

Common Hemoglobin Variants in Southern Taiwan and Their Effect on the Determination of HbA1c by Ion-exchange High-performance Liquid Chromatography

Chih-Hsun Chu^{1,2,3*}, Hing-Chung Lam^{1,3,4}, Jenn-Kuen Lee^{1,3}, Mei-Chun Wang¹, Chih-Chen Lu^{1,3},
Chun-Chin Sun¹, Ming-Ju Chuang¹

¹Division of Endocrinology and Metabolism, Department of Internal Medicine, Kaohsiung Veterans General Hospital, Kaohsiung, ²Department of Rehabilitation Technology, Tzu-Hui Institute of Technology, Pingtung, ³National Yang-Ming University School of Medicine, Taipei, and ⁴Faculty of Nursing, Yuh-Ing Junior College of Health Care and Management, Kaohsiung, Taiwan, R.O.C.

Background: Patients with hemoglobin (Hb) variants may produce false HbA1c measurement. This study aimed to detect the common Hb variants in southern Taiwan and to evaluate their effect on the determination of HbA1c.

Methods: A total of 1,434 samples collected for HbA1c measurement at Kaohsiung Veterans General Hospital in southern Taiwan in March 2008 were submitted for Hb variant analysis by Primus CLC-385. HbA1c measurements were obtained using ion-exchange high-performance liquid chromatography (HPLC) (Tosoh HLC-723 G7) for routine analysis. Patients identified with Hb variants were recalled for boronate-affinity HPLC analysis. The values of estimated average glucose (eAG) were converted from HbA1c. Values of eAG-FPG, calculated by eAG minus fasting plasma glucose (FPG), were compared to estimate the accuracy of HbA1c measurement in patients with Hb variants.

Results: Among the 1,434 patients, the mean \pm standard deviation of FPG was 162.8 ± 60.5 mg/dL, HbA1c was $8.28 \pm 1.97\%$, and eAG was 190.9 ± 56.6 mg/dL. Five Hb variants were detected in 11 patients, the incidence being 0.76%. Hb J was identified in 4 patients, Hb G in 2 patients, Hb E in 1 patient, Hb owari in 3 patients, and high fetal hemoglobin (HbF) in 1 patient. Abnormal HPLC chromatograms were seen among the patients with Hb J, E, G and HbF, but not in the patients with Hb owari. In patients with Hb variants, FPG was 149.5 ± 39.9 mg/dL, HbA1c was $7.29 \pm 2.01\%$, and eAG was 162.5 ± 57.7 mg/dL. Lower values of eAG-FPG may have occurred in the patients with Hb J and E, and in those with high HbF. On scattergrams of the relationship between HbA1c and FPG, the plots of Hb J, E and high HbF lay below the regression line of non-Hb variants. Inconsistent Hb values between both methods were only observed among some samples of patients with Hb variants.

Conclusion: The existence of Hb variants may result in false HbA1c measurement. The possible presence of spuriously low HbA1c levels or abnormal HPLC chromatograms by using ion-exchange methods should be kept in mind. [*J Chin Med Assoc* 2009;72(7):362–367]

Key Words: boronate-affinity HPLC, fetal hemoglobin, glycohemoglobin, Primus CLC-385, Tosoh HLC-723 G7

Introduction

Glycohemoglobin (GHb), measured as HbA1c, is an invaluable tool for monitoring long-term glycemic control, and as such is a key issue in diabetes care. HbA1c

is the result of irreversible nonenzymatic glycation at 1 or both N-terminal valines of the Hb β chain.¹ This irreversible nonenzymatic reaction between glucose and HbA, the main type of Hb in normal adults, occurs during the life span of the erythrocyte. The total



*Correspondence to: Dr Chih-Hsun Chu, Division of Endocrinology and Metabolism, Department of Internal Medicine, Kaohsiung Veterans General Hospital, 386, Ta-Chung 1st Road, Kaohsiung 813, Taiwan, R.O.C. E-mail: chchu@isca.vghks.gov.tw • Received: December 10, 2008 • Accepted: June 8, 2009

amount depends on the average glucose concentrations within 2–3 months prior to the measurement.²

Although several methods based on different principles (high-performance liquid chromatography [HPLC], immunoagglutination, boronate-affinity assays, and electrophoresis) have been developed,^{3,4} the designated Diabetes Control and Complications Trial comparison method utilized an ion-exchange HPLC (Diamat; Bio-Rad, Richmond, CA, USA).⁵ Therefore, the majority of HbA1c measurements are still performed by ion-exchange HPLC.

The structural variants and chemical derivatives of Hb may interfere with many methods. Most Hb variants are a single base pair change in the DNA code, resulting in an amino acid substitution and structural alteration.⁶ Such structural variants may demonstrate additional peaks in the chromatograms and be combined with clinically low or high results. However, not all Hb variants affect HbA1c measurement. In the population with Hb variants, misleadingly high or low GHb values have been identified by only certain methods. Among them, the effect of Hb variants is thought to be greater when ion-exchange HPLC is used.^{7,8}

More than 700 characterized Hb variants have been reported.⁶ The majority arise from point mutations in the α , β , γ , or δ Hb chains.⁶ In addition, certain genetic abnormalities can also cause the switch to adult hemoglobin synthesis to fail, resulting in a condition known as the presence of high fetal hemoglobin (HbF).⁹ The widespread measurement of GHb has identified new variants, many of which produce no phenotypic abnormalities.⁶

The common Hb variants occurring in southern Taiwan are not yet clear. Therefore, this study was designed to investigate the occurrence of Hb variants and their influence on the measurement of HbA1c.

Methods

A total of 1,434 samples collected for HbA1c measurement at Kaohsiung Veterans General Hospital in southern Taiwan in March 2008 were submitted for Hb variant analysis. The blood samples were collected in EDTA anticoagulation bottles for HbA1c and Hb variant analysis. This study was approved by the Medical Ethics and Human Clinical Trial Committee of Kaohsiung Veterans General Hospital.

In this study, HbA1c measurement was done using ion-exchange HPLC (Tosoh HLC-723 G7; Tosoh Co., Tokyo, Japan) as routine analysis. The Tosoh HLC-723 G7 is a compact analyzer designed for the

measurement of HbA1c under routine laboratory conditions. The analyzer employed was a fully automated HPLC system using reagents and conditions specifically designed to separate and quantify Hb A2 and HbF in a 7.5-minute run. In addition, ion-exchange HPLC using a Primus CLC-385 Hb variant analyzer (Primus Co., Kansas City, MO, USA) served for Hb variant analysis. Hb variants were identified by ion-exchange HPLC separation of Hb species using a gradient between 2 mobile phases with differences in salt concentration and pH. CLC-385 resolution relative retention time table R3-4000 columns served as reference for the Hb variants obtained. The patients identified with Hb variants were recalled for further HbA1c measurement using CLC 330 boronate-affinity chromatography (Primus CLC 330; Primus Co.).

Plasma glucose concentrations were measured by a hexokinase method using an automatic biochemistry analyzer (Hitachi 7170; Hitachi Co., Tokyo, Japan). The values of fasting plasma glucose (FPG) were the means of all the consecutive values 3 months prior to or during HbA1c analysis. The relationship between HbA1c and estimated average glucose (eAG) was described by the formula:

$$\text{eAG (mg/dL)} = 28.7 \times \text{HbA1c} - 46.7$$

The equation resulted from the *A1c-Derived Average Glucose* study,¹⁰ which affirmed the existence of a linear relationship between HbA1c and average blood glucose levels. The values of eAG minus FPG (eAG-FPG) were calculated for estimation of the accuracy of HbA1c measurement in the patients with Hb variants.

Statistical analysis

Values are expressed as mean \pm standard deviation. Comparisons between different groups were made by Student's *t* test, except for sex, which was compared by 2-sample Kolmogorov-Smirnov test. A value of $p < 0.05$ was considered statistically significant.

Results

Among the 1,434 patients, 11 patients were found to have Hb variants. The incidence of Hb variants was 0.76%. The characteristics of all patients and the patients with Hb variants are shown in Table 1. The mean age of all patients was 63.8 years (range, 5–96 years). The female-to-male ratio was 1:1.4. Mean FPG was 162.8 ± 60.5 mg/dL, HbA1c was $8.28 \pm 1.97\%$, and eAG was 190.9 ± 56.6 mg/dL. In patients with Hb variants, mean FPG was 149.5 ± 39.9 mg/dL, HbA1c was $7.29 \pm 2.01\%$, and eAG was 162.5 ± 57.7 mg/dL.

Table 1. Patient characteristics*

	All patients (n = 1,434)	Patients with Hb variants (n = 11)
Sex (F/M)	590/844	6/5
Age (yr)	63.8 ± 14.6	64.1 ± 10.9
FPG (mg/dL)	162.8 ± 60.5	149.5 ± 39.9
HbA1c (%)	8.28 ± 1.97	7.29 ± 2.01
eAG (mg/dL)	190.9 ± 56.6	162.5 ± 57.7
eAG-FPG (mg/dL)	28.1 ± 46.5	13.09 ± 31.6

*No significant difference for any comparison. Hb = hemoglobin; FPG = fasting plasma glucose; HbA1c = hemoglobin A1c; eAG = estimated average glucose; eAG-FPG = eAG minus FPG.

Table 2. Results of the 11 patients with hemoglobin variants

Patient	Age (yr)	Sex	FPG (mg/dL)	HbA1c (%)	eAG (mg/dL)	eAG-FPG (mg/dL)	Hb variant
1	73	F	117	5.0	97	-20	J
2	64	F	91	4.8	91	0	J
3	74	F	134	6.6	143	9	J
4	67	F	137	6.4	140	3	J
5	72	F	209	10.6	258	49	G
6	74	F	186	10.5	255	69	G
7	61	M	103	5.2	103	0	E
8	71	M	161	7.4	166	5	owari
9	59	M	164	8.7	203	39	owari
10	41	M	134	7.5	169	35	owari
11	49	M	208	7.5	169	-39	High HbF

FPG = fasting plasma glucose; HbA1c = hemoglobin A1c; eAG = estimated average glucose; eAG-FPG = eAG minus FPG; HbF = fetal hemoglobin.

Among the 11 patients with Hb variants, Hb J was identified in 4 patients, Hb G in 2, Hb E in 1, Hb owari in 3, and high HbF in 1 patient (Table 2).

When compared with standard chromatogram (Figure 1A), the HPLC chromatograms from Tosoh HLC-723 G7 showed an additional peak between labile (L) A1c and stable (S) A1c in 2 patients with Hb J (Figure 1B). However, the other 2 Hb J patients presented with normal chromatograms. H-VI peak existed in both patients with Hb G (Figure 1C). An additional peak between SA1c and A0 was seen in the patient with Hb E (Figure 1D). However, there was no abnormal peak in any of the patients with Hb owari (Figure 1E). In the patient with high HbF, an abnormal peak of the mixture of HbF and LA1c was noted (Figure 1F).

When the values of eAG-FPG were compared among the patients with Hb variants (Table 2) and the mean value of the total number of patients (Table 1), lower levels were found in those with Hb J, E and high HbF. The results suggest that spuriously decreased HbA1c levels may have occurred among these patients. On the other hand, patients with Hb G and owari did not show lower eAG-FPG levels.

The relationship between HbA1c and FPG values in the patients with Hb variants and non-Hb variants

were compared on scattergrams (Figure 2). The plots of Hb J, E and high HbF lay below the regression line of non-Hb variants. However, the plots of Hb G and owari lay beside the regression line of non-Hb variants.

Ten of the 11 patients with Hb variants were recalled for HbA1c measurement by boronate-affinity HPLC (Table 3). Inconsistent Hb values were observed among some, but not all, samples of patients with Hb variants when compared with the ion-exchange HPLC. The values were higher or lower.

Discussion

In normal adults, Hb consists of ~97% HbA, 2.5% HbA2, and 0.5% HbF. Fractionation of HbA by chromatography identifies several minor peaks referred to as HbA1, or fast Hbs, which include the glycosylated forms HbA1a, HbA1b, and HbA1c.¹¹ These fast Hbs form as the result of a 2-step reaction. In the first step, a reversible reaction forms a Schiff base. This reversible reaction is followed by an irreversible, nonenzymatic Amadori rearrangement that produces GHb.¹¹ The glycation alters the structure of the Hb molecule and

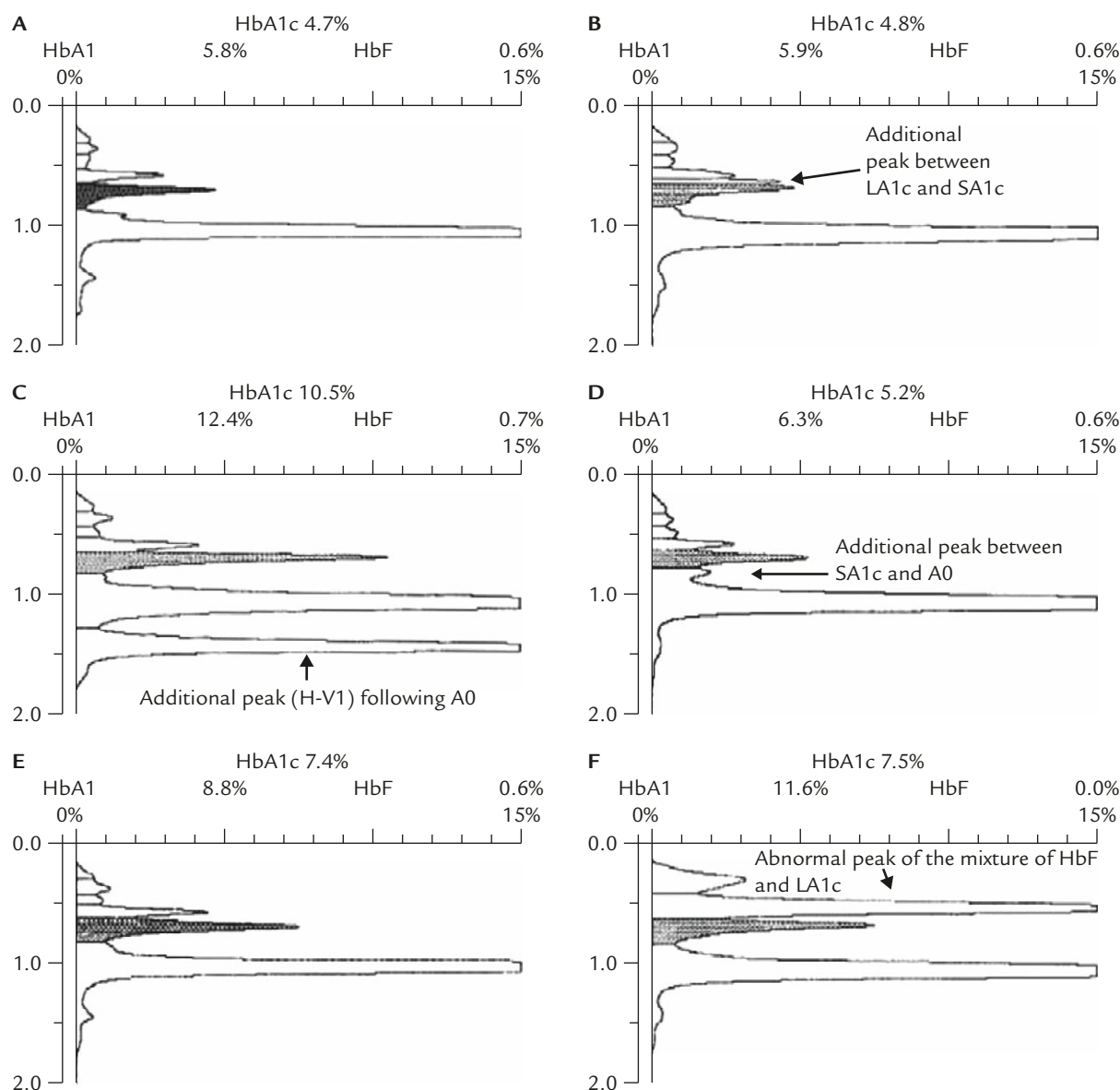


Figure 1. Chromatograms from Tosoh HLC-723 G7. (A) Standard chromatogram. There were several peaks including A1a, A1b, F, labile (L) A1c, stable (S) A1c, and A0. HbA1c was expressed as the percentage of SA1c among total Hb. (B) Chromatogram of Patient 2 with Hb J. An additional peak between LA1c and SA1c was noted. (C) Chromatogram of Patient 6 with Hb G. An additional peak (H-V1) following A0 was noted. (D) Chromatogram of Patient 7 with Hb E. An additional peak between SA1c and A0 was noted. (E) Chromatogram of Patient 8 with Hb owari. There was no abnormal peak identified. (F) Chromatogram of Patient 11 with high HbF. An abnormal peak of the mixture of HbF and LA1c was noted.

decreases its net positive charge. Many methods of testing use 1 or both differences to separate GHb from nonglycated Hbs.

The N-terminal valine of the β chain provides the most common site of glycation within the Hb tetramer, accounting for 80% of HbA1.¹¹ The *International Federation of Clinical Chemistry and Laboratory Medicine* defines HbA1c as Hb that is irreversibly glycosylated at 1 or both N terminal valines of the β chains. Although all commercially available methods include HbA1c in

GHb measurements, they vary in their ability to detect non-A1c GHb.

Depending on the method of determination, the concentration of HbA1c is approximately 4–6% in healthy patients without diabetes. However, the presence of Hb variants may falsely produce low values for HbA1c or spuriously increased HbA1c values. In addition, high HbF levels have been reported to interfere with the results of some HbA1c methods, including boronate-affinity methods.¹² The identification of Hb

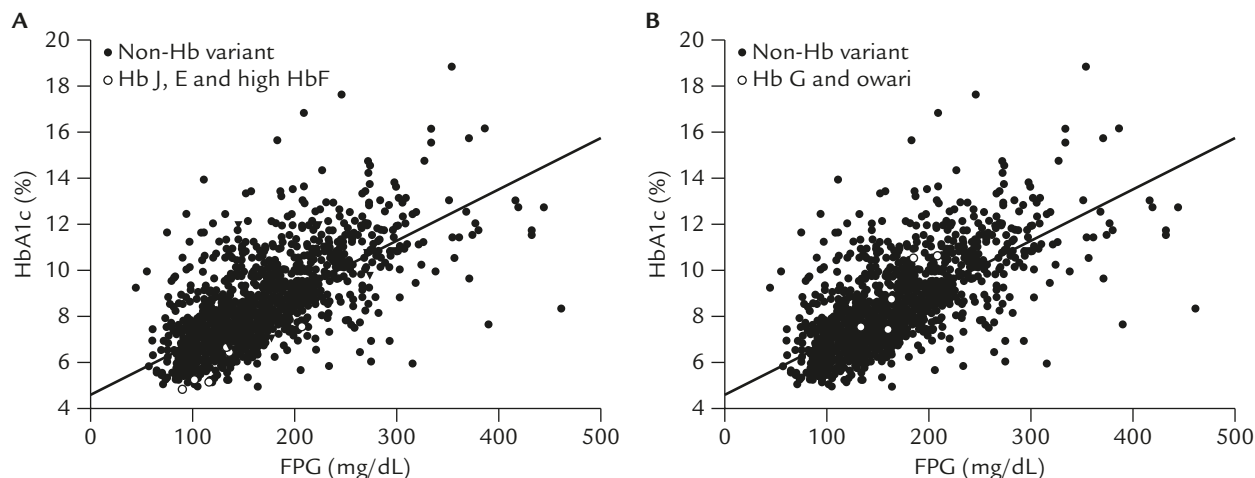


Figure 2. Relationship between HbA1c and fasting plasma glucose (FPG) values in patients with Hb variants and non-Hb variants. (A) The plots of Hb J, E and high HbF lay below the regression line of non-Hb variants. (B) The plots of Hb G and owari lay beside the regression line of non-Hb variants.

Table 3. Comparisons of HbA1c values by ion-exchange HPLC (Tosoh HLC-723 G7) and boronate-affinity HPLC method (Primus CLC 330)

Patient	Hb variant	HbA1c (%)	
		Tosoh HLC-723 G7	Primus CLC 330
1	J	5.2	6.2
2	J	3.9	5.9
3	J	7.3	6.9
4	J	6.3	6.9
5	G	9.5	10.6
7	E	6	6.6
8	owari	7.9	7.6
9	owari	6.5	5.7
10	owari	9.7	9.7
11	High HbF	7.7	7.1

Hb = hemoglobin; HbA1c = hemoglobin A1c; HbF = fetal hemoglobin.

variants is, therefore, important to avoid inaccurate GHb results. In general, an Hb variant can be suspected in patients with HbA1c results >15%, HbA1c measurements below the nondiabetic reference range, or when the HbA1c result varies substantially from other indices of metabolic control and/or clinical impression.

There are more than 30 different GHb assay methods available. The influence of Hb variants on GHb determination is great when using ion-exchange HPLC. With ion-exchange HPLC methods, clinically silent Hb variants may cause additional peaks in chromatograms, resulting in a false HbA1c value. Therefore, if an erroneous result is caused by Hb variants, affinity chromatography may provide a more accurate measure of HbA1c.^{13,14} However, incorrect HbA1c values resulting from affinity chromatography methods have also been described.^{12,15,16} Additionally, the influence

of each Hb variant on HbA1c determinations by affinity chromatography is still not fully investigated. In this study, inconsistently higher or lower HbA1c values were observed among patients with Hb variants when compared with ion-exchange chromatography with affinity chromatography methods. Therefore, affinity HPLC methods could not resolve the interference of Hb variants.

Furthermore, fructosamine can be used as a comparison because nonenzymatic glycation of serum proteins, mainly albumin, is not influenced by Hb variants. However, fructosamine only reflects an average of blood glucose levels over a shorter period of 2–3 weeks.

A large-scale survey of hemoglobinopathies was carried out in China and involved 902,204 people in 28 provinces using electrophoretic techniques; a total of 2,936 pedigrees with abnormal Hb were found.¹⁷

The incidence of abnormal Hb was 0.33%, and the incidence in various regions ranged from 0.04% to 6.06%. In our study, the incidence of Hb variants was 0.76%. Hb J, G, E, owari and high HbF accounted for the common Hb variants in southern Taiwan. Abnormal HPLC chromatograms and spuriously decreased HbA1c levels might possibly have occurred among some of the patients with Hb variants.

The values of eAG were converted from HbA1c. Postprandial hyperglycemia made a large contribution to overall HbA1c levels. Therefore, the values of FPG and eAG could be mismatched. In our study, the deviation of eAG-FPG might be caused by the interference of Hb variants. Nonetheless, the incompatibility of FPG and HbA1c could not be excluded.

It is essential that clinical laboratories be aware of the limitations of their HbA1c assays as well as the importance of visual inspection of ion-exchange chromatograms to detect abnormalities (extraordinary peaks or non-separation of each peak) caused by Hb variants. These results also underline the need for additional investigations of interference caused by Hb variants in all newly developed HbA1c assays.

In conclusion, though the prevalence of Hb variants in southern Taiwan is low, the possible presence of spuriously decreased HbA1c levels or abnormal HPLC chromatograms by using ion-exchange methods should be kept in mind. In diabetic patients with Hb variants, adjustment of medications should depend on serial changes rather than on 1 single HbA1c value.

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