Letter to the Editor

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Detection of Hb Phnom Penh by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry during the measurement of glycated hemoglobin

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To the Editor,

Hemoglobin (Hb) variant is a well-known cause of erroneous results of glycated hemoglobin (HbA_{1c}) [1]. During routine clinical HbA_{1c} analysis with Variant II Turbo 2.0 (VII-T 2.0; Bio-Rad, USA), we encountered seven patients over a 3-year period who showed no HbA_2 peak in chromatograms, but a roughly normal Hb pattern (Supplementary Figure 1A, B). In addition, a marked discordance was observed between the results of HbA_{1c} and fasting plasma glucose (Table 1). In this study, we performed further investigation on the seven samples. This study was approved by the Ethics Committee of Peking University Shenzhen Hospital and written informed consent was obtained from all patients involved in this study.

Subsequent Hb analysis by a capillary electrophoresis (CE) method (CAPILLARYS 2, Hb program; Sebia, France) and a cation exchange high-performance liquid chromatography (HPLC) method (Variant II, β-thalassemia short program) showed no signs of abnormal peaks in samples 1–7 (Supplementary Figure 1C, D). Further HbA_{1c} analysis was conducted by a boronate affinity HPLC system (Premier Ultra^2; Trinity Biotech, Ireland) CAPILLARYS 2, and a matrix-assisted laser desorption/ionization time time-of-flight mass spectrometry (MALDI-TOF MS) system (QuanTOF; Intelligene Biosystems, China) respectively. All operations were conducted according to the manufacturers' instructions. For QuanTOF, HbA_{1c} was measured following the protocol described in our previous study [2].

As shown in Supplementary Table 1, for five external quality control samples assigned with HbA_{1c} reference method from the National Center for Clinical Laboratories in China, the relative differences of five external quality control samples between measured HbA_{1c} values and target values indicate excellent accuracy and consistency of these methods. For samples 1–7, unacceptable positive biases (National Glycohemoglobin Standardization Program [NGSP] criterion, relative bias $<\pm5\%$) were only observed for VII-T 2.0 using Ultra^2 as a comparative method (Table 1) [3]. In addition, only mass spectrogram of QuanTOF revealed a variant globin peak ($m/z=15240.1$), abundance ratios of which accounted for 18.6–20.3% of the total α-globin for samples 1–6 (Figure 1A), with respect to a larger ratio of 36.8% for sample 7 (Figure 1C). The measured difference of the mass-to-charge ratio (m/z) between the variant α-globin and normal α-globin is 113.1 Da (Figure 1A, C).

Sanger DNA sequencing revealed no genetic alterations for the *HBA2* and *HBB* genes using an ABI PRISMTM 3730 XL Sequencer (Applied Biosystems, USA). However, for the *HBA1* gene, compared with the normal sequence (Supplementary Figure 2B), a heterozygous mutation Hb Phnom Penh (*HBA1*:p.Phe118_Thr119ins ATC [isoleucine]) with overlapping peaks was observed for samples 1–6 (Figure 1B). On the contrary, the same homozygous mutation without overlapping peaks was found for sample 7 (Figure 1D). Moreover, the insertion of isoleucine residue (theoretical molecular weight, 113.17 Da) was consistent with the measured difference of m/z of 113.1 Da between the variant α-globin and normal α-globin by QuanTOF.

The normal erythrocyte parameters of samples with a heterozygous mutation determined by a Sysmex XN900 hematology analyzer (Sysmex, Japan) indicated the presence of a clinically silent Hb variant (Supplementary Table 2). However, hematological data of sample 7 showed a classical microcytic hypochromic anemia profile, which suggested that he may suffer from thalassemia. As expected, subsequent gene confirmation by gap

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Table 1: Samples 1–7 were measured by four HbA_{1c} methods.

	Ultra²	VII-T 2.0		CAPILLARYS 3 TERA		QuanTOF		Fasting plasma glucose, mmol/L
	HbA_{1c}, %	HbA_{1c}, %	Bias	HbA_{1c}, %	Bias	HbA_{1c}, %	Bias	
Sample 1	5.9	7.0	18.6%	5.8	−1.7%	5.7	−3.4%	5.2
Sample 2	5.2	6.9	32.7%	5.0	−3.8%	5.0	−3.8%	4.4
Sample 3	5.5	6.2	12.7%	5.4	−1.8%	5.3	−3.6%	5.0
Sample 4	5.8	7.3	25.9%	5.7	−1.7%	5.7	−1.7%	5.6
Sample 5	5.1	6.3	23.5%	5.3	3.9%	5.2	2.0%	4.6
Sample 6	5.3	6.8	28.3%	5.1	−3.8%	5.2	−1.9%	5.1
Sample 7	5.0	6.7	34.0%	4.8	−4.0%	5.0	0.0%	4.5

HbA_{1c} values are expressed in NGSP units. The biases between each method and the comparative method (Ultra²) were calculated.

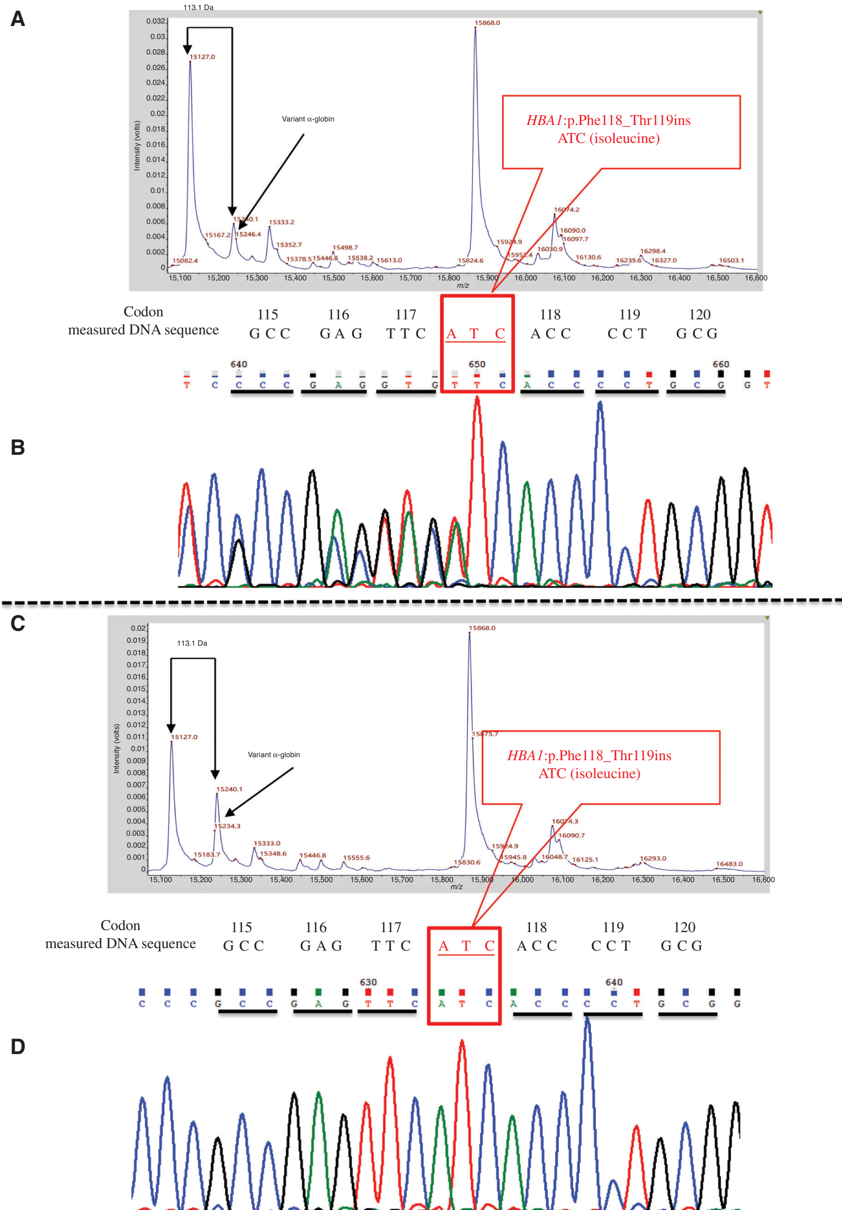

Figure 1: MALDI-TOF mass spectra and Sanger sequencing for samples 1–7.
HbA_{1c} analyzed by QuanTOF showed a variant globin (m/z=15240.1) for samples 1–6 (A) and sample 7 (C). Sanger sequencing data showed a heterozygous or homozygous mutation (*HBA1*:p.Phe118_Thr119ins ATC [isoleucine]) for samples 1–6 (B) and sample 7 (D).

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polymerase chain reaction (gap-PCR) revealed that the patient also carried a Southeast Asian type α-thalassemia, which explained the larger proportion of variant α-globin with respect to that of samples 1–6. Additionally, the deletion of the *HBA1* gene led to the existence of only one *HBA1* gene with mutation, resulting in a falsely homozygous result of Sanger sequencing.

HbA_{1c}, defined as glucose attachment to the N-terminal valine residue of β-globin, plays an important role in monitoring long-term blood glucose levels in diabetes mellitus [4]. HPLC and CE are the most common methods for HbA_{1c} measurement and Hb analysis. However, cation exchange HPLC and CE methods faced challenges because of limited resolving power in detecting the Hb variant discovered in this study. Alternatively, MALDI-TOF MS was able to distinguish the variant by the *m/z* difference between normal and variant globin.

Previous reports demonstrated that MALDI-TOF MS can estimate the quantity of both α- and β-globin chains, as well as glycated forms of each globin chain modified by attachment of a single glucose moiety resulting in a 162-Da increase in globin mass [5, 6]. In the current study, as compared with $Ultra^2$ results, unacceptable positive biases for HbA_{1c} results were observed for VII-T 2.0, suggesting that the Hb variant interferes with the quantitation of HbA_{1c} by VII-T 2.0. On the contrary, the biases for the other methods, such as QuanTOF, were acceptable. Based on 741 Da of *m/z* difference (*m/z* of α-globin = 15,868 Da; *m/z* of β-globin = 15,127 Da) between α-globin and β-globin (Supplementary Figure 2A), we suppose that MALDI-TOF MS may not be prone to interference of variant α-globin because the sufficient *m/z* difference can eliminate overlap of variant α-globin and β-globin. However, further investigation with more clinical data is warranted.

In conclusion, we described a variant Hb detected by MALDI-TOF MS during the quantitation of HbA_{1c}, which interfered with HbA_{1c} measurement by VII-T 2.0; the *m/z* difference determined by MALDI-TOF MS could support the result of Sanger sequencing. Nevertheless, this Hb variant could not be detected by traditional HPLC and CE. Additionally, this study suggests that MALDI-TOF MS-based HbA_{1c} determination may not be prone to interference of variant α-globin.

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