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Hematological and electrophoretic characterization of β-thalassemia in Yunnan province of Southwestern China

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ABSTRACT

Objectives: β-thalassemia is widely distributed in Southwestern China. Characterization of β-thalassemia can improve screening and prenatal diagnosis for at-risk populations.

Design: In this study, the levels of Hb A_2 and Hb A was analysed by gender for a total of 15,067 subjects screened by capillary electrophoresis. Cut-off value with the highest accuracy was established to identify β-thalassemia in 723 suspected β-thalassemia patients. Hematological and electrophoretic characterization of 8 types of common β-thalassemias were analyzed in 486 β-thalassemia subjects.

Setting: Genetic Diagnosis Center, Yunnan Provincial Key Laboratory For Birth Defects and Genetic Diseases, the First People's Hospital of Yunnan Province, Kunming 650032, Republic of China.

Results: Hb A levels was significantly higher in males than females, but there was no significant difference in Hb A_2 levels. A new cut-off (Hb A_2 $\geqslant$4.0%) with the highest accuracy is proposed for the studied population. Hb was significantly higher in males compared to females (p <0.05), whereas no statistically significant differences was found for MCV, MCH, Hb A, and Hb A_2 values. The Hb E group showed comparatively higher values of hematological indices (Hb, MCV, and MCH) than those of other genotype of heterozygous β-thalassemia groups. And -28 (A>G) (HBB:c.-78A>C) had significantly higher Hb A_2 values compared to other

β-thalassemia.

Conclusions: Ethnic groups have diverse β-globin gene mutations and considerable hematologic variations. Our study will lay the foundation for screening programs and clinical management of thalassemia in Southwestern China.

Strengths and limitations of this study

- First study to estimate the characterization of β-thalassemia in Yunnan province, which may be useful for improve screening and prenatal diagnosis in Southwestern China.
- A higher cut-off point of Hb A_2 levels 4.0% (Hb A_2 $\geqslant$4.0%) was recommended for the β-thalassemia screening in Yunnan province.
- Participants used for cut-off value calculation and characterization of β-thalassemias were not collected at the same time.

INTRODUCTION

β-thalassemia is an inherited anemia resulting from genome variants in β-globin chains, and Africa,[1] Asia,[2] Mediterranean[3] and the Middle-East[4] have the greatest prevalence for this disease. Quantification of hemoglobin A_2 (Hb A_2) percent by capillary electrophoresis and routine blood tests is currently a common technique for screening thalassemia and hemoglobinopathies.[5] Low mean cell hemoglobin (MCH) and low mean cell volume (MCV) with a Hb A_2 values exceeding 3.5% is a hallmark of β-thalassemia carriers, and knowing the hemoglobin concentration (Hb) and Hb A_2

range in a population offers a critical screening tool for thalassemia. [6-7] However, there was few report on the reference intervals of β-thalassemia established by Chinese. So it is important to determine values considered appropriate for cut-off points and reference to accommodate the need for screening and control of β-thalassemia.

More than 800 different β-globin gene mutations have been elucidated worldwide. Two subtypes are defined by totally absent (β^0) or partially reduce (β^+) production of normal β-chains, respectively. β-thalassemia severity varies according to the thalassemia mutation, ranging from asymptomatic anemia to severe transfusion-dependent disorder. Populations in different regions have diverse β-globin gene mutations[8] and the ability to identify and characterize these β-thalassemia mutations can assist genetic counseling and prenatal diagnoses.[9] In this study, we established a Hb A_2 dataset for testing β-thalassemia patients of Southwestern China, and we correlated these data to hematological and biochemical values.

MATERIALS AND METHODS

Participants

A total of 15,067 subjects (3,678 men and 11,389 women, 18–45 years-of-age) who sought thalassemia screening at The First Peoples' Hospital of Yunnan Province, during September 2011 to July 2014 were enrolled for capillary electrophoresis. Individuals (n=723) with suspected β-thalassemia (Hb A_2 $\geqslant$3.5%) or abnormal hemoglobin variants were randomly selected from October 2010 to April 2014 (210

men and 513 women, 18–45 years-of-age) for cut-off value calculation. Unrelated β-thalassemia heterozygous patients (n=486) from June 2009 to April 2013 (151 males and 335 females, 19–58 years-of-age), were included in the study to analyze hematological and biochemical parameter values (not the same group used for cut-off value mentioned above).

Capillary electrophoresis

After written and informed consent were obtained from the patients, complete blood counts were measured by an automated cell counter (Sysmex, Tokyo, Japan) for 15,067 subjects. Hemoglobin analysis was performed using capillary electrophoresis (Sebia, Paris, France). Internal quality control was performed by analyzing the control materials provided by the manufacturer. Means comparisons were made among Hb A_2 and Hb A by gender (in three groups: 18–45; 20–29; and 30–39 years-of-age).

Cut-off value calculation

Detection of 17 known β-globin gene mutations (97.3% of known β-thalassemic alleles) in Chinese populations was performed by PCR-reverse dot-blot method as previously described[10] for 723 individuals. Samples without detected mutations were sequenced as previously described.[10] Sensitivity, specificity, Youden's index (YI), likelihood ratio positive (LRP), and likelihood ratio negative (NRP) of Hb A_2 measurements within the interval of 3.5%–4.5% were evaluated. A receiver operating characteristic (ROC) analysis was performed to calculate the area under the curve

(AUC). In addition, we calculated the new cut-off value with the greatest accuracy to identify β-thalassemia in our population. All statistics were computed with SPSS version 16 for Windows.

Hematological and electrophoretic characterization of β-thalassemias

Histograms and tables of descriptive statistics of Hb, MCV, MCH, Hb A and Hb A_2 were generated and comparaed by gender (n >15) for 486 unrelated β-thalassemia heterozygous patients. Also, Hb A, Hb A_2, MCV, MCH and Hb were compared among different genotypes of β-thalassemia (n >10). Continuous variables were compared using independent-sample analysis of variance (ANOVA). For multiple comparisons, a *post hoc* analysis was used when appropriate. All reported *p*-values are two-sided and were statistically significant if $p < 0.05$.

RESULTS

Subjects screened by capillary electrophoresis

The distribution of the whole data of Hb A and Hb A_2 measurements performed in the study was shown in table 1. The mean Hb A among the subjects was 96.83% (95% CI for mean: 96.81%–96.84%) while the mean Hb A_2 was 2.91% (95% CI for mean: 2.90%–2.92%). Of these subjects, 337 cases (2.24%, 337/15,067) had Hb A_2 levels <2.4%, the majority had Hb A_2 levels ranging from 2.4% to 3.5% (95.45%, 14,382/15,067), while 348 cases (2.31%, 348/15,067) had Hb A_2 levels >3.5%. Six subjects without Hb A band and five subjects without Hb A_2 band were found in this

study. Hb F band was only present in 4,381 subjects (29.08%, 4,381/15,067) with the mean 1.17% (95% CI for mean: 1.02%–1.33%), which was not suitable for the description of mean±SD. There was no significant differences in Hb A_2 levels in all three age groups (figure 1). However, Hb A levels was significantly higher in males than females in three age groups (p <0.01) (figure 1).

Cut-off value calculation

Among a total of 723 specimens investigated for β-globin gene mutations, twenty two different mutations were found in 566 cases (78.28%, 566/723) with Hb A_2 levels ranging from 1.8 to 7.9%, including a total of 563 β-thalassemia heterozygotes, one Hb E homozygosity, and two compound heterozygote (table 2). While β-globin mutation was not detected in the remaining 237 subjects. Among β-thalassemia heterozygotes, Hb A_2 values ranged from 1.8% to 4.0% in 69 of 566 subjects (69/566, 12.19%), while the majority (497/566, 87.81%) had Hb A_2 values ⩾4.0%. The sensitivity, specificity, YI, LRP and NRP of each selected cut-off point in screening for β-thalassemia were summarized (table 3). As for hematologic parameters, Hb A_2 at the new cut-off value 4.0% (Hb A_2 ⩾4.0%) yielded high values (0.898, 95% CI: 0.874 to 0.919) of AUC and YI (0.75). The new cut-off with the highest accuracy, the point that sum of sensitivity (85.16%) and specificity (89.81%), was a suitable discriminator indice which can be considered in the screening of β-thalassemia for the studied population, while the old cut-off (Hb A_2 ⩾3.5%) only yielded sensitivity and specificity of 96.64% and 6.37%, respectively.

Hematological and electrophoretic characterization of β-thalassemia mutation

As shown in table 4 and table 5, hematological and molecular characteristics of 486 unrelated heterozygous β-thalassemia mutations were demonstrated, including CD 17 (A>T) (HBB:c.52A>T) (n=125), CD 41-42 (-TCTT) (HBB:c.126_129delCTTT) (n=108), -28 (A>G) (HBB:c.-78A>C) (n=11), IVS-II-654 (C>T) (HBB:c.316-197C>T) (n=51), Hb E (CD 26, G>A) (HBB:c.79G>A) (n=178), CD 27/28 (+C) (HBB:c.84_85insC) (n=5) , CD 71/72 (+A) (HBB:c.216_217insA) (n=5), IVS-I-1 (G>T) (HBB:c.92+1G>T) (n=3). The mean values of hematological and electrophoretic indices (Hb A, Hb A_2, Hb, MCV, MCH) among participants with different genotypes of thalassemia were analysed. Significant difference between gender was observed in Hb (n >15), with males having higher Hb compared to females (p <0.05) (figure 2), whereas no statistical significant differences was found for MCV, MCH, Hb A, and Hb A_2 values between different genders (figure 2 and 3). The Hb E group showed comparatively higher values of hematological indices (Hb, MCV, and MCH) than those of other four genotype of heterozygous β-thalassemia groups (table 5). And -28 (A>G) (HBB:c.-78A>C) had significantly higher Hb A_2 values compared to other β-thalassemia (table 5).

DISCUSSION

β-thalassemia is one of the most common genetic disorders worldwide and each ethnic group has a mutation set of considerable hematologic variations. Clinical

features and hematological presentations of β-thalassemia patients are unique due to race, lifestyle, and altitude. In China, CD 17 (A>T) (HBB:c.52A>T) , CD 41-42 (-TCTT) (HBB:c.126_129delCTTT), -28 (A>G) (HBB:c.-78A>C), IVS-II-654 (C>T) (HBB:c.316-197C>T), Hb E (CD 26, G>A) (HBB:c.79G>A) and CD 71/72 (+A) (HBB:c.216_217insA) mutations accounted for more than 90% of all β-thalassemia mutations in the Chinese population.[11] Characterization and screening of these mutations offers a data basis for prevention and control of thalassemia. However, until now, few studies exist regarding detection and quantification of electrophoretic and hematological parameters for screening thalassemia in Southwestern China. Thus, our report is the first characterization of β-thalassemia mutations as a hematological reference for people in this region.

Women are prone to refer for prenatal diagnosis and genetic counseling in China, so the majority of individuals screened in this study were female. Six subjects without the Hb A band and five subjects without the Hb A_2 band were identified. Among these subjects, four were Hb E homozygous, one was β-thalassemia/Hb E compound heterozygous, and one had hemoglobin variant mutation. Hb A levels was significantly higher in male than female subjects in three age groups (figure 1). However, no significant difference was observed in Hb A_2 in all age groups. In our study, most of the subjects (95.45%, 14,382/15,067) had Hb A_2 within a narrow range (2.4%–3.5%), which agrees with previous observations in Nigeria.[12]

Identification of β-thalassemia carriers is characterized by increased Hb A_2 values. Of the 723 subjects analyzed, the β-globin gene defect was identified in 78.28%

of cases (566/723; range 1.8%–7.9%). One case without the β-globin mutation had the highest Hb A_2 value (7.9%), which may be due to a hemoglobin variant co-eluting with Hb A_2 peak.[13] Few major β-thalassemia (only one case) was found in this study, which was mainly resulted from the fact that children was excluded from this study, and the majority of severe β-thalassemia died before the age of 5.[14]

Hb A_2 greater than 3.5% (Hb A_2 $\geqslant$3.5%) is the diagnostic criterion for the β-thalassemia trait,[15] and values greater than 4.0% (Hb A_2 $\geqslant$4.0%) are also used to identify β-thalassemia carriers in some regions.[16] In this study, the higher cut-off point of Hb A_2 levels 4.0% (Hb A_2 $\geqslant$4.0%) determined by ROC curves displayed more accurate to identify β-thalassemia carriers. This result was somewhat different from previously report, which may be explained by the reason: different population has its own region-wise spectrum and cut-off point of β-thalassemia mutations.[17-18] In our study, most β-thalassemia mutations (497/566, 87.81%) had a Hb A_2 exceeding 4.0% (Hb A_2 $\geqslant$4.0%). And silent β-thalassemia mutations with borderline Hb A_2 values (3.5%–4.0%) were rare in our population.[19] So, the diagnostic thresholds may be increased. Electrophoretic screening criteria for β-thalassemia should be higher than 4.0% (Hb A_2 $\geqslant$4.0%) or individuals with abnormal bands for the population in this study.

Subjects with a β-globin mutation usually had less Hb and erythrocyte indices compared to those without an identifiable mutation.[20] The clinical and hematological parameters in patients of β-thalassemia varied widely.[21] This study was conducted in Kunming, the capital of Yunnan province (elevation: 1,895 meters), and high altitudes

elevate Hb. Males had high Hb values in all five genotype groups compared to females, whereas, no statistical sex-based significant differences were observed for MCV, MCH, Hb A, and Hb A_2 values. These results were similar to previously reported data.[22] Hb E (CD 26, G>A) (HBB:c.79G>A) is clinically asymptomatic with minimal erythrocyte morphological abnormalities in a heterozygote form and this type had the highest MCV, MCH, and Hb values in this study. In Yunnan, Hb E (CD 26, G>A) (HBB:c.79G>A), an abnormal hemoglobin, is the most prevalent β-globin gene mutation (with a frequency of 30.5%)[23] with comparatively lower Hb A_2 values. The mean Hb A_2 of Hb E (%) was 3.87±0.44 in this study (table 5), which was similar to the results of Americans.[24] Subjects with the -28 (A>G) (HBB:c.-78A>C) mutation (β^+-thalassemia), a mutation in the TATA box of the proximal promoter region had higher MCH, MCV, and Hb A_2 values compared to other β-thalassemia types (CD 17 (A>T) (HBB:c.52A>T) , CD 41-42 (-TCTT) (HBB:c.126_129delCTTT), IVS-II-654 (C>T) (HBB:c.316-197C>T)). The -28 (A>G) (HBB:c.-78A>C) phenotype is similar to other β-globin gene promoter mutations.[25]

In conclusion, we offer a retrospective analysis of β-thalassemia that can be used for prenatal diagnosis, genetic counseling, thalassemia control and screening. Characterization of β-thalassemia and diagnostic thresholds for β-thalassemia can be used to identify patients more precisely.

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Contributors JZ and BSZ designed the experiments. JZ, XQM, JH and XHZ performed the experiments. JZ, HC, JS and BSZ analyzed the data. JZ Wrote the paper. All authors critically revised and approved the final manuscript.

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Competing interests None declared.

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Table 1 Distribution of Hb A and Hb A_2 in a total of 15,067 subjects

Parameters	Sex	Age	n	Mean (%)	SD (%)	95% CI for mean
Hb A	Male	18–45	3675	96.95	0.74	96.93%–96.97%
		20–29	1433	96.98	0.59	96.95%–97.01%
		30–39	2045	96.92	0.85	96.88%–96.96%
	Female	18–45	11386	96.79	1.01	96.77%–96.81%
		20–29	6408	96.79	1.02	96.77%–96.82%
		30–39	4574	96.78	1.01	96.75%–96.81%
	total	18–45	15061	96.83	0.96	96.81%–96.84%
Hb A_2	Male	18–45	3676	2.91	0.42	2.90%–2.92%
		20–29	1433	2.90	0.34	2.88%–2.92%
		30–39	2046	2.92	0.44	2.90%–2.94%
	Female	18–45	11386	2.91	0.43	2.90%–2.92%
		20–29	6408	2.90	0.42	2.89%–2.91%
		30–39	4574	2.92	0.45	2.90%–2.93%
	total	18–45	15062	2.91	0.43	2.90%–2.92%

SD: Standard Deviation; CI: confidence interval. Hb F was only detected in 29.08% (4,381/15,067) of the subjects, which was not suitable for the description of mean±SD.

Table 2 Number of β-globin mutations found in this study

Mutation	Type	n	Number of Alleles	Allele Frequency (%)
CD 17 (A>T) (HBB:c.52A>T)	β^0/β^A	166	166	29.04%
CD 41-42 (–TCTT) (HBB:c.126_129delCTTT)	β^0/β^A	145	146	26.26%
CD 26 (G>A) (HBB:c.79G>A)	β^+/β^A	107	110	19.13%
IVS-II-654 (C>T) (HBB:c.316-197C>T)	β^+/β^A	90	92	16.00%
–28 (A>G) (HBB:c.-78A>C)	β^+/β^A	20	20	3.48%
CD 71/72 (+A) (HBB:c.216_217insA)	β^0/β^A	12	12	2.09%
CD 27/28, +C (HBB:c.84_85insC)	β^0/β^A	7	7	1.22%
IVS-I-1 (G>T) (HBB:c.92+1G>T)	β^+/β^A	5	5	0.87%
IVS-I-5 (G>C) (HBB:c.92+5G>C)	β^+/β^A	1	1	0.17%
CD 5 (–CT) (HBB:c.17_18delCT)	β^0/β^A	1	1	0.17%
Hb Dieppe, CD 127 (A>G) (HBB:c.383A>G)	β^0/β^A	1	1	0.17%
Initiation CD (T>C) (HBB:c.2T>C)	β^0/β^A	1	1	0.17%
CD121 (G>T) (HBB:c.364G>T).	β^0/β^A	1	1	0.17%
–31 (A>C) (HBB:c.-81A>G)	β^+/β^A	1	1	0.17%

-29 (A>G) (HBB:c.-79A>G)	β^+/β^A	1	1	0.17%
CD 43 (G>T) (HBB:c.130G>T)	β^0/β^A	1	1	0.17%
CD 113 (T>A) (HBB:c.341T>A)	HbVar	1	1	0.17%
CD 22 (A>C) (HBB:c.68A>C)	HbVar	1	1	0.17%
CD 47 (G>A) (HBB:c.142G>A)	HbVar	1	1	0.17%
CD 41-42/IVS-II-654	β^0/β^0	1	-	-
IVS-II-654/CD 26	β^0/β^+	1	-	-
CD 26/CD 26	β^+/β^+	1	-	-
Total number of alleles	-	566	569	100

β^0: production of β-globin chain is entirely eliminated; β^+: production of β-globin chain is reduced.

Table 3 Predictive value of evaluated indices of the ROC analysis for β-thalassemia

Hb A_2 (%)	A	B	C	D	LRP	NRP	SE	SP	YI
3.5	547	147	19	10	1.03	0.53	96.64	6.37	0.03
3.6	540	85	26	72	1.76	0.10	95.41	45.86	0.41
3.7	531	49	35	108	3.01	0.09	93.82	68.79	0.63
3.8	512	35	54	122	4.06	0.12	90.46	77.71	0.68
3.9	497	25	69	132	5.52	0.14	87.81	84.08	0.72
4.0	482	16	84	141	8.34	0.17	85.16	89.81	0.75
4.1	473	15	93	142	8.78	0.18	83.57	90.45	0.74
4.2	466	13	100	144	9.94	0.19	82.33	91.72	0.74
4.3	456	13	110	144	9.73	0.21	80.57	91.72	0.73
4.4	449	12	117	145	10.38	0.22	79.33	92.36	0.72
4.5	445	12	121	145	10.29	0.23	78.62	92.36	0.71

A: true positive; B: false positive; C: false negative; D: true negative; Likelihood Ratio positive (LRP): sensitivity ÷ (1 - Specificity), Likelihood Ratio Negative (NRP): (1 - Sensitivity)/specificity. Youden's index (YI), Sensitivity (SE): True positive ÷ (true positive + false negative), Specificity (SP): True negative ÷ (true negative + false positive).

Table 4 Characterization of 7 types of β-thalassemia according to sex (mean±SD)

Mutation	Sex (n)	Hb (g/L)	MCV (fl)	MCH (pg)	Hb A (%)	Hb A_2 (%)
CD 17	F (87)	110.30±16.12	64.86±7.96	21.54±3.01	91.80±2.43	5.83±0.52
CD 17	M (38)	136.47±11.55	64.62±4.00	21.46±1.66	92.74±1.55	6.11±0.54
CD 41-42	F (89)	108.71±15.50	65.39±5.76	21.23±1.96	92.04±3.04	5.66±0.57
CD 41-42	M (35)	137.57±11.71	66.82±5.85	22.56±3.74	92.87±2.68	5.87±0.65
CD 26	F (124)	125.18±11.90	77.09±4.74	26.01±1.63	70.64±2.24	3.88±0.45
CD 26	M (54)	151.11±11.07	76.76±4.01	26.18±1.33	70.74±2.37	3.86±0.43
IVS-II-654	F (15)	107.47±14.23	65.35±4.21	21.26±1.13	92.85±1.74	5.53±0.42
IVS-II-654	M (36)	138.40±8.65	63.52±2.90	20.97±0.88	93.57±1.19	5.59±0.53
-28	F (10)	123.5±15.25	72.99±5.00	23.41±1.09	92.49±0.72	6.15±0.44
-28	M (1)*	-	-	-	-	-
CD 27-28	F (5)	106.00±18.61	68.54±2.26	22.22±0.59	88.56±5.09	5.28±0.56
CD 27-28	M (0)*	-	-	-	-	-
CD 71-72	F (4)	105.50±5.07	63.15±2.69	21.45±0.50	92.60±0.50	5.80±0.14
CD 71-72	M (1)*	-	-	-	-	-

*: mean and standard deviation (SD) can not be calculated (n ＜3).

Table 5 Characterization of 8 types of β-thalassemia and comparasion (mean±SD)

Mutation (n)	Hb (g/L)	MCV (fl)	MCH (pg)	Hb A (%)	Hb A_2 (%)
CD 17[1] (125)	118.26±19.14	64.78±6.98	21.52±2.67	92.08±2.24	5.91±0.54
CD 41-42[2] (108)	117.78±19.54	65.84±5.29	21.68±2.56	92.38±2.93	5.75±0.60
CD 26[3] (178)	133.04±16.67	76.99±4.52	26.06±1.55	70.67±2.28	3.87±0.44
IVS-II-654[4] (51)	116.57±19.11	64.81±3.93	21.17±1.06	93.06±1.62	5.55±0.45
-28[5] (11)	125.64±16.11	73.45±4.97	23.61±1.23	92.49±0.68	6.22±0.47
CD 27-28[6] (5)	106.00±18.61	68.54±2.26	22.22±0.59	88.56±5.09	5.28±0.56
CD 71-72[7] (5)	108.80±8.58	64.64±2.60	21.14±0.82	92.76±0.56	5.92±0.29
IVS-I-1[8] (3)	127.00±13.23	72.40±15.98	25.07±6.51	94.20±0.66	5.73±0.57
Comparisons	1 versus 3	1 versus 3	1 versus 3	1 versus 3	1 versus 2
(p <0.05)	2 versus 3	1 versus 5	1 versus 5	1 versus 4	1 versus 3
	3 versus 4	2 versus 3	2 versus 3	2 versus 3	1 versus 4
		2 versus 5	2 versus 5	3 versus 4	2 versus 3
		3 versus 4	3 versus 4	3 versus 5	2 versus 4
		3 versus 5	3 versus 5		2 versus 5
		4 versus 5	4 versus 5		3 versus 4
					3 versus 5
					4 versus 5

P value of signicant differences (p <0.05) between various 5 types of β-thalassemia is listed (n >10). Non-listed comparisons are not significant.

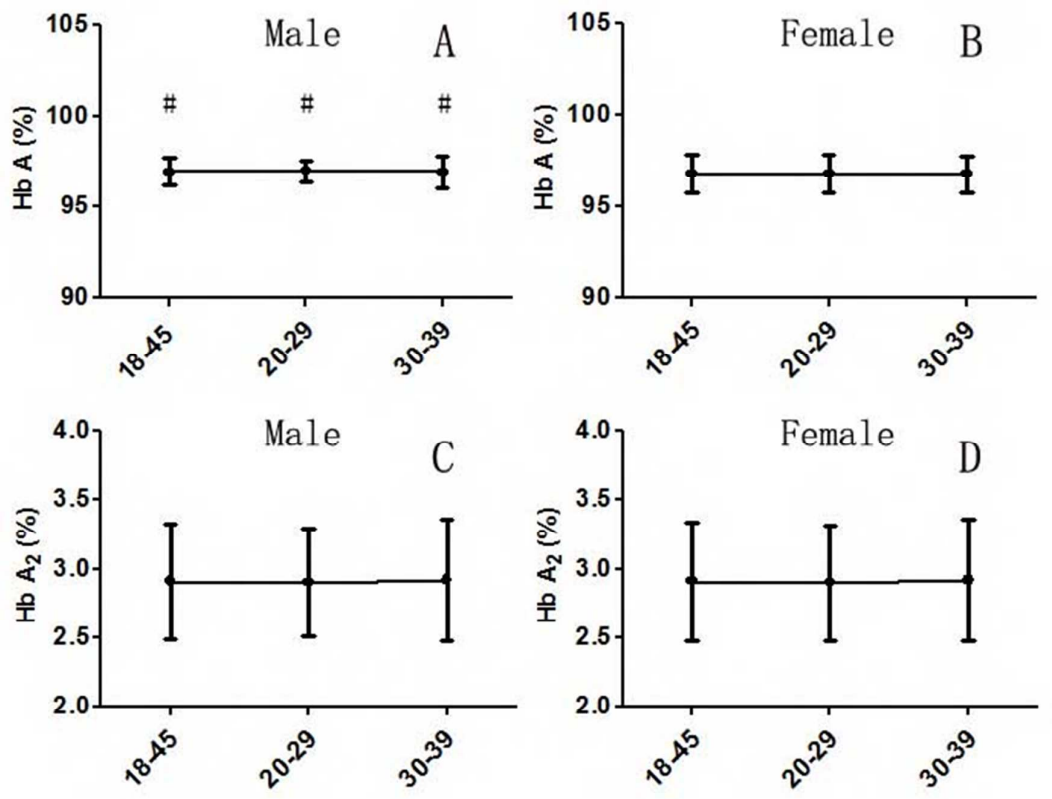

Figure 1 Distribution of Hb A and Hb A_2 in all sample. # Males show significantly higher Hb A values (p <0.05) than females in three age group. There was no significant differences in Hb A_2 levels in all three age groups.

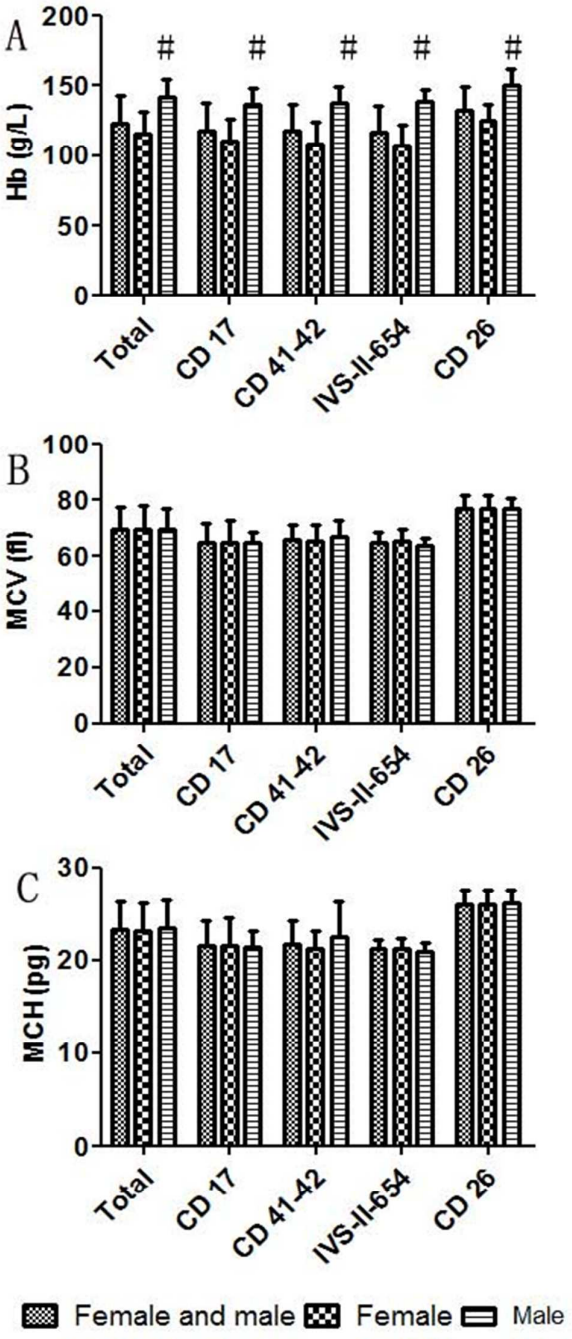

Figure 2 Hematological characterization of β-thalassemia according to sex. # Males show significantly higher Hb values (p <0.05) than females. No difference were observed between gender for MCV and MCH. Total: 486 unrelated heterozygous β-thalassemia.

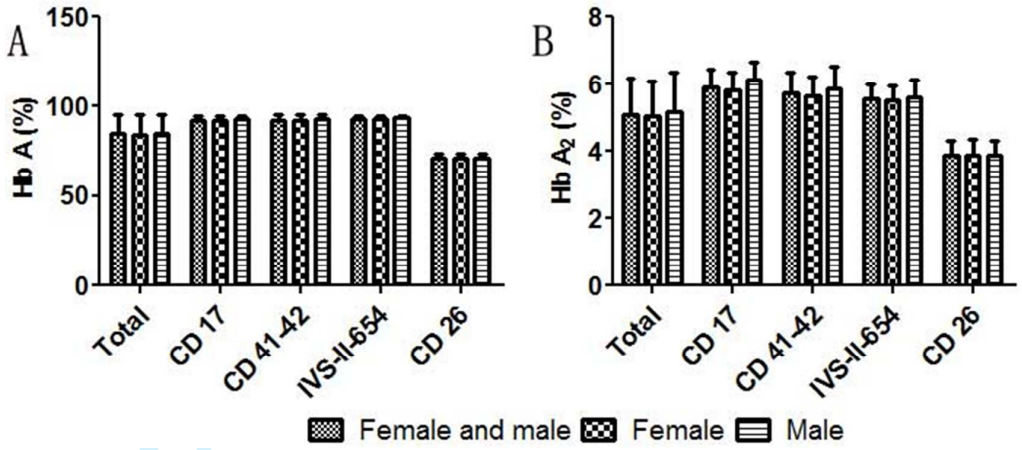

Figure 3 Electrophoretic characterization of β-thalassemia according to sex in the current study. No difference were observed between gender for Hb A and Hb A_2. Total: 486 unrelated heterozygous β-thalassemia.

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Hematological and electrophoretic characterization of β-thalassemia in Yunnan province of Southwestern China

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Hematological and electrophoretic characterization of β-thalassemia in Yunnan province of Southwestern China

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ABSTRACT

Objectives: β-thalassemia is widely found in Southwestern China. Characterization of β-thalassemia can improve screening and prenatal diagnosis for at-risk populations.

Design: A retrospective study.

Methods: In this study, the levels of HbA_2 and HbA were analyzed by gender for a total of 15,067 subjects screened by capillary electrophoresis. The cut-off value with the highest accuracy was established to identify β-thalassemia in 723 patients suspected to have this disease. Hematological and electrophoretic characterization of 8 common types of β-thalassemia were analyzed in 486 β-thalassemia subjects.

Results: HbA levels were significantly higher in males than in females, but there was no significant difference on HbA_2 levels. A new cut-off value for the diagnosis of β-thalassemia (HbA_2 ≥4.0%) with the highest accuracy was proposed for the studied populations. Hb was significantly higher in males compared to females (p <0.05), whereas no statistically significant differences were found for MCV, MCH, HbA, and

HbA_2. The HbE group showed comparatively higher values for hematological indices (Hb, MCV, and MCH) than the other genotypes in heterozygous β-thalassemia groups (p <0.05), and -28 (A>G) (HBB:c.-78A>C) had significantly higher HbA_2 values compared to other β-thalassemia.

Conclusions: Ethnic groups have diversified β-globin gene mutations and considerable hematologic variations. Our study will lay the foundation for screening programs and clinical management of thalassemia in Southwestern China.

Strengths and limitations of this study

- Ours is the first study to characterize β-thalassemia in the Yunnan province, which may be useful to improve screening and prenatal diagnosis in Southwestern China.
- Our study determined that a higher cut-off point of HbA_2 levels (HbA_2 ≥4.0%) should be used for β-thalassemia screening, rather the current value of 3.5%.
- Samples for participants used for capillary electrophoresis, cut-off value calculation and characterization of β-thalassemia were not collected at the same time.

INTRODUCTION

β-thalassemia is an inherited anemia resulting from genome variants in β-globin chains. This disease is most prevalent in Africa, [1] Asia, [2] Mediterranean[3] and the

Middle-East.[4] Quantification of hemoglobin A_2 (HbA_2) percentage by capillary electrophoresis and routine hematology testing are the existing methods for screening thalassemia and hemoglobinopathies.[5] An HbA_2 value exceeding 3.5% combined with a low mean cell volume (MCV) and mean cell hemoglobin (MCH) are the current diagnostic criteria for β-thalassemia carriers. Determination of the hemoglobin concentration (Hb) and HbA_2 range in a population offers a critical screening tool for thalassemia. [6-7] However, there are very few reports on the clinical reference intervals for β-thalassemia patients in China. Therefore, it is important to determine appropriate diagnostic cut-off points and supplement the existing references to improve the screening and control of β-thalassemia.

More than 800 different β-globin gene mutations have been discovered worldwide. Two subtypes are defined by totally absent (β^0) or partially reduced (β^+) production of normal β-chains, respectively. The severity of β-thalassemia varies with the thalassemia mutation, ranging from asymptomatic anemia to a severe transfusion-dependent disorder. Populations in different regions have diversified β-globin gene mutations[8] and the ability to identify and characterize these β-thalassemia mutations can assist in genetic counseling and prenatal diagnoses. [9] In this study, we established an HbA_2 dataset for screening β-thalassemia in people of Southwestern China, and correlated these data to hematological and biochemical values to better understand

thalassemia and to improve its control and prevention.

MATERIALS AND METHODS

Study design

In this study, we performed a retrospective analysis using capillary electrophoresis to determine the HbA_2 dataset, calculated the diagnostic criteria for β-thalassemia and analyzed hematologic parameters to provide an additional characterization of this disease. There were three independent studies conducted during different time periods. The study design is shown in Figure 1.

Setting

This study was conducted in The First Peoples' Hospital of Yunnan Province. This hospital is located in the Jinbi road of Kunming city. It is a general hospital whose annual clinic amount exceeds 1.6 million people with 2000 beds.

Participants

Participants for three independent studies were collected during three different time periods. (1) A total of 15,067 subjects (3,678 men and 11,389 women, 18–45 years-of-age) who sought thalassemia screening during September 2011 to July 2014 had

capillary electrophoresis performed. (2) Individuals (n=723) with suspected β-thalassemia (HbA_2 ≥3.5%) or abnormal hemoglobin variants were randomly selected from October 2010 to April 2014 (210 men and 513 women, 18–45 years-of-age) for cut-off value calculation. (3) Unrelated heterozygous β-thalassemia patients (n=486: 151 males and 335 females, 19–58 years-of-age), were included in the study to analyze hematological and biochemical parameters between June 2009 to April 2013. Written and informed consent were obtained from the patients. The information consent and protocol for these studies were approved by the medical ethics committee of The First Peoples' Hospital of Yunnan Province, PRC. The exclusion criteria were: (1) incomplete information, (2) consanguinity, (3) lack of informed consent, and (4) children (below the age of 18 years).

Capillary electrophoresis

Venous blood samples were collected from subjects (n=15,067) in tubes containing EDTA. Hemoglobin analysis was performed using capillary electrophoresis (Sebia, Paris, France). Internal quality control was performed by analyzing the control materials provided by the manufacturer. Mean comparisons were made among HbA_2 and HbA by gender, comparing male vs. female in each of three age groups: 18–45, 20–29, and 30–39 years using independent-sample analysis of variance (ANOVA).

DNA extraction and detection of β-globin mutations

DNA was extracted from whole blood using the Blood DNA Extraction System (Tianlong Bioscience Shenzhen Ltd., Xian, China). Detection of 17 known β-globin gene mutations (97.3% of known β-thalassemia alleles in Chinese populations) for 723 individuals with suspected β-thalassemia or abnormal hemoglobin variants were performed by PCR-reverse dot-blot method as previously described.[10] These 17 β-globin mutations are as follows: CD 41-42 (–TCTT), IVS-II-654 (C>T), -28 (A>G), CD 71/72 (+A), CD 17 (A>T), HbE (CD 26, G>A), CD 31 (–C), CD 27/28 (+C), CD 43 (G>T), -32 (C>A), -29 (A>G), 30 (T>C), CD 14/15 (+G), Cap +40 to +43 (–AAAC), Initiation CD (T>G), IVS-I-1 (G>T) and IVS-I-5 (G>T). Samples without detected mutations were sequenced on an ABI 3700 automated sequencer using primers that flanked the entire β-globin gene, as previously described.[10]

Cut-off value calculation

Sensitivity, specificity, Youden's index (YI), likelihood ratio positive (LRP), and likelihood ratio negative (NRP) of HbA_2 measurements within the interval of 3.5%–4.5% were evaluated. A receiver operating characteristic (ROC) analysis was performed to calculate the area under the curve (AUC). In addition, we calculated the

cut-off value with the greatest accuracy to identify β-thalassemia in our population.

Characterization of β-thalassemias

Complete blood counts were performed on 486 unrelated β-thalassemia heterozygous patients that were diagnosed in our center using an automated cell counter (Sysmex, Tokyo, Japan). Histograms and tables of descriptive statistics of Hb, MCV, MCH, HbA and HbA_2 were generated and compared by gender (n >15). Also, HbA, HbA_2, MCV, MCH and Hb were compared among different genotypes of β-thalassemia (n >10). Continuous variables were compared using ANOVA. For multiple comparisons, a *post hoc* analysis was used when appropriate. All reported *P*-values are two-sided and were statistically significant if $p < 0.05$. All statistics in this study were computed with SPSS version 16 for Windows.

RESULTS

Subjects screened by capillary electrophoresis

The data distribution of HbA and HbA_2 measurements performed in the study is shown in Figure S1 and Table S1. The mean HbA among the subjects was 96.83% (95% CI for mean: 96.81%–96.84%), while the mean HbA_2 was 2.91% (95% CI for mean: 2.90%–2.92%). Out of these subjects, the majority had HbA_2 levels ranging

from 2.4 to 3.5% (95.45%, 14,382/15,067, while 337 cases (2.24%, 337/15,067) had HbA_2 levels <2.4%, and 348 cases (2.31%, 348/15,067) had HbA_2 levels >3.5%. Six subjects lacking an HbA band and five subjects without an HbA_2 band were identified. The HbF band was present in 4,381 subjects (29.08%, 4,381/15,067) with a mean percentage of 1.17% (95% CI for mean: 1.02%–1.33%). There were no significant differences in HbA_2 levels between the three age groups (18-45, 20-29 and 30-39) (Figure S1). However, HbA levels were significantly higher in males than in females in all three age groups (18-45, 20-29 and 30-39) (p <0.01) (Figure S1).

Cut-off value calculation

Among the total 723 specimens investigated for β-globin gene mutations, twenty-two different mutations were found in 566 cases (78.28%, 566/723), with HbA_2 levels ranging from 1.8 to 7.9%. These included a total of 563 β-thalassemia heterozygotes, one HbE homozygosity, and two compound heterozygotes (Table 1). β-globin mutations were not detected in the remaining 237 subjects. Among β-thalassemia heterozygotes, HbA_2 values ranged from 1.8% to 4.0% in 69 of 566 subjects (69/566, 12.19%), while the majority (497/566, 87.81%) had HbA_2 values ≥4.0%. The sensitivity, specificity, YI, LRP and NRP of each selected cut-off point in screening for β-thalassemia are summarized in Table 2. Regarding hematologic parameters,

HbA_2 at the new cut-off value of 4.0% yielded high values (0.898, 95% CI: 0.874 to 0.919) for AUC and YI (0.75) (Figure S2). The new cut-off had the highest accuracy, with a sensitivity of 85.16% and a specificity of 89.81%, and is therefore a suitable discriminator for screening of β-thalassemia in this population. Using the currently established cut-off (HbA_2 ≥3.5%) only yielded sensitivity and specificity values of 96.64% and 6.37%, respectively.

Hematological and electrophoretic characterization of β-thalassemia mutation

As shown in Tables 3 and S2, hematological and molecular characteristics of 486 unrelated heterozygous β-thalassemia mutations were demonstrated. The mean values for hematological and electrophoretic indices (HbA, HbA_2, Hb, MCV, and MCH) among participants with different genotypes of thalassemia are shown in Table 3. A significant difference between genders was observed for Hb, with males having higher Hb compared to the females (p <0.05), whereas no statistically significant differences were found for MCV, MCH, HbA, and HbA_2 values between the two genders (Figures 2 and 3). The HbE group showed comparatively higher values of hematological indices (Hb, MCV, and MCH) than the other four genotypes of heterozygous β-thalassemia groups, and -28 (A>G) (HBB:c.-78A>C) had significantly higher HbA_2 values compared to other β-thalassemias (Table 3).

DISCUSSION

β-thalassemia is one of the most common genetic disorders worldwide, and each ethnic group has a mutation set with considerable hematologic variations. Clinical features and hematological parameters in β-thalassemia patients vary with race, lifestyle, and altitude. In China, CD 17 (A>T) (HBB:c.52A>T), CD 41-42 (-TCTT) (HBB:c.126_129delCTTT), -28 (A>G) (HBB:c.-78A>C), IVS-II-654 (C>T) (HBB:c.316-197C>T), HbE (CD 26, G>A) (HBB:c.79G>A) and CD 71/72 (+A) (HBB:c.216_217insA) mutations account for more than 90% of all β-thalassemia mutations in the Chinese population.[11] Characterization and screening of these mutations provides a database to aid in the prevention and control of thalassemia. There are very few studies describing the detection and quantification of electrophoretic and hematological parameters for screening thalassemia in the Chinese population. Thus, our report is the first characterization of β-thalassemia mutations in people of Southwestern China and will serve as a key reference for this population.

The majority of individuals screened in this study were female, since patients seeking prenatal diagnosis and genetic counseling represent a large portion of the study participants. Six subjects lacking an HbA band and five subjects without an

HbA_2 band were identified. Among these subjects, four were HbE homozygous, one was β-thalassemia/HbE compound heterozygous, and one had a hemoglobin variant mutation. HbA levels were significantly higher in male than female subjects in the three age groups (Figure S1). However, no significant difference was observed in HbA_2 in all age groups. In our study, most of the subjects (95.45%, 14,382/15,067) had HbA_2 levels that were within a narrow range (2.4%–3.5%), similar to previous observations in Nigerian patients.[12]

Carriers of β-thalassemia have increased HbA_2 values. Of the 723 subjects analyzed, the β-globin gene defect was identified in 78.28% of cases (566/723; range 1.8%–7.9%). One case without the β-globin mutation had the highest HbA_2 value (7.9%), which may be due to a hemoglobin variant co-eluting with HbA_2 peak.[13] Only one case of major β-thalassemia was found in this study, which was due to the fact that children were excluded. The majority of severe β-thalassemia carriers die before reaching the age of 5.[14]

Having an HbA_2 level greater than 3.5% is the current diagnostic criterion for the β-thalassemia trait,[15] while values greater than 4.0% are also used to identify β-thalassemia carriers in some regions.[16] In this study, a higher cut-off point of HbA_2 levels (HbA_2 ≥4.0%) determined by ROC curves had the highest accuracy in identifying β-thalassemia carriers. This result differed from previous reports,[17-19]

which may be explained by regional differences in the spectrum and cut-off values for β-thalassemia mutations. In our study, most β-thalassemia mutations (497/566, 87.81%) had a mean HbA_2 exceeding 4.0%, while silent β-thalassemia mutations with near normal hematological indices and borderline HbA_2 values (3.5%–4.0%) were rare in our population. Therefore, the diagnostic thresholds should be increased for this population.

The cut-off point was established for all samples, including those from β-thalassemia patients and those having abnormal hemoglobins. And Hb variant fraction could be easily screened by abnormal band for capillary electrophoresis.[20] In Yunnan, HbE (CD 26, G>A) (HBB:c.79G>A), an abnormal hemoglobin, is the most prevalent β-globin gene mutation (with a frequency of 30.5%),[21] with comparatively lower HbA_2 values. Here, the mean percentage of HbA_2 in patients with HbE was 3.87±0.44, which was similar to that reported for Americans.[22] HbE and other abnormal hemoglobins, such as CD 22 (A>C) (HBB:c.68A>C) (HbG-Coushatta), have been reported to decrease the expression of HbA_2.[23] In this study, most β-thalassemia were characterized by increased HbA_2 values ranging from 4.3% to 6.6% (93.86%, 428/456).

Subjects with a β-globin mutation usually have lower Hb and erythrocyte indices compared to those without an identifiable mutation[24] and the clinical and

hematological parameters in patients having β-thalassemia are widely variable.[25] This study was conducted in Kunming, the capital of Yunnan province, which has an altitude of 1,895 meters. Regional altitude differences are a contributing factor in the variation of these parameters, as high altitudes are known to elevate Hb values. Males had higher Hb values in all five genotype groups compared to females, whereas, no statistical sex-based significant differences were observed for MCV, MCH, HbA, and HbA_2 values. These results were similar to previously reported data.[26] HbE (CD 26, G>A) (HBB:c.79G>A) is clinically asymptomatic with minimal erythrocyte morphological abnormalities in heterozygous individuals; this type had the highest MCV, MCH, and Hb values in this study. Subjects with the -28 (A>G) (HBB:c.-78A>C) mutation (β^+-thalassemia), a mutation in the TATA box of the proximal promoter region, had higher MCH, MCV, and HbA_2 values compared to other β-thalassemia types (CD 17 (A>T) (HBB:c.52A>T), CD 41-42 (-TCTT) (HBB:c.126_129delCTTT), IVS-II-654 (C>T) (HBB:c.316-197C>T)). The -28 (A>G) (HBB:c.-78A>C) phenotype is similar to other β-globin gene promoter mutations.[27]

In conclusion, we offered a retrospective analysis of β-thalassemia that can be used for prenatal diagnosis, genetic counseling, thalassemia control and screening. Characterization of β-thalassemia and diagnostic thresholds for β-thalassemia can be used to identify patients more precisely.

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Contributors JZ and BSZ designed the experiments. JZ, XQM, JH and XHZ performed the experiments. JZ, HC, JS and BSZ analyzed the data. JZ Wrote the paper. All authors critically revised and approved the final manuscript.

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Competing interests None declared.

Ethics approval The protocol and information consent for this study were approved by the medical ethics committee of The First Peoples' Hospital of Yunnan Province in

accordance with the Declaration of Helsinki.

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Table 1 Number of β-globin mutations found in this study.

Mutation	Type	n	Number of Alleles	Allele Frequency (%)
CD 17 (A>T) (HBB:c.52A>T)	β^0/β^A	166	166	29.04%
CD 41-42 (–TCTT) (HBB:c.126_129delCTTT)	β^0/β^A	145	146	26.26%
CD 26 (G>A) (HBB:c.79G>A)	β^+/β^A	107	110	19.13%
IVS-II-654 (C>T) (HBB:c.316-197C>T)	β^+/β^A	90	92	16.00%
–28 (A>G) (HBB:c.-78A>C)	β^+/β^A	20	20	3.48%
CD 71/72 (+A) (HBB:c.216_217insA)	β^0/β^A	12	12	2.09%
CD 27/28, +C (HBB:c.84_85insC)	β^0/β^A	7	7	1.22%
IVS-I-1 (G>T) (HBB:c.92+1G>T)	β^+/β^A	5	5	0.87%
IVS-I-5 (G>C) (HBB:c.92+5G>C)	β^+/β^A	1	1	0.17%
CD 5 (–CT) (HBB:c.17_18delCT)	β^0/β^A	1	1	0.17%
Hb Dieppe, CD 127 (A>G) (HBB:c.383A>G)	β^0/β^A	1	1	0.17%
Initiation CD (T>C) (HBB:c.2T>C)	β^0/β^A	1	1	0.17%
CD121 (G>T) (HBB:c.364G>T).	β^0/β^A	1	1	0.17%
–31 (A>C) (HBB:c.-81A>G)	β^+/β^A	1	1	0.17%
-29 (A>G) (HBB:c.-79A>G)	β^+/β^A	1	1	0.17%
CD 43 (G>T) (HBB:c.130G>T)	β^0/β^A	1	1	0.17%

CD 113 (T>A) (HBB:c.341T>A)	HbVar	1	1	0.17%
CD 22 (A>C) (HBB:c.68A>C)	HbVar	1	1	0.17%
CD 47 (G>A) (HBB:c.142G>A)	HbVar	1	1	0.17%
CD 41-42/IVS-II-654	β^0/β^0	1	-	-
IVS-II-654/CD 26	β^0/β^+	1	-	-
CD 26/CD 26	β^+/β^+	1	-	-
Total number of alleles	-	566	569	100

β^0: production of β-globin chain is entirely eliminated; β^+: production of β-globin chain is reduced; HbVar: hemoglobin variant.

Table 2 Predictive value of evaluated indices of the ROC analysis for β-thalassemia.

HbA_2 (%)	TP	FN	FP	TN	LRP	NRP	Sn	Sp	YI
3.5	547	147	19	10	1.03	0.53	96.64	6.37	0.03
3.6	540	85	26	72	1.76	0.10	95.41	45.86	0.41
3.7	531	49	35	108	3.01	0.09	93.82	68.79	0.63
3.8	512	35	54	122	4.06	0.12	90.46	77.71	0.68
3.9	497	25	69	132	5.52	0.14	87.81	84.08	0.72
4.0	**482**	**16**	**84**	**141**	**8.34**	**0.17**	**85.16**	**89.81**	**0.75**
4.1	473	15	93	142	8.78	0.18	83.57	90.45	0.74
4.2	466	13	100	144	9.94	0.19	82.33	91.72	0.74
4.3	456	13	110	144	9.73	0.21	80.57	91.72	0.73
4.4	449	12	117	145	10.38	0.22	79.33	92.36	0.72
4.5	445	12	121	145	10.29	0.23	78.62	92.36	0.71

TP: true positive; FP: false positive; FN: false negative; TN: true negative; Likelihood Ratio positive (LRP): sensitivity ÷ (1 - Specificity), Likelihood Ratio Negative (NRP): (1 - Sensitivity)/specificity. Youden's index (YI), Sensitivity (Sn): True positive ÷ (true positive + false negative), Specificity (Sp): True negative ÷ (true negative + false positive).

Table 3 Characterization of 8 types of β-thalassemia and comparison (mean ± SD)

Mutation (*n*)	Hb (g/L)	MCV (fl)	MCH (pg)	HbA (%)	HbA_2 (%)
CD 17[1] (125)	118.26±19.14	64.78±6.98	21.52±2.67	92.08±2.24	5.91±0.54
CD 41-42[2] (108)	117.78±19.54	65.84±5.29	21.68±2.56	92.38±2.93	5.75±0.60
CD 26[3] (178)	133.04±16.67	76.99±4.52	26.06±1.55	70.67±2.28	3.87±0.44
IVS-II-654[4] (51)	116.57±19.11	64.81±3.93	21.17±1.06	93.06±1.62	5.55±0.45
-28[5] (11)	125.64±16.11	73.45±4.97	23.61±1.23	92.49±0.68	6.22±0.47
CD 27-28[6] (5)	106.00±18.61	68.54±2.26	22.22±0.59	88.56±5.09	5.28±0.56
CD 71-72[7] (5)	108.80±8.58	64.64±2.60	21.14±0.82	92.76±0.56	5.92±0.29
IVS-I-1[8] (3)	127.00±13.23	72.40±15.98	25.07±6.51	94.20±0.66	5.73±0.57
Comparisons	1 versus 3	1 versus 3	1 versus 3	1 versus 3	1 versus 2
(p <0.05)	2 versus 3	1 versus 5	1 versus 5	1 versus 4	1 versus 3
	3 versus 4	2 versus 3	2 versus 3	2 versus 3	1 versus 4
		2 versus 5	2 versus 5	3 versus 4	2 versus 3
		3 versus 4	3 versus 4	3 versus 5	2 versus 4
		3 versus 5	3 versus 5		2 versus 5
		4 versus 5	4 versus 5		3 versus 4
					3 versus 5
					4 versus 5

P value of significant differences (p <0.05) between various 5 types of β-thalassemia is listed (n >10). Non-listed comparisons are not significant.

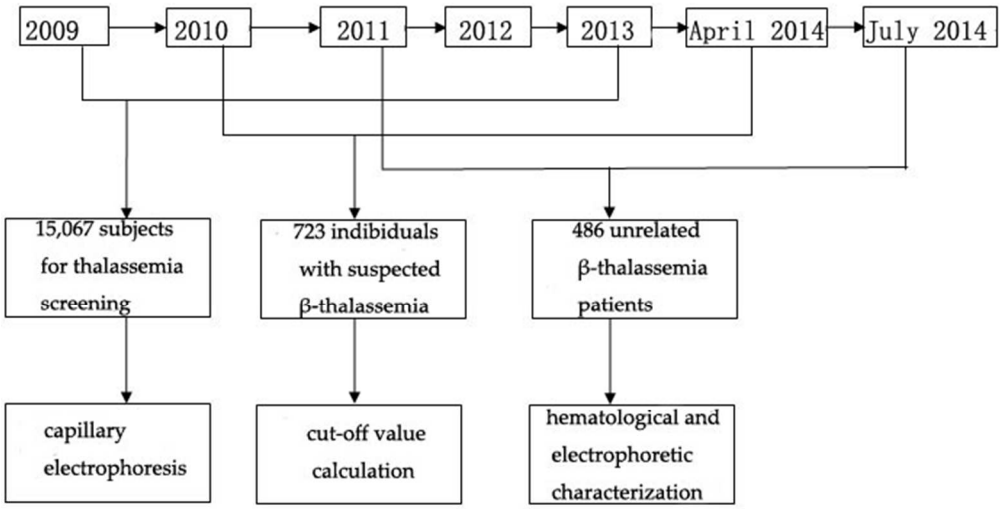

Figure 1 Flow diagram and participant numbers in this study. Capillary electrophoresis, cut-off value calculation, hematological and electrophoretic characterization of β-thalassemia mutation were three independent studies conducted during different time periods.

182x94mm (96 x 96 DPI)

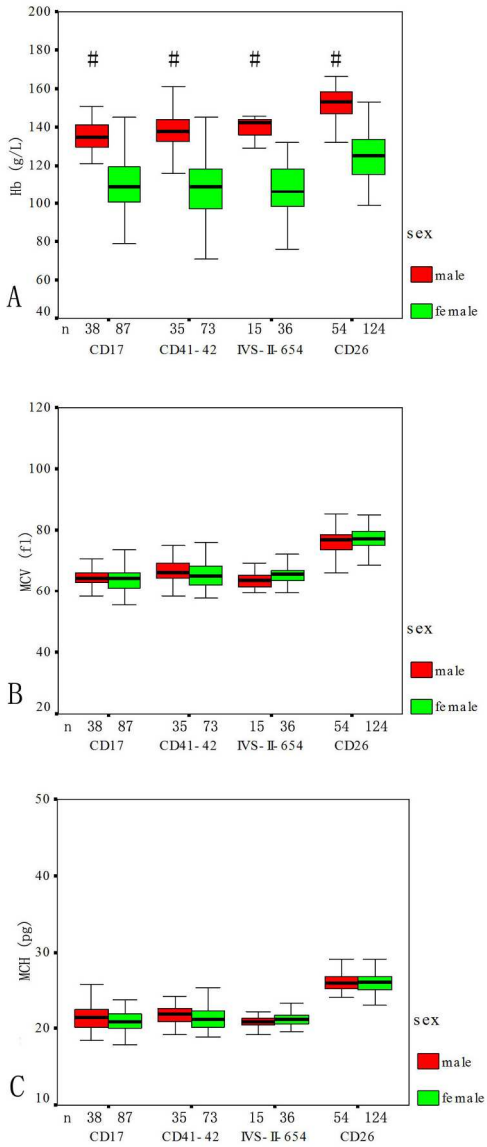

Figure 2 Hematological characterization of β-thalassemia according to sex. # Males have significantly higher Hb values ($P < 0.05$) than females. No differences were observed between genders for MCV and MCH. (n=486 total unrelated heterozygous β-thalassemia cases).

280x659mm (96 x 96 DPI)

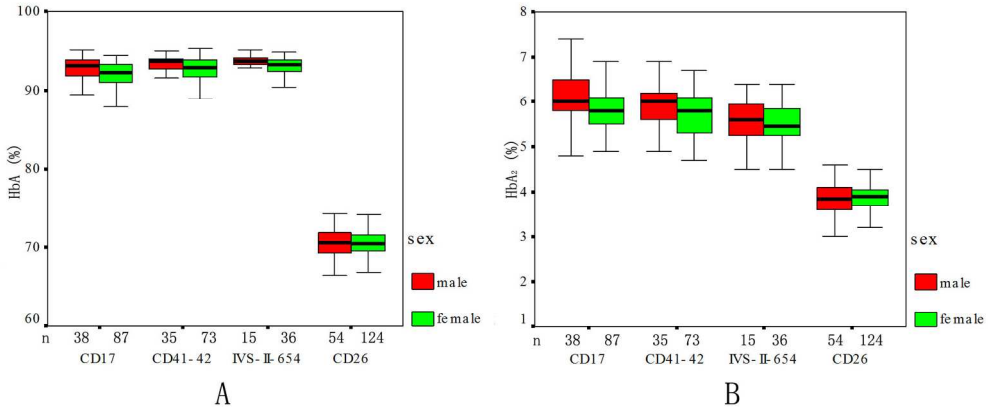

Figure 3 Electrophoretic characterization of β-thalassemia according to sex in the current study. No differences were observed between genders for Hb A and Hb A2. (n= 486 total unrelated heterozygous β-thalassemia).

532x227mm (96 x 96 DPI)

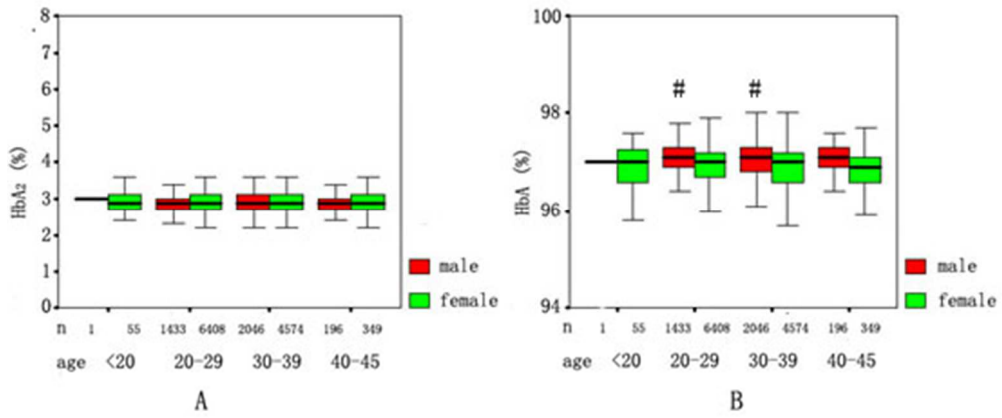

Figure S1 Distribution of HbA2 (A) and HbA (B) in all samples. The data distribution of HbA and HbA2 were measured according to age. # Males show significantly higher Hb A values ($p < 0.05$) than females in three age group (18–45, 20–29, and 30–39 years). There was no significant differences in Hb A2 levels in all three age groups. The sample size of the other two age categories (<20, 40-45) were small, which was not suitable to compare.

195x83mm (72 x 72 DPI)

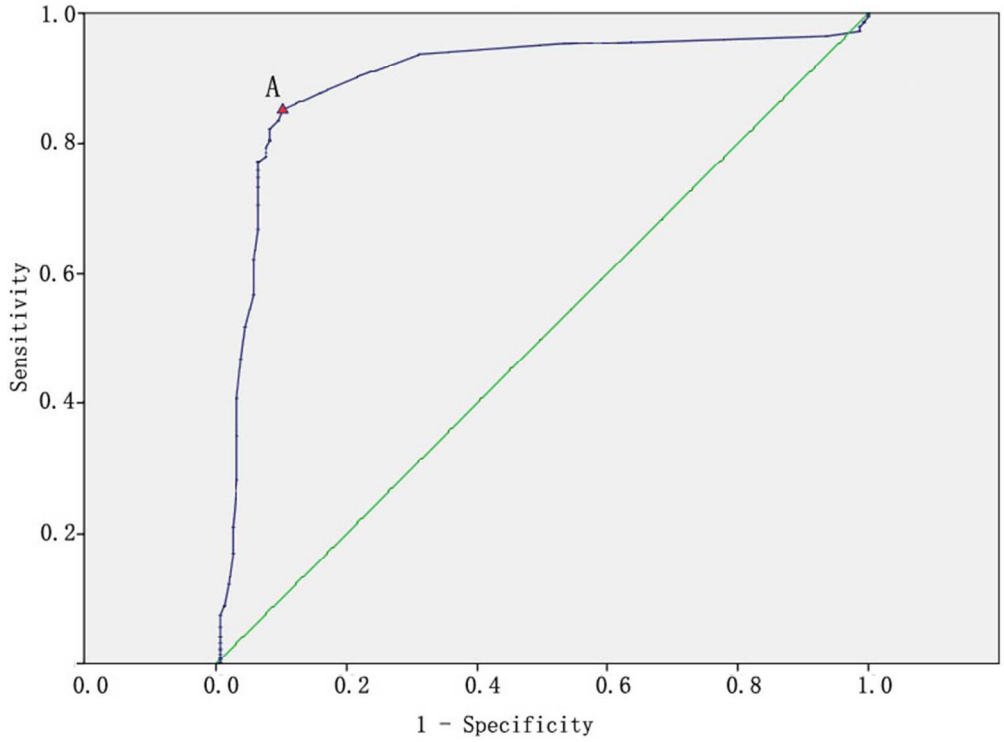

Figure S2 Receiver operative characteristic curves (ROC) of the cut-off point calculation. A: cut-off value at HbA2 4.0%. HbA2 at the new cut-off value of 4.0% yielded high values (0.898, 95% CI: 0.874 to 0.919) for AUC and YI (0.75).

282x222mm (72 x 72 DPI)

Table S1 Distribution of HbA and HbA_2 in all samples

Parameters	Sex	Age	n	Mean (%)	SD (%)	95% CI for mean
HbA	Male	18–45	3675	96.95	0.74	96.93%–96.97%
		<20	1	-	-	-
		20–29	1433	96.98	0.59	96.95%–97.01%
		30–39	2045	96.92	0.85	96.88%–96.96%
		40-45	196	97.02	0.50	96.95%–97.09%
	Female	18–45	11386	96.79	1.01	96.77%–96.81%
		<20	55	96.92	0.55	96.77-97.07%
		20–29	6408	96.79	1.02	96.77%–96.82%
		30–39	4574	96.78	1.01	96.75%–96.81%
		40-45	349	96.71	0.95	96.61%–96.84%
	total	18–45	15061	96.83	0.96	96.81%–96.84%
HbA_2	Male	18–45	3676	2.91	0.42	2.90%–2.92%
		<20	1	-	-	-
		20–29	1433	2.90	0.34	2.88%–2.92%
		30–39	2046	2.92	0.44	2.90%–2.94%
		40-45	196	2.89	0.38	2.84%–2.95%
	Female	18–45	11386	2.91	0.43	2.90%–2.92%
		<20	55	2.91	0.42	2.89-2.91%
		20–29	6408	2.90	0.42	2.89%–2.91%
		30–39	4574	2.92	0.45	2.90%–2.93%
		40-45	349	2.95	0.47	2.90%–2.96%
	total	18–45	15062	2.91	0.43	2.90%–2.92%

SD: Standard Deviation; CI: confidence interval. Hb F was only detected in 29.08%

(4,381/15,067) of the subjects, which was not suitable for the description of mean±SD.

Table S2 Characterization of 7 types of β-thalassemia according to sex (mean ± SD)

Mutation	Sex (*n*)	Hb (g/L)	MCV (fl)	MCH (pg)	HbA (%)	HbA_2 (%)
CD 17	F (87)	110.30±16.12	64.86±7.96	21.54±3.01	91.80±2.43	5.83±0.52
CD 17	M (38)	136.47±11.55	64.62±4.00	21.46±1.66	92.74±1.55	6.11±0.54
CD 41-42	F (73)	108.71±15.50	65.39±5.76	21.23±1.96	92.04±3.04	5.66±0.57
CD 41-42	M (35)	137.57±11.71	66.82±5.85	22.56±3.74	92.87±2.68	5.87±0.65
CD 26	F (124)	125.18±11.90	77.09±4.74	26.01±1.63	70.64±2.24	3.88±0.45
CD 26	M (54)	151.11±11.07	76.76±4.01	26.18±1.33	70.74±2.37	3.86±0.43
IVS-II-654	F (15)	107.47±14.23	65.35±4.21	21.26±1.13	92.85±1.74	5.53±0.42
IVS-II-654	M (36)	138.40±8.65	63.52±2.90	20.97±0.88	93.57±1.19	5.59±0.53
-28	F (10)	123.5±15.25	72.99±5.00	23.41±1.09	92.49±0.72	6.15±0.44
-28	M (1)*	-	-	-	-	-
CD 27-28	F (5)	106.00±18.61	68.54±2.26	22.22±0.59	88.56±5.09	5.28±0.56
CD 27-28	M (0)*	-	-	-	-	-
CD 71-72	F (4)	105.50±5.07	63.15±2.69	21.45±0.50	92.60±0.50	5.80±0.14
CD 71-72	M (1)*	-	-	-	-	-

*: mean and standard deviation (SD) cannot be calculated ($n < 3$).

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No.	Recommendation	Page No.	Relevant text from manuscript
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	2	
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2	
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3	
Objectives	3	State specific objectives, including any prespecified hypotheses	4	
Methods				
Study design	4	Present key elements of study design early in the paper	5	
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5	
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5-6	
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	NA	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	NA	
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6-8	
Bias	9	Describe any efforts to address potential sources of bias	NA	
Study size	10	Explain how the study size was arrived at	5-6	

Continued on next page

Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6-8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6-8
		(*b*) Describe any methods used to examine subgroups and interactions	6-8
		(*c*) Explain how missing data were addressed	NA
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	6-7
		(*e*) Describe any sensitivity analyses	7-8
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8-9
		(b) Give reasons for non-participation at each stage	NA
		(c) Consider use of a flow diagram	25
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8-9
		(b) Indicate number of participants with missing data for each variable of interest	NA
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	NA
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	NA
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	NA
		Cross-sectional study—Report numbers of outcome events or summary measures	8-10
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	8-9
		(*b*) Report category boundaries when continuous variables were categorized	NA
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA

Continued on next page

Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	8-10
Discussion			
Key results	18	Summarise key results with reference to study objectives	11-14
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12-13
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	11
Generalisability	21	Discuss the generalisability (external validity) of the study results	11
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	15

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Hematological and electrophoretic characterization of β-thalassemia in Yunnan province of Southwestern China

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Primary Subject Heading:	Genetics and genomics
Secondary Subject Heading:	Haematology (incl blood transfusion), Diagnostics
Keywords:	thalassemia, capillary electrophoresis, hematological and biochemical parameter values, mutation, cut-off value

Hematological and electrophoretic characterization of β-thalassemia in Yunnan province of Southwestern China

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ABSTRACT

Objectives: β-thalassemia is widely found in Southwestern China. Characterization of β-thalassemia can improve screening and prenatal diagnosis for at-risk populations.

Design: A retrospective study.

Methods: In this study, the levels of HbA_2 and HbA were analyzed by gender for a total of 15,067 subjects screened by capillary electrophoresis. The cut-off value with the highest accuracy was established to identify β-thalassemia in 723 patients suspected to have this disease. Hematological and electrophoretic characterization of 8 common types of β-thalassemia were analyzed in 486 β-thalassemia subjects.

Results: HbA levels were significantly higher in males than in females, but there was no significant difference on HbA_2 levels. A new cut-off value for the diagnosis of β-thalassemia (HbA_2 ≥4.0%) with the highest accuracy was proposed for the studied populations. Hb was significantly higher in males compared to females ($p < 0.05$),

whereas no statistically significant differences were found for MCV, MCH, HbA, and HbA_2. The HbE group showed comparatively higher values for hematological indices (Hb, MCV, and MCH) than the other genotypes in heterozygous β-thalassemia groups ($p < 0.05$), and -28 (A>G) (HBB:c.-78A>C) had significantly higher HbA_2 values compared to other β-thalassemia.

Conclusions: Ethnic groups have diversified β-globin gene mutations and considerable hematologic variations. Our study will lay the foundation for screening programs and clinical management of thalassemia in Southwestern China.

Strengths and limitations of this study

- Ours is the first study to characterize β-thalassemia in the Yunnan province, which may be useful to improve screening and prenatal diagnosis in Southwestern China.
- Our study determined that a higher cut-off point of HbA_2 levels ($HbA_2 \geq 4.0\%$) should be used for β-thalassemia screening, rather the current value of 3.5%.
- Samples for participants used for capillary electrophoresis, cut-off value calculation and characterization of β-thalassemia were not collected at the same time.

INTRODUCTION

β-thalassemia is an inherited anemia resulting from genome variants in β-globin

chains. This disease is most prevalent in Africa, [1] Asia, [2] Mediterranean[3] and the Middle-East.[4] Quantification of hemoglobin A_2 (HbA_2) percentage by capillary electrophoresis and routine hematology testing are the existing methods for screening thalassemia and hemoglobinopathies.[5] An HbA_2 value exceeding 3.5% combined with a low mean cell volume (MCV) and mean cell hemoglobin (MCH) are the current diagnostic criteria for β-thalassemia carriers. Determination of the hemoglobin concentration (Hb) and HbA_2 range in a population offers a critical screening tool for thalassemia. [6-7] However, there are very few reports on the clinical reference intervals for β-thalassemia patients in China. Therefore, it is important to determine appropriate diagnostic cut-off points and supplement the existing references to improve the screening and control of β-thalassemia.

More than 800 different β-globin gene mutations have been discovered worldwide. Two subtypes are defined by totally absent (β^0) or partially reduced (β^+) production of normal β-chains, respectively. The severity of β-thalassemia varies with the thalassemia mutation, ranging from asymptomatic anemia to a severe transfusion-dependent disorder. Populations in different regions have diversified β-globin gene mutations[8] and the ability to identify and characterize these β-thalassemia mutations can assist in genetic counseling and prenatal diagnoses. [9] In this study, we established an HbA_2 dataset for screening β-thalassemia in people of Southwestern China, and

correlated these data to hematological and biochemical values to better understand thalassemia and to improve its control and prevention.

MATERIALS AND METHODS

Study design

In this study, we performed a retrospective analysis using capillary electrophoresis to determine the HbA_2 dataset, calculated the diagnostic criteria for β-thalassemia and analyzed hematologic parameters to provide an additional characterization of this disease. There were three independent studies conducted during different time periods. The study design is shown in Figure 1.

Setting

This study was conducted in The First Peoples' Hospital of Yunnan Province. This hospital is located in the Jinbi road of Kunming city. It is a general hospital whose annual clinic amount exceeds 1.6 million people with 2000 beds.

Participants

Participants for three independent studies were collected during three different time periods. (1) A total of 15,067 subjects (3,678 men and 11,389 women, 18–45

years-of-age) who sought thalassemia screening during September 2011 to July 2014 had capillary electrophoresis performed. (2) Individuals (n=723) with suspected β-thalassemia (HbA_2 ≥3.5%) or abnormal hemoglobin variants were randomly selected from October 2010 to April 2014 (210 men and 513 women, 18–45 years-of-age) for cut-off value calculation. (3) Unrelated heterozygous β-thalassemia patients (n=486: 151 males and 335 females, 19–58 years-of-age), were included in the study to analyze hematological and biochemical parameters between June 2009 to April 2013. Written and informed consent were obtained from the patients. The information consent and protocol for these studies were approved by the medical ethics committee of The First Peoples' Hospital of Yunnan Province, PRC. The exclusion criteria were: (1) incomplete information, (2) consanguinity, (3) lack of informed consent, and (4) children (below the age of 18 years).

Capillary electrophoresis

Venous blood samples were collected from subjects (n=15,067) in tubes containing EDTA. Hemoglobin analysis was performed using capillary electrophoresis (Sebia, Paris, France). Internal quality control was performed by analyzing the control materials provided by the manufacturer. Mean comparisons were made among HbA_2 and HbA by gender, comparing male vs. female in each of three age groups: 18–45,

20–29, and 30–39 years using t-test.

DNA extraction and detection of β-globin mutations

DNA was extracted from whole blood using the Blood DNA Extraction System (Tianlong Bioscience Shenzhen Ltd., Xian, China). Detection of 17 known β-globin gene mutations (97.3% of known β-thalassemia alleles in Chinese populations) for 723 individuals with suspected β-thalassemia or abnormal hemoglobin variants were performed by PCR-reverse dot-blot method as previously described.[10] These 17 β-globin mutations are as follows: CD 41-42 (–TCTT), IVS-II-654 (C>T), -28 (A>G), CD 71/72 (+A), CD 17 (A>T), HbE (CD 26, G>A), CD 31 (–C), CD 27/28 (+C), CD 43 (G>T), -32 (C>A), -29 (A>G), 30 (T>C), CD 14/15 (+G), Cap +40 to +43 (–AAAC), Initiation CD (T>G), IVS-I-1 (G>T) and IVS-I-5 (G>T). Samples without detected mutations were sequenced on an ABI 3700 automated sequencer using primers that flanked the entire β-globin gene, as previously described.[10]

Cut-off value calculation

Sensitivity, specificity, Youden's index (YI), likelihood ratio positive (LRP), and likelihood ratio negative (NRP) of HbA_2 measurements within the interval of 3.5%–4.5% were evaluated. A receiver operating characteristic (ROC) analysis was

performed to calculate the area under the curve (AUC). In addition, we calculated the cut-off value with the greatest accuracy to identify β-thalassemia in our population.

Characterization of β-thalassemias

Complete blood counts were performed on 486 unrelated β-thalassemia heterozygous patients that were diagnosed in our center using an automated cell counter (Sysmex, Tokyo, Japan). Histograms and tables of descriptive statistics of Hb, MCV, MCH, HbA and HbA_2 were generated and compared by gender (n >15). Also, HbA, HbA_2, MCV, MCH and Hb were compared among different genotypes of β-thalassemia (n >10). Continuous variables were compared using ANOVA. For multiple comparisons, a *post hoc* analysis was used when appropriate. Duncan's multiple range test was used to decrease type I error rates. All reported p-values are two-sided and were statistically significant if $p < 0.05$. All statistics in this study were computed with SPSS version 16 for Windows.

RESULTS

Subjects screened by capillary electrophoresis

The data distribution of HbA and HbA_2 measurements performed in the study is shown in Figure S1 and Table S1. The mean HbA among the subjects was 96.83%

(95% CI for mean: 96.81%–96.84%), while the mean HbA_2 was 2.91% (95% CI for mean: 2.90%–2.92%). Out of these subjects, the majority had HbA_2 levels ranging from 2.4 to 3.5% (95.45%, 14,382/15,067, while 337 cases (2.24%, 337/15,067) had HbA_2 levels <2.4%, and 348 cases (2.31%, 348/15,067) had HbA_2 levels >3.5%. Six subjects lacking an HbA band and five subjects without an HbA_2 band were identified. The HbF band was present in 4,381 subjects (29.08%, 4,381/15,067) with a mean percentage of 1.17% (95% CI for mean: 1.02%–1.33%). There were no significant differences in HbA_2 levels between male and female in each three age groups (18-45, 20-29 and 30-39) (Figure S1). However, HbA levels were significantly higher in males than in females in all three age groups (18-45, 20-29 and 30-39) ($p < 0.01$) (Figure S1).

Cut-off value calculation

Among the total 723 specimens investigated for β-globin gene mutations, twenty-two different mutations were found in 566 cases (78.28%, 566/723), with HbA_2 levels ranging from 1.8 to 7.9%. These included a total of 563 β-thalassemia heterozygotes, one HbE homozygosity, and two compound heterozygotes (Table 1). β-globin mutations were not detected in the remaining 237 subjects. Among β-thalassemia heterozygotes, HbA_2 values ranged from 1.8% to 4.0% in 69 of 566 subjects (69/566,

12.19%), while the majority (497/566, 87.81%) had HbA_2 values ≥4.0%. The sensitivity, specificity, YI, LRP and NRP of each selected cut-off point in screening for β-thalassemia are summarized in Table 2. Regarding hematologic parameters, HbA_2 at the new cut-off value of 4.0% yielded high values (0.898, 95% CI: 0.874 to 0.919) for AUC and YI (0.75) (Figure S2). The new cut-off had the highest accuracy, with a sensitivity of 85.16% and a specificity of 89.81%, and is therefore a suitable discriminator for screening of β-thalassemia in this population. Using the currently established cut-off (HbA_2 ≥3.5%) only yielded sensitivity and specificity values of 96.64% and 6.37%, respectively.

Hematological and electrophoretic characterization of β-thalassemia mutation

As shown in Tables 3 and S2, hematological and molecular characteristics of 486 unrelated heterozygous β-thalassemia mutations were demonstrated. The mean values for hematological and electrophoretic indices (HbA, HbA_2, Hb, MCV, and MCH) among participants with different genotypes of thalassemia are shown in Table 3. A significant difference between genders was observed for Hb, with males having higher Hb compared to the females ($p < 0.05$), whereas no statistically significant differences were found for MCV, MCH, HbA, and HbA_2 values between the two genders (Figures 2 and 3). The HbE group showed comparatively higher values of hematological

indices (Hb, MCV, and MCH) than the other four genotypes of heterozygous β-thalassemia groups, and -28 (A>G) (HBB:c.-78A>C) had significantly higher HbA_2 values compared to other β-thalassemias (Table 3).

DISCUSSION

β-thalassemia is one of the most common genetic disorders worldwide, and each ethnic group has a mutation set with considerable hematologic variations. Clinical features and hematological parameters in β-thalassemia patients vary with race, lifestyle, and altitude. In China, CD 17 (A>T) (HBB:c.52A>T), CD 41-42 (-TCTT) (HBB:c.126_129delCTTT), -28 (A>G) (HBB:c.-78A>C), IVS-II-654 (C>T) (HBB:c.316-197C>T), HbE (CD 26, G>A) (HBB:c.79G>A) and CD 71/72 (+A) (HBB:c.216_217insA) mutations account for more than 90% of all β-thalassemia mutations in the Chinese population.[11] Characterization and screening of these mutations provides a database to aid in the prevention and control of thalassemia. Samples for participants used for capillary electrophoresis, cut-off value calculation and characterization of β-thalassemia were not collected at the same time and this is a limitation of this study. There are very few studies describing the detection and quantification of electrophoretic and hematological parameters for screening thalassemia in the Chinese population. Thus, our report is the first characterization of

β-thalassemia mutations in people of Southwestern China and will serve as a key reference for this population.

The majority of individuals screened in this study were female, since patients seeking prenatal diagnosis and genetic counseling represent a large portion of the study participants. Six subjects lacking an HbA band and five subjects without an HbA_2 band were identified. Among these subjects, four were HbE homozygous, one was β-thalassemia/HbE compound heterozygous, and one had a hemoglobin variant mutation. HbA levels were significantly higher in male than female subjects in the three age groups (Figure S1). However, no significant difference was observed in HbA_2 in all age groups. In our study, most of the subjects (95.45%, 14,382/15,067) had HbA_2 levels that were within a narrow range (2.4%–3.5%), similar to previous observations in Nigerian patients.[12]

Carriers of β-thalassemia have increased HbA_2 values. Of the 723 subjects analyzed, the β-globin gene defect was identified in 78.28% of cases (566/723; range 1.8%–7.9%). One case without the β-globin mutation had the highest HbA_2 value (7.9%), which may be due to a hemoglobin variant co-eluting with HbA_2 peak.[13] Only one case of β-thalassemia major was found in this study, which was due to the fact that children were excluded. The majority of β-thalassemia major patients die before reaching the age of 5.[14]

Having an HbA_2 level greater than 3.5% is the current diagnostic criterion for the β-thalassemia trait,[15] while values greater than 4.0% are also used to identify β-thalassemia carriers in some regions.[16] In this study, a higher cut-off point of HbA_2 levels (HbA_2 ≥4.0%) determined by ROC curves had the highest accuracy in identifying β-thalassemia carriers. This result differed from previous reports,[17-19] which may be explained by regional differences in the spectrum and cut-off values for β-thalassemia mutations. In our study, most β-thalassemia mutations (497/566, 87.81%) had a mean HbA_2 exceeding 4.0%, while silent β-thalassemia mutations with near normal hematological indices and borderline HbA_2 values (3.5%–4.0%) were rare in our population. Therefore, the diagnostic thresholds should be increased for this population.

The cut-off point was established for all samples, including those from β-thalassemia patients and those having abnormal hemoglobins. And Hb variant fraction could be easily screened by abnormal band for capillary electrophoresis.[20] In Yunnan, HbE (CD 26, G>A) (HBB:c.79G>A), an abnormal hemoglobin, is the most prevalent β-globin gene mutation (with a frequency of 30.5%),[21] with comparatively lower HbA_2 values. Here, the mean percentage of HbA_2 in patients with HbE was 3.87±0.44, which was similar to that reported for Americans.[22] HbE and other abnormal hemoglobins, such as CD 22 (A>C) (HBB:c.68A>C) (HbG-Coushatta), have been

reported to decrease the expression of HbA_2.[23] In this study, most β-thalassemia were characterized by increased HbA_2 values ranging from 4.3% to 6.6% (93.86%, 428/456).

Subjects with a β-globin mutation usually have lower Hb and erythrocyte indices compared to those without an identifiable mutation[24] and the clinical and hematological parameters in patients having β-thalassemia are widely variable.[25] This study was conducted in Kunming, the capital of Yunnan province, which has an altitude of 1,895 meters. Regional altitude differences are a contributing factor in the variation of these parameters, as high altitudes are known to elevate Hb values. Males had higher Hb values in all five genotype groups compared to females, whereas, no statistical sex-based significant differences were observed for MCV, MCH, HbA, and HbA_2 values. These results were similar to previously reported data.[26] HbE (CD 26, G>A) (HBB:c.79G>A) is clinically asymptomatic with minimal erythrocyte morphological abnormalities in heterozygous individuals; this type had the highest MCV, MCH, and Hb values in this study. Subjects with the -28 (A>G) (HBB:c.-78A>C) mutation (β^+-thalassemia), a mutation in the TATA box of the proximal promoter region, had higher MCH, MCV, and HbA_2 values compared to other β-thalassemia types (CD 17 (A>T) (HBB:c.52A>T), CD 41-42 (-TCTT) (HBB:c.126_129delCTTT), IVS-II-654 (C>T) (HBB:c.316-197C>T)). The -28 (A>G)

(HBB:c.-78A>C) phenotype is similar to other β-globin gene promoter mutations.[27]

In conclusion, we offered a retrospective analysis of β-thalassemia that can be used for prenatal diagnosis, genetic counseling, thalassemia control and screening. Characterization of β-thalassemia and diagnostic thresholds for β-thalassemia can be used to identify patients more precisely.

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Contributors JZ and BSZ designed the experiments. JZ, XQM, JH and XHZ performed the experiments. JZ, HC, JS and BSZ analyzed the data. JZ Wrote the paper. All authors critically revised and approved the final manuscript.

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Competing interests None declared.

Ethics approval The protocol and information consent for this study were approved by the medical ethics committee of The First Peoples' Hospital of Yunnan Province in accordance with the Declaration of Helsinki.

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Table 1 Number of β-globin mutations found in this study.

Mutation	Type	n	Number of Alleles	Allele Frequency (%)
CD 17 (A>T) (HBB:c.52A>T)	β^0/β^A	166	166	29.04%
CD 41-42 (–TCTT) (HBB:c.126_129delCTTT)	β^0/β^A	145	146	26.26%
CD 26 (G>A) (HBB:c.79G>A)	β^+/β^A	107	110	19.13%
IVS-II-654 (C>T) (HBB:c.316-197C>T)	β^+/β^A	90	92	16.00%
–28 (A>G) (HBB:c.-78A>C)	β^+/β^A	20	20	3.48%
CD 71/72 (+A) (HBB:c.216_217insA)	β^0/β^A	12	12	2.09%
CD 27/28, +C (HBB:c.84_85insC)	β^0/β^A	7	7	1.22%
IVS-I-1 (G>T) (HBB:c.92+1G>T)	β^+/β^A	5	5	0.87%
IVS-I-5 (G>C) (HBB:c.92+5G>C)	β^+/β^A	1	1	0.17%
CD 5 (–CT) (HBB:c.17_18delCT)	β^0/β^A	1	1	0.17%
Hb Dieppe, CD 127 (A>G) (HBB:c.383A>G)	β^0/β^A	1	1	0.17%
Initiation CD (T>C) (HBB:c.2T>C)	β^0/β^A	1	1	0.17%
CD121 (G>T) (HBB:c.364G>T).	β^0/β^A	1	1	0.17%
–31 (A>C) (HBB:c.-81A>G)	β^+/β^A	1	1	0.17%
-29 (A>G) (HBB:c.-79A>G)	β^+/β^A	1	1	0.17%
CD 43 (G>T) (HBB:c.130G>T)	β^0/β^A	1	1	0.17%

CD 113 (T>A) (HBB:c.341T>A)	HbVar	1	1	0.17%
CD 22 (A>C) (HBB:c.68A>C)	HbVar	1	1	0.17%
CD 47 (G>A) (HBB:c.142G>A)	HbVar	1	1	0.17%
CD 41-42/IVS-II-654	β^0/β^0	1	-	-
IVS-II-654/CD 26	β^0/β^+	1	-	-
CD 26/CD 26	β^+/β^+	1	-	-
Total number of alleles	-	566	569	100

β^0: production of β-globin chain is entirely eliminated; β^+: production of β-globin chain is reduced; HbVar: hemoglobin variant.

Table 2 Predictive value of evaluated indices of the ROC analysis for β-thalassemia.

HbA_2 (%)	TP	FN	FP	TN	LRP	NRP	Sn	Sp	YI
3.5	547	147	19	10	1.03	0.53	96.64	6.37	0.03
3.6	540	85	26	72	1.76	0.10	95.41	45.86	0.41
3.7	531	49	35	108	3.01	0.09	93.82	68.79	0.63
3.8	512	35	54	122	4.06	0.12	90.46	77.71	0.68
3.9	497	25	69	132	5.52	0.14	87.81	84.08	0.72
4.0	**482**	**16**	**84**	**141**	**8.34**	**0.17**	**85.16**	**89.81**	**0.75**
4.1	473	15	93	142	8.78	0.18	83.57	90.45	0.74
4.2	466	13	100	144	9.94	0.19	82.33	91.72	0.74
4.3	456	13	110	144	9.73	0.21	80.57	91.72	0.73
4.4	449	12	117	145	10.38	0.22	79.33	92.36	0.72
4.5	445	12	121	145	10.29	0.23	78.62	92.36	0.71

TP: true positive; FP: false positive; FN: false negative; TN: true negative; Likelihood Ratio positive (LRP): sensitivity ÷ (1 - Specificity), Likelihood Ratio Negative (NRP): (1 - Sensitivity)/specificity. Youden's index (YI), Sensitivity (Sn): True positive ÷ (true positive + false negative), Specificity (Sp): True negative ÷ (true negative + false positive).

Table 3 Characterization of 8 types of β-thalassemia and comparison (mean ± SD)

Mutation (*n*)	Hb (g/L)	MCV (fl)	MCH (pg)	HbA (%)	HbA_2 (%)
CD 17[1] (125)	118.26±19.14	64.78±6.98	21.52±2.67	92.08±2.24	5.91±0.54
CD 41-42[2] (108)	117.78±19.54	65.84±5.29	21.68±2.56	92.38±2.93	5.75±0.60
CD 26[3] (178)	133.04±16.67	76.99±4.52	26.06±1.55	70.67±2.28	3.87±0.44
IVS-II-654[4] (51)	116.57±19.11	64.81±3.93	21.17±1.06	93.06±1.62	5.55±0.45
-28[5] (11)	125.64±16.11	73.45±4.97	23.61±1.23	92.49±0.68	6.22±0.47
CD 27-28[6] (5)	106.00±18.61	68.54±2.26	22.22±0.59	88.56±5.09	5.28±0.56
CD 71-72[7] (5)	108.80±8.58	64.64±2.60	21.14±0.82	92.76±0.56	5.92±0.29
IVS-I-1[8] (3)	127.00±13.23	72.40±15.98	25.07±6.51	94.20±0.66	5.73±0.57
Comparisons	1 versus 3	1 versus 3	1 versus 3	1 versus 3	1 versus 2
(p <0.05)	2 versus 3	1 versus 5	1 versus 5	1 versus 4	1 versus 3
	3 versus 4	2 versus 3	2 versus 3	2 versus 3	1 versus 4
		2 versus 5	2 versus 5	3 versus 4	2 versus 3
		3 versus 4	3 versus 4	3 versus 5	2 versus 4
		3 versus 5	3 versus 5		2 versus 5
		4 versus 5	4 versus 5		3 versus 4
					3 versus 5
					4 versus 5

P value of significant differences (p <0.05) between various 5 types of β-thalassemia is listed (n >10). Non-listed comparisons are not significant.

Figure legends

Figure 1 Flow diagram and participant numbers in this study. Capillary electrophoresis, cut-off value calculation, hematological and electrophoretic characterization of β-thalassemia mutation were three independent studies conducted during different time periods.

Figure 2 Hematological characterization of β-thalassemia according to sex. # Males have significantly higher Hb values ($p < 0.05$) than females. No differences were observed between genders for MCV and MCH. (n=486 total unrelated heterozygous β-thalassemia cases).

Figure 3 Electrophoretic characterization of β-thalassemia according to sex in the current study. No differences were observed between genders for Hb A and Hb A_2. (n= 486 total unrelated heterozygous β-thalassemia).

Figure S1 Distribution of HbA_2 (A) and HbA (B) in all samples. The data distribution of HbA and HbA_2 were measured according to age. # Males show significantly higher Hb A values ($p < 0.05$) than females in three age group (18–45, 20–29, and 30–39 years). There was no significant differences in HbA_2 levels in all three age groups. The sample size of the other two age categories (<20, 40-45) were small, which was not suitable to compare.

Figure S2 Receiver operative characteristic curves (ROC) of the cut-off point

calculation. A: cut-off value at HbA_2 4.0%. HbA_2 at the new cut-off value of 4.0% yielded high values (0.898, 95% CI: 0.874 to 0.919) for AUC and YI (0.75).

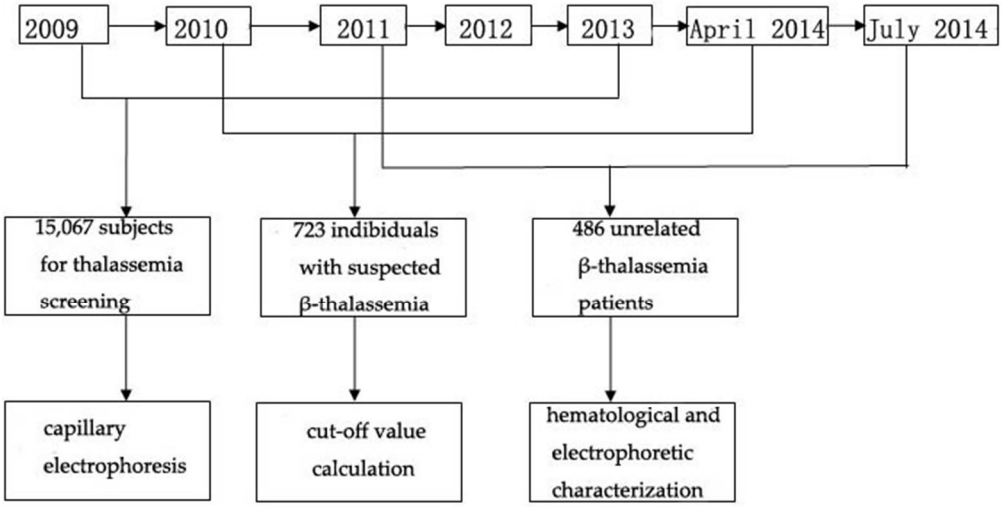

Figure 1 Flow diagram and participant numbers in this study. Capillary electrophoresis, cut-off value calculation, hematological and electrophoretic characterization of β-thalassemia mutation were three independent studies conducted during different time periods.

58x30mm (300 x 300 DPI)

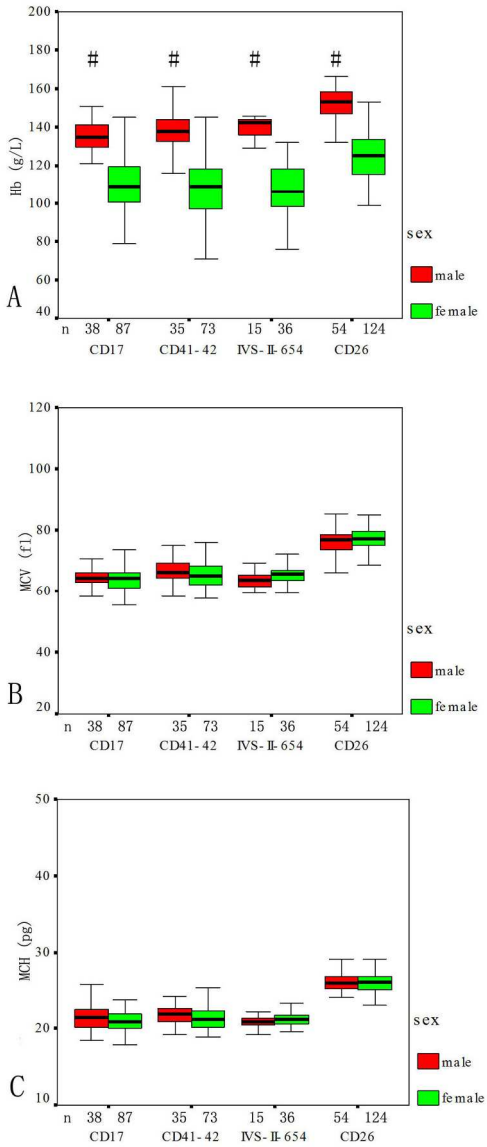

Figure 2 Hematological characterization of β-thalassemia according to sex. # Males have significantly higher Hb values ($p < 0.05$) than females. No differences were observed between genders for MCV and MCH. (n=486 total unrelated heterozygous β-thalassemia cases).

89x211mm (300 x 300 DPI)

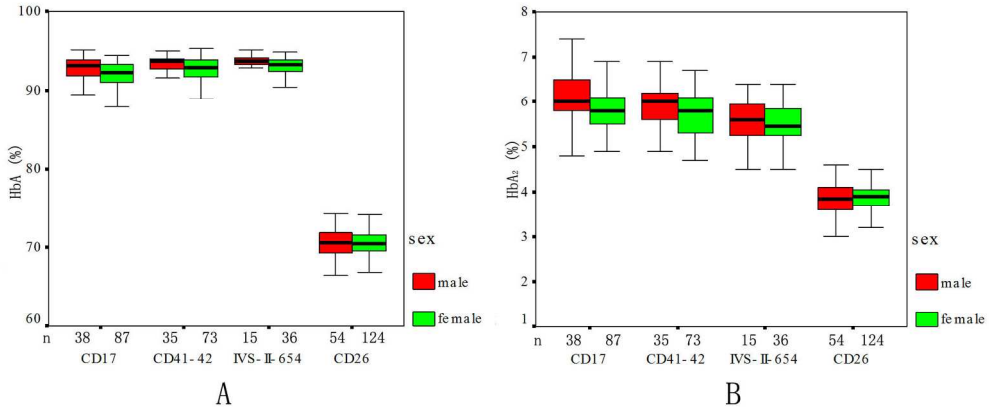

Figure 3 Electrophoretic characterization of β-thalassemia according to sex in the current study. No differences were observed between genders for Hb A and Hb A2. (n= 486 total unrelated heterozygous β-thalassemia).

170x72mm (300 x 300 DPI)

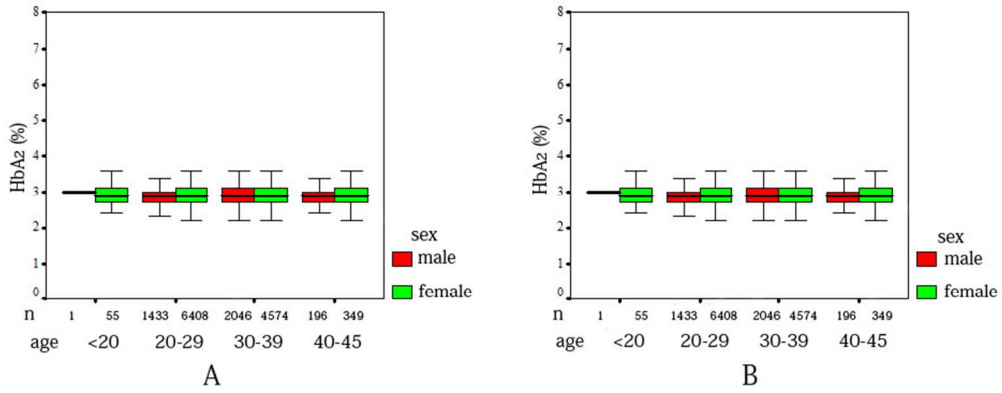

Figure S1 Distribution of HbA2 (A) and HbA (B) in all samples. The data distribution of HbA and HbA2 were measured according to age. # Males show significantly higher Hb A values ($p < 0.05$) than females in three age group (18–45, 20–29, and 30–39 years). There was no significant differences in HbA2 levels in all three age groups. The sample size of the other two age categories (<20, 40-45) were small, which was not suitable to compare.

78x30mm (300 x 300 DPI)

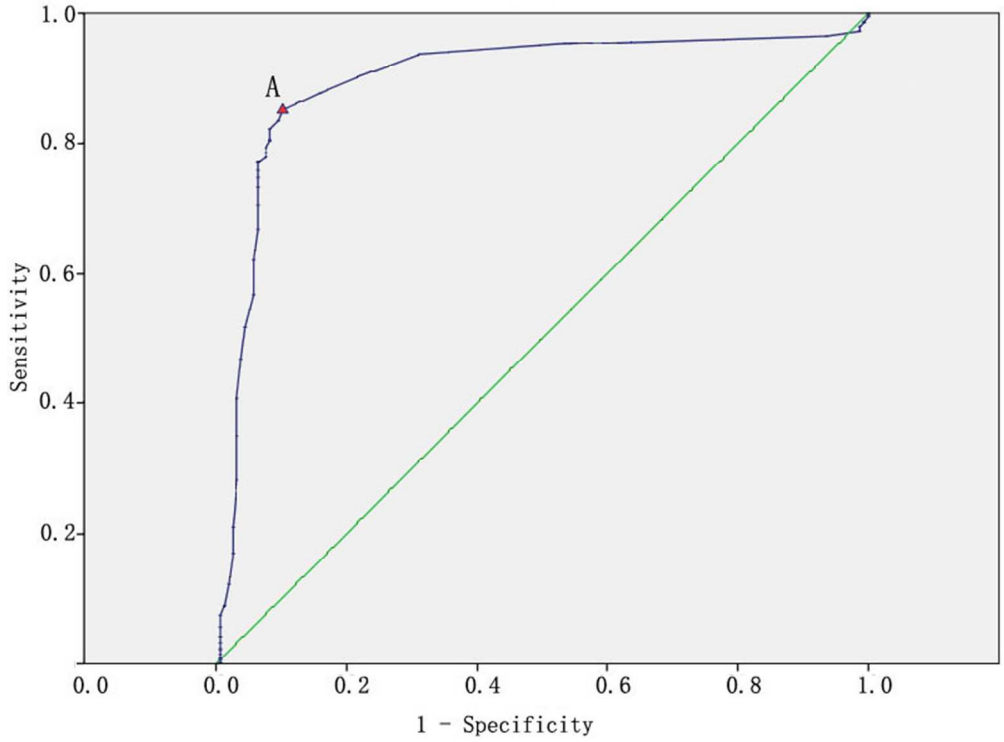

Figure S2 Receiver operative characteristic curves (ROC) of the cut-off point calculation. A: cut-off value at HbA2 4.0%. HbA2 at the new cut-off value of 4.0% yielded high values (0.898, 95% CI: 0.874 to 0.919) for AUC and YI (0.75).

67x53mm (300 x 300 DPI)

Table S1 Distribution of HbA and HbA_2 in all samples

Parameters	Sex	Age	n	Mean (%)	SD (%)	95% CI for mean
HbA	Male	18–45	3675	96.95	0.74	96.93%–96.97%
		<20	1	-	-	-
		20–29	1433	96.98	0.59	96.95%–97.01%
		30–39	2045	96.92	0.85	96.88%–96.96%
		40-45	196	97.02	0.50	96.95%–97.09%
	Female	18–45	11386	96.79	1.01	96.77%–96.81%
		<20	55	96.92	0.55	96.77%-97.07%
		20–29	6408	96.79	1.02	96.77%–96.82%
		30–39	4574	96.78	1.01	96.75%–96.81%
		40-45	349	96.71	0.95	96.61%–96.84%
	total	18–45	15061	96.83	0.96	96.81%–96.84%
HbA_2	Male	18–45	3676	2.91	0.42	2.90%–2.92%
		<20	1	-	-	-
		20–29	1433	2.90	0.34	2.88%–2.92%
		30–39	2046	2.92	0.44	2.90%–2.94%
		40-45	196	2.89	0.38	2.84%–2.95%
	Female	18–45	11386	2.91	0.43	2.90%–2.92%
		<20	55	2.91	0.42	2.89%-2.91%
		20–29	6408	2.90	0.42	2.89%–2.91%
		30–39	4574	2.92	0.45	2.90%–2.93%
		40-45	349	2.95	0.47	2.90%–2.96%
	total	18–45	15062	2.91	0.43	2.90%–2.92%

SD: Standard Deviation; CI: confidence interval. HbF was only detected in 29.08%

(4,381/15,067) of the subjects, which was not suitable for the description of mean±SD.

Table S2 Characterization of 7 types of β-thalassemia according to sex (mean ± SD)

Mutation	Sex (n)	Hb (g/L)	MCV (fl)	MCH (pg)	HbA (%)	HbA_2 (%)
CD 17	F (87)	110.30±16.12	64.86±7.96	21.54±3.01	91.80±2.43	5.83±0.52
CD 17	M (38)	136.47±11.55	64.62±4.00	21.46±1.66	92.74±1.55	6.11±0.54
CD 41-42	F (73)	108.71±15.50	65.39±5.76	21.23±1.96	92.04±3.04	5.66±0.57
CD 41-42	M (35)	137.57±11.71	66.82±5.85	22.56±3.74	92.87±2.68	5.87±0.65
CD 26	F (124)	125.18±11.90	77.09±4.74	26.01±1.63	70.64±2.24	3.88±0.45
CD 26	M (54)	151.11±11.07	76.76±4.01	26.18±1.33	70.74±2.37	3.86±0.43
IVS-II-654	F (15)	107.47±14.23	65.35±4.21	21.26±1.13	92.85±1.74	5.53±0.42
IVS-II-654	M (36)	138.40±8.65	63.52±2.90	20.97±0.88	93.57±1.19	5.59±0.53
-28	F (10)	123.5±15.25	72.99±5.00	23.41±1.09	92.49±0.72	6.15±0.44
-28	M (1)*	-	-	-	-	-
CD 27-28	F (5)	106.00±18.61	68.54±2.26	22.22±0.59	88.56±5.09	5.28±0.56
CD 27-28	M (0)*	-	-	-	-	-
CD 71-72	F (4)	105.50±5.07	63.15±2.69	21.45±0.50	92.60±0.50	5.80±0.14
CD 71-72	M (1)*	-	-	-	-	-

*: mean and standard deviation (SD) cannot be calculated (n ＜3).

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No.	Recommendation	Page No.	Relevant text from manuscript
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	2	
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2	
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3	
Objectives	3	State specific objectives, including any prespecified hypotheses	4	
Methods				
Study design	4	Present key elements of study design early in the paper	5	
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5	
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5-6	
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	NA	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	NA	
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6-8	
Bias	9	Describe any efforts to address potential sources of bias	NA	
Study size	10	Explain how the study size was arrived at	5-6	

Continued on next page

Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6-8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6-8
		(*b*) Describe any methods used to examine subgroups and interactions	6-8
		(*c*) Explain how missing data were addressed	NA
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	6-7
		(*e*) Describe any sensitivity analyses	7-8
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8-9
		(b) Give reasons for non-participation at each stage	NA
		(c) Consider use of a flow diagram	25
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8-9
		(b) Indicate number of participants with missing data for each variable of interest	NA
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	NA
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	NA
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	NA
		Cross-sectional study—Report numbers of outcome events or summary measures	8-10
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	8-9
		(*b*) Report category boundaries when continuous variables were categorized	NA
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA

Continued on next page

Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	8-10
Discussion			
Key results	18	Summarise key results with reference to study objectives	11-14
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12-13
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	11
Generalisability	21	Discuss the generalisability (external validity) of the study results	11
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	15

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.