A Rare Hemoglobin Variant (Hb Iraq-Halabja) Causing Spuriously Low Hemoglobin A1c Values

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Abstract

Various laboratory and patient-related factors can affect the measurement of hemoglobin A1c (HbA1c). We herein present the case of a diabetic patient with spuriously low HbA1c values on ion-exchange high-performance liquid chromatography (HPLC). Further investigations revealed that the patient was heterozygous for a rare Hb variant, namely Hb Iraq-Halabja ($\beta 10 \text{ Ala} \rightarrow \text{Val}$). This is the second report of this variant published in the literature. Clinicians should be aware of the limitations of HbA1c assays because inaccurate values may lead to the inappropriate management of diabetes. Unusual or discrepant HbA1c test results should prompt further investigations for potentially interfering factors, including rare Hb variants.

Key words: diabetes, HbA1c, Hb variant, HPLC, Hb Iraq-Halabja

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Introduction

The hemoglobin A1c (HbA1c) value is a valuable tool in the management of diabetes that most accurately reflects the glycemic control over the preceding two to three months. The relationship between the HbA1c value and the estimated average glucose level is widely applied in daily clinical practice. Since 2010, the HbA1c value has been further recommended to be a diagnostic criterion for diabetes (1, 2). The importance of the HbA1c value in predicting the risk and complications of diabetes is well established (3, 4). However, the accuracy of commonly used HbA1c assays, particularly ion-exchange high-performance liquid chromatography (HPLC), may be adversely affected by the presence of hemoglobin variants (5), and spurious test results can mislead clinicians to inappropriately managing diabetes.

Hemoglobinopathies are the most frequent monogenic alterations, the majority of which can interfere with the measurement of HbA1c due to point mutations in the β chains of Hb. Many Hb variants produce no phenotypic abnormalities, and their detection relies on the fortuitous observation of a discrepancy between the HbA1c and blood glucose levels. To explore the nature of the interference, additional meas-

urements using different HbA1c analyzers and alternative glycemic indices based on different principles are usually indicated. Further investigations of Hb, in particular, some rare Hb variants, require the DNA analysis of the relevant Hb gene.

We herein report an extremely rare Hb variant, Hb Iraq-Halabja ($\beta 10 \text{ Ala} \rightarrow \text{Val}$), in a Taiwanese man with diabetes. We demonstrate, for the first time, that this clinically and electrophoretically silent Hb variant causes spuriously low HbA1c values, as measured using ion-exchange HPLC.

Case Report

A 53-year-old man with newly diagnosed type 2 diabetes presented to our diabetes clinic for regular follow-up. Although this patient was born in Taiwan, his grandparents were immigrants from Mainland China. The initial laboratory tests revealed a fasting plasma glucose level of 117 mg/dL with an unexpectedly low HbA1c value of 4.0% (reference range: 4-6%), which was analyzed using the ion-exchange HPLC method (Tosoh HLC-723 G8 variant analysis mode; Tosoh, Tokyo, Japan). The patient was receiving a combination of metformin and gliclazide. He denied any manifestations suggestive of hypoglycemia during the pre-

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Figure 1. Chromatograms from Tosoh HLC-723 G8. (A) Standard chromatogram. There are several peaks, including A1a, A1b, F, labile A1c, stable A1c and A0. (B) Chromatogram of the patient with Hb Iraq-Halabja. The arrow indicates the abnormal peak between stable A1c and A0.

ceding three months. The follow-up results showed a further lower HbA1c value of 3.1% with a simultaneously measured fasting plasma glucose level of 122 mg/dL. The postprandial blood glucose level ranged between 120 and 180 mg/dL. The peripheral blood indices showed an Hb level of 16.1 g/dL with a red cell count of $4.52 \times 10^6/\mu\text{L}$, a mean corpuscular volume (MCV) of 100 fL and a mean corpuscular hemoglobin (MCH) of 35.5 pg.

Compared with the standard chromatogram of Tosoh G8, there was an abnormal peak between stable A1c and A0 (Fig. 1). The integrator identified the peak at a retention time of 0.61 minutes as HbA1c. According to the manufacturer's instructions, the extra peak appearing between the A1c and A0 peaks was non-reportable and caused erroneous HbA1c results. Therefore, a different method that is not affected by the presence of Hb variants was employed to quantify the HbA1c value. Using boronate affinity chromatography (Primus CLC 385; Primus, Kansas City, MO, USA), the HbA1c value was found to be 6.9%, which was more consistent with the patient's blood glucose concentration. In addition, we measured the level of fructosamine (Roche Cobas Mira; Mannheim, Germany) as an alternative marker of glycemic control; the value was 249 $\mu\text{mol/L}$, which corresponded to the estimated average glucose level of 120 mg/dL over the most recent two to three weeks. Taken together, these findings suggested that the Hb variant accounted for the spuriously low HbA1c values.

Figure 2. A DNA sequence analysis shows the presence of Hb Iraq-Halabja in which a heterozygous mutation of GCC to GTC produces the Ala→Val substitution at codon 10 of the β -globin gene.

Figure 3. The size of the amplified DNA (144 bp) and the *MwoI*-digested fragments (106 and 38 bp) of the $\beta^{\text{Iraq-Halabja}}$ gene are depicted. Lane P and N: *MwoI*-digested amplified DNA of a positive and normal control; Lane S: *MwoI*-digested amplified DNA of our patient with Hb Iraq-Halabja. M: 100 bp ladder.

However, an Hb analysis using capillary electrophoresis (Sebia, Norcross, GA, USA) and HPLC (Primus CLC 385; Primus, Kansas City, MO, USA) identified no abnormal hemoglobin, quantifying HbA2 at 2.5% and HbA at 97.5%. Hence, genetic testing was performed after obtaining the patient's written informed consent. Genomic DNA was extracted from the peripheral leukocytes using a commercially available kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). To identify Hb variants, the α 1-, α 2- and β -globin genes were amplified using the protocols of Chang et al. (6, 7). The polymerase chain reaction (PCR) products were purified and directly sequenced as previously described (6, 7). The DNA analysis revealed a single-base heterozygous mutation (GCC to GTC) at codon 10 of the β -globin gene, producing a Ala→Val substitution, previously termed Hb Iraq-Halabja (Fig. 2). In addition, the mutation was identified using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay with *MwoI* restriction digestion. This mutation abolished the *MwoI* restriction enzyme site (Fig. 3). After identifying the rare Hb variant, we attempted to trace the genetic origin; however, we had difficulty in obtaining genetic information from the patient's family.

Discussion

This rare Hb variant (Iraq-Halabja) has not been previously reported in a Taiwanese individual. To our knowledge, this is the second case report of this variant published in the literature (8). The β -chain Hb variant was first identified in a 36-year-old man originating from Halabja in northern Iraq with multiple Hb disorders in his family, including β^0 -thalassemia, α -thalassemia and α -globin triplication. Routine hematologic tests in this Iraqi man revealed a microcytic and slightly anemic phenotype with an elevated HbA2 level suggestive of β -thalassemia. Functional studies of the variant showed a normal oxygen affinity, normal heat stability and isopropanol precipitation tests; the variant is a non-pathological β -globin variant. However, the glycemic status of the study subject was not mentioned; thus, the possible impact of the variant on HbA1c measurement was not investigated. In contrast, our patient with type 2 diabetes was not anemic, and genetic screening excluded other associated hemoglobin gene disorders. Therefore, the identification of the phenotype in the present case allowed for a clearer assessment of the features associated with the β 10 Ala $\rightarrow$ Val mutation.

In addition to the blood glucose concentrations, the HbA1c values in patients with diabetes can be adversely affected by two major factors, namely the altered lifespan of erythrocytes and the presence of Hb variants (9). Any condition that shortens erythrocyte survival, such as hemolytic anemia or recovery from acute blood loss, may falsely lower HbA1c test results, regardless of the assay method used. In contrast, the effects of genetic variants on HbA1c determination vary considerably among commercially available methods. In general, an Hb variant should be suspected in patients with an HbA1c value of $>15\%$, HbA1c test results below the nondiabetic reference range or a discrepancy between the HbA1c value and blood glucose profile or clinical presentation. At present, there are more than 30 different glycohemoglobin assay methods. HbA1c assays can be divided into methods based on molecular charge (e.g. ion-exchange HPLC and electrophoresis) and those based on molecular structure (e.g. immunoassay and affinity chromatography). Among these methods, the effects of Hb variants on HbA1c measurement are thought to be greater on ion-exchange HPLC (5, 9), the currently most commonly used method in Taiwan. Hb variants that cannot be separated from HbA or HbA1c produce spurious results in ion-exchange HPLC assays. On the other hand, boronate affinity chromatography measures glycated hemoglobin based on the interaction of cis-glycols with boronic acid, regardless of the glycation site. This method has been proven to exhibit the least interference with respect to HbA1c assays in subjects with Hb variants; however, similar to immunoassays, it cannot detect the presence of hemoglobin variants (5, 9). In the present case, the HbA1c value determined using boronate affinity chromatography was more plausible than that meas-

ured using ion-exchange HPLC. This comparison between the two assays confirmed the nature of spurious low values on ion-exchange HPLC and thus highly raised our suspicion of the presence of an Hb variant.

More than 1,000 Hb variants have been reported to date (10). Diverse and specific Hb variants have been found in different racial and ethnic groups. In early studies, the common Hb variants in Taiwan have included Hb G-Taiwan-Amis, Hb J-Meinung, Hb G-Taichung and Hb E, with an estimated incidence of 0.57%, 0.065%, 0.049% and 0.027%, respectively (11). Based on a recent screening program in southern Taiwan, Hb J, Hb E, high Hb F and Hb H were identified in 4,239 diabetic subjects using Tosoh G5. The prevalence of overall Hb variants in this group was estimated to be 0.35% (12). Among these common Hb variants, Hb E and Hb J have been demonstrated to be associated with falsely low HbA1c values measured using Tosoh G8 (13, 14). In this report, we described a case of spuriously low HbA1c values measured using the same instrument. Nevertheless, the same Hb variant may yield different HbA1c results depending on the method used. For example, Hb E produces falsely high HbA1c values on Bio-Rad VariantTM II Turbo (15) and factitiously low values on Tosoh G8.

In subjects with factors interfering with the interpretation or measurement of HbA1c, glycemic assessments based on different methods should be considered. Determination of the fructosamine level depends on the glycation of serum proteins, primarily albumin and is thus not influenced by Hb variants. In this case, a fructosamine test provided a result that was more consistent with the measured blood glucose concentration than the HbA1c value measured using Tosoh G8. However, as albumin has a turnover of approximately two weeks (compared to the 100-120 days of erythrocytes), the fructosamine level reflects the degree of glycemia over this far shorter period. In addition, the higher within-patient variation and inadequate evidence for its efficacy in predicting the risk of diabetes and diabetic complications limit its use (16). Measurement of the glycated albumin (GA) level was developed to address some of the weak points of fructosamine measurement, particularly the instability (17). Indeed, physicians generally measure the GA level instead of the fructosamine level in Japan. Nevertheless, the GA test is not available in Taiwan. At present, frequent capillary blood glucose monitoring combined with boronate affinity chromatography appeared to be the best alternative for glycemic assessment in this case.

In conclusion, we identified a rare β -globin silent variant (Hb Iraq-Halabja) as a new factor affecting HbA1c measurement. It is important for clinicians to be aware of the limitations of HbA1c assays. Unusual or discrepant HbA1c test results should prompt further investigations of interfering factors, including rare Hb variants.

The authors state that they have no Conflict of Interest (COI).

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