

# Abnormal haemoglobins, Hb Takamatsu and Hb G-Szuhu, detected during the analysis of glycosylated haemoglobin (HbA<sub>1c</sub>) by high performance liquid chromatography

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## Abstract

**Background**—During medical checkups of two unrelated female outpatients during their annual health examination and one male inpatient suffering from cardiac failure the glycosylated haemoglobin (HbA<sub>1c</sub>) concentrations measured by high performance liquid chromatography (HPLC) were low, in spite of normal fasting plasma glucose concentrations. However, HbA<sub>1c</sub> concentrations measured by latex immunoagglutination 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 HbA<sub>1a+b</sub>, which resulted in falsely low HbA<sub>1c</sub> concentrations. Isoelectric focusing analysis of the patients' haemoglobin disclosed abnormal haemoglobins, which migrated faster than normal HbA<sub>1</sub> in the two female patients and slower in the male patient. The cDNA sequence and amino acid analyses of the haemoglobin  $\alpha$ -chains and  $\beta$ -chains indicated the presence of the haemoglobin variant  $\beta$  120 Lys→Gln in the two female patients and  $\beta$  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 HbA<sub>1c</sub> concentration readings when using HPLC.

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

Glycosylated haemoglobin (HbA<sub>1c</sub>) has been shown to be increased in diabetes mellitus<sup>1</sup> and is widely used as a marker for long term glycaemic status. Analysis of HbA<sub>1c</sub> is performed by high performance liquid chromatography (HPLC), electrophoresis, immunoassay, and colorimetry, as well as other methods. Among them, HPLC is generally used as the standard method for the determination of HbA<sub>1c</sub> concentrations.<sup>2,3</sup> However, the HbA<sub>1c</sub> concentration is affected by several factors other than blood glucose concentrations, such as uraemia,<sup>4</sup> alcohol abuse,<sup>5</sup> high dose aspirin intake,<sup>6</sup> hyperbilirubinaemia,<sup>7</sup> and the decreased life span of erythrocytes<sup>8</sup> (massive

bleeding, haemolytic anaemia, and anaemia in pregnancy). Moreover, several kinds of haemoglobinopathy are known to result in falsely high or low HbA<sub>1c</sub> values when measured by means of HPLC. In this report, the detection of two abnormal haemoglobins, Hb Takamatsu ( $\beta$ 120 Lys→Gln) and Hb G-Szuhu ( $\beta$ 80 (EF4) Asn→Lys), during HPLC analysis for HbA<sub>1c</sub> 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 (HbA<sub>1c</sub> analyser, Model HA-8110; Daiichi, Kyoto, Japan; HbA<sub>1c</sub> reference range, 4.0–5.4%, % coefficient of variation (%CV),  $\pm$  0.2%) with a cation exchange column was used for the measurement of the HbA<sub>1c</sub> 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.*<sup>9</sup>

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 extraction<sup>10</sup> 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 1 Primers used in direct sequencing of Hb cDNA

<i><math>\alpha</math>-chain</i>	
Forward 5'	> ACT CTT CTG GTC CCC ACA GA < 3'
Reverse 5'	> TTC AAA GAC CAG GAA GGG CC < 3'
<i><math>\beta</math>-chain</i>	
Forward 5'	> GCT TCT GAC ACA ACT GTG < 3'
Reverse 5'	> CCC CAG TTT AGT AGT TGG AC < 3'

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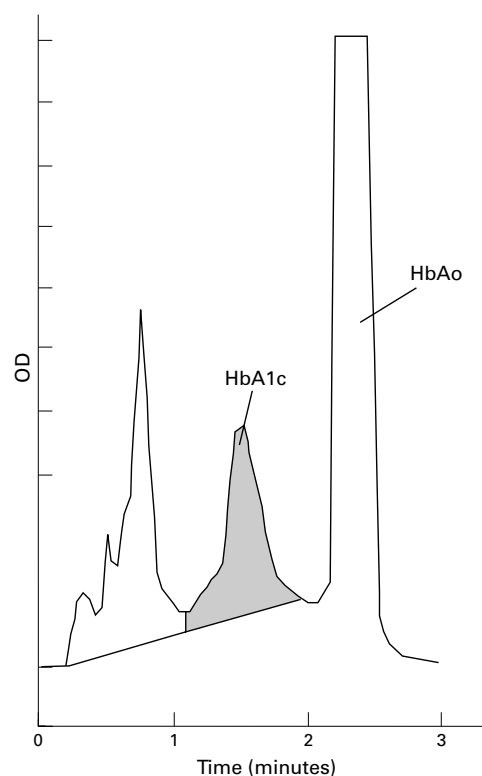


Figure 1 High performance liquid chromatography (HPLC) chromatogram of erythrocyte haemolysates of Hb Takamatsu (patient KM). An additional peak near HbA<sub>1c</sub> caused an underestimation of HbA<sub>1c</sub> by this method.

PCR was carried out in a volume of 25 µl containing 50 mM KCl, 10 mM Tris/HCl (pH 8.8), 1.5 mM MgCl<sub>2</sub>, 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 glycosylated haemoglobin were low: 1.2% in KM, 0.9% in TY, and 2.7% in ES.

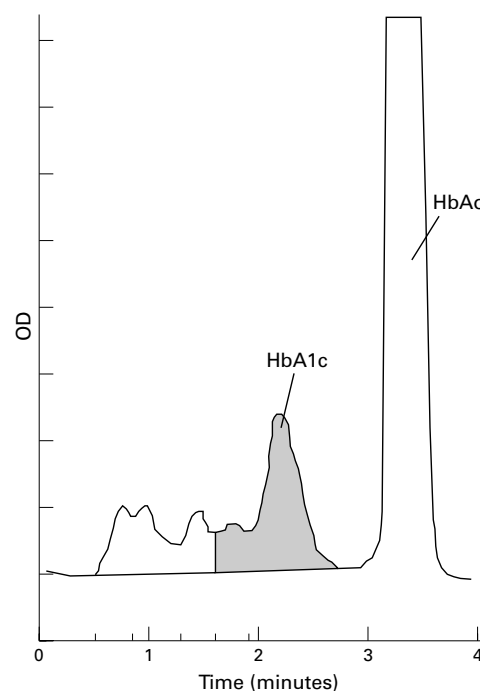


Figure 2 High performance liquid chromatography (HPLC) chromatogram of erythrocyte haemolysates of Hb G-Szuhu (patient ES). An additional peak near HbA<sub>1c</sub> caused an underestimation of HbA<sub>1c</sub> by this method.

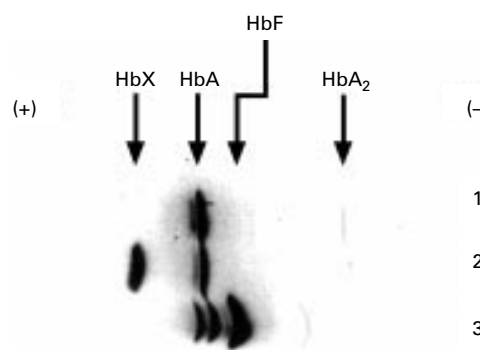


Figure 3 Isoelectric focusing gel electrophoresis of a control subject, cord blood, and the patient with Hb Takamatsu. Lane 1, electrophoretic pattern of control subject; lane 2, electrophoretic pattern of Hb Takamatsu; lane 3, electrophoretic pattern of cord blood. Abnormal haemoglobin migrating faster than HbA<sub>1</sub> is indicated by HbX.

HbA<sub>1c</sub> concentrations measured by latex immunoagglutination 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 HbA<sub>1c</sub> (fig 1, Hb Takamatsu; fig 2, Hb G-Szuhu). Isoelectric focusing gel electrophoresis demonstrated an additional haemoglobin migrating faster than HbA<sub>1</sub> in KM and TY (fig 3), and an additional haemoglobin migrating slower than HbA<sub>1</sub> in ES (fig 4). Abnormal haemoglobin accounted for 43% (KM), 45% (TY), and 44% (ES) of the total haemoglobin. cDNA sequence and amino acid analyses of the α-chain and β-chain of the patients' haemoglobin disclosed that lysine at position 120 of the β-chain was replaced by glutamine in KM and TY (fig 5), and asparagine at position 80 of the β-chain was replaced

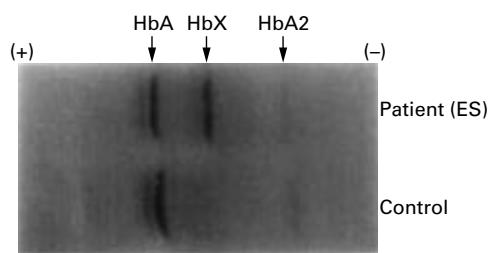


Figure 4 Isoelectric focusing gel electrophoresis of a control subject and the patient with Hb G-Szuhu. Abnormal haemoglobin migrating slower than HbA<sub>1</sub> is indicated by HbX.

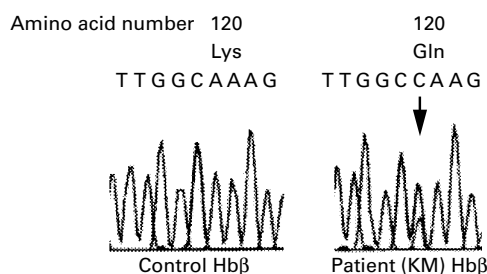


Figure 5 Mutation of amino acid sequence of the Hb $\beta$  gene at position 120 obtained by means of an ABI PRISM sequencer. The arrow indicates the first nucleotide of the codons for amino acid 120.

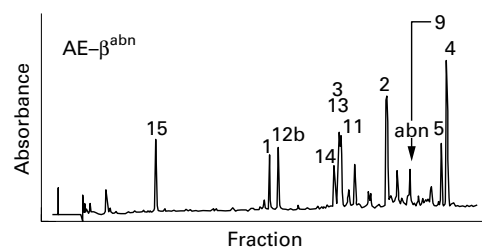


Figure 6 Elution profile of the tryptic digests of aminoethyl abnormal haemoglobin  $\beta$  of the patient ES (Hb G-Szuhu) by reversed phase high performance liquid chromatography (HPLC).

by lysine in ES (fig 6; table 2), confirming the presence of the abnormal haemoglobins, Hb Takamatsu<sup>11</sup> and Hb G-Szuhu,<sup>12</sup> respectively.

### 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 HbA<sub>1c</sub> determination.

HbA<sub>1c</sub> 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 uraemia,<sup>4</sup> alcohol abuse,<sup>5</sup> high dose aspirin intake,<sup>6</sup> and massive bleeding, as well as various kinds of haemoglobinopathies. Uraemia, alcohol abuse, and high dose aspirin intake cause falsely high HbA<sub>1c</sub> concentrations, whereas bleeding, haemolytic anaemia, and pregnancy cause falsely low concentrations. However, in the present cases, the factors that typically cause low HbA<sub>1c</sub> concentrations were excluded, and it was shown that the disparity between low HbA<sub>1c</sub> concentration and normal fasting blood sugar could be ascribed to abnormal concentrations of haemoglobins Hb Takamatsu and Hb G-Szuhu. Among the methods available for HbA<sub>1c</sub> 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 HbA<sub>1c</sub> concentrations as estimated by HPLC. In HPLC, the peak of Hb Takamatsu and Hb G-Szuhu is included in the Hb<sub>1a+b</sub> peak calculation, leading to falsely low HbA<sub>1c</sub> results. In Hb Takamatsu, lysine at position 120 of the  $\beta$ -chain is substituted for glutamine, while in Hb G-Szuhu, asparagine at position of 80 of the  $\beta$ -chain is substituted for lysine. However, these haemoglobinopathies disturb neither the oxygenation/deoxygenation function, nor the stability of the haemoglobin molecule<sup>11</sup> because these mutations do not affect heme contacts or  $\alpha_1\beta_1$  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.<sup>13</sup> 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.<sup>11</sup> 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,<sup>12</sup> 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.<sup>14</sup> One fifth of the haemoglobinopathies show symptoms such as cyanosis,<sup>15,16</sup> polycythaemia,<sup>17</sup> haemolysis and/or anaemia,<sup>18-20</sup> 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 HbA<sub>1c</sub> concentrations. Others include HbC and HbS, as well as HbO

Table 2 Amino acid composition of abnormal peptide and amino acid sequence of the Tp-9 peptide

Amino acid	Analytical value (molar ratio)	Theoretical value (Tp-9)
Asp	1.90?	3
Ser	1.09	1
Gly	2.08	2
Ala	1.98	2
Val	1.06	1
Leu	2.93?	4
Phe	0.93	1
Lys	1.04	1
His	0.92	1

Normal Tp-9: Val-Leu-Gly-Ala-Phe-Ser-Asp-Gly-Leu-Ala-His-Leu-Asp-Asn-Leu-Lys  
 Abnormal Tp-9: ↓ Lys

Padova,<sup>21</sup> Hb Himeji,<sup>22</sup> Hb Camden,<sup>23</sup> Hb Riyadh,<sup>24</sup> Hb J-Meerut,<sup>25</sup> Hb Sherwood Forest,<sup>26</sup> Hb Manitoba, and Hb G-Coushatta.<sup>27</sup> Thus, abnormal haemoglobin should be taken into consideration when a disparity between normal fasting blood sugar and HbA<sub>1c</sub> concentration is seen because silent haemoglobinopathy is not rare in the elderly. In addition, it seems necessary to investigate HbA<sub>1c</sub> concentrations by other methods, such as latex immunoagglutination and isoelectric focusing gel electrophoresis, in cases showing discrepant HbA<sub>1c</sub> and blood sugar concentrations,<sup>28</sup> especially in patients with diabetes whose expected HbA<sub>1c</sub> concentration is disproportionately low compared with their blood sugar concentrations, to avoid misinterpretation of the glycaemic state.

Some medical checkups in Japan include HbA<sub>1c</sub> measurement routinely. Therefore, HbA<sub>1c</sub> measurement by HPLC can serve as an aid to detecting some additional silent haemoglobinopathies.

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