

HbA mutation causing false-normal HbA1c results determined by HPLC in a patient with type 2 diabetes mellitus: A case report

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Abstract. Glycated hemoglobin A1c (HbA1c) is a convenient measure of long-term blood glucose concentrations and it is an accepted diagnostic test for type 2 diabetes mellitus (T2DM). The present study reported on a female patient with T2DM, whose fasting blood glucose and glycated albumin levels were elevated, while the HbA1c levels were in the normal range, which was inconsistent with the patient's clinical diagnosis. In the subsequent analysis, genomic DNA was extracted from the patient's blood and the HbA genes were analyzed by Sanger sequencing. The results indicated that the patient's HbA α 1/2-chain genes had no mutations, while two HbA β -chain gene mutations were present, including an HBB:c.9T>C variant and a Hb G-Coushatta variant. The HBB:c.9T>C variant is a silent mutation that has no effect on HbA1c levels when detected by ion-exchange high-performance liquid chromatography (HPLC), while the Hb G-Coushatta variant may cause a discrepancy between blood glucose control and HbA1c levels when detected by ion-exchange HPLC. These results suggested that the Hb G-Coushatta variant gave rise to the false-normal result regarding HbA1c levels when detected

by ion-exchange HPLC that was inconsistent with the clinical manifestations in this patient.

Introduction

Diabetes mellitus (DM) is a group of clinical syndromes characterized by disorders of glucose metabolism caused by genetic and environmental factors; these syndromes are divided into type 1 DM (T1DM), T2DM, other special types and gestational diabetes. T1DM is caused by the destruction of islet β cells and absolute insulin deficiency, while T2DM is characterized by insufficient insulin secretion and insulin resistance. The current diagnostic criteria for DM primarily include the World Health Organization-recommended 75-g oral glucose tolerance test and the American Diabetes Association-recommended level of glycated hemoglobin A1c (HbA1c) (1).

HbA1c is a product of the Hb in red blood cells (RBCs) that binds to glucose in serum, is formed by the nonenzymatic attachment of glucose (known as glycation) to Hb and normally reflects the ambient glucose concentration of the previous 2 to 3 months, which is the lifespan of an RBC, suggesting that HbA1c is a convenient measure of long-term blood glucose concentrations. In addition, HbA1c is not affected by several factors, such as time, fasting and exercise. Given these characteristics, the measurement of HbA1c is among the most important laboratory medical advances in diabetes care in decades and this measurement is now an accepted diagnostic test for T2DM and is used for monitoring glycemic control in patients with DM (2).

However, it has been indicated that numerous factors may influence HbA1c levels, including variability in erythrocyte life span and erythropoiesis (3). In addition, severe hypertriglyceridemia and chronic alcoholism may interfere with the measurement of HbA1c (3). Previous studies suggested that Hb variants may also affect the detection of HbA1c (1,4,5). In the present study, the HbA1c levels in a patient diagnosed with T2DM were observed to be in the normal range, which is inconsistent with the clinical diagnosis; this discrepancy was investigated and discussed in the present study.

Case report

A 55-year-old female patient presented at the Department of Endocrinology and Metabolism of the Affiliated Hospital

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Cre, creatinine; DM, diabetes mellitus; FBG, fasting blood glucose; GA, glycated albumin; Hb, hemoglobin; HbA1c, glycated Hb A1c; HCT, hematocrit; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MCH, mean corpuscular Hb; MCHC, MCH concentration; MCV, mean corpuscular volume; PLT, platelet count; RBC, red blood cells; RDW, RBC distribution width; T2DM, type 2 DM; TC, total cholesterol; TG, triglyceride; UA, uric acid; WBC, white blood cell count

Key words: glycated hemoglobin A1c, diabetes mellitus, point mutation, variant

of Qingdao University (Qingdao, China) in Laoshan District of Qingdao on January 16, 2019 and requested assistance to manage her T2DM. She was diagnosed with T2DM 12 months previously based on fasting blood glucose (FBG) levels of 11 mmol/l. Prescribed metformin (500 mg) was given for 12 months and the FBG level decreased to 7 mmol/l, while the postprandial blood glucose was 8-9 mmol/l. Selected laboratory test data for the patient are presented in Table I. The results suggested that the proportion of HbA1c obtained for this patient during a routine examination using the Variant II (Bio-Rad Laboratories, Inc.) was 3.8%, which was in the normal range and inconsistent with the clinical diagnosis. Subsequently, to further confirm whether the values obtained at our laboratory were correct, the levels of HbA1c, FBG and glycated albumin were further measured at another clinical laboratory at the headquarters of the Affiliated Hospital of Qingdao University (Qingdao, China) in Shinan District of Qingdao 3 days later. The resulting laboratory test data are provided in Table II. These tests provided a proportion of HbA1c of 3.7%, which was also measured during a routine examination using the Variant II (Bio-Rad Laboratories, Inc.); this result was slightly below the normal range and was still inconsistent with the clinical diagnosis.

Next, genomic DNA was extracted from the patient's blood using the Ezup Column Blood Genomic DNA Purification kit (Sangon Biotech Co., Ltd.) and genomic DNA was used to amplify HbA genes with specific forward and reverse primers by targeting the HbA α 1/2- and β -chain genes (HbA- α 1 forward, 5'-CCACCACCAAGACCTACT-3' and reverse, 5'-TCACAGAAGCCAGGAAGT-3'; HbA- α 2 forward, 5'-CCACCACCAAGACCTACT-3' and reverse, 5'-TCACAG AAGCCAGGAAGT-3'; HbA- β 1-forward, 5'-ACTCCTAAG CCAGTGCCAGA-3' and 1-reverse, 5'-AGATCCCCAAAG GACTCAAAG-3'; HbA- β 2-forward, 5'-TGAGGAGAAGTC TGCCGTTAC-3' and 2-reverse, 5'-AAAACGATCCTGAGA CTTCCAC-3'; HbA- β 3-forward, 5'-TGATAATTTCTGGGT TAAGGCAA-3' and HbA- β 3-reverse, 5'-TAACCTCCAAAT CAAGCCTCTAC-3'). PCR was performed in 50 μ l of solution containing 1-2 μ l DNA (20-50 ng/ μ l), 5 μ l Taq buffer (10X), 2 μ l dNTPs (10 mM), 2.5 U Taq DNA polymerase (Sangon Biotech Co., Ltd.) and 2 μ l of each of the two primers (10 μ M). ddH₂O was then added to increase the reaction volume to 50 μ l. The following thermocycling conditions were used: 95°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 30 sec at 55-60°C, and 50 sec at 72°C, finishing with 8 min at 72°C. The PCR products were detected by gel electrophoresis. In addition, the HbA genes were analyzed using Sanger sequencing. The PCR products were purified using the SanPrep Column PCR Product Purification kit (Sangon Biotech Co., Ltd.). Sequencing PCR was subsequently performed in 20 μ l of solution containing 10 ng purified PCR products, 4 μ l BigDye (2.5X; Thermo Fisher Scientific, Inc.), 2 μ l BigDye Seq Buffer (5X; Thermo Fisher Scientific, Inc.) and 1 μ l sequencing primers (3.2 pmol/ μ l), with 12 μ l ddH₂O added to increase the reaction volume to 20 μ l. The thermocycling conditions were as follows: 1 min at 96°C, followed by 25 cycles of 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C, finishing at 4°C. The sequencing PCR products were purified using the following method: 2 μ l EDTA (125 mM) and 2 μ l NaAc (3 M) were

Table I. Summary of selected laboratory test data measured at the Affiliated Hospital of Qingdao University in Laoshan District of Qingdao.

| Parameter | Patient's value | Reference range |
|-------------------------|-------------------|-----------------|
| Hb, g/l | 142.0 | 115.0-150.0 |
| MCH, pg | 30.6 | 27.0-34.0 |
| MCHC, g/l | 349.0 | 316.0-354.0 |
| MCV, fl | 87.7 | 82.0-100.0 |
| HCT, % | 40.7 | 35.0-45.0 |
| RBC, $\times 10^{12}/l$ | 4.64 | 3.8-5.1 |
| RDW, % | 11.4 ^a | 11.6-16.5 |
| PLT, $\times 10^9/l$ | 249 | 125-350 |
| WBC, $\times 10^9/l$ | 6.42 | 3.5-9.5 |
| FBG, mmol/l | 7.63 ^a | 3.90-6.16 |
| HbA1c, % | 3.8 | 3.6-6.0 |
| ALT, U/l | 16.0 | 7.0-40.0 |
| AST, U/l | 17.0 | 13.0-35.0 |
| TG, mmol/l | 1.34 | 0.30-1.92 |
| TC, mmol/l | 4.29 | 2.32-5.62 |
| HDL, mmol/l | 1.31 | 0.80-2.35 |
| LDL, mmol/l | 2.57 | 1.90-3.12 |
| BUN, mmol/l | 6.14 | 2.6-7.5 |
| Cre, μ mol/l | 88.0 | 31.0-132.0 |
| UA, μ mol/l | 280.0 | 89.2-339.0 |
| GA, % | 21.7 ^a | 10.4-15.7 |

^aAbnormal results. Hb, hemoglobin; MCH, mean corpuscular Hb; MCHC, MCH concentration; MCV, mean corpuscular volume; HCT, hematocrit; RBC, red blood cells; RDW, RBC distribution width; PLT, platelet count; WBC, white blood cell count; FBG, fasting blood glucose; HbA1c, glycated Hb A1c; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BUN, blood urea nitrogen; Cre, creatinine; UA, uric acid; GA, glycated albumin.

Table II. Summary of selected laboratory test data measured at the headquarters of the Affiliated Hospital of Qingdao University in the Shinan District of Qingdao.

| Parameter | Patient's value | Reference range |
|-------------|-------------------|-----------------|
| FBG, mmol/l | 7.40 ^a | 3.90-6.16 |
| HbA1c, % | 3.7 | 4.3-6.3 |
| GA, % | 18.1 | 10.4-15.7 |

^aAbnormal result. FBG, fasting blood glucose; HbA1c, glycated hemoglobin A1c; GA, glycated albumin.

added per well to a 96-well plate, after which 50 μ l alcohol (100%) was added and the solution was mixed and incubated for 15 min at room temperature. The plate was centrifuged at 3,000 \times g at 4°C for 30 min, after which the plate was inverted. A subsequent centrifugation was performed at 4°C and the

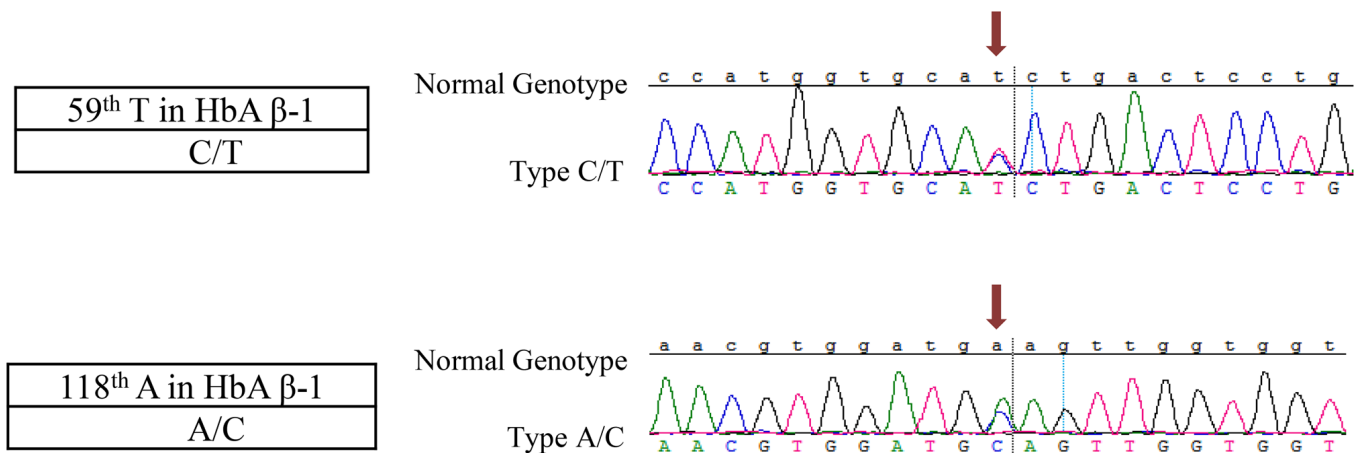


Figure 1. HbA1c sequence was tested using Sanger sequencing. The patient's HbA β-chain contains gene mutations, including a C/T point mutation at point 59 and an A/T point mutation at point 118. The mutation sites are indicated by red arrows. HbA1c, glycated hemoglobin A1c.

power was turned off as soon as the speed was up to 185 x g. Next, 70 μ l alcohol (70%) was added to the plate and samples were centrifuged at 3,000 x g at 4°C for 15 min. After the plate was inverted, samples were further centrifuged at 4°C and the power was turned off as soon as the speed was up to 185 x g. Once the alcohol had evaporated at room temperature, 10 μ l Hi-Di Formamide (Sangon Biotech Co., Ltd.) was added to dissolve the DNA. The DNA was subsequently denatured at 95°C for 4 min and 4°C for 4 min in the PCR instrument. Samples were then sequenced on an ABI 3730XL DNA analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The Sanger sequencing results were then analyzed using SeqMan DNASTAR Lasergene 11.0 software (DNASTAR Inc.). The results of the Sanger sequencing indicated that the patient's HbA α 1/2-chains had no gene mutations (data not shown). However, gene sequencing of the HbA β -chain PCR products suggested that the patient had two HbA β -chain gene mutations (Fig. 1). The first mutation was a C/T point mutation that occurred at point 59, which comprises CAT>CAC at codon 2 in the HbA β -chain and is named HBB:c.9T>C variant described in the human hemoglobin variant (HbVar) database (<http://globin.bx.psu.edu/hbvar>). The next mutation was an A/T point mutation that occurred at point 118, which comprises GAA>GCA at codon 22 in HbA β -chain and is named the Hb G-Coushatta variant, also known as the Hb G Hsin Chu variant, Hb G-Saskatoon variant or Hb G-Taegu variant described in HbVar. As ion-exchange high-performance liquid chromatography (HPLC) for HbA1c quantification is based on charge differences and is known to be susceptible to interference from Hb variants (6). The HBB:c.9T>C variant is a silent mutation, resulting in no change in the charge of the patient's HbA β -chain; thus, the HBB:c.9T>C variant has no effect on the HbA1c level when detected by ion-exchange HPLC. However, previous studies have indicated that the Hb G-Coushatta variant usually leads to underestimation of HbA1c (7-9). HbA variants constitute a well-known cause of analytical interference in HbA1c measurements (10). Thus, it is most likely that the Hb G-Coushatta variant leads to HbA1c levels that are clearly inconsistent with the clinical manifestations.

Discussion

The measurement of HbA1c levels is an accepted diagnostic test for T2DM. The interindividual variation in HbA1c, defined as the glycation gap, is not attributable to mean blood glucose values and has been indicated to be associated with diabetic nephropathy (3,11). Several studies have described low HbA1c levels in patients with Hb variants (9,12,13). According to the laboratory test data obtained for the patient of the present study, numerous factors that may lead to low HbA1c levels were excluded, except for Hb variants.

In the present study, to determine why the HbA1c level was clearly inconsistent with the clinical manifestations, the HbA1c sequence of this patient was analyzed using Sanger sequencing and the results suggested that the patient's HbA β -chain had two gene mutations. To confirm the Hb variants of the patient, the mutations were searched in HbVar. According to HbVar, the first gene mutation is named the HBB:c.9T>C variant, which is a silent mutation that results in no change in the charge of the patient's HbA β -chain and the data from the ClinVar Miner website (<https://clinvarminer.genetics.utah.edu>) also indicated that the HBB:c.9T>C variant is considered to be benign and is not related to any pathological conditions; In addition, ion-exchange HPLC for HbA1c quantification are based on charge differences (6), thus, the HBB:c.9T>C variant has no effect on the HbA1c level when detected by ion-exchange HPLC. The second gene mutation is named the Hb G-Coushatta variant, which has been observed to occur in certain populations, such as native Americans, Chinese, Koreans, Japanese, Turks and Algerians. Previous studies have indicated that this variant usually leads to underestimation of HbA1c and HbA variants constitute a well-known cause of analytical interference in the measurement of HbA1c levels (7-10). One of these studies suggested that the Hb G-Coushatta variant affected the determination of HbA1c levels, with the ion-exchange HPLC method measuring an HbA1c level that was 45% lower than that obtained by the immunoturbidimetric assay method (9). Thus, it may be concluded that the Hb G-Coushatta variant is the most likely cause of the HbA1c measured by HPLC appearing normal, which was clearly inconsistent with the clinical manifestations.

An unusually low level of HbA1c or discordance between blood sugar and HbA1c values should alert clinicians to the possibility of Hb variants. Since the effect of a particular Hb variant on HbA1c results is frequently method-dependent (e.g., ion-exchange HPLC methods are frequently affected by the presence of Hb variants), repeat analysis should ideally be performed using an alternative method based on a different analytical principle from the initial assay (14); these alternative methods may include electrophoresis, immunoassays and enzymatic analysis. Among the methods, the principle of electrophoresis is based on the fact that colloidal particles with different electric charges under certain conditions move through an electric field by electrostatic attraction, which may identify abnormal Hb (12). In the future, when the HbA1c levels of patients are clearly inconsistent with the clinical manifestations, electrophoresis should be used to measure HbA1c levels, which may help with the monitoring of HbA1c levels when HbA variants are present in those patients.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available in a public curated database named figshare (https://figshare.com/articles/figure/HbA_mutations_in_a_T2DM_patient/12922502; DOI: 10.6084/m9.figshare.12922502). In addition, the .sqd file contains the high-throughput sequencing data.

Authors' contributions

JW analyzed and interpreted of the data, wrote the initial draft and revised the manuscript. YW, MH and QW contributed to the acquisition and analysis of the data. WL and LY contributed to evaluation and interpretation of the data. MH and QW made substantial contributions to the conception and design of the current study. JW and QW confirmed the authenticity of all the raw data. JW and QW agreed to be accountable for all aspects of the work. All authors critically reviewed the manuscript and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (Qingdao, China). The patient provided written informed consent.

Patient consent for publication

The patient had provided written informed consent regarding the publication of the case details.

Competing interests

The authors declare that they have no competing interests.

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