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BRIEF REPORT

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Hb I: A α-globin chain variant causing unexpected HbA_{1c} results

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Background: HbA_{1c} is the standard bio-marker for glycemic control in patients with diabetes. Here, we report a α-globin chain variant and evaluate its effect on HbA_{1c} measurements.

Methods: A 21-year-old female was suspected of harboring a hemoglobin variant following HbA_{1c} measurement during a routine examination using Variant II Turbo 2.0 (Bio-Rad). An oral glucose tolerance test was performed using an AU5800 clinical chemistry system (Beckman Coulter). HbA_{1c} was reanalyzed using D10 (Bio-Rad), Capillarys 2 Flex Piercing (Sebia), and Premier Hb9210 (Trinity Biotech). Hemoglobin analysis was performed using high-performance liquid chromatography (HPLC) on the Bio-Rad Variant II (β-thalassemia short program) and capillary electrophoresis (CE, Capillarys 2 Flex Piercing, Hb program). Sanger sequencing of α and β genes was also conducted.

Results: HbA_{1c} was initially measured at 24.2% using Variant II Turbo 2.0. For the oral glucose tolerance test, fasting glucose, 1-hour, and 2-hour levels were recorded as 4.25, 7.89, and 5.34 mmol/L, respectively. Subsequently, HbA_{1c} values determined by D10, Capillarys 2 Flex Piercing (HbA_{1c} program), and Premier Hb9210 were 4.5% (26 mmol/mol), no HbA_{1c} value, and 4.8 (29 mmol/mol), respectively. Hemoglobin analyzed using CE and HPLC revealed an abnormal hemoglobin. Sanger sequencing identified a transversion mutation of the α2 gene [CD16(AAG>GAG), Lys>Glu, *HBA2*: c.49 A>G], corresponding to a Hb I variant.

Conclusion: An unusually high HbA_{1c} or discordance between blood sugar and HbA_{1c} values should alert about the possibilities of hemoglobin variants.

KEYWORDS

capillary electrophoresis, Hb I, HbA_{1c}, hemoglobin variants, HPLC

1 | INTRODUCTION

Hemoglobin (Hb) variants are a group of common inherited genetic defects resulting in structural changes in the hemoglobin molecule.[1] HbA_{1c} is used as the gold standard for monitoring long-term glycemic control in people with diabetes mellitus and is characterized by nonenzymatic binding of glucose to the N-terminal valine residue on the β-chain of the HbA.[2] Previously, studies have shown that hemoglobin variants can affect the detection of HbA_{1c}.[3,4] In this study, we incidentally found a variant of the α2 chain in a Chinese individual following HbA_{1c} analysis. Here, the Hb variant interfered with the quantification of HbA_{1c} based on the popular methods of high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE).

2 | METHODS

2.1 | HbA_{1c} analysis

This study was approved and conducted in accordance with the protocol of the Institutional Medical and Ethics Committee of Peking University Shenzhen Hospital. Written informed consent was obtained from the subject.

A 21-year-old Chinese female was suspected of having a hemoglobin variant after HbA_{1c} measurement during a routine examination using Variant II Turbo 2.0 [VII-T 2.0] (Bio-Rad, Hercules, CA, USA). An oral glucose tolerance test was performed using an AU5800 clinical chemistry system (Beckman Coulter, Brea, CA, USA). Her high HbA_{1c} value was inconsistent with the results of oral glucose tolerance test. We, therefore, hypothesized that a hemoglobin variant could be interfering with the HbA_{1c} analysis. Next, a HPLC method (D10, HbA_{1c} mode; Bio-Rad, Hercules, CA, USA), a CE method (Capillarys 2 Flex Piercing [C2FP], HbA_{1c} program; Sebia, Lisses, France), and a boronate affinity HPLC system (Premier Hb9210, Trinity Biotech, Bray, Ireland) were used to confirm the accuracy of the HbA_{1c} results.

2.2 | Hemoglobin variant investigation

Further test for hemoglobin analysis was performed using C2FP (Hb mode) and Bio-Rad Variant II (β-thalassemia short program). Hematological parameters were measured using a Sysmex XN9000 hematology analyzer (Sysmex Co., Kobe, Japan).

Genomic DNA was extracted from the patient's blood with a genomic DNA isolation Kit (Tiangen Biotech, Beijing, China), and the genomic DNA was used to amplify the *HBA1*, *HBA2*, and *HBB* genes, with specific forward/reverse primers. *HBA1* forward primer: 5′-TGG AGG GTG GAG ACG TCC TG - 3′, *HBA1* reverse primer: 5′-TCC ATC CCC TCC TCC CGC CCC TGC CTT TTC-3′ (NCBI Accession No.: NC_000016.10, Region: 176478-177658, amplicon length: 1181 bp); *HBA2* forward primer: 5′-TGG AGG GTG GAG ACG TCC TG-3′, *HBA2* reverse primer: 5′-CCA TTG TTG GCA CAT TCC GG-3′(NCBI Accession No.: NC_000016.10, Region:172674- 173758, amplicon length: 1085 bp); *HBB* forward primer: 5′-AAC TCC TAA GCC AGT GCC AGA AGA GC-3′, *HBB* reverse primer: 5′-ATG CAC TGA CCT CCC ACA TTC CC-3′ (NCBI Accession No.: NC_000011.10, Region: 5225403-5227235, amplicon length: 1833 bp). Sanger sequencing of the polymerase chain reaction (PCR) products was performed on an ABI PRISM™ 3730 XL Sequencer (Applied Biosystems, Foster City, CA, USA). Both forward and reverse sequencing were performed. Alpha thalassemia gene detection was performed using thalassemia gene kit (Yishengtang, Shenzhen, China).

3 | RESULTS

3.1 | HbA_{1c} analysis

For the oral glucose tolerance test, fasting glucose, 1-hour, and 2-hour levels were recorded as 4.25, 7.89, and 5.34 mmol/L, respectively. HbA_{1c} initially measured using Variant II Turbo 2.0 was 24.2% (Figure 1A). In follow-up analyses, HbA_{1c} values measured

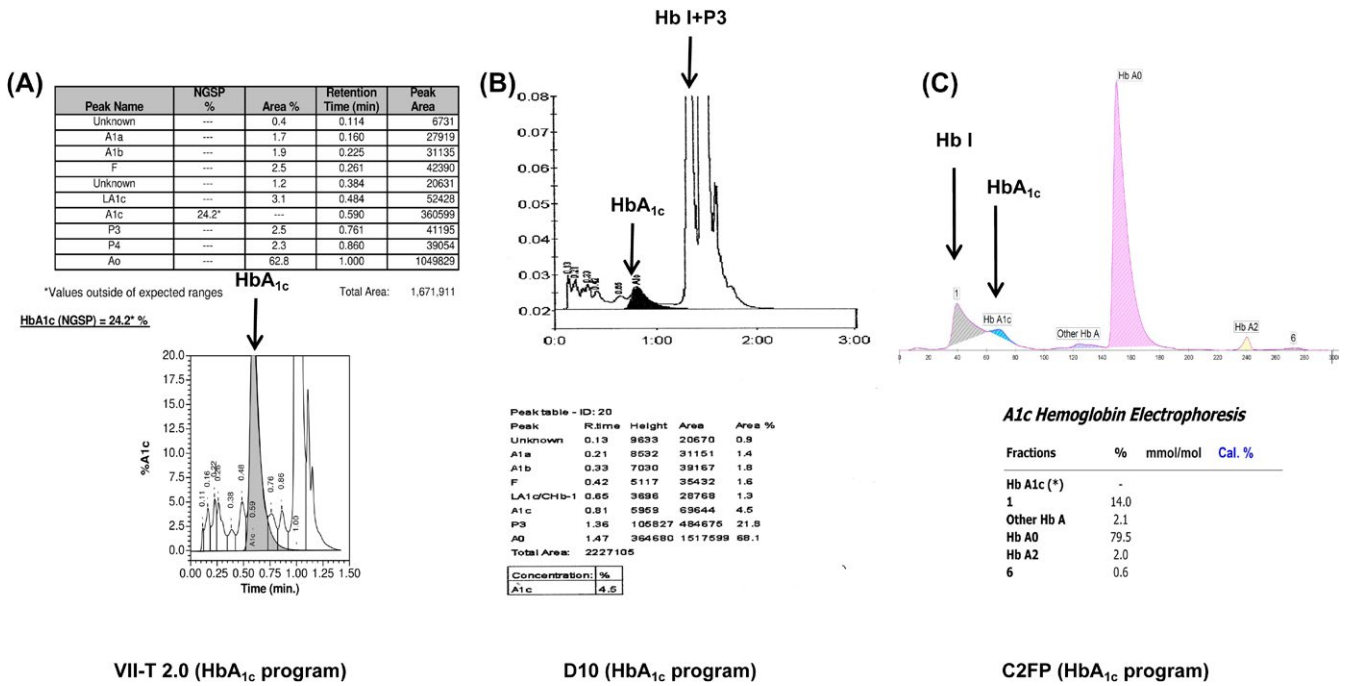

FIGURE 1 HbA_{1c} analysis using Variant II Turbo 2.0 (A), D10 (B), and Capillarys 2 Flex Piercing (C). An abnormal peak (Hb I) was observed on chromatograms of D10 and Capillarys 2 Flex Piercing. Arrows indicate the presence of Hb I

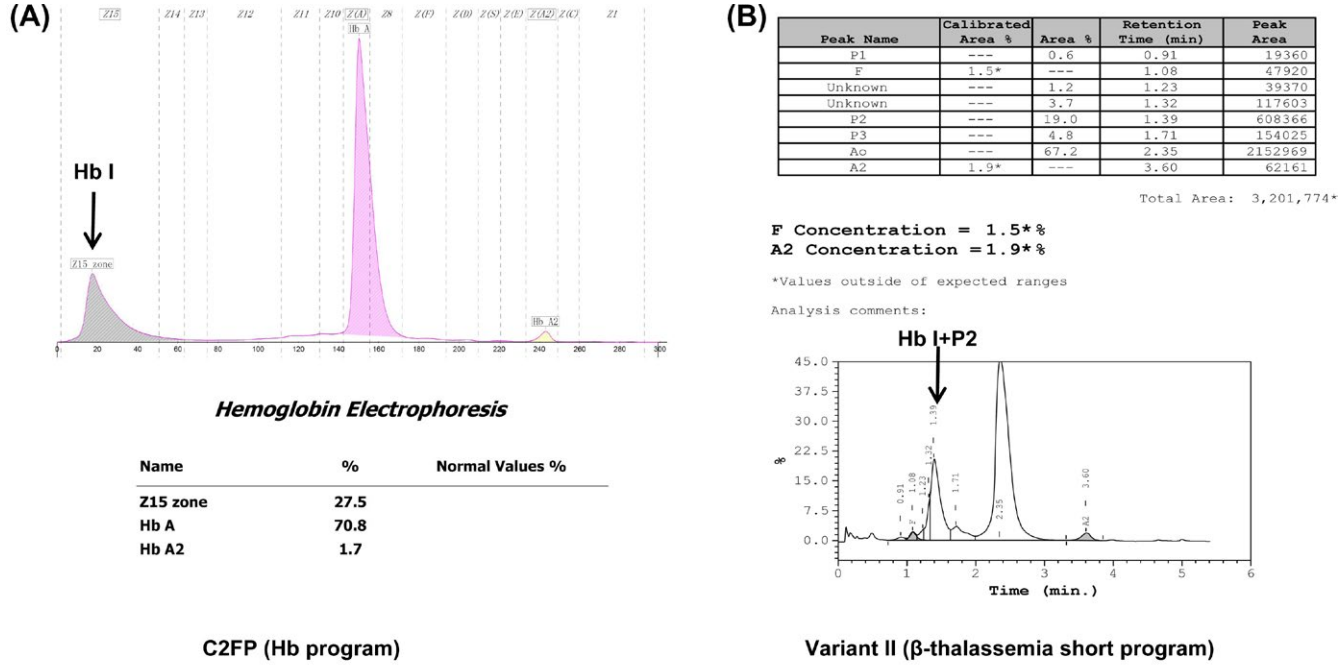

FIGURE 2 Confirmation of the hemoglobin variant. Hemoglobin analysis determined using Bio-Rad Variant II (β-thalassemia Short Program) (A) and C2FP (Hb program) (B). Arrows indicate the presence of Hb I. Sanger sequencing analysis showing the missense mutation [alpha2 16(A14), Lys>Glu, HBA2:c.49 A>G] at nucleotide 260 (C)

using D10, C2FP, and Hb9210 were 4.5% (26 mmol/mol), no HbA_{1c} value, and 4.8% (29 mmol/mol), respectively. Simultaneously, the D10 and C2FP both detected an abnormal peak, chromatogram of D10 showed the abnormal peak was co-eluted with P3 (Figure 1B,C). However, C2FP yielded no HbA_{1c} value because C2FP cannot completely separate HbA_{1c} from the variant (Figure 1C). Significantly, bias was observed for VII-T 2.0 (bias: 404.2%), when compared with the result obtained from Hb9210.

3.2 | Hemoglobin analysis and DNA sequencing

Hemoglobin analyzed using C2FP revealed a significantly abnormal band (Hb I), comprising 27.5% of the total hemoglobin in zone 15 (Figure 2A). Chromatogram of the Bio-Rad Variant II (β-thalassemia short program) showed that Hb I was co-eluted with P2, with the retention time of 1.39 minutes (Figure 2B). Hemoglobin analysis revealed that the co-elution of Hb I and HbA_{1c} led to the falsely elevated HbA_{1c} value of 24.2% using VII-T 2.0.

The hematological data of the patient showed no signs of anemia, with a RBC count of 4.51 $x10^{12}$/L (ref: 3.8-5.1 $x10^{12}$/L), Hb of 136 g/L (ref: 115-150 g/L), mean corpuscular volume of 91.4 fL (ref: 81.0-100.0 fL), and mean corpuscular Hb of 30.2 pg (ref: 27-34 pg).

Sanger sequencing revealed that the α1 and β genes were normal. α-thalassemia deletion and nondeletion were also ruled out. However, it showed a single base substitution in the α2 globin gene, a A>G transversion in the exon one at nucleotide 260, resulting in the replacement of a lysine by a glutamic acid at codon 16 in the heterozygous state (Figure 2C). This mutation [alpha2 16(A14), Lys>Glu, *HBA2*: c.49 A>G] was reported as Hb I according to the record in the HbVar database.

4 | DISCUSSION

Hb variants constitute a well-known cause of analytical interference in HbA_{1c} measurements.[5] Hb I is the α2 globin variant change

in amino acids (Lys>Glu) in codon 16. To the best of our knowledge, this is the first report of Hb I in Chinese people. Previous studies showed Hb I interfered with HbA_{1c} measurement by HPLC.[6,7] However, the interference of Hb I on HbA1c has not been determined by CE in previous studies. In the current study, systematically analysis was performed to determine both the HbA_{1c} result and the separation of hemoglobin variant by different methods including cation exchange HPLC, boronate affinity HPLC, and capillary electrophoresis.

In this case, oral glucose tolerance testing suggested that the female did not have diabetes mellitus that appeared to contradict her initially high HbA_{1c} value as measured using VII-T 2.0. C2FP yielded no HbA_{1c} value because C2FP cannot completely separate HbA_{1c} from the variant. Hemoglobin analysis revealed that the co-elution of Hb I and HbA_{1c} led to the falsely elevated HbA_{1c}. Meanwhile, our study confirms that this hemoglobin can be separated by HPLC and CE. Results obtained from VII (β-thalassemia short program) and C2FP (Hb program) showed that HbA and HbA_2 peaks rather than Hb I peak accounted for similar percentage in the chromatograms. We speculated that the percentage difference in Hb I between the two methods may mainly because that the Hb I separated by C2FP may contain other components (eg P1, P2, P3, and unknown peaks and so on) which indicated in the chromatogram of VII-T (Hb program).

Boronate affinity HPLC is commonly used as the comparative method because it is thought to have minimal or no interference from Hb variants.[8] The results of the boronate affinity method suggested that the woman actually had a normal HbA_{1c} value. Significantly, biases indicated the variant seriously affected HbA_{1c} quantity by VII-T 2.0. Furthermore, reporting erroneous HbA_{1c} results could potentially lead to missed diabetes diagnoses and inappropriate treatment of known diabetics.

Chromatograms of VII-T 2.0 showed the analyzer based on HPLC were not able to separate variants from HbA or HbA_{1c}. As such, Hb variants can be suspected in patients with unusually high HbA_{1c} values, or that the HbA_{1c} results can vary substantially from other indices of metabolic control. In this case, HbA_{1c} should be verified by repeat analysis. Since the effect of a particular Hb variant on HbA_{1c} results is often method dependent, repeat analysis should ideally be performed using a method based on a different analytical principle from the initial assay. HbA_{1c} chromatograms should be carefully checked to detect the possible presence of variants that can cause measurement interference.

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