Letter to the Editor

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Detection of a novel hemoglobin variant Hb Liaoning by matrix assisted laser desorption/ionization-time of flight mass spectrometry

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To the Editor,

Hemoglobin (Hb) variants, characterized by structural changes in the Hb molecules, are a group of common inherited disorders caused by mutations in the globin genes. To date, more than 1300 variants have been identified and over 150 unstable Hb variants are documented to be responsible for hemolytic anemia with various severity [1]. Moreover, many studies have demonstrated that Hb variants could bring about analytical interference in HbA_{1c} measurements. Therefore, the identification and characterization of clinically relevant Hb variants is of paramount importance to make a correct diagnosis.

Classical methods for the detection of Hb variants such as electrophoresis and cation exchange high-performance liquid chromatography (HPLC) rely on the protein charge difference induced by the gene mutation [2]. However, difficulties arise in the case of co-eluting variants or components exhibiting unmatched retention times. Sophisticated methods such as electrospray ionization tandem mass spectrometry, liquid chromatography-mass spectrometry and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) were regarded as alternative methods for the detection of Hb variants [3–5]. Generally, Hb variants should be finally confirmed using DNA sequencing analysis, which provides accurate sequence variation information.

Here, we report a novel Hb variant namely Hb Liaoning, which was firstly identified by MALDI-TOF MS.

This study was approved by the Institutional Medical and Ethics Committee of Peking University Shenzhen Hospital. Written informed consent was obtained from the subject. The proband, a 36-year-old man of Chinese Han nationality from Liaoning Province of China, was referred to our hospital for a routine health check. The HbA_{1c} level was initially measured using a capillary electrophoresis (CE) analyzer (capillaryS 2, HbA_{1c} program, Sebia, France), which yielded no HbA_{1c} value with an atypical electropherogram showing no apparent abnormal peak (Supplementary material, Figure 1). The phoresis software identified profiles as "atypical" mostly due to the presence of additional peaks [6]. Therefore, we hypothesized that a Hb variant might interfere with the HbA_{1c} analysis. HbA_{1c} was further quantified using a HPLC method (Variant II Turbo 2.0, Bio-Rad, USA), a boronate affinity HPLC system (Premier Hb9210, Trinity Biotech, Ireland), an immunoassay method (Cobas Tina-quant Hemoglobin A1c Gen.3, Cobas c501, Roche Diagnostics, Switzerland) and a MALDI-TOF analyzer (QuanTOF, Intelligene Biosystems Co. Ltd, China), respectively. For QuanTOF, whole blood samples collected in EDTA tubes, as well as the standards, were diluted at 1:200 in distilled water. The samples were further mixed with 10 mg/mL sinapinic acid (Sigma-Aldrich, USA) to achieve a final concentration of 10% (v/v). Then, 2.5 μL of this mixture was spotted onto a stainless steel MALDI target. MS was performed after the mixture was dry following the manufacturer's instruction. The result of HbA_{1c} by QuanTOF was calculated by the percentage of glycated-βHb vs. total βHb. HbA_{1c} results are presented via the standard curve established by the reference materials traceable to the National Glycohemoglobin Standardization Program (NGSP).

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Accordingly, the HbA_{1c} results were 5.2% (33 mmol/mol, by Variant II Turbo 2.0), 5.1% (32 mmol/mol, by Hb9210), 4.9% (30 mmol/mol, by Cobas c501) and 4.9% (30 mmol/mol, by Quan TOF). Acceptable biases (National Glycohemoglobin Standardization Program [NGSP] criterion, bias within ±5.0%) were observed for Variant II Turbo 2.0 (2.0%), Cobas c501 (–3.9%) and QuanTOF (–3.9%), when compared with the result obtained from Hb9210 [7]. The chromatogram of Variant II Turbo 2.0 showed no indication of variant (Supplementary material, Figure 2). Whereas, mass spectrogram of QuanTOF showed an abnormal Hb chain (m/z=15,169.4), with a relative intensity accounting for 26.0% of the total αHb (Figure 1B). The normal Hb chains, including the αHb subunit (m/z=15,127.9), βHb subunit (m/z=15,868.0), and glycated-βHb (m/z=16,030.0) were also be found in the spectrogram (Figure 1A, B).

Subsequent Hb analyses using CE (CapillaryS2, Hb program) and ion exchange HPLC (β-thalassemia short program, Variant II, Bio-Rad, USA) were conducted. Surprisingly, no sign of abnormal peaks or atypical chromatogram presented (Supplementary material, Figures 3, 4). Sanger sequencing was performed to confirm the presence as well as the nature of Hb variant. The genomic DNA was extracted from the patient's peripheral blood for PCR amplification. *HBA1*, *HBA2* and *HBB* genes were amplified with specific primers (Supplementary material, Table 1). Then, the PCR products was conducted on an ABI PRISMTM 3730 XL Sequencer (Applied Biosystems, USA). The sequencing data revealed a novel heterozygous mutation [α15(A13)(GGT > GTT), Gly > Val, *HBA2*:c.47 G > T] in the *α2* gene, resulting in a coding transition from glycine (molecular weight: 75.1 Da) to valine (molecular weight: 117.1 Da) at codon 15 (Supplementary material, Figure 5). As shown in Figure 1B, the variation of relative molecular weight induced by the substitution from glycine to valine (42.0 Da) could also be found from the m/z changes between αHb subunit and variant Hb subunit (41.5 Da). As

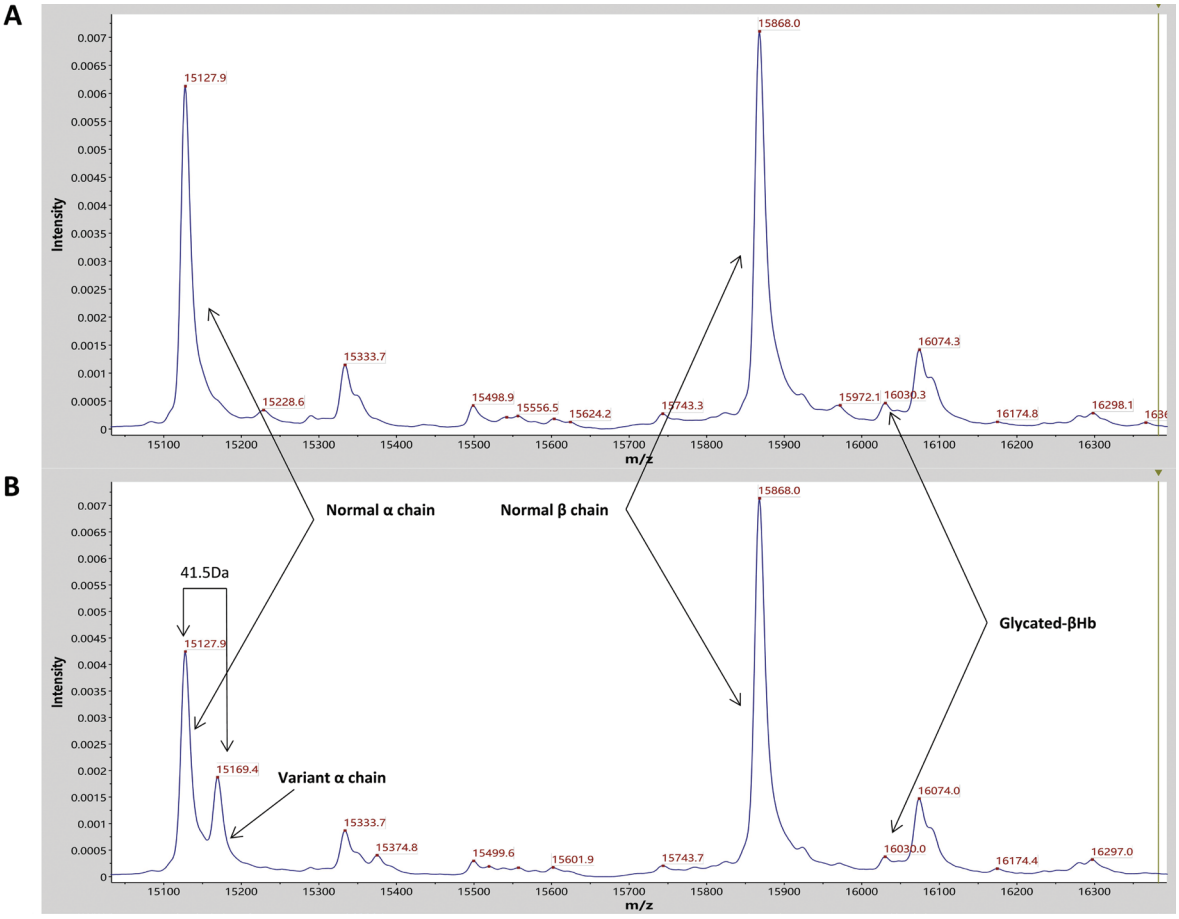

Figure 1: MALDI-TOF mass spectra of control hemoglobin and Hb Liaoning.
(A) Control hemoglobin from normal adult and (B) Hb Liaoning from the proband, respectively. The occurrence of two peaks separated by 41.5 Da clearly indicate the presence of a variant α chain (m/z=15,169.4). Arrows indicate the presence of normal α chain (m/z=15,127.9), normal β chain (m/z=15,868.0), and glycated-βHb (m/z=16,030.0).

the variant has not been reported previously, we named it Hb Liaoning based on the region where the patient originated from.

To determine the patient's hematological characteristics associated with Hb Liaoning, the hematological data was measured using a Sysmex XN900 hematology analyzer (Sysmex Co., Japan). His hematological indices were as follows: red blood cell count, 5.24×10^{12} cells/L (reference: $3.8–5.1\times10^{12}$ cells/L); Hb, 144 g/L (reference: 115–150 g/L); mean corpuscular volume, 87.8 fL (reference: 81.0–100.0 fL); and mean corpuscular Hb, 27.5 pg (reference: 27–34 pg). No sign of anemia was found indicating a non-pathological Hb variant.

Hb variant is one of the causes of hemolytic anemia, as well as analytical interference in HbA_{1c} measurements. In our case, Hb Liaoning showed no significant clinical presentation. However, this variant caused interference in HbA_{1c} measurement using CE. In previous studies, a boronate affinity HPLC method was often used as the comparative method, as it is assumed to be unaffected by most Hb variants as total glycohemoglobin is measured regardless of Hb species [8]. Acceptable biases mentioned in the results suggesting that Hb Liaoning has no significant effect on HbA_{1c} measurements for Variant II Turbo 2.0 and Quan TOF.

HPLC and CE are the first-line methods for HbA_{1c} measurements and Hb analysis. However, limited studies showed the usage of MALDI-TOF MS in HbA_{1c} measurements. Previous studies demonstrated that MALDI-TOF MS can estimate the quantity of both the αHb and βHb, as well as glycated forms of each Hb subunit modified by attachment of a single glucose moiety [9, 10].

In the current study, cation exchange HPLC and electrophoresis methods faced challenges in detecting Hb Liaoning because the charge difference is inapparent and beyond detection limitation. Alternatively, MALDI-TOF MS was able to distinguish Hb Liaoning by the *m/z* difference. Nevertheless, MALDI-TOF MS may not be able to distinguish all types of Hb variants, especially when the *m/z* difference is slightly and beyond the instruments' resolution. Last but not least, detection of Hb variants and recognition of the interference in HbA_{1c} testing are crucial, especially in high prevalence regions of Hb variants.

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