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CASE REPORT

A case of variant hemoglobin (Hb Agenogi) with type 2 diabetes mellitus showed high HbA1c levels measured by immunoassay due to enhanced antigenicity

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Abstract
HbA1c is a widely utilized biomarker for the management of diabetes mellitus. The presence of variant hemoglobins might interfere with HbA1c measurement using different methods. We herein describe Hb Agenogi (β90Glu → Lys) with type 2 diabetes mellitus whose HbA1c measured by immunoassay (IA) showed falsely high levels while HbA1c measured by high-performance liquid chromatography (HPLC) in the standard mode (SM) showed falsely low levels. To clarify the cause of the falsely high-IA-HbA1c levels, HbA1c was measured by various methods. Glycated albumin was slightly higher than the reference range. HbA1c measured by HPLC in the variant mode, affinity assay and enzymatic assay showed the range (mean ± 2SD: 6.0–6.8%) in this case. However, HbA1c measured by several HPLC-SMs showed falsely low levels (4.1–4.4%). IA-HbA1c using an antibody manufactured by Fujirebio Inc. showed falsely high levels (7.3–8.2%); whereas, IA-HbA1c using an antibody manufactured by Roche Ltd. showed the expected range (6.2–6.5%). In the case of Hb Agenogi, IA-HbA1c using an antibody manufactured by Fujirebio yielded falsely high levels. The mutation at codon 90 of the β-globin chain might enhance antigenicity of the N-terminal peptide region and, therefore, lead to falsely high-HbA1c levels in IA-HbA1c.

Keywords Variant hemoglobin · Hb Agenogi · HbA1c · Immunoassay

Introduction

Currently, HbA1c is widely used for the target value of glycemic control or for diagnosis of diabetes mellitus, the gold standard indicator of glycemic control [1], but there are both biological and analytical interference. It is known that inaccurate HbA1c values are observed in various hematologic diseases and that HbA1c does not accurately reflect glycemic control in such diseases [2]. Variant hemoglobin is one of the several conditions with inaccurate HbA1c values [3].

To date, more than 1300 types of variant hemoglobin have been reported [4]. Among hematologic diseases such as anemia and polycythemia, variant hemoglobin was originally found as a disease caused by a mutation of globin gene. Increasing numbers of subjects are being detected with variant hemoglobin that is associated with inaccurate HbA1c values; however, most such subjects are asymptomatic [3, 5, 6]. Inaccurate HbA1c values observed in subjects with variant hemoglobin are mostly due to a difference in mobility in high-performance liquid chromatography (HPLC). Therefore, if HbA1c is measured by immunoassay, correct values may be obtained in the most variant hemoglobin. However, when variant hemoglobin is accompanied by unstable hemoglobin, abnormal glycation, or abnormal antigenicity, inaccurate HbA1c results are also observed when they are measured by immunoassay [3, 5, 7, 8].

We herein describe a case of variant hemoglobin with type 2 diabetes mellitus showing falsely high-HbA1c levels measured by an immunoassay (IA-HbA1c) using Determiner L HbA1c (Kyowa Medex, Co., Tokyo, Japan). IA-HbA1c value in this case was higher than HbA1c estimated by the average plasma glucose suggesting the presence of a falsely

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high value shown in the Results section. To determine the cause of the falsely high-IA-HbA1c levels, we measured the HbA1c levels in this case by various methods.

Patient and methods

Patient

The patient is a 77-year-old male. In 2010, he visited Yamane Hospital because he was pointed out 130 mg/dl of fasting plasma glucose (FPG) and 7.8% of immunoassay (IA)-HbA1c in health checkup. He was diagnosed as type 2 diabetes mellitus and improved his lifestyle by diet therapy and exercise therapy. Although glycemic control appeared to improve based on the lower random plasma glucose and the glycated albumin results, HbA1c results among different methods were discordant.

Laboratory analysis

PCR amplification was performed with DNA extracted from white blood cells for sequencing all α1-, α2-, and β-chain exons in the globin gene by direct sequencing assay.

HbA1c was measured by the following systems and reagents (Table 1). HA-8170, HA-8181 (Arkray, Inc., Kyoto, Japan), HLC-723G8, HLC-723G9, HLC-723G11 (Tosoh, Co., Tokyo, Japan), and Rapid column A1c (Sekisui Medical, Co., Tokyo, Japan) were used for HPLC assay in standard mode (SM). HA-8180T (Arkray Inc., Kyoto, Japan), HLC-723G8, HLC-723GX (Tosoh, Co., Tokyo, Japan), and the Variant II Turbo system (Bio-Rad Laboratories, Inc., CA, USA) were used for HPLC assay in variant mode (VM). Determiner L HbA1c (Kyowa Medex, Co., Tokyo, Japan), Rapidia Auto HbA1c (Fujirebio, Inc., Tokyo, Japan), Banalyst HbA1c (Arkray, Inc., Kyoto, Japan), Celltac Chemi (Nihon Kohden, Co., Tokyo, Japan), Cobas Reagent HbA1c III (Roche Diagnostics, K.K., Tokyo, Japan), and Vitros chemistry products HbA1c reagent kit (Ortho Clinical Diagnostics, Inc., NJ, USA) were used for immunoassay. Antibodies in the former 4 kits (Determiner L HbA1c, Rapidia Auto HbA1c, Banalyst HbA1c, and Celltac Chemi) and the latter 2 kits (Cobas Reagent HbA1c III and Vitros chemistry products HbA1c reagent kit) were manufactured by Fujirebio Inc. and Roche K.K., respectively. Norudia N HbA1c (Sekisui Medical Co., Tokyo, Japan), CinQ HbA1c (Arkray Inc., Kyoto, Japan), and Metabolead HbA1c (Kyowa Medex, Co., Tokyo, Japan) were used for enzymatic assay. HLC-723G8 in affinity mode (Tosoh, Co., Tokyo, Japan) was used for affinity assay. HbA1c values were subsequently converted to National Glycohemoglobin Standardization

Table 1 HbA1c measurement methods, manufacturers, kits (instruments) and HbA1c value of Hb Agenogi

Method type	Mode or manufacturer of antibody	Company	Product name	HbA1c (%)
HPLC	Standard mode	Arkray Inc.	HA-8181	4.4
HPLC	Standard mode	Arkray Inc.	HA-8170	4.2
HPLC	Standard mode	Tosoh Co.	HLC-723G8	4.1
HPLC	Standard mode	Tosoh Co.	HLC-723G9	4.1
HPLC	Standard mode	Tosoh Co.	HLC-723G11	4.0
HPLC	Standard mode	Sekisui Medical Co.	Rapid column A1c	4.4
HPLC	Variant mode	Arkray Inc.	HA-8180T	6.2
HPLC	Variant mode	Tosoh Co.	HLC-723G8	6.6
HPLC	Variant mode	Tosoh Co.	HLC-723GX	6.5
HPLC	Variant mode	Bio-Rad Laboratories Inc.	Variant II Turbo	6.3
Immunoassay	Fujirebio	Kyowa Medex Co.	Determiner L HbA1c	8.2
Immunoassay	Fujirebio	Fujirebio Inc.	Rapidia Auto HbA1c-L	7.8
Immunoassay	Fujirebio	Arkray Inc.	Banalyst Ace HbA1c	7.3
Immunoassay	Fujirebio	Nihon Kohden Co.	Celltac Chemi	7.5
Immunoassay	Roche	Roche Diagnostics K.K.	Cobas Reagent HbA1c III	6.2
Immunoassay	Roche	Ortho Clinical Diagnostics Inc.	Vitros chemistry products HbA1c reagent kit	6.5
Enzymatic assay	–	Sekisui Medical Co.	Norudia N HbA1c	6.4
Enzymatic assay	–	Arkray Inc.	CinQ HbA1c	6.7
Enzymatic assay	–	Kyowa Medex Co.	Metabolead HbA1c	6.4
Affinity assay	Affinity mode	Tosoh Co.	HLC-723G8	6.7

Program (NGSP) equivalent values in compliance with the official equation [9].

Ethical approval to perform the aforementioned tests was obtained from the Ethical Committee of Yamane Hospital (date of approval at 20th June 2016; approval no. Ya-201660A). The patient received a sufficient explanation about the significance and method of these analyses including globin gene analysis and the measurement of HbA1c by various methods before agreeing to sign the consent form.

Results

In the clinical course of this case, a falsely high value of IA-HbA1c was suggested. Therefore, diurnal fluctuations of plasma glucose levels (measured 7 times a day; before and 2 h after every meal as well as at bedtime) were examined and the average plasma glucose level was 158 mg/dl. The HbA1c levels estimated from the average plasma glucose level by the Nathan's formula [estimated HbA1c (%) = mean plasma glucose (mg/dl) + 46.7/28.7] [10] was 7.1%. IA-HbA1c at that time was 8.1%. This finding suggested falsely high-IA-HbA1c levels in this case.

Variant hemoglobin was strongly suspected from the dissociation between IA-HbA1c and HPLC-SM-HbA1c in this case. Analysis of the globin gene revealed a heterozygous mutation at β codon 90 of Glu (GAG) → Lys (AAG) and the patient was diagnosed with Hb Agenogi [11].

When HbA1c was measured by various methods, glycated albumin level was 20.4% (the reference range: 11–16%). HbA1c levels measured using HPLC-VM (HPLC-VM-HbA1c) (HA-8180T, HLC-723G8, HLC-723GX, and the Variant II Turbo) were 6.2–6.6% (Table 1). Although abnormal peak was not observed in HPLC-SM (HLC-723G8) chromatogram (Fig. 1b), abnormal peak of variant hemoglobin (HbX0) was observed later than HbA0 in HPLC-VM (HLC-723GX) chromatogram (Fig. 1d). As Hb Agenogi is slow-moving variant hemoglobin [11], HPLC-VM-HbA1c is more likely to produce a correct value. HPLC-VM-HbA1c measured using 4 different instruments was 6.4 ± 0.2% (mean ± SD). The expected range for HbA1c values in this case was set to 6.0–6.8% (mean ± 2SD), and values less than 6.0% and more than 6.8% were considered to be falsely low and falsely high, respectively. HbA1c values measured by enzymatic assay (EA-HbA1c) and affinity assay (Af-HbA1c) were found to be 6.4–6.7% and 6.7%, respectively, both being the expected range. HPLC-SM-HbA1c was found to be falsely low (4.0–4.4%). Due to difference in antibodies utilized, IA-HbA1c levels showed falsely high (7.3–8.2%) when an antibody manufactured by Fujirebio was used (Determiner L HbA1c, Rapidia Auto HbA1c, Banalyst HbA1c and Celltac Chemi) and showed the expected range (6.0–6.8%) when an antibody manufactured by Roche was used (Cobas Reagent HbA1c III and BistroMicrochip HbA1c).

Discussion

Falsely high-immunoassay results with methods using the Fujirebio antibody and falsely low HPLC in standard mode were observed in a case of Hb Agenogi. Hb Agenogi is asymptomatic variant hemoglobin which was first reported in 1966 by Miyaji et al. [11]. They already reported that Hb Agenogi is slow-moving variant hemoglobin. However, HbA1c value in Hb Agenogi has not been reported until now. Most cases of variant hemoglobin show falsely low-HPLC-SM-HbA1c levels and normal IA-HbA1c levels.

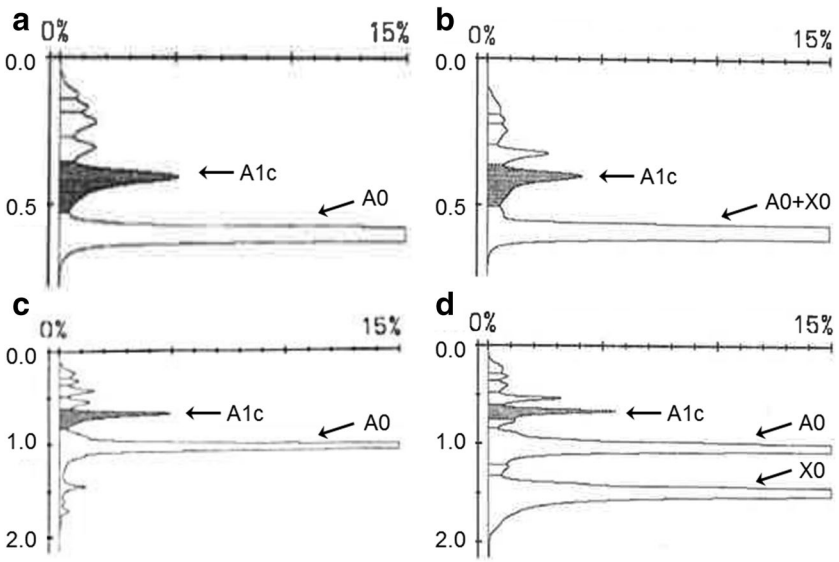

Fig. 1 Standard versus variant mode HPLC chromatograms: **a** HPLC-SM, HLC-723G9, control, **b** HPLC-SM, HLC-723G9, Hb Agenogi, **c** HPLC-VM, HLC-723GX, control, **d** HPLC-VM, HLC-723GX, Hb Agenogi

To elucidate the mechanism that underlies falsely high-IA-HbA1c values in a case of Hb Agenogi, various HbA1c measurements were performed on the same specimen. EA-HbA1c and IA-HbA1c using an antibody manufactured by Roche showed the expected results in this case; whereas, IA-HbA1c using an antibody manufactured by Fujirebio showed falsely high. These results lead to the hypothesis that the mutation at the 90th amino acid position of the β-globin chain modified the antigenicity of the N-terminal peptide resulting in falsely high-IA-HbA1c values.

Enhanced glycation or enhanced antigenicity is potential mechanism underlying falsely high-IA-HbA1c values. The former would lead EA-HbA1c values and Af-HbA1c values to be similarly influenced and the later would only affect IA-HbA1c values. In this case, only IA-HbA1c values were observed to be falsely high making the enhanced antigenicity hypothesis more likely. Enzymatic assay depend on enzymatic reactions after cleavage at the 2nd and 3rd amino acid position; therefore, mutations affecting amino acids after the third position would not be expected to affect EA-HbA1c values. Consequently, EA-HbA1c values were within the expected range in this case.

For most variant hemoglobin, IA-HbA1c and EA-HbA1c values provide similar results. An examination of the literature reveals IA-HbA1c and EA-HbA1c values to be different exclusively in the variant hemoglobin Hb Himeji (β140Ala → Asp) [7] and HbC (β6Glu → Lys) [8]. Hb Himeji has been reported as variant hemoglobin with enhanced glycation [12]. Consequently, both IA-HbA1c and EA-HbA1c values should theoretically be identically elevated; however, the authors found that the results that were higher using IA in cases of Hb Himeji [7]. HbA1c values measured by the IFCC method results agreeing with EA-HbA1c and IA-HbA1c values were found to be comparatively high. This indicates that Hb Himeji enhances not only glycation, but also antigenicity [7].

Adult hemoglobin has a tetramer structure composed of two α-subunits and two β-subunits. The α-subunit consists of 141 amino acids and the β-subunit consists of 146 amino acids. Hb Agenogi has a mutation at the 90th amino acid of the β-globin chain, and the 90th amino acid is close enough to the N-terminal peptide [13] to affect results using some IA methods, depending on the specificity of the antibody. The substitution at the 90th amino acid position appears to enhance, therefore, presumed to enhance the antigenicity of the N-terminal peptide. Accurate IA-HbA1c levels using antibodies manufactured by Roche hint that the antigenicity in the peptide region proximal to the 6th position has possibly increased.

Diagnostic guidelines in Europe and the United State recently allow a diagnosis of diabetes mellitus based solely on HbA1c. However, the susceptibility of HbA1c measurements to falsely high values creates a risk of non-diabetic subjects being prescribed diabetes medication [1]. Moreover, even in cases of diabetes, using HbA1c to guide glycemic control might lead to over treatment and subsequent iatrogenic hypoglycemia. Diagnostic guidelines of diabetes mellitus in Japan do not permit a diabetes diagnosis based solely on HbA1c values and it is essential to recognize hyperglycemia of fasting plasma glucose, 75 g OGTT 2-h, or casual plasma glucose [14]. However, there exist reports of falsely elevated HbA1c values leading the prescription of oral diabetes medication in non-diabetic subjects in Japan [8, 15]. Immunoassays permit analysis of a large number of specimens in a short time. However, physicians need to be aware of interferences of HbA1c measurement by variant hemoglobin and compare HbA1c results to other patient information or test results to avoid misdiagnosis or incorrect treatment.

Glycated albumin is known to be unaffected in cases of variant hemoglobin and anemia [2]. Glycated albumin accurately reflected plasma glucose levels in these cases. Most variant hemoglobins are clinically asymptomatic with blood tests revealing no abnormalities. The mechanisms that underlie inaccurate HbA1c values in variant hemoglobin are too many to allow a universal HbA1c measurement method in all the variant hemoglobins. Therefore, glycemic control in cases of variant hemoglobin should be guided by glycated albumin. An alternative recourse is to accurately diagnose the variant hemoglobin and find an unaffected HbA1c measurement method.

IA-HbA1c values in Hb Agenogi were found to be falsely high. HbA1c measurements were performed using a variety of methods. EA-HbA1c and IA-HbA1c using an antibody manufactured by Roche yielded correct values; whereas, IA-HbA1c using an antibody manufactured by Fujirebio yielded falsely high values. The above results suggest that the mutation at the 90th amino acid position of the β-globin chain modified the antigenicity of the N-terminal peptide (around the 6th amino acid) resulting in falsely high-IA-HbA1c values.

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Compliance with ethical standards

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

Human rights 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 Helsinki Declaration of 1964 and later versions. Informed consent or

a substitute for it was obtained from all patients prior to their participation in this study.

References

1. American Diabetes Association. Standards of medical care in diabetes; glycemic targets. Diabetes Care. 2017;40(Suppl 1):S48–56.
2. Koga M. Glycated albumin; clinical usefulness. Clin Chim Acta. 2014;433:96–104.
3. Bry L, Chen PC, Sacks DB. Effects of hemoglobin variants and chemically modified derivatives on assays for glycohemoglobin. Clin Chem. 2001;47:154–63.
4. Wajcaman H. Human hemoglobin variants, the electric versions. http://globin.cse.psu.edu. Accessed 24 Oct 2018.
5. Schnedl WJ, Krause R, Halwachs-Baumann G, et al. Evaluation of HbA1c determination methods in patients with hemoglobinopathies. Diabetes Care. 2000;23:339–44.
6. Thomas LB, Agosti SJ, Man MA, et al. Screening for hemoglobinopathies during routine hemoglobin A1c testing using the Tosoh G7 Glycohemoglobin Analyzer. Ann Clin Lab Sci. 2007;37:251–5.
7. Nishihara E, Koga M, Kadowaki S, et al. Method-dependant HbA1c values in a family with hemoglobin Himeji. Clin Chem Acta. 2011;412:1689–92.
8. Furuya M, Koga M, Ishibashi M, et al. A non-diabetic subject with variant hemoglobin HbC who was administered anti-diabetic drugs due to falsely high HbA1c values measured using an immunoassay. J Jpn Diabetes Soc. 2016;59:463–8.
9. Committee on the Standardization of Diabetes Mellitus-Related Laboratory Testing of Japan Diabetes Society. International clinical harmonization of glycated hemoglobin in Japan: from Japan Diabetes Society to National Glycohemoglobin Standardization Program values. Diabetol Int. 2010;3:8–10.
10. Nathan DM, Kuenen J, Borg R, Zheng H, Schoenfeld D, Heine RJ, A1c-Derived Average Glucose Study Group. Translating the A1C assay into estimated average glucose values. Diabetes Care. 2008;31:1473–8.
11. Miyaji T, Suzuki H, Ohba Y, et al. Hemoglobin Agenogi (alpha 2 beta 2-90 Lys), a slow-moving hemoglobin of a Japanese family resembling Hb-E. Clin Chim Acta. 1966;14:624–9.
12. Ohba Y, Miyaji T, Murakami M, et al. Hb Himeji or β140 (H18) Ala → Asp a slightly unstable hemoglobin with increased β N-terminal glycation. Hemoglobin. 1986;10:109–25.
13. Perutz MF. Regulation of oxygen affinity of hemoglobin: influence of structure of the globin on the heme iron. Annu Rev Biochem. 1979;48:327–86.
14. The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus. Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. Diabetol Int. 2010;1:2–20.
15. Shimizu S, Taira A, Hatazaki M, et al. Two cases of non-diabetic variant hemoglobin who received the administration of oral hypoglycemic agent(s) because of the spurious high levels of HbA1c. J Jpn Diabetes Soc. 2015;58:121–7.