Abnormal haemoglobins, Hb Takamatsu and Hb G-Szuhu, detected during the analysis of glycated haemoglobin (HbA1c) by high performance liquid chromatography

Y Moriwaki, T Yamamoto, Y Shibutani, T Harano, S Takahashi, T Hada

Abstract
Background—During medical checkups of two unrelated female outpatients during their annual health examination and one male inpatient suffering from cardiac failure the glycated haemoglobin (HbA1c) concentrations measured by high performance liquid chromatography (HPLC) were low, in spite of normal fasting plasma glucose concentrations. However, HbA1c concentrations measured by latex immunooagglutination and fructosamine concentrations were within the normal range.

Method—Investigations were performed to elucidate the reasons for these discrepancies.

Results—Abnormal haemoglobins, Hb Takamatsu and Hb G-Szuhu, were found. The HPLC chromatogram showed an additional peak near HbA1c, which resulted in falsely low HbA1c concentrations. Isoelectric focusing analysis of the patients’ haemoglobin disclosed abnormal haemoglobins, which migrated faster than normal HbA, in the two female patients and slower in the male patient. The cDNA sequence and amino acid analyses of the haemoglobin α-chains and β-chains indicated the presence of the haemoglobin variant β 120 Lys → Gln in the two female patients and β 80 Asn → Lys in the male patient; that is, Hb Takamatsu and Hb G-Szuhu.

Conclusions—These cases show how these silent haemoglobin variants can result in falsely low HbA1c concentration readings when using HPLC.

Keywords: abnormal haemoglobin; high performance liquid chromatography; glycated haemoglobin

Glycated haemoglobin (HbA1c) has been shown to be increased in diabetes mellitus1 and is widely used as a marker for long term glycaemic status. Analysis of HbA1c is performed by high performance liquid chromatography (HPLC), electrophoresis, immunomassay, and colorimetry, as well as other methods. Among them, HPLC is generally used as the standard method for the determination of HbA1c concentrations.2 However, the HbA1c concentration is affected by several factors other than blood glucose concentrations, such as ureaemia,1 alcohol abuse,3 high dose aspirin intake,4 hyperbilirubinaemia,5 and the decreased life span of erythrocytes6 (massive bleeding, haemolytic anaemia, and anaemia in pregnancy). Moreover, several kinds of haemoglobinopathy are known to result in falsely high or low HbA1c values when measured by means of HPLC. In this report, the detection of two abnormal haemoglobins, Hb Takamatsu (β120 Lys→Gln) and Hb G-Szuhu (β80 Asn→Lys), during HPLC analysis for HbA1c in two unrelated women and one man is described.

Patients, materials, and methods
Blood samples were obtained from two female outpatients during an annual medical checkup and one male inpatient. Haematological data were obtained by means of an automated analyser. The subjects were KM, a 60 year old woman whose blood pressure was 200/100 mm Hg; TY, a 65 year old woman with mitral valvular disease; and ES, a 76 year old man admitted for the treatment of cardiac failure. KM and TY are not related, although their parents are from the Kagawa prefecture. Automated HPLC (HbA1c analyser, Model HA-8110; Daiichi, Kyoto, Japan; HbA1c reference range, 4.0–5.4%, % coefficient of variation (%CV), ±0.2%) with a cation exchange column was used for the measurement of the HbA1c concentrations. Isoelectric focusing gel electrophoresis using a commercially available kit (haemoglobin IEF gel; Joko, Tokyo, Japan) was performed to detect abnormal haemoglobin in KM and TY. In ES, isoelectric focusing gel electrophoresis was performed using the method of Righetti et al.7,8

Direct sequencing of haemoglobin cDNA in KM and TY was performed according to the following method. Total RNA was obtained from peripheral blood cells by guanidium thiocyanate/phenol/chloroform extraction9 and then cDNA was reverse transcribed from the total RNA. Table 1 shows the primers for the polymerase chain reaction (PCR), which were used under the following conditions: cDNA was denatured at 94°C for five minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for one minute, with a final extension step of seven minutes.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used in direct sequencing of Hb cDNA</th>
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<tbody>
<tr>
<td>α-chain</td>
<td>Forward 5’ &gt; ACT CTT CTG TGT CCC ACA GA &lt; 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ &gt; TTC AAA GAC CAG GAA GGG GC &lt; 3’</td>
</tr>
<tr>
<td>β-chain</td>
<td>Forward 5’ &gt; CCT TCT GAC ACA ACT GTG &lt; 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ &gt; CCC CAG TTT AGT GTG AC &lt; 3’</td>
</tr>
</tbody>
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PCR was carried out in a volume of 25 µl containing 50 mM KCl, 10 mM Tris/HCl (pH 8.8), 1.5 mM MgCl2, 0.1% Triton X-100, 200 µM each of dATP, dCTP, dGTP, and dTTP, 0.1 µg of cDNA, 10 pmol of each primer, and 1.5 U of Taq polymerase in a DNA thermal cycler (Perkin Elmer, Foster City, California, USA). Amplified DNA products were sequenced by the dye termination method using a DNA sequencing kit (Perkin Elmer) with the same primers as for the PCR, and analysed by means of an ABI PRISM 310 (Applied Biosystems, Foster City, California, USA).

Amino acid structural analysis of abnormal haemoglobin in ES was performed as follows. Isolation of the abnormal haemoglobin β-chain was carried out by means of CM-52 cellulose column chromatography, and peptide obtained by tryptic digestion of the aminoethylated abnormal haemoglobin was analysed by reversed phase HPLC (TSK gel 80Ts, 4.6 × 250 mm, Tosoh; elution buffer, 0.1% TFA/50% acetonitril–0.1% TFA/50 min; flow rate, 0.7 ml/min; detection wavelength, 214 nm). The amino acid composition of the hydrolysates of the abnormal peptide was analysed using an automatic amino acid analyser.

Results

Laboratory data revealed normal fasting blood sugar (850 mg/litre in KM, 950 mg/litre in TY, and 990 mg/litre in ES), although the concentrations of glycated haemoglobin were low: 1.2% in KM, 0.9% in TY, and 2.7% in ES. HbA1c concentrations measured by latex immunooagglutination were normal, as were routine haematological data. Indices suggesting haemolysis, such as indirect bilirubin or lactate dehydrogenase, were normal. HPLC chromatograms showed an additional peak near HbA₃ useful (fig 1, Hb Takamatsu; fig 2, Hb G-Szuhu). Isoelectric focusing gel electrophoresis demonstrated an additional haemoglobin migrating faster than HbA in KM and TY (fig 3), and an additional haemoglobin migrating slower than HbA in ES (fig 4).

Abnormal haemoglobin in ES was detected during HbA₁c analysis.
Table 2 Amino acid composition of abnormal peptide and amino acid sequence of the Tp-9 peptide

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Analytical value (molar ratio)</th>
<th>Theoretical value (Tp-9)</th>
</tr>
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<tbody>
<tr>
<td>Asp</td>
<td>1.907</td>
<td>3</td>
</tr>
<tr>
<td>Ser</td>
<td>1.09</td>
<td>1</td>
</tr>
<tr>
<td>Gly</td>
<td>2.08</td>
<td>2</td>
</tr>
<tr>
<td>Ala</td>
<td>1.98</td>
<td>2</td>
</tr>
<tr>
<td>Val</td>
<td>1.06</td>
<td>1</td>
</tr>
<tr>
<td>Leu</td>
<td>2.937</td>
<td>4</td>
</tr>
<tr>
<td>Phe</td>
<td>0.93</td>
<td>1</td>
</tr>
<tr>
<td>Lys</td>
<td>1.04</td>
<td>1</td>
</tr>
<tr>
<td>His</td>
<td>0.92</td>
<td>1</td>
</tr>
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Abnormal Tp-9: Lys

Discussion

We report two unrelated non-diabetic patients and one patient with cardiac failure with the silent haemoglobin variants Hb Takamatsu and Hb G-Szuhu. They were found incidentally during a medical checkup that included HbA1C determination.

HbA1c has been used as a routine clinical laboratory marker for evaluating long term glycaemic control. However, several factors are known to affect its concentration. They include ureaemia, alcohol abuse, high dose aspirin intake, and massive bleeding, as well as various kinds of haemoglobinopathies. Ureaemia, alcohol abuse, and high dose aspirin intake cause falsely high HbA1c concentrations, whereas bleeding, haemolytic anaemia, and pregnancy cause falsely low concentrations. However, in the present cases, the factors that typically cause low HbA1c concentrations were excluded, and it was shown that the disparity between low HbA1c concentrations and normal fasting blood sugar could be ascribed to abnormal concentrations of haemoglobins Hb Takamatsu and Hb G-Szuhu. Among the methods available for HbA1c determination, HPLC with a cation exchange column is widely used as the standard method. The haemoglobins (Hb Takamatsu and Hb G-Szuhu) from our patients caused falsely low HbA1c concentrations as estimated by HPLC. In HPLC, the peak of Hb Takamatsu and Hb G-Szuhu is included in the HbA1c peak calculation, leading to falsely low HbA1c results. In Hb Takamatsu, lysine at position 120 of the β-chain is substituted for glutamine, while in Hb G-Szuhu, asparagine at position 80 of the β-chain is substituted for lysine. However, these haemoglobinopathies disturb neither the oxygenation/deoxygenation function, nor the stability of the haemoglobin molecule because these mutations do not disturb neither the heme contacts or αβ1 contacts. In fact, one carrier of Hb Takamatsu complained of dyspnea on exertion because of mitral valvular disease, but the other was asymptomatic and showed no haematological abnormalities. The male patient with Hb G-Szuhu in our study suffered from cardiac failure as a result of aortic and mitral regurgitation. However, these valve dysfunctions were not considered to be symptoms of this haemoglobinopathy, although the patient described by Imai et al showed mild polycythaemia. Therefore, both Hb Takamatsu and Hb G-Szuhu are considered to be asymptomatic haemoglobin variants.

Hb Takamatsu was first reported by Iuchi et al in 1980. The subject was a 38 year old woman who underwent a surgical operation for uterine cancer in 1979, and by 1987 16 families with Hb Takamatsu had been described in Japan. Hb G-Szuhu was first reported by Blackwell et al in 1969 and 24 cases of Hb G-Szuhu had been found in Japan by 1996 (T Harano, 1996, personal communication). Over 700 abnormal haemoglobins have been described throughout the world. One fifth of the haemoglobinopathies show symptoms such as cyanosis, polycythaemia, haemolysis and/or anaemia, whereas the remaining four fifths are asymptomatic. Most of the cases are accompanied by changes in electric charge because of the substitution of amino acids, resulting in an abnormal HPLC elution pattern. Hb Takamatsu and Hb G-Szuhu are not the only haemoglobinopathies that result in falsely low HbA1c concentrations. Others include HbC and HbS, as well as HbO...
Abnormal haemoglobins detected during HbA1c analysis

Abnormal haemoglobins detected during HbA1c analysis, haemoglobinopathy is not rare in the elderly. In HbA1c concentration is seen because silent parity between normal fasting blood sugar and should be taken into consideration when a dis-diabetes whose expected HbA1c concentration lent technical assistance.

The authors are grateful to Ms Asako Yamamoto for her excel-

HbA1c measurement routinely. Therefore, HbA1c measurement by HPLC can serve as an

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