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Monitoring novel modified hemoglobin using mass spectrometry contributes to accurate blood glucose management of the Han Chinese population

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ABSTRACT

Background: The goal of this study was to detect novel modified forms of hemoglobin using mass spectrometry (MS) and to investigate the effect of modified hemoglobin on HbA1c and fasting plasma glucose (FPG).
Methods: This study was conducted on 1200 subjects aged > 25 years. Hemoglobin from the above-mentioned subjects was detected using direct-infusion electrospray ionization-MS, and HbA1c and FPG were measured according to the manufacturer's instructions. Regression analysis was performed to estimate the correlations and interactions among HbA1c, FPG, and modified hemoglobin.
Results: Multiple modified forms (α_1, α_2, α_3, β_1, β_2, and β_3) of hemoglobin were observed using MS. Statistical analyses indicated that modified hemoglobin was significantly correlated with FPG ($p \leq .01$). The association of FPG with α_1% ($p = .021$) and β_3% ($p < .001$) values was independent of HbA1c% and other modified forms of hemoglobin. Interaction analyses implied two significant interaction effects of HbA1c% with gender ($\beta = -0.184$, $p = .007$) and α_3% ($\beta = -0.104$, $p < .001$) on FPG. The relationship between HbA1c% and FPG was stronger in males than in females, and a decreased level of α_3% also affected the association of HbA1c% and FPG.
Conclusions: This MS-based method is an effective tool for monitoring glycated forms of hemoglobin than traditional approaches. For the Han Chinese population, multiple-glycated hemoglobin affects the association of FPG with HbA1c%, and the correlation between FPG and HbA1c% in females is different from that in males. These data suggest that the HbA1c criteria for the diagnosis and monitoring of diabetes should be established according to genders and modified types of hemoglobin.

1. Introduction

Diabetes is a disease characterized by chronic hyperglycemia, insulin resistance, and β-cell dysfunction, which leads to long-term complications. Due to increased nutrition conditions and reduced activity levels, the prevalence of type 2 diabetes has continued to climb [1]. As the complications of diabetes are irreversible and difficult to detect at an early stage [2], discovering novel biomarkers for the diagnosis and monitoring of diabetes is important.

Glycated hemoglobin, the product of the nonenzymatic glycation of hemoglobin, is regarded as an important biomarker for long-term glycemic control because it reflects the average blood glucose levels during the previous 2 to 3 months [3]. HbA1c, glycated hemoglobin with a stable adduct of glucose linked to the N-terminal valine of the β-chain, has been used in the diagnosis and management of diabetes [3]. Although there are various approaches to HbA1c measurement [4–6], the

Abbreviations: HbA1c, hemoglobin A1c; FA, formic acid; FPG, fasting plasma glucose; MS, mass spectrometry; DIESI-FTICR MS, direct-infusion electrospray ionization-Fourier transform ion cyclotron mass spectrometry; SD, standard deviation

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glycated hemoglobin test is now an international standard for reporting valid HbA1c concentrations [7,8]. However, traditional methods of glycated hemoglobin measurement have various limitations [9]. First, the association of HbA1c with glycemia can be interfered by several factors, such as genetic variants [10], ethnicity-related factors [11], chronic kidney disease [12], and hemoglobinopathies [13]. Second, as a diagnostic molecule, HbA1c is less sensitive than fasting plasma glucose (FPG) and 2-hour plasma glucose [14,15]. Additionally, traditional methods cannot measure different subtypes of hemoglobin simultaneously.

Mass spectrometry (MS), an accurate method of measuring the mass, level, and structure of molecules, has been proven to be an alternative approach for assessing different types of glycated hemoglobin [16–19] due to several advantages. First, MS can provide accurate mass of various hemoglobin species and all forms of glycated hemoglobin simultaneously [20,21]. Second, MS-based methods are able to identify novel modified products of hemoglobin, which might be useful as novel biomarkers for diabetes [22]. For the Chinese population, the association of modified hemoglobin with blood glucose has not been evaluated, and a better understanding of this association will help in the development of more specific and accurate methods for diabetes management.

To evaluate the associations and interactions of modified hemoglobin with blood glucose, in the present study, we enrolled 1200 subjects from the Beijing Physical Examination Center and measured modified hemoglobin using direct-infusion electrospray ionization-Fourier transform ion cyclotron mass spectrometry (DIESI-FTICR MS). Several types of modified hemoglobin were observed, and the associations of the glycated hemoglobin subtypes with HbA1c levels measured by traditional methods were analyzed. We also performed an interaction analysis to evaluate the interactions among different subtypes of modified hemoglobin.

2. Materials and methods

2.1. Participants and chemicals

A total of 1200 participants who received physical examinations at Beijing Physical Examination Center between July 2015 and November 2016 were enrolled in this study. After excluding participants, who did not examine the levels of FPG and HbA1c simultaneously during their physical examination, venous blood samples from 1112 of the remaining participants were used in the following study. This study was approved by the Ethics Review Board of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences in accordance with principles of the 1975 Helsinki Declaration and its amendments, and informed consent was acquired from each participant.

LC-MS grade methanol and formic acid (FA) were purchased from Fisher Scientific Corporation (Fair Lawn, NJ, USA). Ultrapure water was prepared using a Millipore synergy ultrapure water purification system (Millipore, France). Analytical grade acetone was purchased from Beijing Chemical Works (Daxing, Beijing, China).

2.2. Clinical biomarkers

FPG was examined using reagents supplied by Abbott diagnostics on the Abbott ARCHITECT c16000 analyzer (Abbott Diagnostics, Abbott Park, IL, USA), and HbA1c was measured using AdamsA1c HA-8160 (ARKRAY Inc., Kyoto, Japan) based on cationic exchange high-performance liquid chromatography according to International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) standardized protocol [7].

2.3. Sample preparation

The same blood sample used to examine HbA1c and FPG were used to perform the MS analyses. Red cells (100 μL) was diluted to 200 μL with ultrapure water, and the mixture was vortexed for 10 s. After centrifugation at 15,000*g* for 5 min at 4 °C, the supernatant was transferred to a new tube. Then, 10 μL of the resulting sample solution was further diluted with 1000 μL of acetone containing 3% (*v*/v) FA and vortexed for 10 s. After centrifugation at 2000*g* for 3 min at 4 °C, the supernatant was discarded, and the pellet was diluted with 1000 μL of ultrapure water. Then, 25 μL of the resulting solution was diluted with 475 μL of methanol:water(*v*/v/v, 7:3) containing 0.2% FA. The solution was centrifuged at 15,000*g* for 5 min at 4 °C, and the supernatant was transferred to a new tube.

2.4. Mass spectrometric analysis

All mass spectrometric analyses were performed on a 9.4 T hybrid DIESI-FTICR MS (Bruker Daltonics, Billerica, MA, USA). The operating parameters in positive ion mode are as follows: nebulizing gas flowrate, 1.5 L/min; drying gas flowrate, 2.5 L/min; drying gas temperature, 180 °C; capillary voltage, 4600 V; spray shield voltage, 3800 V. Each mass spectrum was acquired with a total of 95 scans accumulated over the *m/z* range of 500 to 2600 with a resolution of 41,000 at *m/z* 400. The instrument was calibrated with ESI TuningMix standard solution in positive ion mode. The solution of each sample was infused directly into the electrospray ion source at a flowrate of 3 μL/min using a syringe pump. The quality control sample from the mixed sera of five patients with diabetes was analyzed once every twenty test samples to monitor the experimental reproducibility and stability.

2.5. Statistical analysis

In the current study, we have transferred high-performance liquid chromatography-based measured values of HbA1c in International System of units (SI, mmol/mol) to the National Glycohemoglobin Standardization Program (NGSP) units based on the following equation:

$$NGSP - HbA1c(\%) = 0.915 \times SI - HbA1c(mmol/mol) + 2.15\%$$

As the levels of the modified hemoglobin in this study are expressed as “percentage units”, we used NGSP units of HbA1c for statistical analyses to compare the values of HbA1c and different forms of modified hemoglobin.

Linear regression was performed to evaluate the associations of the subtypes of modified hemoglobin with HbA1c or FPG. The values of modified hemoglobin were calculated based on the following formula:

$$a_i\% = 100 \times \frac{a_i}{\sum_{i=0}^{3} a_i}\%$$

where a_i indicate the levels of the unmodified α (or β) subunit of hemoglobin and the three major modified forms detected in the current study. Additional analyses were also performed to examine the interactions among different forms of modified hemoglobin, which was calculated based on the counterfactual framework-based approach of causal mediation analysis proposed by Hayes [23], which is a statistical strategy focused on identifying and quantifying the interactions of biological factors. Impaired fasting glucose was defined as FPG ≥ 6.1 mmol/L. Statistical analysis was performed using SPSS version 23.0 software (SPSS Inc., Chicago, IL, USA) with the PROCESS Procedure for SPSS Release 2.12 [23]. Data are reported as the mean ± standard deviation (SD) and the mean (95% CI) unless otherwise indicated.

3. Results

3.1. Modified hemoglobin profiling

The general characteristics of the participants are listed in Table 1. The average age of the eligible participants was 45.4 ± 11.4 years,

Table 1
Characteristics of the participants in this study.

Variables	Molecular weight (Da)	Modified forms of hemoglobin	Mean ± SD
Age, y	\	\	45.4 ± 11.4
Male, %	\	\	60.9
BMI, kg/m^2	\	\	25.5 ± 3.8
FPG, mmol/L	\	\	6.96 ± 2.74
HbA1c, mmol/mol(%)	\	\	45 ± 16(6.27 ± 1.49)
α_1, %	15,224.6	α-chain + 98.1 Da	10.04 ± 6.15
α_2, %	15,288.4	Glycated α-chain	2.77 ± 1.02
α_3, %	15,451.8	Glycated α-chain + glucose residue	2.01 ± 1.37
β_1, %	15,965.7	β-chain + 98.3 Da	7.54 ± 4.73
β_2, %	16,028.5	Glycated β-chain	5.52 ± 1.79
β_3, %	16,174.5	Glycated β-chain + fucose residue	4.54 ± 1.78

BMI, body mass index; FPG, fasting plasma glucose; SD, standard deviation; α_1, α_2, α_3, β_1, β_2, and β_3 are the major modified forms of hemoglobin detected by mass spectrometry in this study. Reference range of BMI for Chinese Han population: low body weight, < 18.5 kg/m^2; normal weight, 18.5–23.9 kg/m^2; overweight, 24–27.9 kg/m^2; obese, ≥28 kg/m^2. Reference range of FPG for Chinese Han population: hypoglycemia, < 4 mmol/L; normal, 4–7 mmol/L; hyperglycemia, > 7 mmol/L. HbA1c: normal, 20–53 mmol/mol (4–6%).

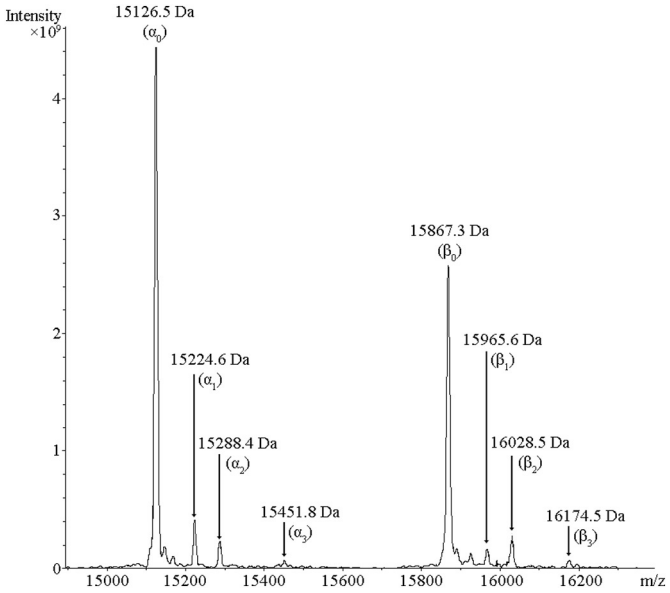

Fig. 1. The deconvoluted spectrum of hemoglobin α-and β-chains and the corresponding modified forms of hemoglobin. α_0 (MW 15126.5 Da): unmodified α-chain; β_0 (MW 15867.3 Da): unmodified β-chain; α_1 (MW 15224.6 Da): a novel modified form of the α-chain (α-chain + 98.1 Da); β_1 (MW 15965.6 Da): a novel modified form of the β-chain (β-chain + 98.3 Da:); α_2 (MW 15288.4 Da): the glycated α-chain (α-chain + glucose residue (161.9 Da)); β_2 (MW 16028.5 Da):the glycated β-chain (β-chain + a mass of glucose residue, 161.2 Da); α_3 (MW 15451.8 Da): a modified form of the glycated α-chain plus one glucose residue (163.3 Da); β_3 (MW 16174.5 Da): a modified form of the glycated β-chain plus one fucose residue (145.2 Da).

60.9% of the participants were males, and the average FPG was relatively high, 6.96 ± 2.74 mmol/L, as were the corresponding the values of HbA1c (45 ± 16 mmol/mol or 6.27 ± 1.49%). A representative deconvoluted mass spectrum is shown in Fig. 1. As shown in **Fig. 1**, the hemoglobin α- and β-chains and three major modified forms were observed. The unmodified α-and β-chains were named α_0(molecular weight (MW), 15,126 Da) and β_0(MW, 15867.3 Da), respectively. Three additional modified forms of the α- and β-chains were also observed. The unmodified α- and β-chains plus 98 Da were named α_1 (MW, 15224.6 Da) and β_1 (MW, 15965.6 Da), respectively; the unmodified α- and β-chains plus one glucose residue were named α_2 (MW, 15288.4 Da) and β_2 (MW,16028.5 Da), respectively; and the unmodified α-chain plus 325.3 Da and β-chains plus 308.9 Da were named α_3 (MW, 15451.8 Da) and β_3 (MW, 16176.2 Da), respectively. It should be noted that this is the first report of the presence of α_1, α_3, β_1, and β_3 in the Han Chinese population. Based on the masses detected in this study, α_3 and β_3 may be multiple-glycated hemoglobin, corresponding to an additional glucose residue on the glycated α-chain and an additional fucose residue on the glycated β-chain, respectively.

3.2. Correlation of modified hemoglobin with FPG or HbA1c

To reveal the associations of the modified forms of hemoglobin with blood glucose, linear regression was performed. Statistical analysis indicated that the values of HbA1c%, α_1%, α_2%, α_3%, β_1%, β_2%, and β_3% were all strongly associated with FPG. For example, beta = 1.588, r^2 = 0.736 for HbA1c%; beta = 2.149, r^2 = 0.636 for α_2%; and beta = 1.179, r^2 = 0.588 for β_2%, all with p < .001 (Table 2). However, after adjustment for the levels of all forms of modified hemoglobin, the values of α_2%, α_3%, β_1%, and β_2% were not independently associated with the level of FPG.

As HbA1c% is regarded as a biomarker of average blood glucose, the associations of the modified hemoglobin with the values of HbA1c% were investigated. The values of α_1%, α_2%, β_1%, β_2%, and β_3% were significantly correlated with the values of HbA1c%; for example, beta = 1.272, r^2 = 0.762, p < .001 for α_2% and beta = 0.711, r^2 = 0.736, p < .001for β_3%. When other modified hemoglobin forms were controlled, the interactions between the values of HbA1c% and the values of α_1%, α_2%, α_3%, β_2%, or β_3% were still significant. Interestingly, the values of α_3% or β_3% were negatively correlated with the FPG levels (α_3%: beta = −0.039, p = .199; β_3%: beta = −0.287, p < .001) but positively associated with the values of HbA1c% (α_3%: beta = 0.033, p = .016; β_3%: beta = 0.054, p < .001) (Table 2).

3.3. Associations of HbA1c% values with different modulators

Interactions between the HbA1c% and potential modulators were also analyzed, and the results are shown in Table 3. The interaction between HbA1c% and FPG was significantly modified by gender (beta = −0.184 (−0.318 to −0.050), p = .007) and α_3% (beta = −0.104 (−0.152 to −0.055), p < .001). After further adjustment for age and gender, the interaction of HbA1c% with α_3% also remained significant (beta = −0.106 (−0.154 to −0.059, p < .001). The association of HbA1c% with FPG was stronger in males (beta = 1.596 (1.532 to 1.661), p < .001) than in females (beta = 1.412 (1.295 to 1.530), p < .001) (Fig. 2a). We then divided the levels of α_3% into three different levels: low (α_3% levels < mean + SD), intermediate (mean-SD ≤ α_3% levels < mean + SD), and high (α_3% levels ≥ mean + SD). Increasing strength of the interaction of HbA1c% with FPG was observed with decreasing levels of α_3% (low level of α_3%: beta = 1.673 (1.604 to 1.742), p < .001; intermediate

Table 2
Linear correlation of modified hemoglobin with fasting plasma glucose (FPG) or HbA1c%.

Modified hemoglobin	Model type	FPG			HbA1c%		
		Beta[a]	*P*	r^2	Beta[a]	*P*	r^2
HbA1c%	model 1[b]	1.588	< **0.001**	0.736	\	\	\
	model 2[c]	1.351	< **0.001**	\	\	\	\
α_1%	model 1[b]	0.004	**0.006**	0.007	0.031	< **0.001**	0.016
	model 2[c]	−0.005	**0.021**	\	0.149	**0.002**	\
α_2%	model 1[b]	2.149	< **0.001**	0.636	1.272	< **0.001**	0.762
	model 2[c]	0.238	0.200	\	0.585	< **0.001**	\
α_3%	model 1[b]	−0.156	**0.010**	0.006	−0.049	0.130	0.001
	model 2[c]	−0.039	0.199	\	0.033	**0.016**	\
β_1%	model 1[b]	0.005	**0.010**	0.006	0.033	< **0.001**	0.011
	model 2[c]	0.005	0.091	\	−0.087	0.072	\
β_2%	model 1[b]	1.179	< **0.001**	0.588	0.711	< **0.001**	0.736
	model 2[c]	−0.002	0.644	\	0.357	< **0.001**	\
β_3%	model 1[b]	−0.766	< **0.001**	0.244	−0.318	< **0.001**	0.144
	model 2[c]	−0.287	< **0.001**	\	0.054	< **0.001**	\

[a] Coefficient of the linear regression.
[b] Unadjusted model.
[c] Model adjusted for the values of other modified forms of hemoglobin.

Table 3
Interactions of HbA1c% with age, gender, and different forms of modified hemoglobin.

Modulator	Model type	CV (95% CI)	p
Age	Model 1[b]	0.001 (−0.005 to 0.006)	0.789
	Model 2[c]	\	\
Gender[a]	Model 1[b]	−0.184 (−0.318 to −0.050)	**0.007**
	Model 2[c]	\	\
α_1%	Model 1[b]	0.004 (−0.005 to 0.012)	0.403
	Model 2[c]	0.004 (−0.004 to 0.013)	0.329
α_2%	Model 1[b]	−0.017 (−0.052 to 0.019)	0.356
	Model 2[c]	0.013 (−0.025 to 0.050)	0.504
α_3%	Model 1[b]	−0.104 (−0.152 to −0.055)	< **0.001**
	Model 2[c]	−0.106 (−0.154 to −0.059)	< **0.001**
β_1%	Model 1[b]	0.000 (−0.012 to 0.011)	0.965
	Model 2[c]	0.001 (−0.011 to 0.012)	0.931
β_2%	Model 1[b]	−0.004 (−0.024 to 0.016)	0.713
	Model 2[c]	0.011 (−0.010 to 0.031)	0.303
β_3%	Model 1[b]	−0.017 (−0.053 to 0.019)	0.354
	Model 2[c]	−0.027 (−0.064 to 0.010)	0.148

[a] "male = 1" and "female = 2" were assigned to gender for statistical analyses.
[b] Unadjusted model.
[c] Model adjusted using age and gender.

level of α_3%: beta = 1.613 (1.555 to 1.670), p < .001; and high level of α_3%: beta = 1.486 (1.414 to 1.558), *p* < .001) (Fig. 2b).

4. Discussion

In the current study, the MS-based approach detected three major modified forms of the α- and β-chains of hemoglobin (α_1, α_2, α_3, β_1, β_2, and β_3). We found that the levels of all these modified forms of hemoglobin were associated with FPG levels. The interaction analyses revealed that the interaction between HbA1c% and FPG was modified by gender and the level of α_3%.

Glycated hemoglobin, especially HbA1c%, is regarded as a useful biomarker for monitoring the average blood glucose in the previous 2 to 3 months [3]. However, the effects of factors including genetic variants [10], ethnicity-related factors [11], chronic kidney disease [12], and hemoglobinopathies [13] on traditional biochemical approaches might interfere with the associations of HbA1c% values with blood glucose levels. The MS-based method, which is able to detect all species of hemoglobin, is believed to be a better alternative method [16–19]. In the current study, we detected three additional modified forms of

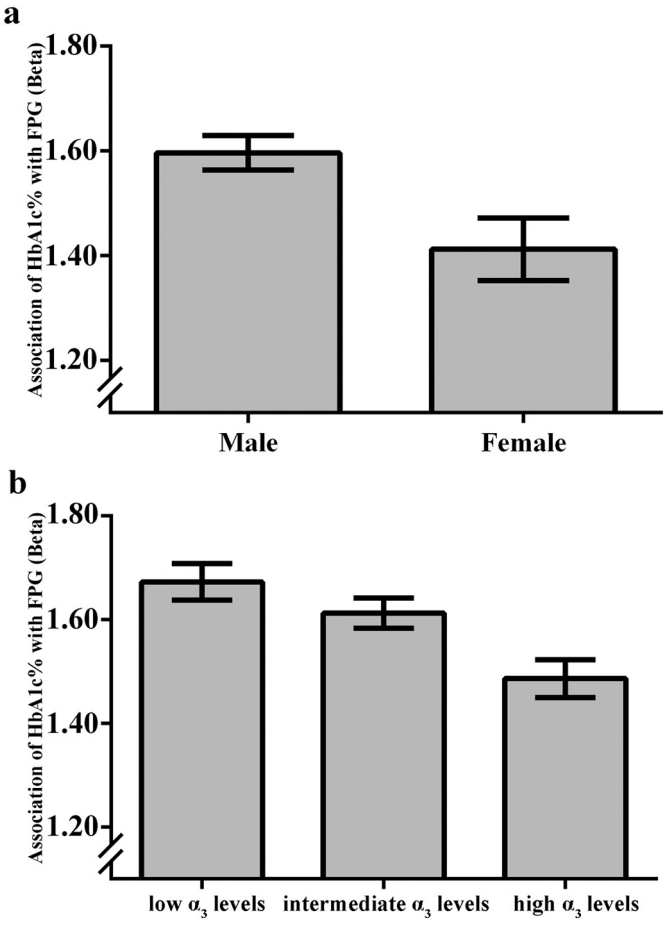

Fig. 2. Associations of HbA1c% with FPG according to gender and level of α_3%. (a) The relationship between HbA1c and FPG is stronger in males than in females. (b) The correlation of FPG with HbA1c% is associated with the level of α_3%. An increase in the correlation is detected with a decrease in the level of α_3%: low level (α_3% < mean - SD), intermediate level (mean - SD ≤ α_3% < mean + SD), and high level (α_3% ≥ mean + SD).

hemoglobin, α_1, α_2, α_3, β_1, β_2, and β_3 (Table 1). In terms of the detected MWs of these chemicals, α_1 (or β_1) is the α-chain(or β-chain) plus a mass of 98 Da covalently linked to hemoglobin, which were first reported for the Han Chinese population in the current study; α_2 and β_2

are the glycated α-chain and the glycated β-chain, respectively, which are the α-chain and β-chain plus a mass of 162 Da (one glucose residue), respectively; and α_3 and β_3 correspond to the glycated α-chain plus one glucose residue (162 Da) and the glycated β-chain plus a fucose residue (145.2 Da), respectively (Table 1). However, it should be noted that the exact structures of these forms of modified hemoglobin need to be further investigated.

As average blood glucose is difficult to monitor using conventional methods, the clinical significance of monitoring the levels of these modified forms of hemoglobin simultaneously was further evaluated using FPG. The values of α_1%, α_2%, $\alpha\%_3$, β_1%, β_2%, and β_3% were significantly associated with the levels of FPG (Table 2). By controlling for other types of modified hemoglobin (including HbA1c%), we found that the values of α_1% and β_3% are independently associated with the levels of FPG. Our findings suggest that all modified forms of hemoglobin detected in our study could help monitor the level of blood glucose more accurately. However, in our study, HbA1c, the most common form of glycated hemoglobin, with the N-terminal valine of the β-chain glycated, was not highly correlated with β_2% ($r^2 = 0.736$). This result suggests that β_2, through a nonenzymatic process, may involve multiple glycation reactions to produce different forms of the modified hemoglobin simultaneously in the Han Chinese population, which was also observed in a previous study [16].

In addition, interaction analyses among the detected modified forms of hemoglobin indicated that gender and α_3% interacted significantly with HbA1c% (Table 3); thus, gender and the levels of α_3% could affect the associations of HbA1c% with FPG (Fig. 2). The linear coefficient of HbA1c% with FPG is significantly lower in females with high levels of α_3%. These findings suggest that glycation of the β-chain in females might be more difficult and that the double-glycated α-chain would also affect the glycation of the β-chain, resulting in different effects of gender on the association between FPG and HbA1c. The reasons may be: 1) estrogen might affect structure of red blood cell, which further lead to changes in glucose concentration in the red cells [24]; 2) glycation of α-chain might affect the structure of β-chain and the formation of HbA1c.Therefore, using the same diagnostic criteria and monitoring targets based on HbA1c% for both males and females might not be reasonable due to the presence of a difference in the association of HbA1c% with blood glucose. On the other hand, these results also suggest that the levels of α_3% should also be considered when using HbA1c% for blood glucose monitoring. Therefore, using MS-based method to monitor different forms of modified hemoglobin is important.

Previous studies revealed that genetic variants [10], ethnicity-related factors [11], chronic kidney disease [12], and hemoglobinopathies [13] might interfere with the association of HbA1c% with blood glucose. Our study is the first to find that other forms of modified hemoglobin also affect the correlation between HbA1c% and FPG. It should be noted that these glycated forms of hemoglobin were first found in the Han Chinese population, which differs from western populations [25]. Our study has many strengths. First, we established the MS method to detect multiple modified forms of hemoglobin simultaneously, which will help to accurately monitor blood glucose. Second, a large sample size was used to confirm our conclusions. Third, our study is the first to report the interactions between two(or multiple) subtypes of glycated hemoglobin, which provides novel insights for understanding glycated forms of hemoglobin in addition to HbA1c%. However, our study also has some limitations. First, we used FPG rather than average blood glucose in the current study. Second, the exact structures of the modified forms of hemoglobin were not identified in the present study.

5. Conclusions

In conclusion, the MS-based method, which detects various forms of modified hemoglobin, is an effective tool for monitoring glycated forms of hemoglobin. Multiple forms of glycated hemoglobin affect the association of FPG with HbA1c. For the Han Chinese population, the correlation between blood glucose and HbA1c% in females differs from that in males. These data suggest that the HbA1c criteria for the diagnosis and monitoring of diabetes should be established according to genders and modified types of hemoglobin.

Conflicts of interest

The authors declare no financial/non-financial competing interests.

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