Detection of hemoglobin variant HbS on the basis of discrepant HbA1c values in different measurement methods

Yusuke Takeda¹ · Daiji Kawanami¹ · Kazunori Utsunomiya¹

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Abstract Glycated hemoglobin (HbA1c) is commonly used to assess long-term glycemic control in patients with diabetes mellitus. Numerous conditions, including hemoglobinopathies, can alter HbA1c measurements and cause misleading results. More than 20 methods for determining HbA1c are commercially available to clinical laboratories. Herein, we report a diabetic patient in whom the HbS variant was detected on the basis of discrepant HbA1c levels estimated using immunonephelometry or high-performance liquid chromatography (HPLC). The patient, a 48-year-old African man with a 10-year history of type 2 diabetes, was referred to our hospital with an HbA1c level estimated at 13.3 % by immunonephelometry and 7.6 % by HPLC, whereas the glycoalbumin level was 47.5 %. These discrepancies prompted us to carry out genetic sequence analysis in which we identified an A → T transversion in codon 6 of the patient's *HBB* gene, corresponding to a predicted E6V substitution (βCD6) characteristic of HbS. Our results indicate that redundant measurements of HbA1c using diverse methods may be useful when the presence of abnormal Hb is suspected.

Keywords Abnormal hemoglobin · HbS · Glycoalbumin · HbA1c · HPLC · Immunonephelometry

Introduction

Levels of hemoglobin A1c (HbA1c) are a retrospective indicator of glycemic status over a 1–2 month period and, as such, are used to evaluate long-term glycemic control in patients with diabetes mellitus [1]. HbA1c is characterized by irreversible nonenzymatic glycation at the NH₂ moieties of valine residues in the hemoglobin (Hb) β chain, encoded by the *HBB* gene [2, 3]. The accuracy of HbA1c measurement as a marker of hyperglycemia can be affected adversely by the presence of Hb variants that reduce red blood cell viability [3–5]. These variants are occasionally detected on the basis of discrepancies between actual plasma glucose levels and HbA1c values that arise due to a reduction in exposure of Hb to plasma glucose. Moreover, the altered electrophoretic mobility of certain Hb variants can give rise to artifacts in the measurement of HbA1c using high-performance liquid chromatography (HPLC) [3, 5–9]. Here we report an African patient with diabetes in whom an Hb variant was incidentally detected on the basis of a discrepancy between HbA1c values measured by HPLC and immunonephelometry.

Case report

A 48-year-old Nigerian man with a 10-year history of type 2 diabetes was referred to our hospital for glycemic control. He had no evidence of family history of anemia or diabetes. Although he had been treated with metformin, pioglitazone, and glimepiride by his general practitioner since the age of 38 years, his glycemic control was poor, and his HbA1c had been maintained at around 10 %, as measured by immunonephelometry. His HbA1c level was measured at 13.3 % by immunonephelometry (CHM-4100,

Yusuke Takeda and Daiji Kawanami have equally contributed to this work.

✉ Daiji Kawanami
daijika@jikei.ac.jp

¹ Division of Diabetes, Metabolism and Endocrinology, Department of Internal Medicine, Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan

Nihon Kohden, Tokyo, Japan) in a visit to his general practitioner just prior to being referred to our hospital.

Table 1 shows the laboratory data on admission. His glycoalbumin (GA), measured enzymatically (JCA-BM2250, JEOL, Tokyo, Japan), was 47.5 % (normal range 12.4–16.3 %), indicating severe hyperglycemia, which was corroborated by a casual plasma glucose level of 536 mg/dl. In contrast, his HbA1c level, measured by HPLC (HLC-723 G7 analyzer, Toso, Tokyo, Japan), was 7.6 %. The mean corpuscular volume (MCV) of his red blood cells

was slightly below the normal range, although serum iron and ferritin were within normal ranges. Serum haptoglobin levels, which were evaluated to investigate the presence of hemolysis, were also within normal range. The difference between low HbA1c levels measured by HPLC and higher levels measured by immunonephelometry, along with GA value, strongly suggested the presence of an abnormal Hb species with altered electrophoretic mobility.

Figure 1 shows the HPLC elution pattern of Hb of a healthy individual and the patient in this case. The elution pattern of the patient's Hb contains a peak at ~40 min that was not present in that of the healthy individual. Similarly, isoelectric focusing indicates the presence of an abnormal band between HbA2 and HbA0 in the patient sample, which was not present in the healthy sample (Fig. 2), confirming the existence of the variant HbS species.

These results led us to analyze the *HBB* DNA sequence to identify the specific variant. Genetic testing was carried out with the consent of the patient in conformity with the Declaration of Helsinki. Sequence analysis (Fukuyama

Table 1 Laboratory data on admission

Variable analyzed	Results
Complete blood count	
WBC	$3.6 \times 10^3/\text{mm}^3$
RBC	$482 \times 10^4/\text{mm}^3$
Hb	13.2 g/dl
Ht	38.0 %
MCV	80.1 fL
MCH	27.0 pg
PLT	$13.3 \times 10^4/\text{mm}^3$
Chemistry	
TP	6.8 g/dl
Alb	2.6 g/dl
T-Bil	0.5 mg/dl
ALT	21 IU/l
AST	23 IU/l
LDH	274 IU/l
ALP	269 IU/l
CK	79 IU/l
γ -GTP	61 IU/l
AMY	62 IU/l
TC	244 mg/dl
TG	110 mg/dl
HDL-C	108 mg/dl
BUN	16.1 mg/dl
Cr	0.89 mg/dl
Na	135 mmol/l
K	4.3 mmol/l
Cl	103 mmol/l
FPG	536 mg/dl
HbA1c(HPLC)	7.6 %
Glycoalbumin	47.5 %
Urinalysis	
Protein	(–)
Glucose	(4+)
Protein	(–)
Ketone bodies	(–)
Others	
Urinary CPR	59.4 $\mu\text{g/day}$

a Normal subject

Fig. 2 Detection of abnormal hemoglobin by isoelectric focusing. In this case, an abnormal band was found between HbA2 and HbA0, corresponding to HbS (arrow). We first generated the hemolysate, then mixed it with potassium cyanide (KCN) solution, followed by gel electrophoresis (4 h at 10 °C). Coomassie brilliant blue (CBB) staining was then performed to detect the abnormal band

Rinsho, Co. Ltd, Hiroshima, Japan) of the patient's *HBB* gene identified an A → T transversion in codon 6 (Fig. 3), giving rise to a predicted V → A substitution characteristic of HbS.

Discussion

Here we report an African diabetic patient in whom an Hb variant was detected on the basis of discrepant HbA1c values measured by immunonephelometry and HPLC. To date, four basic methods are available to measure HbA1c: immunoassay (immunonephelometry), ion-exchange HPLC, boronate affinity chromatography, and enzymatic assay [3]. Methods for detecting HbA1c are subject to interference by, for example, chemical structure and/or

Fig. 3 Sequence analysis of the *HBB* gene. Identification of nucleotide substitutions through sequencing of amplified DNA of exon 3 of the *HBB* gene from this case. Codon 6 shows a GAG → GTG substitution in the sense strand

molecular charge, potentially resulting in inaccurate HbA1c measurements [5–8]. HPLC separates Hb species based on charge differences. *HBB* mutations in certain hemoglobinopathies give rise to changes in the Hb β amino acid sequence that result in an altered charge state and in turn to incorrect measurements of HbA1c by HPLC [9]. Immunonephelometry measures inhibition of latex agglutination by antibodies that recognize the N-terminal glycosylated amino acids [3]. First-generation antibodies recognize amino acids 4–10 on the β chain of Hb, resulting in analytic interference by the globally prevalent HbS and HbC chains, in addition to other Hb variants that may span this epitope [3]. This interference prompted the development of second- and third-generation antibodies that recognize the extreme N-terminal Hb β chain amino acids [3]. These antibodies are believed to bind HbS, providing analytically accurate HbA1c values in patients with HbS trait and other heterozygous Hb variant conditions in which the RBC lifespan may be normal [3]. For this reason, results obtained from immunoassay and HPLC can be different. Boronate affinity is a structurally specific method that recognizes the *cis*-diol groups of glucose bound to Hb [3]. In this method, affinity separation of glycated Hb is performed by selecting the *cis*-diol groups of glycated Hb bound with a boronate affinity resin. This method provides for chromatographic separation of glycated and nonglycated Hb [3]. For enzymatic assay, whole-blood samples are lysed and digested by extensive proteolysis, resulting in the release of N-terminal amino acids from the Hb β chains of glycated Hb. The signal produced by glycated valines in the subsequent chromogenic reaction is used to calculate HbA1c [3]. Although neither boronate affinity chromatography nor enzymatic assays are affected by Hb variants, they are not designed to discern the presence of Hb variants [3].

An increasing number of hemoglobinopathies cause false results in glycated Hb determinations due to hemolysis or altered detectability in glycated Hb assays. While most factors altering HbA1c measurements are likely to be associated with clinical suspicion, most hemoglobinopathies are clinically silent [10]. In the case reported here, discrepancies between measurements of HbA1c by HPLC and immunonephelometry prompted us to pursue further investigations.

In most adults, Hb comprises four polypeptide chains—two α and two β—each of which binds one heme group [11]. More than 700 Hb variants have been identified worldwide [8], the most common being HbS, HbE, HbC, and HbD [5]. In Japan alone, 200 Hb variants have been characterized, 31.9 % of which involve abnormalities in α globin, and 65.5 % in β globin [12]. Many Hb variants worldwide have single amino acid substitutions in the β chain [5].

HbS is commonly found in equatorial Africa and eastern Saudi Arabia [13] and has an overall prevalence in the USA of $\sim 1:5000$. Most cases in the USA are in American people of Sub-Saharan-African descent, in whom the prevalence is about 1:500 [14]. In contrast, Hb variants are rare in Japan. The prevalence of hemoglobinopathies in the general population of Japan is estimated to be $\sim 1:2350$ [15]. Similar to our case, Harano et al. reported a case of HbS an African individual living in Japan [15].

HbS results from a substitution of a glutamic acid at position 6 in the β chain, which was the exact amino acid substitution in the case reported here. Persons carrying a single copy (heterozygous) of the *HBB* S variant (HbAS) are described as having sickle cell trait; those with two defective copies (homozygous) of the gene (HbSS) have sickle cell disease. Sickle cell disease is characterized by the formation of long, inflexible chains of Hb that cause RBCs to be retarded in small capillaries, giving rise to significant organ damage. Dosage of the S variant is associated with anemia due to its impact on RBC life span, which ranges from ~ 120 days for HbAA to ~ 93 days for HbAS [16] and ~ 10 – 20 days for HbSS.

Although most Hb variants are clinically silent, some cause biochemical aberrations that can falsely increase or decrease HbA1c levels, depending on the method of assay and the type of Hb variant [5]. Moreover, the degree of these effects varies according to the measuring device. The effects of Hb variants on HbA1c values determined by HPLC have been evaluated previously [17]. While an abnormal peak in HPLC suggests the existence of Hb variants, on occasion, the peak cannot be detected. When the presence of Hb variants cannot be ruled out, it is important to compare measurements of HbA1c levels obtained using different methods.

Two large prospective randomized clinical trials—the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS)—demonstrated that risks for diabetic complications are strongly related to HbA1c [5, 18]. Therefore, many diabetes organization worldwide recommend specific HbA1c targets in their guidelines. As such, the current situations is of significant concern in patients with Hb variants. Although other glycated proteins such as GA and fructosamine are available, their clinical utility has not been established compared with HbA1c, and there is no convincing evidence that relates their values to diabetic complications [18]. Even in countries with elevated prevalence of Hb variants, such as the USA, the problem of assessing glycemic control by measuring HbA1c in patients with Hb variants has not been solved [18]. When unexpected results are obtained, it is recommended that additional HbA1c methods be used that are less likely to encounter interference [5]. It is suggested that laboratories serving patient

populations with a high prevalence of Hb may consider to: (1) establish algorithms in order to help determine an appropriate method for HbA1c measurement based on medical history [3, 19]; and (2) implement laboratory processes that can identify analytic interferences in such populations [3, 5]. A report from Ireland shows that HbA1c interference by Hb variants is a potential global concern due to the increasing numbers of emigrants from Africa and Southeast Asia, countries with a high prevalence of Hb variants [20]. Future studies are required to establish guidelines and algorithms regarding HbA1c measurement methods in patients with Hb variants.

In this case, although the accuracy of HbA1c values by immunonephelometry remains unclear, this measurement method appears to be relatively accurate compared with HPLC. That said, it is an inappropriate indicator for glycemic control in this situation. To obtain accurate glycemic control in this situation, we will use GA, and fructosamine that are alternative indicators of glycemic control in patients with Hb variants [21].

In most cases, abnormal Hb should be considered in patients in whom discrepancies between levels of HbA1c and glycoalbumin are detected. It should be borne in mind, however, that different measurements of HbA1c can lead to diagnosis of abnormal Hb.

Compliance with Ethical Standards

Human Rights Statement and Informed Consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Declaration of Helsinki, 1964, and later versions. Informed consent was obtained for being included in the study.

Conflict of interest The authors declare that they have no conflicts of interest.

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