

DIAGNOSTIC PERFORMANCE OF HEMOGLOBIN A2 AND HEMOGLOBIN E FOR THALASSEMIA SCREENING IN A SOUTHERN CHINESE POPULATION**DIJAGNOSTIČKA EFIKASNOST HEMOGLOBINA A2 I HEMOGLOBINA E U SKRININGU NA TALASEMIJU KOD POPULACIJE JUŽNE KINE**Junyi Wang[†], Siyang Yu[†], Dengqing Si, Shiqiong Niu*

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Summary

Background: Hemoglobin A2 (HbA2) is an established laboratory biomarker for thalassemia screening; however, its diagnostic performance may be influenced by demographic characteristics and hemoglobin variants. Hemoglobin E (HbE), a common structural hemoglobin variant in Southern China, may affect the interpretation of HbA2 results and reduce screening accuracy when conventional cut-off values are applied. This study aimed to evaluate the clinical utility of HbA2 and HbE in thalassemia screening and to establish optimized HbA2 cut-off values for improving diagnostic performance in a Southern Chinese population.

Methods: A total of 1,352 individuals were retrospectively enrolled, including 355 α -thalassemia carriers, 174 β -thalassemia carriers, and 823 genetically normal controls. Complete blood count parameters, capillary electrophoresis (CE) results, and thalassemia genotypes determined by polymerase chain reaction–reverse dot blot (PCR-RDB) analysis were collected. Receiver operating characteristic (ROC) curve analysis was performed to determine optimal HbA2 cut-off values for thalassemia screening in men, non-pregnant women, and pregnant women.

Results: Thirteen α -thalassemia genotypes and ten β -thalassemia genotypes were identified. For α -thalassemia screening, the optimal HbA2 cut-off values were $<2.5\%$, $<2.4\%$, and $<2.6\%$ in men, non-pregnant women, and pregnant women, respectively. HbE carriers exhibited significantly lower HbA2 levels than other β -thalassemia carriers, indicating that HbE should be interpreted as an independent laboratory marker during screening. After

Kratak sadržaj

Uvod: Hemoglobin A2 (HbA2) predstavlja utvrđeni laboratorijski biomarker za skrining talasemije; međutim, na njegovu dijagnostičku efikasnost mogu uticati demografske karakteristike i varijante hemoglobina. Hemoglobin E (HbE), česta strukturna varijanta hemoglobina u južnoj Kini, može uticati na tumačenje rezultata za HbA2 i smanjiti tačnost skrininga kada se primenjuju uobičajene granične vrednosti. Cilj ove studije bio je da se proceni klinički značaj HbA2 i HbE u skriningu talasemije i odrede optimizovane granične vrednosti za HbA2 radi poboljšanja dijagnostičke efikasnosti kod populacije južne Kine.

Metode: Retrospektivno je uključeno ukupno 1.352 ispitanika, uključujući 355 nosilaca gena za alfa-talasemiju, 174 nosioca gena za beta-talasemiju i 823 genetski zdrave osobe (kontrolna grupa). Prikupljeni su podaci o parametrima kompletne krvne slike, rezultatima kapilarne elektroforeze (CE) i genotipovima talasemije utvrđenim analizom metodom lančane reakcije polimeraze i »reverse dot blot« tehnike (PCR-RDB). Sprovedena je analiza ROC krive (Receiver Operating Characteristic) kako bi se odredile optimalne granične vrednosti HbA2 za skrining talasemije kod muškaraca, žena koje nisu u drugom stanju i trudnica.

Rezultati: Identifikovano je trinaest genotipova α -talasemije i deset genotipova β -talasemije. Za skrining alfa-talasemije, optimalne granične vrednosti HbA2 iznosile su $<2\%$, 5% , $<2,4\%$ i $<2,6\%$ za muškarce, žene koje nisu trudne i trudnice, respektivno. Nosioci HbE imali su značajno niže nivoe HbA2 u odnosu na druge nosioce beta-talasemije, što ukazuje na to da HbE treba tumačiti kao nezavisni laboratorijski marker tokom skrininga. Nakon isključivanja osoba sa genotipom β^E (GAG>AAG)/ β^N , optimalne granične

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exclusion of β^E (GAG>AAG)/ β^N individuals, the optimal HbA2 cut-off values for β -thalassemia screening were >4.0%, >4.1%, and >4.7% in men, non-pregnant women, and pregnant women, respectively. The optimized screening model demonstrated improved positive and negative predictive values compared with conventional HbA2-based criteria.

Conclusion: HbA2 demonstrates good diagnostic accuracy for thalassemia screening when population-specific thresholds are applied. Combined assessment of HbA2 and CE-detected HbE improves screening performance and enhances the identification of both α - and β -thalassemia carriers. These findings support the clinical application of individualized laboratory screening strategies and provide evidence for optimizing thalassemia screening protocols in high-prevalence populations.

Keywords: thalassemia, hemoglobin A2, hemoglobin E, diagnostic performance, biomarker, capillary electrophoresis

Introduction

Thalassemia is one of the most common monogenic genetic diseases caused by the defective synthesis of globin chains of hemoglobin (Hb). The imbalance of globin chain expression could lead to hemolysis and erythropoiesis impairment (1, 2). The carrier rates of thalassemia vary across different regions of China, with genetic heterogeneity and geographic regional differences, highest in Guangxi, Hainan and Guangdong Province (3–5). The incidence of thalassemia carriers was 19.48% in Guangdong Province, of which α -thalassemia, β -thalassemia and co-inherited α - and β -thalassemia account for 15.07%, 3.8% and 0.61% (5). The primary screening methods for thalassemia are automated hematology analyzers and CE. $MCV < 82$ fl and $MCH < 27$ pg are the positive screening thresholds for microcytic hypochromic anemia, $HbA2 < 2.5\%$ indicates suspected α -thalassemia carriers and $HbA2 > 3.5\%$ indicates suspected β -thalassemia carriers, despite of gender and pregnancy status (6). Recent studies have reported that HbA2 value was correlated with many factors, such as thalassemia type, geographic region, gender and pregnancy (7). Hemoglobin E (HbE) is one of the most common hemoglobin variants in southern China, caused by a point mutation (GAG > AAG) at codon 26 on the β -globin gene, which leads to the reduced expression of β -globin gene, and therefore functionally belongs to β^+ form of β -thalassemia (8, 9). CE can separate HbA2 and HbE. It has been reported that the mean HbA2 value in HbE heterozygotes is 3.4% (10). This finding is clearly inconsistent with the traditional criterion for diagnosing β -thalassemia, which defines the threshold as $HbA2 > 3.5\%$ (6). Thus, this study collected data from men, non-pregnant women and pregnant women to analyze the correlation between thalassemia genotypes and CE results (HbA2, HbE),

and to establish a gender- and pregnancy-stratified HbA2 cutoff value applicable to the Shenzhen region in southern China, thereby improving the diagnostic accuracy of thalassemia screening.

Zaključak: HbA2 pokazuje dobru dijagnostičku tačnost za skrining talasemije kada se primene populaciono-specifični pragovi. Kombinovana procena HbA2 i CE-detektovanog HbE poboljšava performanse skrininga i unapređuje identifikaciju nosilaca i α - i β -talasemije. Ovi nalazi podržavaju kliničku primenu individualizovanih laboratorijskih strategija skrininga i pružaju dokaze za optimizaciju protokola skrininga talasemije u populacijama sa visokom prevalencijom.

Ključne reči: talasemija, hemoglobin A2, hemoglobin E, dijagnostička efikasnost, biomarker, kapilarna elektroforeza

Materials and Methods

Data collection

Study data were collected from the Laboratory Information System from May 2019 to October 2022. A total of 548 cases diagnosed with thalassemia by genetic test were enrolled in this study (355 α -thalassemia, 174 β -thalassemia and 19 α -thalassemia compounded with β -thalassemia cases) (Figure 1). 823 cases were chosen as healthy controls, with $MCV \geq 82$ fL, $MCH \geq 27$ pg and normal results of thalassemia genetic test. All cases had CBC, CE and thalassemia genetic test results. Information on age, gender and pregnancy status (based on gynecology and obstetrics clinical diagnosis) was collected simultaneously. After excluding 19 patients with α -thalassemia compounded with β -thalassemia, a retrospective analysis of 1352 individuals was performed. The Medical Ethics Committee of the Seventh Affiliated Hospital of Sun Yat-sen University approved the study waiving informed consent.

Laboratory analysis

CBC was carried out on Sysmex XN9000 automated hematology analyzer (Sysmex, Japan). Erythrocyte-related parameters including MCV, MCH, MCHC, RBC and Hb were collected. CE was performed on Capillarys 2 flex piercing (Serbia, France). Genomic DNA was isolated from peripheral blood samples using a commercial DNA

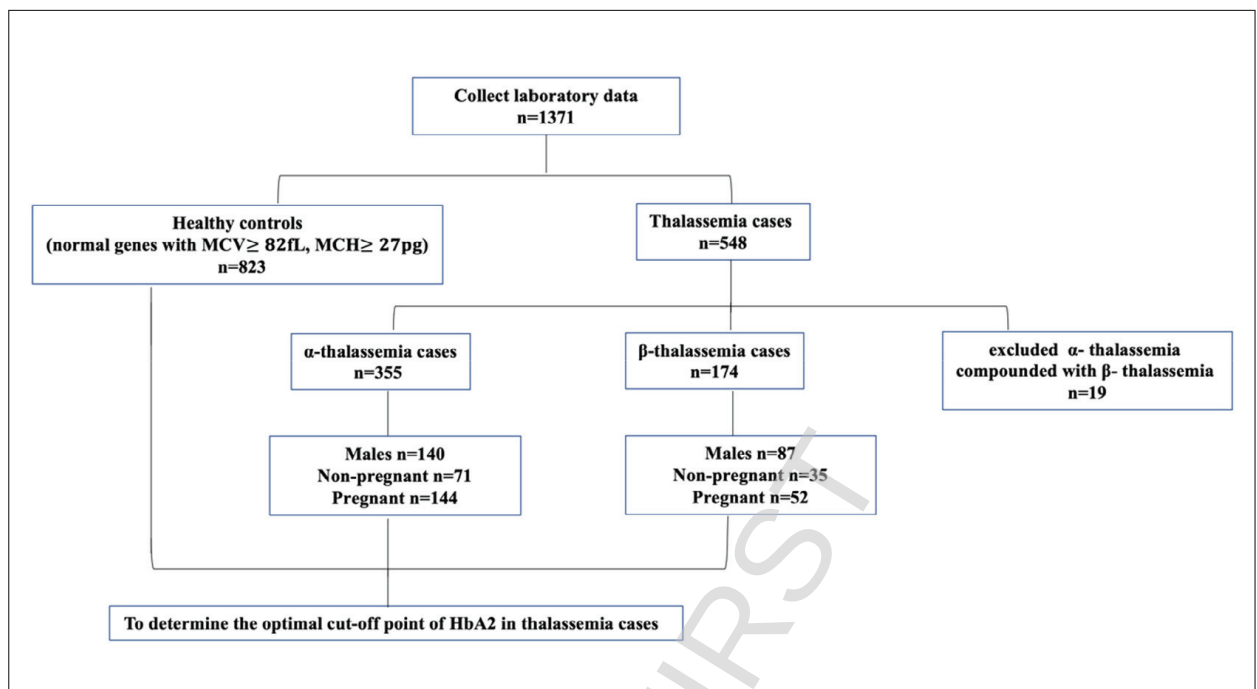


Figure 1 Schematic diagram of data group.

extraction kit (HybriBio, China). Samples with a DNA concentration of 20–40 ng/μL and an OD₂₆₀/OD₂₈₀ ratio of 1.5–2.5 were considered qualified. For thalassemia genotyping, multiplex PCR amplification was performed using biotin-labeled primers to amplify 3 α-globin gene deletions (–SEA, –α^{3.7}, –α^{4.2}), 3 α-globin gene mutations (α^{CS}, α^{QS}, α^{WS}), and 17 β-globin gene mutations including CD41/42 (–TTCT), CD43 (G > T), IVS-II-654 (C > T), CD17 (A > T), CD14/15 (+G), –28 (A>G), –29 (A>G), CD71/72 (+A), βE (GAG > AAG), IVS-I-1 (G>A/T), CD27-28 (+G), IVS-I-5 (G>C), and CAP (A>C). The amplified products were then hybridized via reverse dot blot (RDB), and the results were finally interpreted by chemical color development (HybriBio, China). Judgment criteria for positive hybridization bands: only the normal spot showing color indicates the normal sample; when both the normal and mutant spots show color, it indicates a heterozygous mutation; when only the mutant spot shows color, it indicates a homozygous mutation. All samples collected were peripheral blood.

Statistical analysis

The statistical analyses for this study were performed on Beckman Coulter DxAI platform (<https://www.xsmartanalysis.com/beckman/login/>). Non-normal distributions were uniformly expressed as median (IQR), and differences between groups were compared by the nonparametric Mann-Whitney test. Statistical significance was defined as

$p < 0.05$. Received operating characteristic (ROC) curves were generated to determine the optimal cut-off values of HbA2 in men, non-pregnant, and pregnant women. All ROC curves analyses were performed with Python.

Results

Data characteristics of enrolled individuals

A total of 355 α-thalassemia cases (118 cases of α-thalassemia silent, 226 cases of α-thalassemia trait, 11 cases of α-thalassemia intermedia), 174 β-thalassemia cases and 823 healthy controls were enrolled in this study. Age and blood erythrocyte-related parameters of study participants were shown in Table 1. All α-thalassemia cases were classified into α-thalassemia silent, α-thalassemia trait and α-thalassemia intermedia. The results showed that MCH and MCHC were reduced, and RBC was higher sequentially in α-thalassemia silent, trait and intermedia patients, with statistically significant differences compared to healthy controls. Hb decreased significantly in α-thalassemia trait and α-thalassemia intermedia cases. All blood erythrocyte-related parameters of β-thalassemia cases showed significant differences compared to the healthy controls.

Table I Hematological Data of enrolled individuals.

Characteristic	Healthy control	α -thalassemia silent	α -thalassemia trait	α -thalassemia intermedia	β -thalassemia
Number of cases	823	118	226	11	174
Age (years)	30 (27, 33)	29 (26, 34)	30 (26, 36)	44 (32, 71)**	30 (26, 35)
MCV (fl)	90.6 (87.7, 93.5)	82.7 (79.5, 86.3)***	69.4 (67.1, 71.3)***	70.1 (61.3, 74.8)***	64.8 (62.8, 70.3)***
MCH (pg)	30.4 (29.3, 31.5)	26.8 (25.7, 28.2)***	21.5 (20.6, 22.2)***	19.2 (16.8, 20.7)***	20.4 (19.4, 22.2)***
MCHC (g/L)	335 (329, 341)	324.00 (317, 329)***	310 (304, 316)***	288 (274, 294)***	313 (307, 319)***
RBC ($\times 10^{12}/L$)	4.20 (3.79, 4.80)	4.70 (4.20, 5.60)***	5.60 (4.81, 6.21)***	4.91 (3.90, 5.60)	5.60 (4.64, 6.29)***
Hb (g/L)	127 (115, 143)	127 (113, 146)	120 (106, 132)***	88 (80, 110)***	114 (100, 131)***

MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RBC: red blood cell, Hb: hemoglobin, Mann-Whitney U *** $p < 0.001$

Genetic diagnosis and genotype distribution of thalassemia

In this study, 13 α -thalassemia genotypes were identified. The proportions of silent, trait and intermedia α -thalassemia were 33.24%, 63.66% and 3.10%, respectively (Table II). Among these, $\alpha\alpha^{SEA}/\alpha\alpha$ genotype was the most prevalent accounting for 62.26%.

10 β -thalassemia mutation genotypes were identified in our study, all of which were classified as β -thalassemia trait. The $\beta^{CD41/42(-TTCT)}/\beta^N$ genotype was the most common (37.93%), followed by $\beta^{IVS-II-654(C > T)}/\beta^N$ (32.18%) (Table II).

HbA2 proportion in different thalassemia types

In α^+/α , α^0/α , α^+/α^+ and α^+/α^0 group, HbA2 proportions were 2.4%(IQR:2.3-2.6), 2.3%(IQR:2.2-2.4), 2.4%(IQR:2.2-2.5), 0.9%(IQR:0.7-1.2), respectively (Fig.2). HbA2 level in patients with genotype α^+/α^0 was lower than α^+/α , α^+/α^+ and α^0/α , with statistically significant differences. $\beta^E(GAG > AAG)/\beta^N$ functionally belongs to β -thalassemia trait, while it could result in structural β -globin variant, with a detectable HbE band. Therefore, $\beta^E(GAG > AAG)/\beta^N$ was classified as a separate group, along with β^+/β and β^0/β . It was worth noting that proportion of HbA2 3.3%(IQR:3.0-3.4) in genotype $\beta^E(GAG > AAG)/\beta^N$ cases

was significantly lower than β^+/β 5.2%(IQR:4.9-5.5) and β^0/β 5.3%(IQR:5.1-5.5) (Figure 2). 12 cases with genotype $\beta^E(GAG > AAG)/\beta^N$ showed apparently elevated HbE content in CE results 25.0%(IQR:24.3-25.4) (Supplement Table I, Supplement Figure 1).

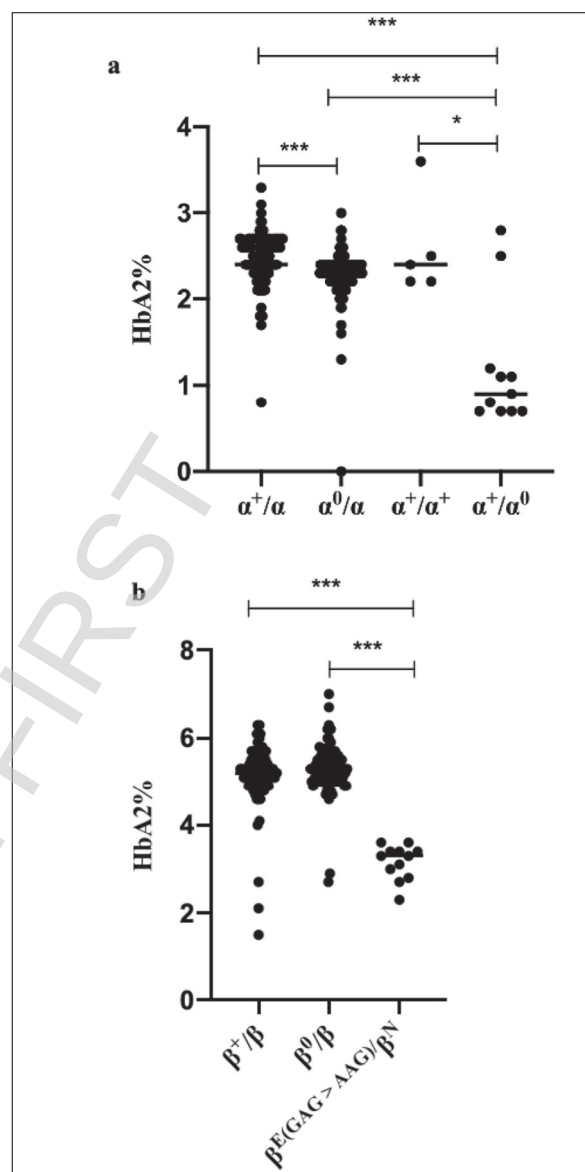
Determination of optimal HbA2 cut-off threshold in thalassemia cases

ROC curves were estimated by gender and pregnant state group to determine a specific optimal cut-off threshold for thalassemia. In α -thalassemia, the cut-off threshold was set at 2.5% for men; at 2.4% for non-pregnant women; and at 2.6% for pregnant women. The AUCs of all groups were more than 0.70. The specificity was 0.76, 0.73, and 0.78, and sensitivity was 0.68, 0.70, and 0.62, respectively.

In β -thalassemia without $\beta^E(GAG > AAG)/\beta^N$ cases, it was set at 4.0% for men; at 4.1% for non-pregnant women; and at 4.7% for pregnant women. The AUC was all close to 1.00, with specificity and sensitivity were all more than 0.90. Therefore, the optimal HbA2 cut-off point was highly capable of screening thalassemia cases in all the gender and pregnant state groups analyzed (Figure 3).

Table II Distribution of α -thalassemia and β -thalassemia genotype.

Genotype	Type	Number of cases	Frequency of all (%)
α -thalassemia			
α -thalassemia silent			
$-\alpha^{3.7}/\alpha\alpha$	α^+/α	66	18.59
$-\alpha^{4.2}/\alpha\alpha$	α^+/α	23	6.48
$\alpha^{CS}\alpha/\alpha\alpha$	α^+/α	15	4.23
$\alpha^{WS}\alpha/\alpha\alpha$	α^+/α	8	2.25
$\alpha^{QS}\alpha/\alpha\alpha$	α^+/α	6	1.69
α -thalassemia trait			
$--^{SEA}/\alpha\alpha$	α^0/α	221	62.26
$-\alpha^{3.7}/-\alpha^{4.2}$	α^+/α^+	2	0.56
$-\alpha^{3.7}/-\alpha^{3.7}$	α^+/α^+	2	0.56
$\alpha^{WS}\alpha/-\alpha^{4.2}$	α^+/α^+	1	0.28
α -thalassemia intermedia			
$-\alpha^{4.2}/--^{SEA}$	α^+/α^0	4	1.13
$-\alpha^{3.7}/--^{SEA}$	α^+/α^0	3	0.85
$\alpha^{WS}\alpha/--^{SEA}$	α^+/α^0	2	0.56
$\alpha^{CS}\alpha/--^{SEA}$	α^+/α^0	2	0.56
β -thalassemia			
$\beta^{CD41/42(-TTCT)}/\beta^N$	β^0/β	66	37.93
$\beta^{IVS-II-654(C>T)}/\beta^N$	β^+/β	56	32.18
$\beta^{-28(A>G)}/\beta^N$	β^+/β	17	9.77
$\beta^{CD17(A>T)}/\beta^N$	β^0/β	14	8.05
$\beta^{E(GAG>AAG)}/\beta^N$	β^+/β	12	6.90
$\beta^{CD71/72(+A)}/\beta^N$	β^0/β	3	1.72
$\beta^{CD43(G>T)}/\beta^N$	β^0/β	2	1.15
$\beta^{CD14/15(+G)}/\beta^N$	β^0/β	2	1.15
$\beta^{CAP+1(A>C)}/\beta^N$	β^+/β	1	0.57
$\beta^{-29(A>G)}/\beta^N$	β^+/β	1	0.57

**Figure 2** Proportion of HbA2 in different thalassemia types. β^+/β , without β^E ($GAG > AAG$)/ β^N genotype ***, $p < 0.001$ *, $p < 0.05$

Verification of CE screening efficiency with novel screening strategy

This study determined that HbA2 cut-off thresholds of 2.5%, 2.4%, and 2.6% should be used for screening for α -thalassemia in males, non-pregnant women, and pregnant women, respectively. For β -thalassemia screening, the HbE band should be considered the primary screening marker, and higher HbA2 cut-off thresholds of 4.0%, 4.1%, and 4.7% should be adopted for these groups, respectively, rather than the diagnostic criteria ($HbA2 < 2.5\%$ and $> 3.5\%$). The efficiency of the novel screening strategy was compared with

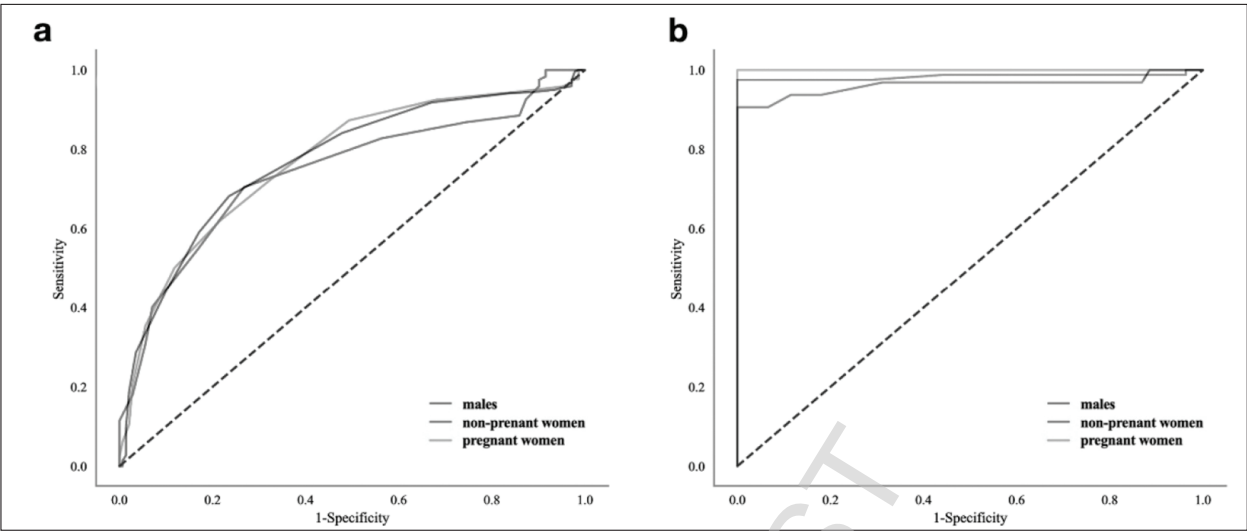


Figure 3 ROC curves for HbA2 screening used for thalassemia cases in men, non-pregnant women and pregnant women. a: Optimal cut-off points of HbA2 used for α -thalassemia b: Optimal cut-off points of HbA2 used for β -thalassemia without β^E ($GAG > AAG$)/ β^N genotype.

Table III Optimal cut-off for HbA2 in thalassemia cases (without β^E ($GAG > AAG$)/ β^N genotype) in men, non-pregnant women, and pregnant women.

Group	Males	Non-pregnant women	Pregnant women	Overall
α -thalassemia				
Cut-off point	2.5	2.4	2.6	2.5
Sensitivity	0.68	0.70	0.62	0.69
Specificity	0.76	0.73	0.78	0.74
Youden's index	0.45	0.44	0.40	0.43
AUC	0.77	0.74	0.77	0.77
β -thalassemia				
Cut-off point	4.0	4.1	4.7	4.0
Sensitivity	0.98	0.91	1.0	0.97
Specificity	1.0	1.0	1.0	1.0
Youden's index	0.98	0.91	1.00	0.97
AUC	0.98	0.96	1.0	0.98

AUC: the area under the ROC curve

Table IV Screening efficiency of HbA2 for thalassemia.

	Traditional Screening		Novel Screening	
	PPV	NPV	PPV	NPV
α -thalassemia				
overall	50.29% (263/523)	88.90% (737/829)	48.23% (272/564)	89.47% (705/788)
Males	60.11% (107/178)	87.73% (236/269)	60.11% (107/178)	87.73% (236/269)
Non-pregnant women	50.86% (59/116)	89.29% (100/112)	57.78% (52/90)	86.23% (119/138)
Pregnant women	42.36% (97/229)	89.51% (401/448)	38.18% (113/296)	92.11% (350/381)
β -thalassemia				
overall	96.95% (159/164)	98.74% (1173/1188)	100% (165/165)	99.24% (1178/1187)
Males	98.75% (79/80)	97.82% (359/367)	100% (84/84)	99.17% (193/197)
Non-pregnant women	100.00% (29/29)	96.98% (193/199)	100% (31/31)	97.97% (193/197)
Pregnant women	92.73% (51/55)	99.84% (621/622)	100% (50/50)	99.68% (625/627)

the conventional screening criteria (Table III). In α -thalassemia, the positive predictive value (PPV) and negative predictive value (NPV) of traditional fixed HbA2 criteria ($HbA2 < 2.5\%$) was 50.29% and 88.90%. The novel HbA2 cut-off threshold showed better PPV in men (60.11%) and non-pregnant women (57.78%), and the NPV improved to 92.11% in pregnant women. In β -thalassemia, the PPV

and NPV of traditional fixed HbA2 criteria (HbA2 > 3.5%) were 96.95% and 98.74%, respectively. After formulating the novel screening strategy, PPV increased to 100% and NPV increased to 99.24% in general.

Discussion

Thalassemia is an autosomal recessive genetic disorder that causes hemolytic anemia due to the decline or lack of globin chain synthesis (4). The prevalence of thalassemia in Guangdong Province was as high as 19.48%, including 15.07% of α -thalassemia and 3.8% of β -thalassemia (5). In our study, a total of 13 α -thalassemia genotypes were identified by genetic test, with $--^{SEA}/\alpha\alpha$ possessing the highest proportion (62.25%), followed by $-\alpha^{3.7}/\alpha\alpha$ (18.59%), $-\alpha^{4.2}/\alpha\alpha$ (6.48%), which were in general agreement with the previous report in Shenzhen (11). However, α -thalassemia genotypes were inconsistent with Hainan (1). 10 β -thalassemia mutation types were detected, among which $\beta^{CD41/42(-TTCT)}/\beta^N$ accounted for the highest percentage (37.93%), followed by $\beta^{IVS-II-654(C>T)}/\beta^N$ (32.18%), it was common with genotypes of β -thalassemia reported in Guangdong (6), while inconsistent with other Provinces such as Hubei area of China (12). These research data indicated that the distribution of thalassemia in China varies by region. As one of the most populous cities in southern China, Shenzhen was characterized by high mobility among young population and complex migration patterns. Analyzing the geno-type distribution of thalassemia in this region and establishing a Shenzhen-specific HbA2 cutoff threshold are of great significance for the prevention and control of thalassemia in the area. However, the genetic testing conducted in this study only covered common subtypes of thalassemia, which may result in the failure to detect rare subtypes.

Changes of hemoglobin components could indicate the subtype of thalassemia. HbA2 is formed by two α chains and two δ chains, and now is widely used for screening of thalassemia. However, factors such as δ gene mutation and iron deficiency anemia could affect the level of HbA2 (13). Mutations that occur on the δ -globin gene will lead to a decreased δ chains and eventually HbA2 levels (14). While the iron deficiency could directly decrease the bone marrow synthesis rate of HbA2 (15). Currently, high-performance liquid chromatography (HPLC) and CE are used in clinical laboratories to measure hemoglobin fractions (16). Studies have confirmed that CE can separate between HbE and HbA2, and that this method was superior to HPLC when HbE is present (17). Domestic and international studies have shown that HbE can be detected via CE in HbE homozygotes, heterozygotes, and compound

with α -thalassemia (9, 11). Furthermore, HbE homozygotes may have normal HbA2 levels (10). In this study, HbE and HbA2 proportions were calculated to be 25.0% (IQR:24.3–25.4) and 3.3% (IQR: 3.0–3.4) in 12 $\beta^E_{(GAG>AAG)}/\beta^N$ cases, which was in accordance with a previous study with 52 cases (HbE 28.3%, HbA2 3.4%) (10). Our results showed that the HbA2 level in $\beta^E_{(GAG>AAG)}/\beta^N$ was significantly lower than in β^+/ β 5.2%(IQR:4.9–5.5) and β^0/ β 5.3%(IQR:5.1–5.5). Therefore, we consider the HbE band in CE to be used as an independent screening marker in β -thalassemia, $\beta^E_{(GAG>AAG)}/\beta^N$ cases were excluded when generating the ROC curve to determine the optimal HbA2 cut-off point. Each presence of HbE band or higher level of HbA2 than the optimal threshold, ought to be treated as evidence of β -thalassemia. Following the implementation of the new β -thalassemia screening strategy, both the PPV and the NPV have improved.

A significant increase in NPV was observed, compared to the routine screening of α -thalassemia, suggesting that the novel screening system could avoid missed diagnosis better, especially in pregnant women. The PPV of the novel screening α -thalassemia in overall population was 48.23%, while it rises to 57.78% in non-pregnant women, which could decrease unnecessary genetic test of them. Therefore, the advantage of the novel screening strategy is mainly reflected in women with grouped by pregnancy status. Our study found that the cutoff value for HbA2 in the diagnosis of α -thalassemia in pregnant women was 2.6% (AUC = 0.77). Qian's study also showed that HbA2 has limited diagnostic value for α -thalassemia in pregnant women (AUC = 0.587), with a cutoff value of 2.71% (18). However, in contrast to Kang's findings, this study indicated that AUC for α -thalassemia in pregnant women was 0.63, with a cutoff value of 2.35% (7). Inconsistencies in CE results may be attributed to sample size, regional variations, and population migration. The limitation is that our study was a single-center retrospective study in Guangming District of Shenzhen. Moreover, the unsatisfactory screening efficiency of HbA2 in α -thalassemia was also owing to the severe iron deficiency anemia or δ -thalassemia (13). Iron metabolism indicators are not routine clinical laboratory indicators, so it cannot be ruled out whether the population in our study has iron deficiency anemia. Future studies should increase the sample size and exclude iron deficiency anemia and δ -thalassemia from healthy controls, to further validate the applicability of this HbA2 threshold in other areas of Shenzhen.

Hb Westmead (HbWS) is a structural variant of Hb, caused by the mutation in codon 122 (CAC > CAG) of the $\alpha 2$ -globin gene, changing histidine to glutamate (19). In this study, two discrete values were observed in α^+/α^0 cases with obvious higher

HbA2 levels (2.5 and 2.8), whose genotypes were both $\alpha^{\text{WS}}\alpha/\text{--}^{\text{SEA}}$ (Figure 2). The MCV of these two cases were 65.0fl and 70.1fl, and MCHs were 20.7pg and 21.3pg, respectively. These values were broadly like the results of the reported 20 cases from Guangdong Province, with HbA2 proportion of 2.55 ± 0.12 , MCV of 68.27 ± 4.20 fl, and MCH of 20.89 ± 1.10 pg (5). The results of these two cases were absent of HbH band, which is consistent with a domestic study (19). Our study included 12 α^{WS} carriers with no Hb variant band observed in CE test. The content above results in the recognition difficulty of α^{WS} in clinical practice.

Therefore, combining erythrocyte-related parameters with CE is crucial for preventing missed diagnoses of α -thalassemia. In our study, a gender- and pregnancy-stratified HbA2 cutoff value was illustrated innovatively, combined with HbE band as an independent laboratory biomarker, to improve the diagnostic performance of thalassemia using CE.

Conclusions

This study demonstrates that HbE can serve as an independent laboratory marker for β -thalassemia screening, and that population-specific HbA2 cutoff thresholds stratified by gender and pregnancy status improve the diagnostic accuracy for α - and β -thalassemia. When HbA2 is $<2.5\%$ or $>4.0\%$ in males, $<2.4\%$ or $>4.1\%$ in non-pregnant women, $<2.6\%$ or $>4.7\%$ in pregnant women, genetic testing is highly recommended. The combined assessment of HbA2 and CE-detected HbE enhances screening performance, increases positive and negative predictive values, and provides a reliable laboratory-based approach for identifying thalassemia carriers. These findings support the application of individualized biomarker-based peripheral blood screening strategies in clinical laboratories, facilitating targeted genetic testing and optimizing the efficiency of thalassemia diagnostics in high-prevalence populations.

CRedit authorship contribution statement

Junyi Wang: Writing – review & editing, Writing – original draft, Data curation, Formal analysis.

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Data statement

The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request.

Ethics in publishing and Informed consent

The study was approved by the Medical Ethics Committee of the Seventh Affiliated Hospital of Sun Yat-sen University, which waived the requirement for informed consent (Grant Number: KY-2023-099-02).

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Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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