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SHORT COMMUNICATION

Screening of β-Thalassemia Carriers based on Matrix Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry

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SUMMARY

Background: **The traditional analytical methods for β-thalassemia carriers are time-consuming and costly. This study takes advantage of matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) to directly analyze human whole blood samples and established a preliminary method for screening β-thalassemia carriers.**
Methods: **A sample of 3 μL whole blood was diluted 200-fold with distilled water, mixed with sinapic acid, and subjected to MALDI-TOF MS analysis. The efficacy of the method for the screening of β-thalassemia carriers was assessed using the β-thalassemia gene results as the standard.**
Results: **β-thalassemia gene results showed 109 β-thalassemia carriers and 110 normal individuals out of 219 samples. We developed a δ-globin peak slope index that can simply and effectively distinguish β-thalassemia carriers from healthy individuals, the best cutoff value for the slope index was -0.819.**
Conclusions: **MALDI-TOF MS has the potential to be a valuable tool for β-thalassemia carriers screening.**
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KEYWORDS

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INTRODUCTION

Thalassemia is a monogenic heritable hemolytic anemia caused by globin synthesis disorder [1,2]. The lack of α or β globin chains will lead to an imbalance in the ratio of α/β, affecting the life span of red blood cells and causing anemia symptoms in patients.
Thalassemia is also one of the most common and harmful genetic diseases in southern China and other Southeast Asian countries [3,4]. The objective of this study was to develop an inexpensive and rapid method for β-thalassemia carriers screening.
Many methods are used to screen β-thalassemia carriers. These include the RBC parameter model [5], hemoglobin electrophoresis (HE) [6], Gap-PCR, and next-generation sequencing (NGS) [7,8]. Mass spectrometry

is a method for the qualitative and quantitative analysis of samples by analyzing the mass to charge ratio (m/z) of ions. MALDI is a soft ionization technique and the most typical application of MALDI-TOF MS is the identification of microorganisms [9,10]. MALDI-TOF MS also demonstrated satisfactory performance in the detection of glycated hemoglobin and hemoglobin variants [11,12]. MALDI-TOF MS has also been used to identify disease markers in serum, such as multiple myeloma and the serologic diagnosis of COVID-19 infection [13]. These studies show that MALDI-TOF MS technology is mature, reliable, and has good prospects for clinical applications.

At present, research on thalassemia based on MALDI-TOF MS mainly focuses on the significance of the α/β ratio in the screening for thalassemia [14-15], while the value of δ chain in the diagnosis of β-thalassemia carriers is rare.

In this study, 219 whole blood samples were analyzed by MALDI-TOF MS. We hypothesized that the δ chains of β-thalassemia carriers and healthy controls had different peak intensities at MALDI-TOF MS detection. Therefore, the δ-chain signal is significantly different between β-thalassemia carriers and healthy controls, which can be used for screening β-thalassemia carrier.

MATERIALS AND METHODS

Sample collection and preparation

Whole blood samples were collected using EDTA-K_2 tubes (Chongqing Sanfeng Medical Instrument Co., Ltd., Chongqing, China). Exclusion criteria: 1) patients with iron deficiency anemia; 2) patients who had received blood transfusion. Written informed consent was obtained from the patient.

The samples were stored at 4℃, and all samples were first subjected to thalassemia gene detection, followed by MALDI-TOF MS detection. All operations were completed within 48 hours.

Reagent A was prepared by mixing acetonitrile and trifluoroacetic acid (TFA) at a volume ratio of 3:7. Reagent B was prepared by dissolving 10 mg sinapic acid (SA) in 1 mL of reagent A. Three microliters of whole blood were diluted 200-fold with distilled water and mixed/vortexed for 30 seconds with shaking. Then, 5μL of diluted hemoglobin was added to 45 μL of reagent B and shaken well. Two microliters of the hemoglobin and auxiliary matrix mixture were added to the mass spectrum target plate and dried at 40℃.

Chemicals and materials

HPLC-grade acetonitrile and MS-grade sinapic acid were purchased from Shanghai Aladdin Company. Trifluoroacetic acid was purchased from Shanghai Minreal Company. Water used in this work was Milli-Q water. Thalassemia gene detection reagents were purchased from Hybribio Limited (Chaozhou, China).

MALDI-TOF-MS analysis

MALDI-TOF MS is Clin-TOF-II. (Bioyong Technologies Inc., Beijing, China). After mass calibration of the mass spectrometer, m/z detection was performed in positive linear mode. The mass spectrometry parameters were set as follows: m/z detection range was 2,000 - 20,000; The focus m/z value was 8,000. Each spectrum was accumulated using 500 laser shots (50 positions per sample spot and 10 laser shots per position). The MALDI-TOF MS raw data were processed using MALDI-MS software (V2.9.3). The m/z and peak intensity values of all peaks were extracted as ASCII text files after smoothing and baseline removal.

Raw mass spectra data in the m/z range of 7,900 - 8,000 were obtained. This section of data includes the m/z and corresponding intensity of β-globin and δ-globin peaks, with values rounded to two decimal places. The data were imported into a self-built slope-index analysis program to calculate the slope index of the δ-globin peak for each sample.

RESULTS

Basic profile of β-thalassemia carriers

Table 1 presents the frequency of different types of β-thalassemia mutations in this study. All carriers of these mutations were heterozygous.

Mass spectra of human whole blood based on MALDI-TOF MS

Figure 1A shows a schematic diagram of a human whole blood sample analyzed by MALDI-TOF MS. Figure 1B shows the mass spectra of the whole blood sample with m/z ranging from 2,000 - 20,000.

Within the range 2,000 - 20,000, there were four distinct peaks: α-chain (+1, +2 charge) and β-chain (+1, +2 charge) (Figure 1B). The m/z values of the four peaks are consistent with other reports; the enlargement of m/z in the range of 7,500 - 8,000 shows the δ peak (Figure 1C). The m/z of the δ peak with +2 charges was 7,962, which was consistent with previous reports.

The characteristics of the δ peak between healthy controls and β-thalassemia carriers

A total of 110 healthy controls and 109 β-thalassemia carriers were identified. There were significant differences in the mass spectrum pattern between the two groups: the curve declined more rapidly in the normal samples after the β-globin peak, and there was no obvious δ-globin peak (red curve in Figure 1D). In β-thalassemia carriers (blue and green curves in Figure 1D), a δ-globin peak was visible after the β-globin peak. The slope index was significantly higher in β-thalassemia carriers compared to the normal group.

Using the ROC curve, the best cutoff value for the slope index was -0.819 (Figure 2A). Among the 110 healthy controls, three samples had a slope index greater than -0.819, indicating three false-positive cases. Similarly,

Table 1. Frequency of different types of β-thalassemia mutations in this study.

HGVS name	β-thal mutation	n
HBB:c.52A>T	CD17 (AAG > TAG)	39
HBB:c.126_129delCTTT	CD41/42 (-TTCT)	27
HBB:c.316-197C>T	IVS-II-654 (C > T)	23
HBB:c.-78A>G	-28 (A > G)	7
HBB:c.84_85insC	CD27/28 (+C)	4
HBB:c.130G>T	CD43 (GAG > TAG)	3
HBB:c.-11_-8delAAAC	Cap (-AAAC)	3
HBB:c.-79A>G	-29 (A > G)	1
HBB:c.216_217insA	CD71-72 (+A)	1
HBB:c.79G>A	βE (GAG > AAG)	1

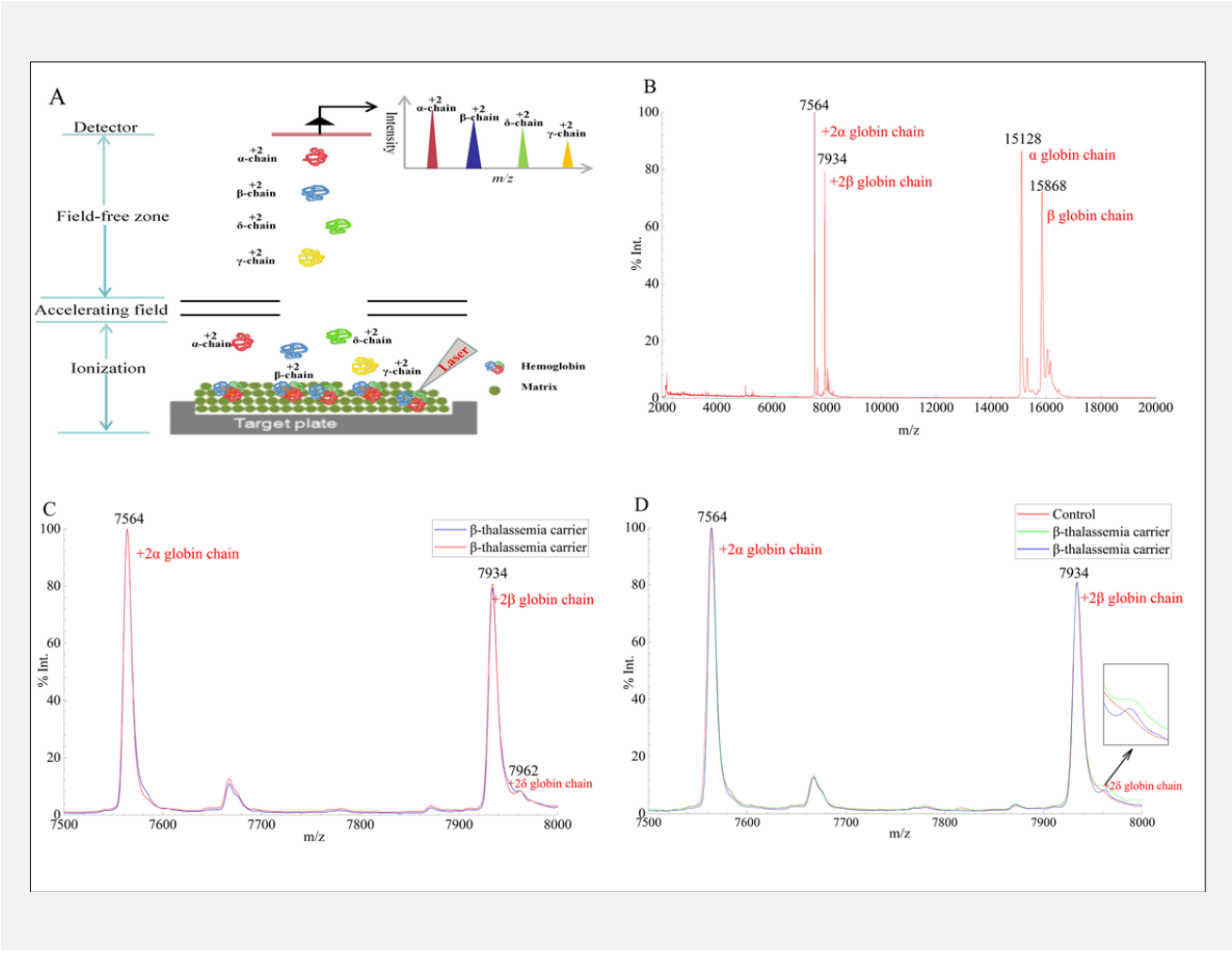

Figure 1. Mass spectra of human whole blood based on MALDI-TOF MS.

A - Schematic diagram of whole blood detection based on MALDI-TOF MS.
B - Mass spectra of whole blood sample with m/z ranging from 2,000 - 20,000. The graph contains four distinct peaks: α-chain (+1, +2 charge) and β-chain (+1, +2 charge).
C - Mass spectra of α, β, and δ globin with +2 charges. Their m/z were 7,564, 7,934, and 7,962, respectively.
D - Differences in δ-globin peaks of three samples. The red curve represents control sample, while the blue and green curves are β-thalassemia carriers.

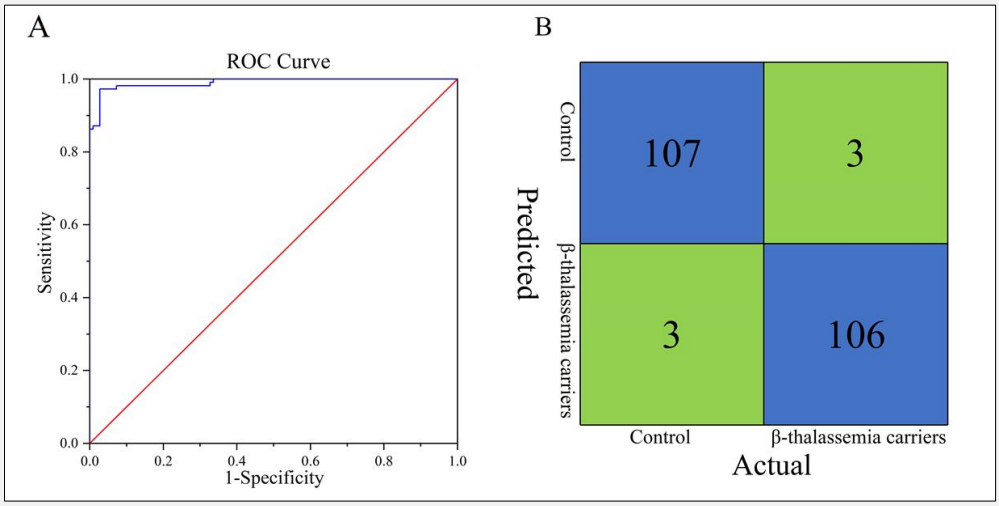

Figure 2. ROC curve of slope Index and the confusion matrix of the classification results.

A - Using the ROC curve, the best cutoff value for the slope index was -0.819.
B - A cutoff value of -0.819 yielded three false-positive results and three false-negative results, respectively.

out of the 109 β-thalassemia carriers, three samples had a slope index less than -0.819, indicating three false-negative cases (Figure 2B). The three false-negative results included two cases of HBB:c.-11_-8delAAAC mutation and a case of HBB:c.79G>A mutation.

Three samples with a negative result were subjected to hemoglobin electrophoresis, yielding HbA2 results of 2.7%, 2.8%, and 3.0%.

DISCUSSION

This study was based on MALDI-TOF MS for microbial identification, and the specific parameters were not modified; therefore, hemoglobin and microorganisms could be identified in the same batch. The results showed that δ-globin peaks with +2 charges were significantly different between the two groups, indicating that the diagnostic efficiency has clinical value.

Some researchers have used ESI-MS to study hemoglobin levels. After ESI, the globin chain carries multiple charges, resulting in a range of peak maps [14]. The analysis of multiple peak maps requires a more complex data-processing flow. Some scholars have also used MALDI-TOF MS to study hemoglobin, which requires the addition of myoglobin or isotope as internal standard and the use of α/β and δ/β area ratio differences to construct diagnostic models, which increases the detection cost [15]. Furthermore, differences in γ-globin levels have been employed as a means of investigating β-thalassemia [16]; however, this method may be inappropriate for infants because part of the hemoglobin in infants is HbF (α2/γ2). In this study, 10 β-thalassemia carriers who were less than one year old were successfully identified on the basis of δ-globin signature peaks. This method only utilizes the slope difference of the δ-globin peak without requiring complex data processing. The results suggest that MALDI-TOF MS analysis of whole blood may represent a potential screening method for β-thalassemia.

The three false-negative samples were rare β-thalassemia carriers of HBB:c.-11_-8delAAAC mutation and HBB:c.79G>A mutation. Unlike common β-thalassemia carriers, these three samples had normal hemoglobin and MCV levels, indicating that the synthesis of β-globin chains was little affected by the site mutation, and δ-globin was not transcribed in a compensatory manner [17]. Analysis of the hematological features of HBB:c.-11_-8delAAAC mutation cases suggests that HBB:c.-11_-8delAAAC mutation may be a polymorphic site with no genetic effect [18]. The efficiency of β-chain synthesis is much higher than that of other β-globin mutants [19].

HbE is a clinical manifestation of HBB:c.79G>A mutation. It is commonly believed that HbE carriers exhibit microcytic anemia. However, a study conducted in southern China demonstrated that 19.81% of HbE carriers exhibited normal blood parameters [20]. The sample of HbE carriers in this study exhibited an MCV of 80.4, which is within the critical range.

The results of hemoglobin electrophoresis demonstrated that the HbA2 of the three false-negative samples were within the normal range and exhibited similar δ peak curves to those of the control samples. This finding sug-

gests a potential limitation of the methodology employed.

CONCLUSION

In conclusion, this study applied the MALDI-TOF MS method to explore the application of δ-globin for the screening of β-thalassemia carriers. Each sample was detected within 1 minute with simple pretreatment, without complex model establishment. In addition, mass spectrometry parameters can be shared with microbial identification, which is convenient for staff to use. However, for β-thalassemia carriers without increased δ-globin expression, this method has the possibility of false negative. For future research, we will collect more samples of β-thalassemia carriers with HBB:c.-11_-8delAAAC and HBB:c.79G>A mutations and focus on exploring their mass spectrometry characteristics.

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Ethical Approval:
The study was approved by the Ethics Committee of People's Hospital of Changshou Chongqing. All experiments were performed in accordance with approved guidelines. Written informed consent was obtained from the patient.

Declaration of Interest:
The authors have no conflicts of interest to declare.

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