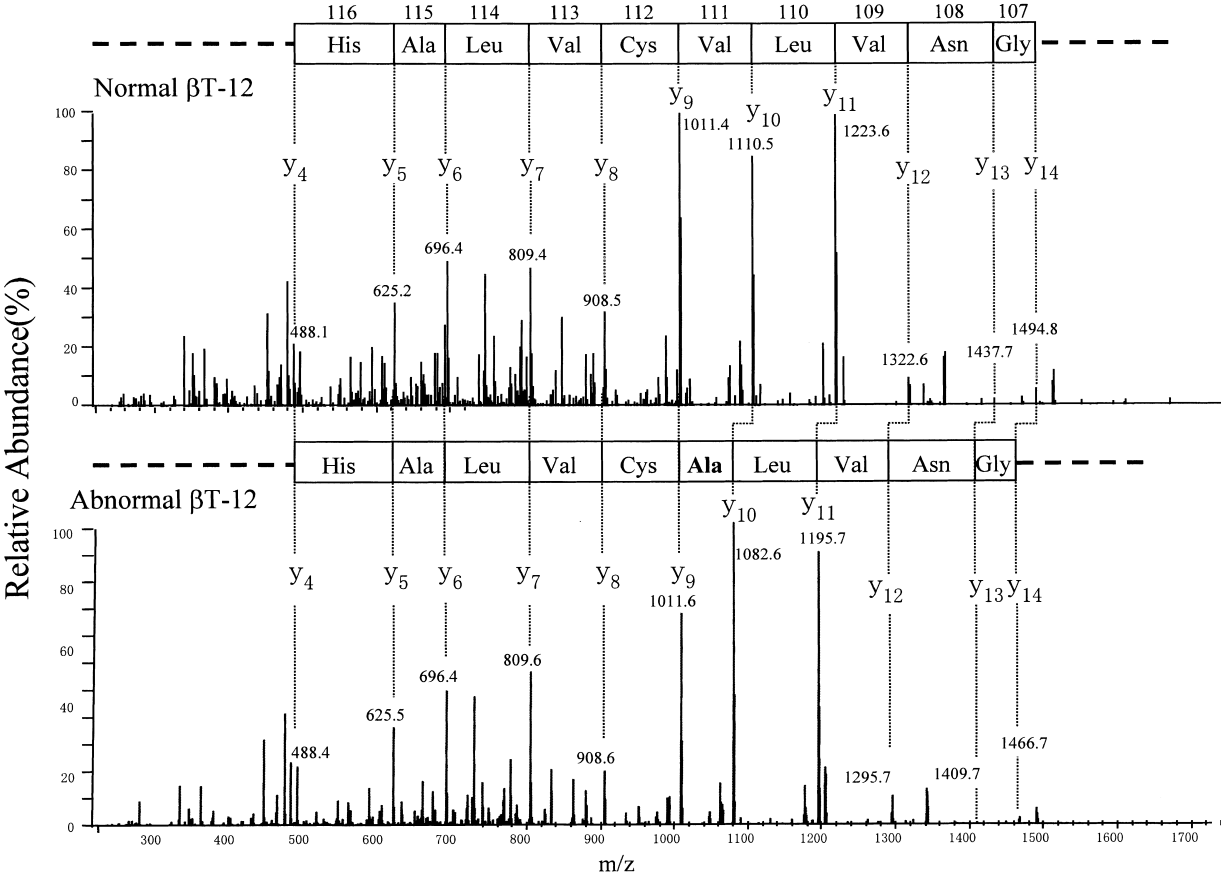

Fig. 6. CID mass spectra of a precursor ion of m/z 861.0, the doubly charged ion of normal βT-12 and m/z 847.1, the doubly charged ion of abnormal βT*-12. The interval of each adjacent y series ion shows the molecular mass of the proposed amino acid, which is shown at the top of the figure.

Acknowledgements

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