

## Research Article

# False measurement of glycated hemoglobin in patients without hemoglobin A

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**Background:** Hemoglobin (Hb) A<sub>1c</sub>, a biochemical marker widely used in monitoring diabetes mellitus, can be quantitatively measured by various examining systems. However, significant errors still exist. In the present study, we evaluated the HbA<sub>1c</sub> level in five patients with compound heterozygotes by five different examining systems and our goal is to identify the existence of erroneous HbA<sub>1c</sub> measurement.

**Methods:** Blood samples collected from normal (no hemoglobin variants) and abnormal (compound heterozygotes) patients were analyzed by capillary electrophoresis technique and sequence analysis. The samples without HbA expression via above methods were further analyzed for HbA<sub>1c</sub> by ion exchange HPLC Variant II/ Variant II Turbo 2.0 (VII and VII-T 2.0), boronate affinity HPLC, capillary electrophoresis, and Tinaquant immunoassay.

**Results:** HbA<sub>1c</sub> expression were unexpectedly detected in the compound heterozygous samples by using additional examining systems: The HPLC VII and VII-T 2.0 detected HbA<sub>1c</sub> expression in two of five samples and failed to detect the abnormal HbA<sub>2</sub> expression; the CE system detected HbA<sub>1c</sub> expression in one of five samples with abnormal HbA<sub>2</sub> expression; the Ultra2 and PPI system detected the HbA<sub>1c</sub> expression of all samples without abnormal HbA<sub>2</sub>.

**Conclusions:** Five human samples without HbA expression were additionally detected with HbA<sub>1c</sub> expression with or without abnormal HbA<sub>2</sub> expression by five analysis systems and the different examining assay potentially affected the test results. These results demonstrated that the limitations of current examining systems for monitoring patients with hemoglobin disorders highlighting the further improvement in the method of clinical HbA examination.

## Introduction

Glycated hemoglobin (HbA<sub>1c</sub>), the glycated fraction of hemoglobin A, is a biochemical marker. The protein is formed via nonenzymatic glycation of the valine residue at the N-terminal of hemoglobin  $\beta$ -chain with glucose. HbA<sub>1c</sub> test is routinely utilized in monitoring long-term glycemic control and assessing the risk of complications [1,2]. In the 2010 guideline of the American Diabetes Association, HbA<sub>1c</sub> was recommended as one criterion for diabetes screening and diagnosis using a cut-off value of 6.5% (48 mmol/mol) [3]. Therapeutic strategies, according to HbA<sub>1c</sub> test, have been established as well [4]. Therefore, the accurate and precise measurement of HbA<sub>1c</sub> is extremely crucial to clinical practices. Currently, a variety of methods based on different principles are used for HbA<sub>1c</sub> measurement in clinical laboratories and these methodologies included cation exchange-high performance liquid chromatography (CE-HPLC), boronate affinity high-performance liquid chromatography (BAC), capillary electrophoresis (CE), and immunoassay. However, the results of HbA<sub>1c</sub> test from these methods may be influenced with patients' pathophysiological conditions, such as hemolysis, reduced erythrocyte life span, technical interference of certain hemoglobin variants, or elevated HbF expression [5]. More recently, several studies, reported the measurement of HbA<sub>1c</sub>, were significantly affected by patients with Hb variants

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(HbAS, HbAE, HbAC, HbAD, HbAJ (Bangkok), HbAG (Taipei)) and increasing evidence suggested that the results of HbA<sub>1c</sub> in Hb variants detected by implementation of BAC method as well as Roche Tinaquant immunoassay were much less affected than the result of International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) reference method [6–9].

Compound heterozygosity is one of the critical elements to cause genetic disease in human being. The measurement of HbA<sub>1c</sub> is closely associated with the screen or diagnosis of compound heterozygosity patients with Hb variants. Notably, few studies were reported in the HbA<sub>1c</sub> measurement from compound heterozygous patients without HbA expression by using these examining assays. In the present study, HbA<sub>1c</sub> in the blood samples collected from five compound heterozygous patients without HbA expression was analyzed by five common HbA<sub>1c</sub> detection systems to better understand the HbA<sub>1c</sub> measurement in clinical application.

## Materials and methods

### Samples

The study was approved by the Ethics Committee of Zhongshan Hospital of Sun Yat-sen University. A total of 40 whole blood samples with EDTA collected from previously tested specimens were conducted with HbA<sub>1c</sub> analysis by a Bio–Rad Variant II Turbo 2.0 analyzer. Those samples were also analyzed by capillary electrophoresis (Capillars 2; Sebia, Lisses, France) to confirm the absence of Hb variants. HbA<sub>1c</sub> in the 40 samples without HA variants (4.4–14.4% HbA<sub>1c</sub>) were then analyzed with different assays. Furthermore, five blood samples without hemoglobin A expression obtained from previously tested clinical specimens underwent hemoglobin analysis by Sebia capillars 2 system and the type of hemoglobin was confirmed by genotyping analysis. Five samples with compound heterozygotes were collected: the genotype of the first sample was  $\alpha\alpha/\alpha\alpha$  and  $\beta^{CD26}/\beta^{CD41-42}$ ; the second sample was  $\alpha\alpha/\alpha\alpha$  and  $\beta^{IVS2-654}/\beta^{NewYork}$ ; the third sample was similar to the second sample,  $\alpha\alpha/\alpha\alpha$  and  $\beta^{CD41-42}/\beta^{NewYork}$ ; the fourth sample was  $\alpha\alpha/\alpha\alpha$  and  $\beta^{CD41-42}/\beta^{Bangkok}$ ; the fifth sample was  $-SEA/-\alpha^{4.2-Q-Thailand}$  and  $\beta/\beta$ . The above five patients were nondiabetic patients with normal fasting blood glucose. All blood samples were aliquot quadruplicate and frozen at  $-70^{\circ}\text{C}$  before analysis.

### Analysis methods

In present study, Bio–Rad Variant II system (Bio–Rad, U.S.A) was used as a comparative method for Hb<sub>1c</sub> measurement given, it gained National Glycohemoglobin Standardization Program Level Laboratory certification and was traceable to the Diabetes Control and Complications Trial Reference method. A total number of 40 normal samples (no Hb variants) and five samples from patients with compound heterozygotes were examined using five routine methods: (1) the HPLC Variant II system (Bio–Rad, U.S.A); (2) the HPLC Variant II Turbo 2.0 system (Bio–Rad, U.S.A); (3) the Ultra2 system (Trinity Biotech, U.S.A) using the BAC principle; (4) the Capillars 2 Flex Piercing (C2FP) system (Sebia, France) using the CE principle; (5) the Roche Modular PPI system (Roche, Germany) using Tina-quant Hemoglobin A<sub>1c</sub> III principle. The mixed whole blood samples were adopted to transferring value-assignment in order to improve the comparability of those systems to Bio–Rad Variant II. To analyze the results detected from samples with five examining assays, Bland–Altman plots were performed. Ordinary linear regression was performed to regression and bias analysis. The percentage deviation plots were used to carry out bias estimation ( $-6.0-6.0\%$ ).

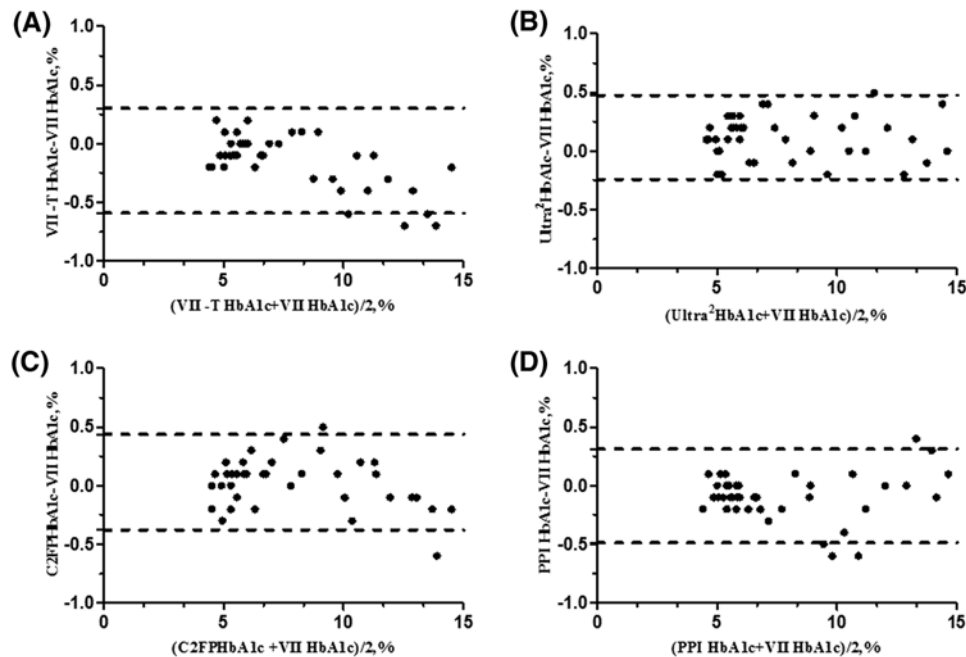
### Statistical analysis

Statistical analyses were carried out using SPSS software version 17.0. The Student's *t*-test was used to compare differences between two groups. Data were presented as means  $\pm$  standard deviations (SDs). Statistically significant difference was defined as *P* value  $<0.05$ .

## Results

### HbA<sub>1c</sub> measurement in the patient without HA variants

The HbA<sub>1c</sub> values of 40 patients without HA variants were correlated with VII system detected by VII, VII-T 2.0, Ultra2, C2FP, and PPI system. The results were further analyzed with corresponding equations:  $y = 1.0514x - 0.2502$  ( $R^2 = 0.9969$ ),  $y = 0.9991x - 0.1105$  ( $R^2 = 0.9965$ ),  $y = 1.0178x - 0.1701$  ( $R^2 = 0.9957$ ), and  $y = 0.9932x + 0.1404$  ( $R^2 = 0.9957$ ). Bland–Altman plots (Figure 1) showed agreement between VII system and VII-T, Ultra2, C2FP, PPI systems and the 95% confidence interval (95% CI) for the deviation between VII system and those systems was  $-7.5\%$  to  $+7.5\%$  (95% CI VII-T: 0.3 to  $-0.59$ ; Ultra2: 0.48 to  $-0.24$ ; C2FP: 0.38 to  $-0.44$ ; PPI: 0.49 to  $-0.31$ ).



**Figure 1. Bland–Altman difference plot applied to comparing HbA1c results of VII system with other systems**  
 X-axis is mean between the test and comparative methods (%). Y-axis is difference between the test and comparative methods (%). Dotted lines represent 95% CI (A) VII-T system compares with VII system; (B) Ultra2 system compares with VII system; (C) C2FP system compares with VII system; (D) PPI system compares with VII system.

## HbA<sub>1c</sub> measurement in the patients with compound heterozygotes

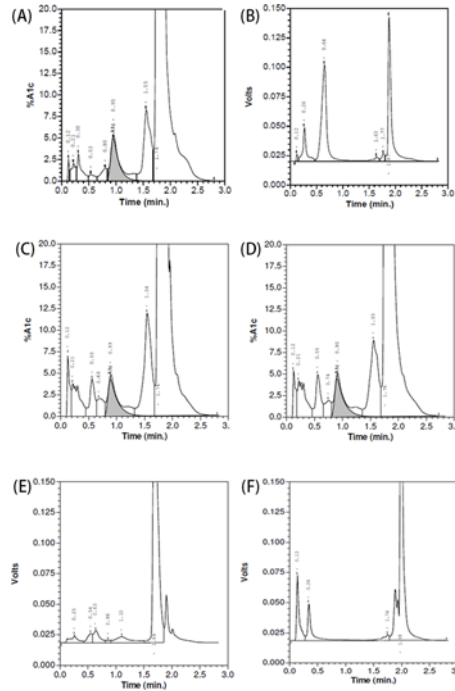
HbA<sub>1c</sub> is formed on the basis of HbA and the change of HbA can impact the formation of HbA<sub>1c</sub>. Blood samples without HbA are defined as no HbA<sub>1c</sub> expression according to the definition of the International Federation of Clinical Chemistry and Laboratory Medicine. Five blood samples with compound heterozygotes in our study were detected without HbA expression, indicating that there was no HbA<sub>1c</sub> expression. However, the five examining systems showed different results.

Two erroneous results for HbA<sub>1c</sub> were attained in the VII analyzer and the chromatograms of the first (Figure 2B), fourth (Figure 2E), and fifth (Figure 2F) samples were all labeled ‘Volts’, which meant abnormal electrophoresis signal and was considered unacceptable according to the manufactures’ instructions. However, the VII chromatograms for the second (Figure 2C) and third (Figure 3D) samples with Hb New York and  $\beta$ -thalassemia (Figure 2C,D) were not easily distinguished from a normal HbAA chromatogram (Figure 3A) and the proportions of the HbA<sub>1c</sub> (Table 1) and HbA<sub>0</sub> (85.5% and 86.3% respectively) were produced. The peak of Hb NewYork0 was all mistakenly identified as HbA<sub>0</sub>.

Similarly, the chromatograms of the VII-T 2.0 analyzer for the first (Figure 3B) and fifth (Figure 3F) samples also did not show the results of HbA<sub>1c</sub> and also labeled ‘Volts’. The VII-T 2.0 chromatograms for the second (Figure 3C) and third samples (Figure 3D) also appeared normal with no indication of variants and the Hb New York appeared to elute as the HbA<sub>0</sub> window. The VII-T 2.0 chromatogram for the fourth (Figure 3E) was different from the VII chromatogram and showed a HbA<sub>1c</sub> value (4.7% in Table 1). Checking the chromatogram, we found the proportion of P4 was very high (84.9%), which was different from normal chromatogram (under 1.5%). So, we assumed that the P4 window was the peak of Hb J-Bangkok.

Of the five heterozygotes analyzed by C2FP system, erroneous results were reported in only one sample (Figure 4F). The electropherograms for the first, second, third and fourth, (Figure 4B–E) all showed the ‘atypical profile’ flag due to identification of the abnormal electropherograms. In addition, four of five electropherograms misidentified the peak of Hb variants as the peak of Hb A<sub>0</sub>. For the fifth sample with Hb Q-H disease (Figure 4F), electropherogram of C2FP analyzer produced an erroneous ‘normal’ HbA<sub>1c</sub> (3.9% in Table 1) result and did not show the peak of HbH.

All of those hemoglobin variants were undetectable on the HbA<sub>1c</sub> principle from PPI and primus Ultra2 systems. It was remarkable that Ultra2 and PPI systems all produced spuriously ‘normal’ HbA<sub>1c</sub> results for all five double heterozygotes (Table 1).



**Figure 2. The chromatogram of five double heterozygous carriers on Bio-Rad V analyzer**  
 (A) Normal sample; (B)  $\beta^{CD26}/\beta^{CD41-42}$  carrier; (C)  $\beta^{IVS2-654}/\beta^{NewYork}$  carrier; (D)  $\beta^{CD41-42}/\beta^{NewYork}$  carrier; (E)  $\beta^{CD41-42}/\beta^{J-Bangkok}$  carrier; (F)  $-SEA/-\alpha^{4.2-Q-Thailand}$  carrier.

**Table 1 HbA1c values for five double heterozygotes carriers on Bio-Rad VII, Bio-Rad VII-T 2.0, Sebia Capillarys 2 Flex Piercing, Trinity Biotech Ultra2, and Roche PPI systems**

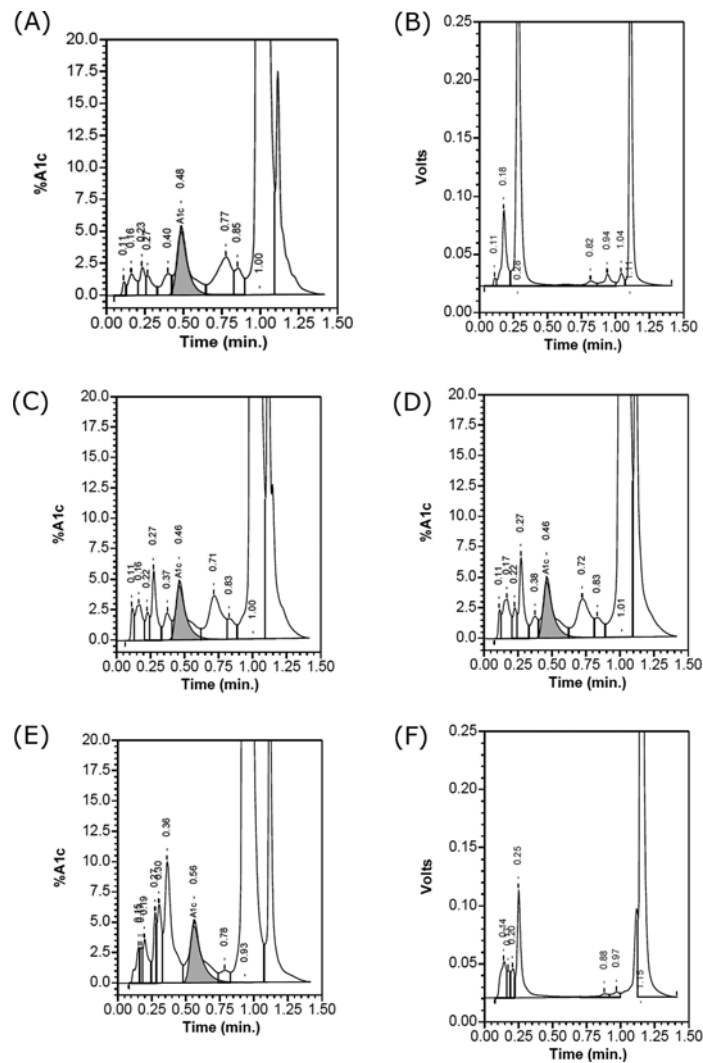
Samples	Genotypes	Bio-Rad VII	Bio-Rad VII-T	Sebia C2FP	Trinity Biotech Ultra2	Roche PPI
1	$\alpha\alpha/\alpha\alpha;$ $\beta^{CD26}/\beta^{CD41-42}$	Nr	Nr	Nr	4.2%	4.5%
2	$\alpha\alpha/\alpha\alpha;$ $\beta^{IVS2-654}/\beta^{NewYork}$	4.3%	4.5%	Nr	22 mmol/mol 4.1%	26 mmol/mol 4.2%
3	$\alpha\alpha/\alpha\alpha;$ $\beta^{CD41-42}/\beta^{NewYork}$	23 mmol/mol 4.5%	26 mmol/mol 4.6%	Nr	21 mmol/mol 4.3%	22 mmol/mol 4.8%
4	$\alpha\alpha/\alpha\alpha;$ $\beta^{CD41-42}/\beta^{J-Bangkok}$	26 mmol/mol Nr	27 mmol/mol 4.7%	Nr	23 mmol/mol 4.7%	29 mmol/mol 3.8%
5	$-SEA/-\alpha^{4.2-Q-Thailand};$ $\beta/\beta$	Nr	28 mmol/mol Nr	3.9%	28 mmol/mol 5.3%	– 5.7%
				–	34 mmol/mol	39 mmol/mol

Nr, no HbA1c value was reported for this sample by the system; ‘–’, no IFCC HbA1c value transferred.

## Discussion

The HbA<sub>1c</sub> value reflects the patient’s mean glycemic level in the past 6–8 weeks. Hemoglobinopathy alters the composition and structure of hemoglobin and may lead to misinterpretation of the HbA<sub>1c</sub> result. Hemoglobin variants have been reported to potentially affect the precision of current examining methods for HbA<sub>1c</sub> measurement [10–12]. However, HbA<sub>1c</sub> values measured in patients with compound heterozygotes are rarely reported.

HbA<sub>1c</sub> has been referred as one of major markers for diabetes diagnosis by the World Health Organization since 2011 [13]. However, HbA<sub>1c</sub> is still not a diagnostic criterion for diabetes in China yet. One of the reasons is that



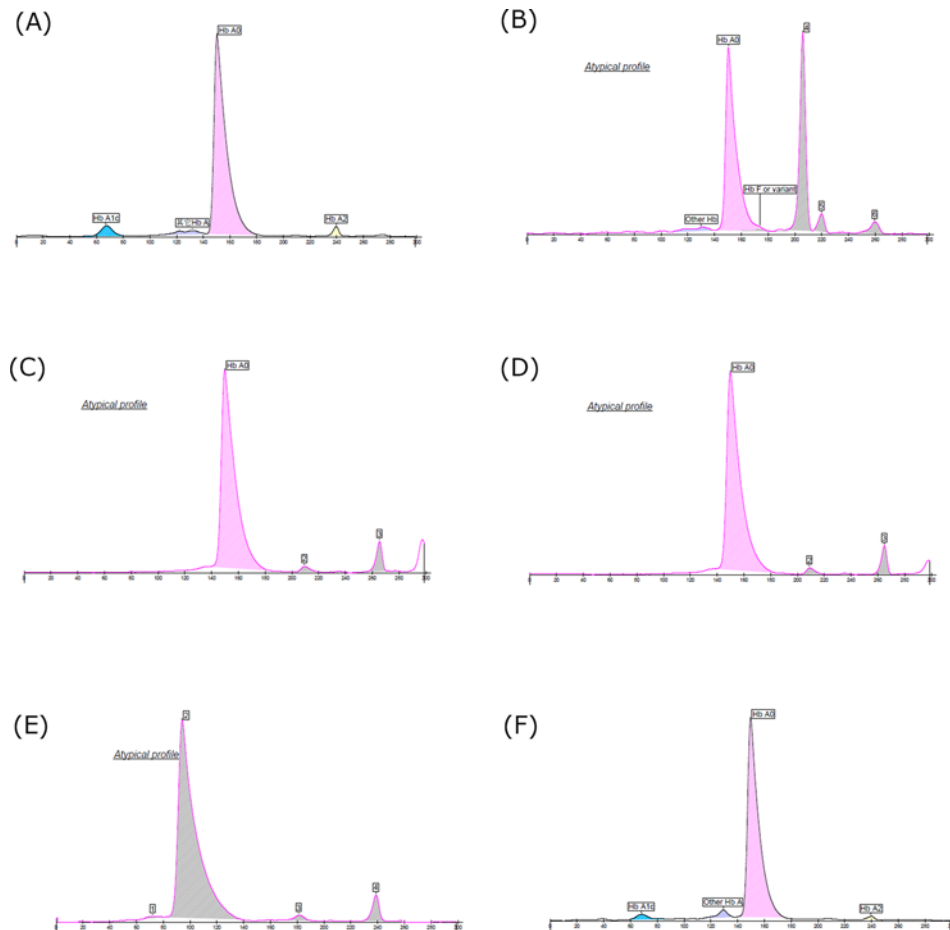
**Figure 3. The chromatogram of five double heterozygous carriers on Bio-Rad V Turbo analyzer**

(A) Normal sample; (B)  $\beta^{\text{CD}26}/\beta^{\text{CD}41-42}$  carrier; (C)  $\beta^{\text{IVS}2-654}/\beta^{\text{NewYork}}$  carrier; (D)  $\beta^{\text{CD}41-42}/\beta^{\text{NewYork}}$  carrier; (E)  $\beta^{\text{CD}41-42}/\beta^{\text{J-Bangkok}}$  carrier; (F) -SEA/- $\alpha^{4.2-Q}$ -Thailand carrier.

China is a population with a high prevalence of hemoglobinopathy and thalassemias [14–17]. The incidence of  $\alpha$ - and  $\beta$ -thalassemias is 8.53% and 2.54% respectively [14]. Of note, the incidence of abnormal Hbs is 0.358% in Chaozhou city [15] and 0.4% in Dongguan city, China. The common  $\alpha$ -globin variants are Hb Constant Spring, Hb Q-Thailand, and Hb G-Honolulu. The common  $\beta$ -globin variant was Hb E, Hb New York, Hb J-Bangkok, et al. [16]. Thalassemias combined with hemoglobin variants are a special physiopathological condition that cannot form HbA. Five cases in our article were double heterozygous samples that do not contain HbA and HbA<sub>1c</sub>. We tested those five samples by five examining systems in order to assess the specificity of common methods in the measurement of HbA<sub>1c</sub>.

HbA<sub>1c</sub> values can be evaluated by various methods based on the molecular charge (cation-exchange high-performance liquid chromatogram (CE-HPLC) and electrophoresis) or molecular structure (immunoassays, boronate affinity chromatography, and mass spectrometry). Both VII and VIIT2.0 belong to CE-HPLC assay, which can separate HbA<sub>1c</sub> from HbA because glycation of the N-terminal valine reduces the positive charge. In present five cases, the two principles reported two of five and three of five spurious HbA<sub>1c</sub> results respectively. Because Hb New York has the similar charge to HbA and can be eluted together with HbA Hb New York in the second and third cases, it was erroneously identified as HbA<sub>0</sub> due to elution in the respective retention and Hb (New York)<sub>1c</sub> misidentified as HbA<sub>1c</sub>. Hb J-Bangkok is another common hemoglobin variant in China and its charge is different from HbA.





**Figure 4. The chromatogram of five double heterozygous carriers on Sebia C2FP analyzer**

(A) Normal sample; (B)  $\beta^{CD26}/\beta^{CD41-42}$  carrier; (C)  $\beta^{IVS2-654}/\beta^{NewYork}$  carrier; (D)  $\beta^{CD41-42}/\beta^{NewYork}$  carrier; (E)  $\beta^{CD41-42}/\beta^{J-Bangkok}$  carrier; (F)  $-SEA/-\alpha^{4.2-Q-Thailand}$  carrier.

Therefore, the chromatograms of VII and VIIT2.0 were shown abnormally. Lo et al. [18] reported that a case of spuriously normal HbA<sub>1c</sub> results due to misidentifying HbG Taipei as HbA<sub>0</sub> by the variant II system. These results showed that CE-HPLC was obviously interfered by Hb variants with different charges and might result in erroneous HbA<sub>1c</sub> concentration.

C2FP is a new assay to evaluate HbA<sub>1c</sub> based on the separation of Hb property and charge by capillary electrophoresis. There is a strong consistency between the results of C2FP and VII [19]. Many literatures have reported that the resolution of C2FP is superior to CE-HPLC resulting from allowing the separation of many common and rare Hb variants from the HbA<sub>0</sub> fraction [19–21]. Of five samples without HbA expression, C2FP system could detect HbA<sub>1c</sub> up to four samples. Although C2FP system misidentified Hb F as HbA<sub>0</sub> in the first sample and Hb (New York) 0 as HbA<sub>0</sub> in the second and third sample, the system did not display HbA<sub>1c</sub> values, which might prompt laboratories to pay more attention to those patients with hemoglobin fractions. However, in the fifth sample, C2FP system detected the HbA<sub>1c</sub> values without showing HbH expression and misidentified HbQ-Thailand as HbA<sub>0</sub>. Literatures had reported that C2FP system produced two inaccurate results of the 18 rare variants (Hb Silver Springs and Hb J-Broussais) [11]. For HbG Coushatta, C2FP system produced a HbA<sub>1c</sub> value different from the results detected by Tandem HPLC-capillary electrophoresis (IFCC reference method) [22]. Although C2FP system could separate many common and rare Hb variants from the HbA<sub>0</sub> fraction, we also must focus on analyzing raw data of the different Hb variants and finding the problems of the electropherograms.

Ultra2 system using boronate affinity HPLC method is based on the fact that glycosylated and non-glycosylated are separated regardless of hemoglobin species and the Ultra2 system for Hb A<sub>1c</sub> seems to have less interference by Hb variants compared with the IFCC Reference Method [23]. PPI system is based on Tinaquant immunoassay using antibodies

to target N-terminal glycosylated amino acids on the  $\beta$  chain for quantifying Hb A<sub>1c</sub> and the Hb A<sub>1c</sub> percentage is calculated according to the Hb A<sub>1c</sub> and Hb concentrations. Ultra2 system and Tinaquant immunoassay had been used as comparative methods in previous articles since these methods were less likely to show interference by Hb variants [11,24]. In our five cases, the Ultra2 system and PPI system measured total glycosylated hemoglobin and detected a normal HbA<sub>1c</sub>. But there was no Hb A in these samples and Hb A<sub>1c</sub> could not be detected. So, we assumed that the HbA<sub>1c</sub> measurement derived from patients without Hb A expression by those examining systems should not be used as an indicator of those people with average blood glucose level to screen and diagnose the diabetes mellitus.

## Conclusions

Each examining system for HbA<sub>1c</sub> measurement could not eliminate the interference of double heterozygous in our work. The implementation of such methods may not generate trustworthy results for clinic application. Though C2FP system may be superior to other systems, it is important to know that the hemoglobinopathy can affect HbA<sub>1c</sub> measurement. Patients with compound heterozygous variants are suggested to use non-Hb-based method, such as fructosamine, glycosylated albumin or continuous glucose monitoring, to assess long-term glycemic control instead of Hb A<sub>1c</sub> measurement. Due to the high frequencies of hemoglobin variants, it is important to clarify these limitations when using these methods to measure Hb A<sub>1c</sub>.

## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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## Author Contribution

M.S. designed and performed the research, analyzed the data, and wrote the paper; D.W. guided and supervised the experiment process; W.W. detected the specimens; D.Z. detected the specimens; S.X. collected the specimens; X.W. detected the specimens; T.H. detected the specimens.

## Abbreviations

BAC, boronate affinity high-performance liquid chromatography; C2FP, Capillars 2 Flex Piercing; CE, capillary electrophoresis; CE-HPLC, cation exchange-high performance liquid chromatography; HbA<sub>1c</sub>, glycosylated hemoglobin.

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