MALDI–TOF–MS for Rapid Screening and Typing of β-Globin Variant and β-Thalassemia through Direct Measurements of Intact Globin Chains

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BACKGROUND: Traditional phenotype-based screening for β-globin variant and β-thalassemia using hematological parameters is time-consuming with low-resolution detection. Development of a MALDI–TOF–MS assay using alternative markers is needed.

METHODS: We constructed a MALDI–TOF–MS-based approach for identifying various β-globin disorders and classifying thalassemia major (TM) and thalassemia intermedia (TI) patients using 901 training samples with known *HBB/HBA* genotypes. We then validated the accuracy of population screening and clinical classification in 2 separate cohorts consisting of 16 172 participants and 201 β-thalassemia patients. Traditional methods were used as controls. Genetic tests were considered the gold standard for testing positive specimens.

RESULTS: We established a prediction model for identifying different forms of β-globin disorders in a single MALDI–TOF–MS test based on δ- to β-globin, γ- to α-globin, γ- to β-globin ratios, and/or the abnormal globin-chain patterns. Our validation study yielded comparable results of clinical specificity (99.89% vs 99.71%), and accuracy (99.78% vs 99.16%) between the new assay and traditional methods but higher clinical sensitivity for the new method (97.52% vs 88.01%). The new assay identified 22 additional abnormal hemoglobins in 69 individuals including 9 novel ones, and accurately screened for 9 carriers of deletional hereditary persistence of fetal hemoglobin or δβ-thalassemia. TM and TI were well classified in 178 samples out of 201 β-thalassemia patients.

CONCLUSIONS: MALDI–TOF–MS is a highly accurate, predictive tool that could be suitable for large-scale screening and clinical classification of β-globin disorders.

Introduction

β-globin disorders are the most pervasive monogenic disorder worldwide, and are prevalent in the tropics and subtropics, which reflects natural selection since

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heterozygotes are protected against malaria (1–3). β-globin disorders are characterized by the underlying molecular defects in β-globin gene clusters that result in defective hemoglobin (Hb) synthesis, accounting for significant morbidity and mortality in a high-risk population. About 1.5% of the global population are carriers of β-thalassemia, and over 40 000 newborns are affected annually (3, 4).

Appropriate population screening strategies for this genetic disorder contribute to early recognition and avoidance of high-risk offspring. The large-scale population-based preventive program for β-globin disorders, comprising carrier screening, genetic counseling, and prenatal diagnosis, is the first of its kind to be documented, and has yielded effective outcomes (1, 3, 5). Nationwide prevention and control programs have been successfully implemented in several Mediterranean countries or regions (6–8). The conventional strategy used in primary screening for β-globin disorders is based on a phenotypic approach using hematological parameters and Hb component analysis, followed by multiple methodologies for genotype characterization in suspected carriers to establish the diagnosis (9–11). Complex turnaround of samples among multiplex detection systems may cause logistical issues. The samples need to be analyzed on at least 2 different sets of instruments, and then interpreted manually; this is time-consuming and labor-intensive. In addition, to ensure the accuracy of results, multiple sets of instruments must be calibrated daily, which increases cost (9–11). In low- and middle-income countries, which account for 90% of the global β-globin disorder patients, conventional screening and prevention strategies for this disease have not been effectively promoted due to resource limitations (12). Hence, more cost-effective and easy-to-implement strategies are needed.

The pathogenesis of α- and β-thalassemia is related to the imbalance between the synthesis of α- and β-globin chains. It is possible to distinguish the various thalassemia subtypes by analysis of absent or reduced production of different globin subunits. Bottom-up mass spectrometry (MS) could determine the abundance ratio of α- and β-globin chains by the respective trypsin digested peptides. Tryptic hydrolysis, however, introduces higher uncertainty and cost to the whole approach (13, 14). A top-down approach has been reported showing that electrospray ionization 21 Tesla Fourier transform ion cyclotron resonance–tandem mass spectrometry can detect β-thalassemia trait by determining intact globin chains (15). Because the instrument is an ultrahigh resolution mass spectrometer, it is expensive and requires highly skilled operators; hence, this method cannot be used widely in routine clinical applications.

Using a Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI–TOF–MS) platform, we proposed here a practical, efficient, and economical top-down approach for screening β-globin variant and β-thalassemia through qualitative and quantitative analysis of intact globin peptide chains. The optimized protocol of the MALDI–TOF–MS assay was established using a training set, and then validated by population screening and clinical classification in 2 separate cohorts consisting of 16 172 healthy participants and 201 patients with thalassemia major (TM) and thalassemia intermedia (TI).

Materials and Methods

STUDY DESIGN

We designed 3 phases (Fig. 1) for establishing and developing the MALDI–TOF–MS-based assay for identifying β-globin variant and β-thalassemia. First, a QuanTOF I (Intelligene Biosystems, Qingdao, China) MALDI–TOF mass spectrometer was used to analyze the intact globin chains. These processed spectra were mass-calibrated using the known masses of Hb α, β, γ, and δ subunits, respectively. The mass spectrometer configuration and quality control are described in the online Data Supplement.

Second, the training set was used to develop a model for testing β-globin variant and β-thalassemia and determining fetal hemoglobin (Hb F) concentration. We recruited 901 training samples with known *HBB*/*HBA* genotypes, which consisted of 598 normal controls and 303 samples with various β-globin disorders (Supplemental Table 1). The training samples with known variants were categorized in 3 groups that included Group 1: abnormal Hb variants (16 cases); Group 2: β-thalassemia traits (152 cases), coinheritance of β- and α-thalassemia (34 cases), and deletional hereditary persistence of fetal hemoglobin (HPFH)/δβ-thalassemia (11 cases) (16); and Group 3: patients with β-thalassemia (90 cases). Group 1 was used for establishing an optimized model for qualitative analyses. The last 2 groups were used for establishing an optimized model for quantitative analyses. Four different forms of globin ratios, the abundance ratio of intact δ-chain to β-chain (δ/β), α-chain to β-chain (α/β), γ-chain to α-chain (γ/α), and γ-chain to β-chain (γ/β), were included as candidate indicators. Group 2 was used for typing carriers of β-thalassemia, and determining deletional HPFH/δβ-thalassemia. Group 3 was used for typing for those patient samples with TM or TI (Supplemental Table 2). The definitions of TM and TI were based on those previously described (3, 17). For establishing an optimized model for quantitative determination of Hb F, we merged 598 normal controls and 197 samples with 3 types of mutations from Group 2, and further subdivided these total 795 samples into 2 subgroups based on hematological parameters and Hb F-associated genotypes: HPFH

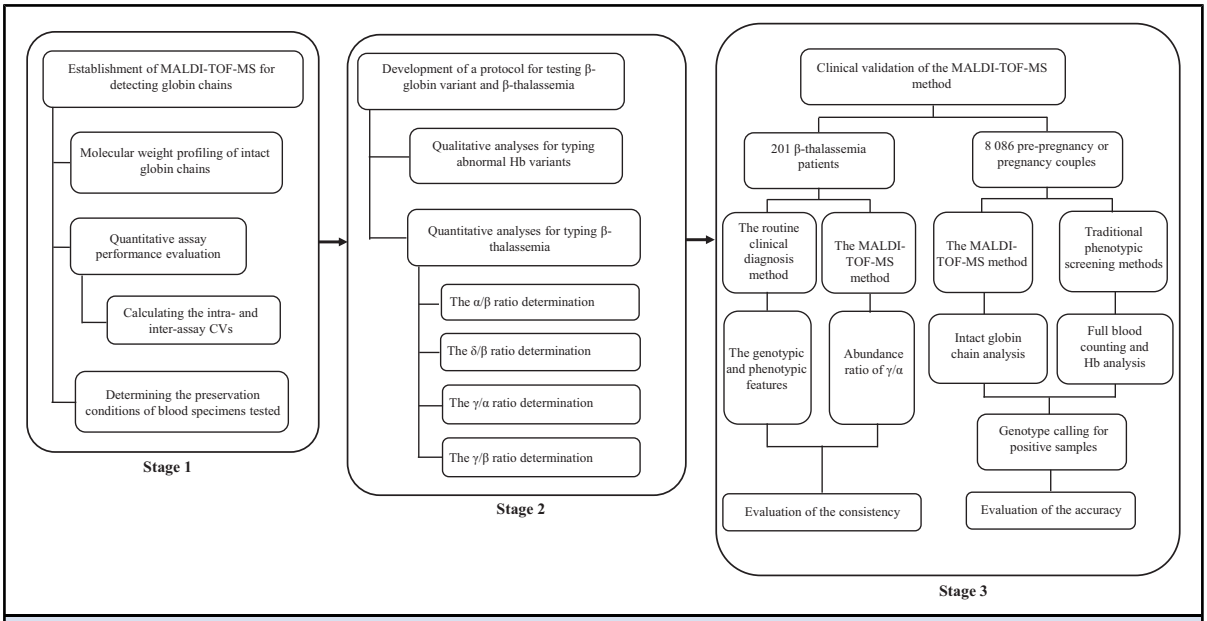

Fig. 1. Schematic overview of study design. CV, coefficient of variation; Hb, hemoglobin.

phenotype with Hb F > 5% (32 cases) and with Hb F ≤ 5% (763 cases) as controls (Supplemental Table 3) (11). We determined the optimal indicator and its cutoff of HPFH phenotype using these 2 groups of samples. The phenotypic and genotypic characteristics of all 32 training samples with HPFH phenotype are summarized in Supplemental Table 4.

Third, to validate the MALDI–TOF–MS prediction model applied for population screening and clinical classification in β-globin disorders, 2 separate cohorts, 16 172 healthy individuals in the general population, and 201 patients with β-thalassemia, were recruited. We performed the absolute and relative quantification of different intact globins by MALDI–TOF–MS in samples from this validation set. To assess the reliability and validity of the model for population screening, a total of 16 172 individuals consisting of 4929 couples for premarital screening and 3157 couples for antenatal screening were enrolled from 5 primary healthcare centers. Each sample was subjected to a blind study simultaneously for traditional methods and the new MALDI–TOF–MS approach. Peripheral blood samples were collected into EDTA anticoagulation tubes and subjected to full blood count (FBC) using a Sysmex KX-21N autoanalyzer (Sysmex Corporation, Kobe, Japan), and on capillary electrophoresis (CE) using a Capillarys II (Sebia, Paris, France), which were used as the first-line screening assays according to traditional methods (11). Samples with positive results in either of the 2 screening methods were verified by genetic testing (Supplemental Fig. 1). After genotype characterization of the suspected positive specimens, we analyzed the accuracy of the results from 2 screening approaches, comprehensively evaluated the feasibility and effectiveness of the new MALDI–TOF–MS phenotypic screening approach, and verified the performance of the new approach on quantification of Hb F. We also evaluated the consistency of clinical classification between the new approach and clinical genetic diagnosis in an independent cohort of 201 patients with TM or TI. Most of the patients received a transfusion, and the blood collection was carried out over 15 days at least after transfusion. Clinical data from these 201 patient samples are summarized in Supplemental Table 2. The research protocol for this study was designed and implemented in accordance with the principles of the Declaration of Helsinki. This study was approved by the Ethics Committee of Liuzhou Maternity and Child Healthcare Hospital (number IRB-2020-001), the Ethics Committee of Dongguan Maternal and Child Health Care Hospital (number IRB-2020-54), the Ethics Committee of Huizhou First Maternal and Child Health Care Hospital (number IRB-2020052), the Ethics Committee of NanFang Hospital of Southern Medical University (number NFEC-2019-039), and the Ethics Committee of Zhuhai Women and Children's Hospital (number IRB-2020081401). Written informed consent was obtained for each participant.

SAMPLE PREPARATION AND MASS SPECTROMETRY

An aliquot of 2 μL EDTA anticoagulated whole blood was diluted 1:200 in deionized water. The diluted blood was mixed 1:9 with matrix fluid (10 mg/mL sinapinic

acid [Sigma-Aldrich, St. Louis, USA], 40% CH3CN, and 0.1% TFA). An aliquot of 2.5 μL of this mixture was spotted onto disposable stainless steel MALDI target plate (2600 μm Slide Type [6 × 16 sample array]) to form a spot of 2.4 mm in diameter. The target plate temperature was maintained at 39 °C during the spotting operation. After spotting, the target plate was left until the droplets dried and crystallized thoroughly (approximately 20 s); we then put the target plate into the chamber for detection. Mass spectra were generated in linear and positive-ion modes with the following parameters: acceleration voltage, 20 kV; laser pulse frequency, 1 kHz; laser pulse energy, 5 μJ; *m/z* range, 2000–35 000 for covering intact globins and globin dimer; focus mass, 16 000; scanning rate, 0.5 mm/s; and 10 rows scan per sample spot. It took 1 minute to detect each sample spot. Each sample acquired at least 30 individual spectra (800 shots/spectra) consisting of >20 000 effective laser shots. The accumulation and averaging of the spectra contributed to the statistical accuracy of the measurement.

QUANTITATIVE ANALYSIS OF INTACT GLOBIN CHAINS

Quantitative analysis of globin chains was conducted by using the QuanHGB software package developed by Intelligene Biosystems (Qingdao, China). Peak detection was performed by using the software's default settings. Each plate/batch included 4 standard samples (including 2 negative controls and 2 positive controls) for peak correction, mass calibration, and peak area ratio normalization. Postacquisition data processing was done to average all spectra that passed a threshold of signal intensity >9.5 mV at each spot. Integrated peak areas calculated by the software at *m/z* = 15 127, 15 868, and 15 925 were used to represent the as abundance of α, β, and δ subunits. γ-1 subunit and γ-2 subunit are at *m/z* = 16 009 and 15 995. Results generated were presented as an abundance ratio of intact globin chains as percentages (%).

The detailed information from MALDI–TOF–MS, statistical analysis, and genotyping of disease-causing and modifier genes (18, 19) is described in the online Data Supplement. The primers used in genotyping are listed in Supplemental Table 5.

Results

ESTABLISHMENT OF MALDI–TOF–MS FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF GLOBIN CHAINS

We separated the 4 types of intact globin chains from hemoglobin in peripheral blood sample using a MALDI–TOF–MS instrument. In accordance with theoretical molecular weight that was calculated from the determined polypeptide sequence, *m/z* ratios of α, β, and δ subunits were 15 127, 15 868, and 15 925, successively. γ subunit consists of 2 globin chains (γ-1 and γ-2 subunits), with *m/z* of 16 009 and 15 995, respectively (Fig. 2, A and B). The results had excellent repeatability and reproducibility for quantifying β-globin and δ-globin chains in a healthy control and a β-thalassemia carrier (Fig. 2C). We calculated 3 principal abundance ratios: δ/β, γ/α, and γ/β via peak area of specific globin chains from the spectra. The intra- and interassay ($n = 10$) coefficient of variation (CV) of the δ/β ratio in a healthy control and a β-thalassemia carrier ranged between 2.60% and 3.51% (Table 1). Because the γ-globin chain was barely detectable in a sample from a healthy person, the γ/α and γ/β ratio were only available for a β-thalassemia carrier, for which intra- and interassay ($n = 10$) CV of γ/α ratio were 7.90% and 8.26%, and for the γ/β ratio 5.30% and 4.19%, respectively (Table 1). We assessed the stability of whole blood samples stored at 4 °C, −20 °C, and −80 °C by comparing the δ/β ratios of samples ($n = 5$) kept at the above temperatures for 3, 7, 14, and 21 d with those of the fresh samples. The samples were deemed stable for 21 days because the ion intensity responses of all preserved samples were between 92.0% and 113.5% (Supplemental Table 6). The δ/β (Pearson correlation coefficient = 0.913; $R^2 = 0.833$, Supplemental Fig. 2, A and C) and γ/β (Pearson correlation coefficient = 0.934; $R^2 = 0.872$, Supplemental Fig. 2, B and D) that were calculated via the peak area from the spectra paralleled those from CE.

SCREENING AND TYPING PROTOCOL FOR β-GLOBIN VARIANT AND β-THALASSEMIA

We conducted MALDI–TOF–MS on a training set composed of 901 pre-typed reference samples (Supplemental Table 1). Samples of abnormal Hb variants were subjected to qualitative analyses of globin chains while the other positive types were subjected to further quantitative analyses. We analyzed the difference of *m/z* between fractions of normal globin and abnormal globin peaks. Comparing against the calculated theoretical molecular mass of the abnormal peptide chains, MALDI–TOF–MS characterized 15 out of 16 α- and β-globin structural variants (Fig. 3A, Supplemental Table 7).

For identifying β-thalassemia traits, clinically sensitive biomarkers were expected to be selected by significantly increasing the numerator, or decreasing the denominator. Kruskal–Wallis test and ROC analysis demonstrated that δ/β ($P < 0.001$; area under the curve [AUC] = 0.998) was more suitable than α/β ($P = 0.169$; AUC = 0.546), γ/α ($P < 0.001$; AUC = 0.388), and γ/β ($P < 0.001$; AUC = 0.397) to distinguish β-thalassemia trait carriers from normal controls

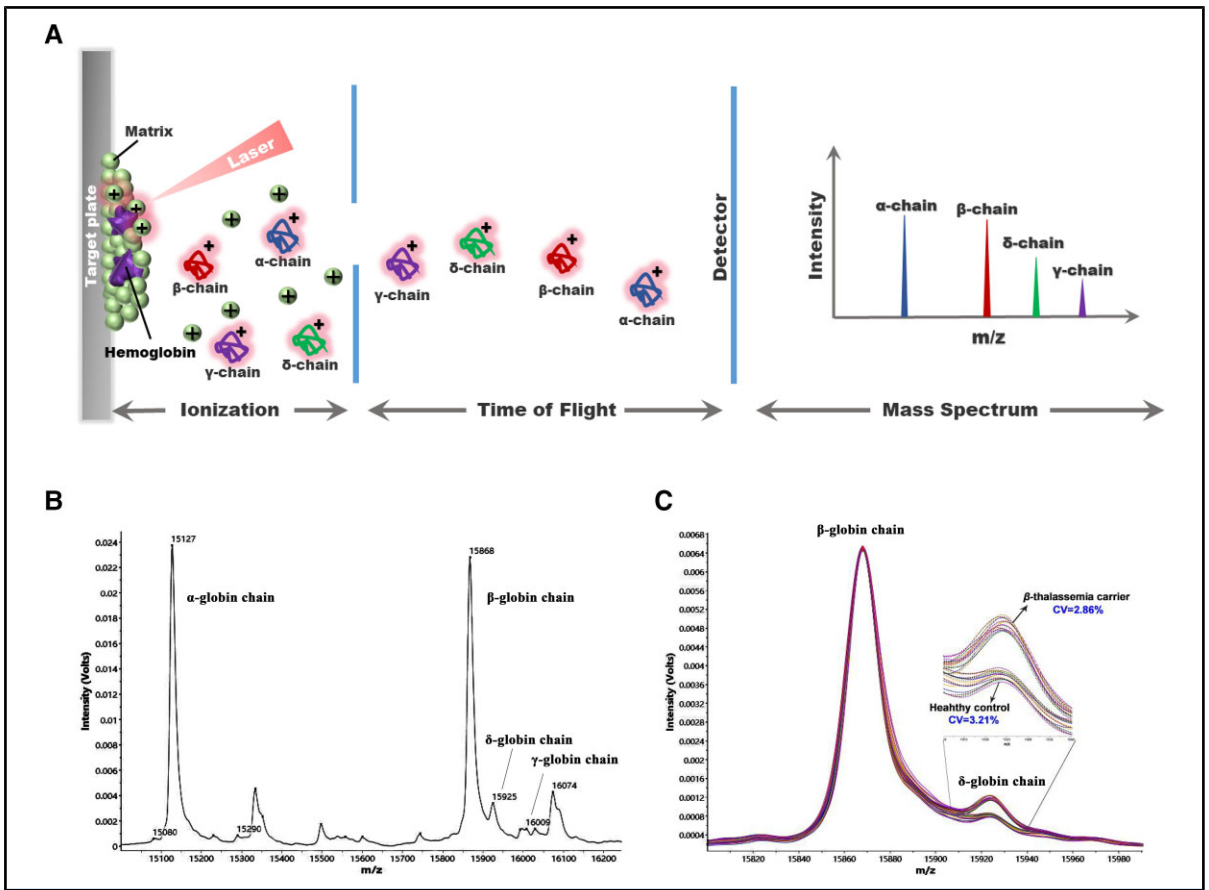

Fig. 2. Establishment of MALDI–TOF–MS for qualitative and quantitative measurements of globin chains. (A), Schematic diagram of 3 steps for qualitative determination of intact globin chains within Quan-TOF mass spectrometry (MS) machine; (B), MALDI–TOF mass spectrum of globin chains for normal individual without any hemoglobin disorder; (C), Reproducibility of Quan-TOF MS machine for quantification of globin peptides.

(Fig. 3B and Supplemental Fig. 3A). The maximum Youden Index was 0.971, with a δ/β cutoff of 16.401%, a clinical sensitivity of 97.3%, and a clinical specificity of 99.8%. The δ/β > 16.401% was the optimal index for discriminating a β-thalassemia trait from a normal control. The indicator described previously also exhibited a high clinical sensitivity in identifying the coinheritance of α- and β-thalassemia, while it was incapable of differentiating a β-thalassemia trait from the coinheritance of α- and β-thalassemia ($P = 0.041$; AUC = 0.388. Fig. 3B, Supplemental Fig. 3B).

Through statistical tests based on 795 training samples (Supplemental Table 3), the γ/β ratio was the best predictive indicator for deletional HPFH/δβ-thalassemia (AUC = 0.997) and HPFH phenotype with Hb F > 5% (AUC = 0.997) among 4 different forms of globin ratios. The optimal cutoff value for deletional HPFH/δβ-thalassemia was 29.293%, with a clinical sensitivity of 100%, and clinical specificity of 99.5%. The optimal cutoff value for HPFH phenotype was set at 9.574%, which demonstrated the highest clinical sensitivity (100%) and Youden Index (0.983), with a specificity of 98.3% (Fig. 3B, Supplemental Fig. 3, C and D).

The optimal indicator and cutoff for typing TM and TI was a γ/α ($P < 0.001$; AUC = 0.865) of 17.835% by analyzing 90 patients with β-thalassemia from a training set. The γ/α had the highest clinical sensitivity of 72.0%, and clinical specificity of 93.8% (Fig. 3C, Supplemental Fig. 3E).

VALIDATION OF POPULATION SCREENING AND CLINICAL CLASSIFICATION USING A MALDI–TOF–MS PROTOCOL

Through direct measurements of intact globin chains in the 16 172 individuals (8 086 couples), we detected 768 samples (4.75%) with 3 major β-globin disorders and

Table 1. Mean and CV for intraassay ($n = 10$) and interassay ($n = 10$) measurements of the 3 different globin-chain ratios in a healthy control and a β-thalassemia carrier.

	Healthy control			β-Thalassemia carrier		
	δ/β	γ/α	γ/β	δ/β	γ/α	γ/β
Intraassay						
Mean ratio (%)	14.56	NA	NA	19.10	7.33	7.03
%CV	3.51	NA	NA	2.92	7.90	5.30
Interassay						
Mean ratio (%)	13.10	NA	NA	17.40	7.53	7.38
%CV	3.08	NA	NA	2.60	8.26	4.19

Abbreviations: NA, not available; CV, coefficient of variation.

structural Hb variants in total, and identified 32 couples (3.96‰) at risk of having affected offspring (Supplemental Tables 8 and 9), which were confirmed by genetic testing. Our study yielded comparable results of clinical specificity (99.89% vs 99.71%) and accuracy (99.78% vs 99.16%) between our new assay and traditional methods (Table 2) but higher clinical sensitivity of our method than that of the control method (97.52% vs 88.01%). By assessing the technical performance of identifying 3 different classes of β-globin disorders, MALDI–TOF–MS showed significantly higher analytical sensitivity than traditional methods for the detection of structural Hb variants (96.33% vs 36.70%). Thus, the new assay identified 22 additional structural Hb variants in 69 individuals including 9 novel ones (Supplemental Tables 10 and 11). Nine carriers of deletional HPFH or δβ-thalassemia were accurately screened. Of the 16 172 screening samples, 43 (2.66‰) were categorized as HPFH phenotype with Hb F > 5%, and major Hb F-associated genotypes were identified in 86.05% (37/43) of the positive samples (Supplemental Tables 12 and 13). The assay correctly recognized 37 true-positive samples while there were 202 false-positive samples, with an accuracy comparable to traditional methods (98.74% vs 99.96%, Supplemental Table 14). The absolute and relative quantification of different intact globins in the different subgroups are provided in Supplemental Fig. 4A.

Using the γ/α ratio of 17.835% as a direct indicator that was optimized by 90 training samples in Stage 2 (Fig. 1), TM and TI were accurately classified in 178 samples (88.56%) out of 201 samples from an additional independent cohort of patients with β-thalassemia. The absolute quantification of intact γ-globin chains was much higher in TI than in TM (Supplemental Fig. 4B), consistent with the results of a higher concentration of Hb F in TI by CE (Supplemental Table 2).

Discussion

Collectively, we have established a MALDI–TOF–MS-based procedure combining qualitative and quantitative tools for precisely screening and typing β-globin variant and β-thalassemia (Fig. 4).

A major limitation in identifying abnormal Hbs through CE or high-performance liquid chromatography is that further genotyping is often necessary. Additionally, a series of "electrophoretically silent" Hb variants are undetectable by CE or high-performance liquid chromatography (20, 21). MALDI–TOF–MS can type abnormal Hb variants by measuring molecular masses of intact globin chains, which affords a high-resolution method for resolving this issue (22). We correctly characterized 36/39 of structural Hb variants using MALDI–TOF–MS while 13/39 were identified by using CE. The actual *m/z* ratio in the spectra were consistent with the theoretical molecular weights of globin variants (Supplemental Tables 7 and 11). MALDI–TOF–MS failed to detect 3 variants for 5 cases: one from a training set for HBB:c.304G>C (Hb Rush, p.Glu102Gln), and 4 from a validating set, of which 3 were for HBA2:c.91G>C (Hb G-Honolulu, p.Glu31Gln) and one for HBB:c.364G>C (Hb D-Los Angeles, p.Glu122Gln), whose molecular mass difference (Δm) with an intact globin chain were 1 Da. This was due to the limited mass resolution attainable on the instrument used in this study. HBB:c.79G>A (Hb E, p.Glu27Lys, $\Delta m = 1$ Da) is very common in Asian descent and may exhibit normal red cell indices in heterozygotes (23). Although an abnormal peptide pattern of Hb E was absent in this study, increased δ/β was present, similar to other β-thalassemia mutations. This showed that we could use δ/β > 16.401% to screen Hb E carriers in a healthy population. Over 1 000 Hb variants that lead to physiological implications with varying severity have been reported. Among them, 113 Hb variants are similar to Hb Rush, whose Δm is ≤1, and 11 that may lead to microcytic anemia (Supplemental Table 15). The minimum Δm of 36 abnormal Hbs characterized by MALDI–TOF–MS was 13 Da (Supplemental Tables 7 and 11). Thus, it will be necessary to expand diversity of abnormal Hbs to identify the minimal detectable Δm with an intact globin chain using our assay.

In view of the multiple potential application scenarios, we designed 3 schemes for sample processing in the population screening study, all of which showed superior capacity in screening for β-globin disorders. This

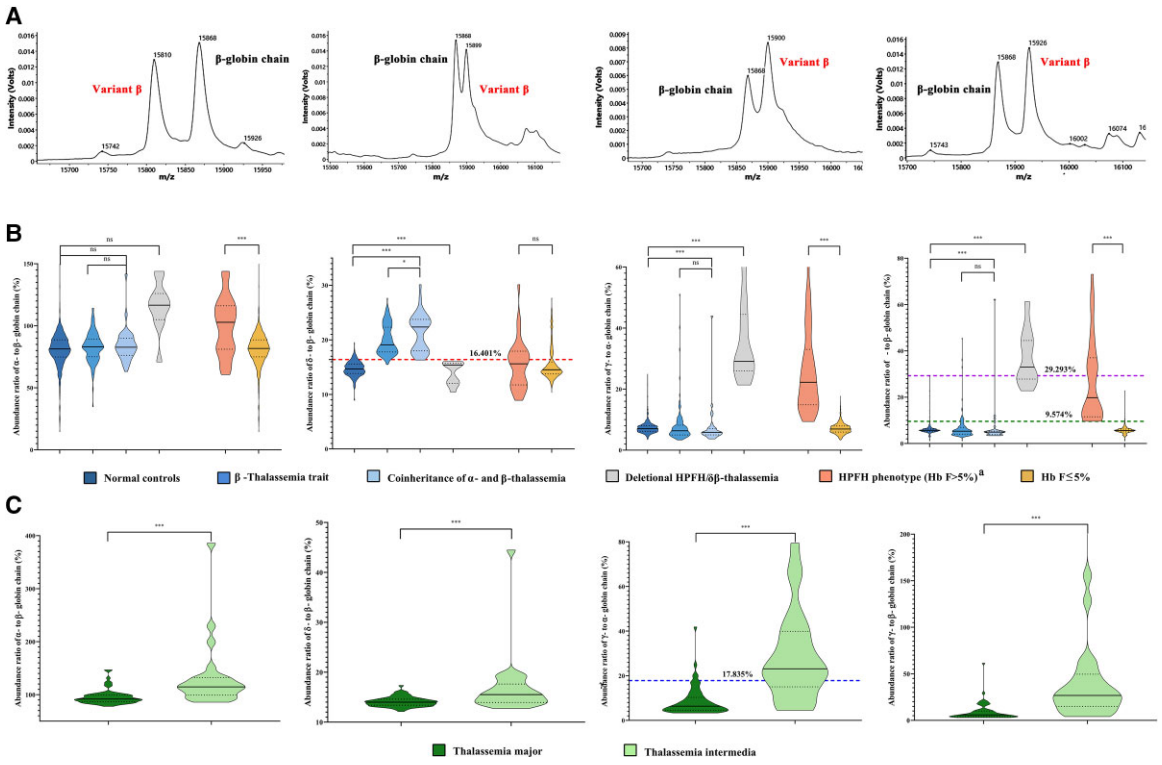

Fig. 3. Development and optimization of the MALDI–TOF–MS protocol in screening and typing β-globin variant and β-thalassemia. (A), Representative mass spectra in determining the abnormal Hb variants. Four structural variants, Hb G-Coushatta (HBB:c.68A>C, p.Glu23Ala), Hb Ty Gard (HBB:c.374C>A, p.Pro125Gln), Hb San Diego (HBB:c.328G>A, p.Val110Met), and Hb J-Bangkok (HBB:c.170G>A, p.Gly57Asp) were accurately detected; (B), Determination of the optimal cutoff values for 4 different forms of globin ratios (α/β, δ/β, γ/α, and γ/β) for typing β-thalassemia trait/HPFH carriers, or for classifying individuals with Hb F > 5% ($n = 32$) and with Hb F≤5%, or (C) for typing the patients with β-thalassemia into TM and TI. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant; Hb F, fetal hemoglobin; HPFH, hereditary persistence of fetal hemoglobin.

demonstrated the flexibility and scalability in clinical settings (Supplemental Tables 16 and 17). Multivariate linear regression analyses revealed no significant effect of sex, age, or pregnancy on the optimal indicators in MALDI–TOF–MS assay: δ/β, γ/β, and γ/α, in either of the groups (Supplemental Table 18).

The reactivation of γ-globin synthesis can ameliorate the severity of β-thalassemia (24, 25). We optimized the abundance ratio of γ/β on determining Hb F increase (Supplemental Fig. 3D), which better enabled us to precisely predict the potential risk of β-thalassemia among high-risk couples (Supplemental Table 9). The major genetic variants responsible for increased Hb F concentrations were identified in the positive samples with increased γ/β ratio, including *KLF1* mutations, rs368698783 in *HBG1*, rs766432 in *BCL11A*, rs9399137 in *HBS1L-MYB,* nondeletional HPFH mutations in *HBG1/HBG2* proximal promoter and the deletional forms of HPFH/δβ-thalassemia (Supplemental Tables 4 and 13) (17, 26–28). Using the γ/β ratio of 9.574% as a screening indicator for HPFH phenotype, 43 out of 16 172 Chinese general individuals (2.66‰) were determined to exhibit increased Hb F (>5%), including 9 samples with deletional HPFH/δβ-thalassemia, 28 samples with nondeletional Hb F-associated genotypes mentioned before, and 6 samples with unknown cause (Supplemental Table 13). We could also screen for the 2 known forms of deletional HPFH/δβ-thalassemia using the γ/β cutoff value of 29.293% (Table 2, Supplemental Table 14). Furthermore, patients with TM and TI clinically manifest different anemia severities and their clinical classification relies on complex clinical data. Generally, reactivation of γ-globin gene could effectively ameliorate the clinical severity of

Table 2. The performance of the MALDI–TOF–MS approach vis-a-vis traditional screening methods for screening β-globin disorders in a large-scale population.

	Abnormal Hb variant				β-Thalassemia trait/coinheritance of α- and β-thalassemia[a]				Deletional HPFH/δβ-thalassemia				β-Globin disorders			
Major classes	MALDI–TOF–MS	FBC + CE	Kappa[b]	p[c]	MALDI–TOF–MS	FBC + CE	Kappa[b]	p[c]	MALDI–TOF–MS	FBC + CE	Kappa[b]	p[c]	MALDI–TOF–MS	FBC + CE	Kappa[b]	p[c]
TN—n	16 062	16 054	0.462	<0.001	15 414	15 416	0.977	0.087	16 044	16 023	0.491	<0.001	15 388	15 361	0.908	<0.001
TP—n	105	40			634	626			9	9[d]			748	675		
FN—n	4	69			15	23			0	0			19	92		
FP—n	1	9			3	1			13	34[d]			17	44		
Clinical sensitivity	96.33%	36.70%			97.69%	96.46%			100%	100%			97.52%	88.01%		
Clinical specificity	99.99%	99.94%			99.98%	99.99%			99.92%	99.79%			99.89%	99.71%		
Accuracy	99.97%	99.52%			99.89%	99.85%			99.92%	99.79%			99.78%	99.16%		

Abbreviations: FBC, full blood counting; CE, capillary electrophoresis; Hb, hemoglobin; HPFH, hereditary persistence of fetal hemoglobin; n, number of samples; TN, true negative; TP, true positive; FN, false negative; FP, false positive.

[a]106 positive samples with abnormal Hb variants identified in a qualitative analyses protocol were excluded from further quantitative testing because of its sample features unsuitable for accurate quantification.

[b]The consistency between MALDI–TOF–MS approach and traditional screening methods (FBC + CE) were assessed with a weighted Kappa test. A high Kappa (>0.75) means high agreement, and a low Kappa (<0.4) means poor agreement.

[c]Comparisons between MALDI–TOF–MS approach and traditional screening methods (FBC + CE) for β-globin disorders screening were performed by using the McNemer test.

[d]This data was obtained by using the γ/β cutoff value of 29.293% and these 9 carriers were involved in a total of 43 positive HPFH individuals tested by using the γ/β cutoff value of 9.574%.

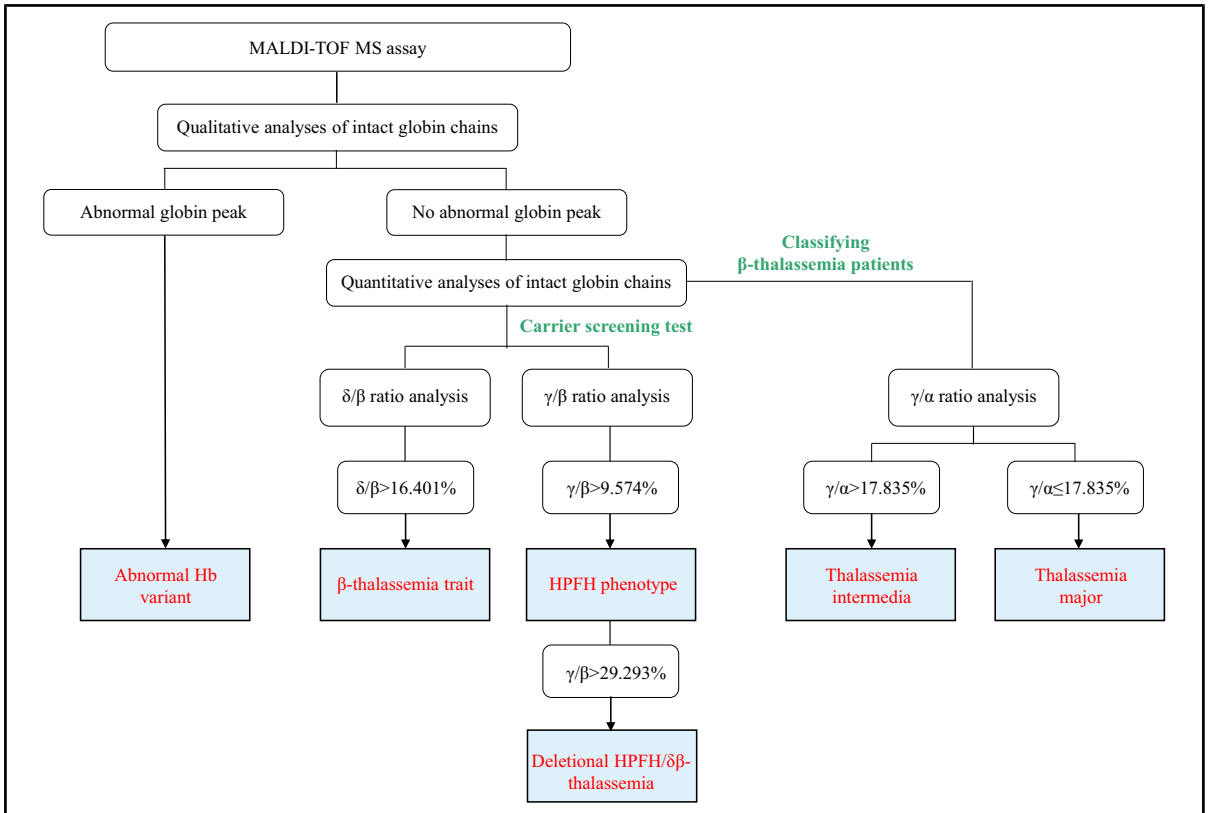

Fig. 4. Overview of analytical procedures for rapid screening and typing of β-globin variant and β-thalassemia using MALDI–TOF–MS assay. The procedure was composed of 2 protocols, a qualitative analysis for detecting abnormal hemoglobins and a quantitative analysis for determining β-thalassemia trait and HPFH/δβ-thalassemia. The quantitative analysis of intact globin chains was further used for carrier screening test using δ/β and γ/β ratios, and classifying β-thalassemia patients using γ/α ratio.

β-thalassemia (24, 25). Patients with β-thalassemia having higher γ-globin production are mostly classified into nontransfusion-dependent TI forms. The γ/α ratio based on intact globin chains was used to clinically differentiate TM and TI patients. We also found an unexpected discrepancy in α/β chain ratios between TM and TI, where the α/β ratios were determined to be higher in TI than in TM (Supplemental Fig. 4B). This was likely due to the higher concentration of β-globin chains in TM, mostly from frequent red blood cell transfusion instead of endogenous production. In addition, TM had a higher proportion of Hb A than TI by CE, consistent with the results of higher β-globin concentration measured by MALDI–TOF–MS. Therefore, the α/β ratios were higher in TI than in TM because of the same background concentrations of α-globin chains.

The advantages of this new technology are ease-of-operation, labor-savings, high-throughput automated detection, fast turnaround time of 1 min, and cost-effective and low reagent consumption (<$1 per test [Supplemental Table 19]). The MALDI–TOF–MS approach also requires fewer operation steps than the bottom-up approach targeting on tryptic peptides. As a consequence, the features of high throughput and low cost make MALDI–TOF–MS a good choice for large-scale clinical applications (29, 30).

We are not the first to apply MALDI–TOF–MS on hemoglobin disorders. A prior patent provided a general description of how to characterize the spectra patterns from Hb variants and thalassemia using a dozen samples (31). However, our study applied MALDI–TOF–MS toward screening and typing of β-globin disorders in a large-scale population, finding that MALDI–TOF–MS was superior to traditional methods when using the cutoff values of 3 different globin ratios we established.

We failed to distinguish α-thalassemia with satisfactory clinical specificity by using α/β, possibly due to the rapid degradation of excess β-globin polypeptide. Other more specific markers such as carbonic anhydrase and intracellular enzyme might be of value here (32, 33).

To summarize, we developed a prediction model for rapid identification of various β-globin disorders based on direct measurements of intact globin chains using MALDI–TOF–MS. MALDI–TOF–MS could serve as an alternative tool for large-scale phenotypic screening and typing of β-globin variant and β-thalassemia.

Supplementary Material

Supplementary material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: TM, thalassemia major; TI, thalassemia intermedia; Hb, hemoglobin; MS, mass spectrometry; Hb F, fetal hemoglobin; HPFH, hereditary persistence of fetal hemoglobin; FBC, full blood counting; CE, capillary electrophoresis; Gap-PCR, gap-PCR; RDB, reverse dot blot; NGS, next-generation sequencing; CV, coefficient of variation; AUC, area under the curve; Δ*m*, mass difference; NA, not available.

Human Genes: *HBB*, hemoglobin subunit beta; *HBA1*, hemoglobin subunit alpha 1; *HBA2*, hemoglobin subunit alpha 2; *HBG1*, hemoglobin subunit gamma 1; *HBG2*, hemoglobin subunit gamma 2; *KLF1*, Kruppel-like factor 1; *BCL11A*, BAF chromatin remodeling complex subunit BCL11A; *HBS1L*, HBS1 like translational GTPase; *MYB*, MYB proto-oncogene, transcription factor.

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