Quantitative evaluation of the clinical severity of hemoglobin H disease in a cohort of 591 patients using a scoring system based on regression analysis

α-thalassemia, a common genetic disorder characterized by decreased or absent synthesis of α-globin chains, frequently occurs in Southeast Asia (SEA) with an estimated carrier rate of 17.3-51.5%, meaning that 74-663 of every 10,000 newborns in the region may be affected by α-thalassemia major or intermedia and will have birth defects without intervention.[1-4] α-thalassemia intermedia, also termed hemoglobin H (Hb H) disease, is typically caused by genetic defects in three of the four α-globin genes, which can be divided into deletional and non-deletional forms.[1,5] The most common deletional Hb H disease genotypes in SEA and southern China are - $-^{SEA}/-\alpha^{3.7}$ and - $-^{SEA}/-\alpha^{4.2}$, while the main non-deletional genotype is - $-^{SEA}/\alpha^{CS}\alpha$ (HCS).[3-7] In this study, we first aimed to evaluate clinical symptoms by collecting multiple phenotypic indicators from a large cohort of Hb H patients. By referring to a validated scoring system for β-thalassemia intermedia and adjusting some parameters for the phenotypic analysis of Hb H patients,[8] we were able to quantitatively determine the clinical severity of Hb H patients' conditions, based on the phenotypic differences among patients with varied α-thalassemic genotypes. The proposed scoring system may support the evaluation of Hb H disease progression and treatment decisions in clinical practice.

A total of 591 patients were recruited via standard sampling based on their basic information, medical history (transfusion dependence, chelation history, etc.), physical examination data, laboratory examination results, and abdominal ultrasound results. We validated the thalassemia (*HBA* and *HBB*) genotypes using conventional molecular diagnostic approaches, including gap-Polymerase Chain Reaction (gap-PCR), Sanger sequencing, and multiplex ligation-dependent probe amplification (MLPA).[7] All patients provided their informed consent to participate in the original study in accordance with the Declaration of Helsinki. The final cohort comprised seven ethnic groups, of which the southern Chinese Han population was the largest. Of the 591 patients, 224 (38%) had deletional Hb H disease, mainly with - $-^{SEA}/-\alpha^{3.7}$ (73.21%) and - $-^{SEA}/-\alpha^{4.2}$ (26.34%) genotypes. The remaining 367 patients (62%) had non-deletional Hb H disease, primarily with HCS (90.74%) and Hb H Quong Sze (HQS) (5.18%) genotypes. The proportion of young patients (aged <18 years) with the non-deletional HCS genotype was significantly higher than that of young patients with the deletional Hb H genotype, indicating that HCS has an earlier age of onset and more severe symptoms. Our analysis of the core indicators of thalassemia symptoms revealed that HCS patients had an earlier age at first blood transfusion, higher serum ferritin levels, etc. (Table 1). Patients with Hb H Westmead (HWS; a non-deletional type of α-thalassemia[7]) exhibited relatively mild clinical symptoms as well as higher Hb levels compared to individuals with the - $-^{SEA}/-\alpha^{3.7}$ and - $-^{SEA}/-\alpha^{4.2}$ genotypes. These results largely support previous findings regarding the heterogeneity of Hb H patients with non-deletional (- $-^{SEA}/\alpha^{CS}\alpha$) or deletional (- $-^{SEA}/-\alpha^{3.7}$ or - $-^{SEA}/-\alpha^{4.2}$) α-thalassemia genotypes.[9,10]

After observing the heterogeneity of Hb H patients in this cohort, we further classified the patients into transfusion-dependent thalassemia (TDT) and non-transfusion-dependent thalassemia (NTDT) groups according to their age at first transfusion and annual transfusion frequency, according to the guidelines of the Thalassemia International Federation.[11,12] Of the 544 patients, 70 were classified as TDT (including 68 HCS patients and 2 deletional Hb H patients) and 474 were classified as NTDT (including 216 deletional Hb H patients and 258 non-deletional Hb H patients). Notably, of the 47 patients who underwent splenectomy, 39 had a history of blood transfusions before surgery. Of those 39 patients, 13 depended on regular blood transfusions, with severe cases requiring transfusions as frequently as every 20 days. Following surgery, only 6 patients received blood transfusions, 4 of whom received transfusions due to unexpected fevers, and 2 of whom required occasional transfusions during menstruation. These results suggest that splenectomy generally reduces the transfusion dependence of Hb H patients. These results demonstrate a high degree of heterogeneity among Hb H diseases and suggest that their clinical severity cannot be solely predicted by genotype or transfusion dependence.

We next attempted to quantitatively evaluate the disease severity of Hb H patients by considering the phenotypic complexity of the disease. To do this, three hematologists first classified the patients' symptoms as mild, moderate, or severe according to the following six key parameters: 1) Hb at a steady state; 2) age at first blood transfusion; 3) requirement for blood transfusion; 4) spleen size; 5) age at thalassemia presentation; and 6) growth and development.[8] To establish and evaluate the scoring system, we randomly divided the 591 patients into a training cohort with 298 cases and a validation cohort with 293 cases (*Online Supplementary Figure S1A, B*). We used a univariate logistic regression model to assess the associations between the severity classifications and 18 candidate parameters,

Table 1. Phenotypic characterization of 591 hemoglobin H patients based on two major genotypic categories.

Parameter	Deletional Hb H disease, N=224			Non-deletional Hb H disease, N=367						
	--^{SEA}/-$\alpha^{3.7}$	--^{SEA}/-$\alpha^{4.2}$	--^{THAI}/-$\alpha^{3.7}$	--^{SEA}/$\alpha^{CS}\alpha$	--^{SEA}/$\alpha^{QS}\alpha$	--^{SEA}/$\alpha^{WS}\alpha$	--^{THAI}/$\alpha^{CS}\alpha$	--^{SEA}/$\alpha^{CD30}\alpha$	--^{SEA}/$\alpha^{intA-G}\alpha$	*P*[a]
N (%)	164 (73.21)	59 (26.34)	1 (0.45)	333 (90.74)	19 (5.18)	10 (2.73)	2 (0.54)	2 (0.54)	1 (0.27)	-
Age, N (%)										<0.001
< 18 years	94 (57.32)	32 (54.24)	1 (100)	280 (84.10)	15 (78.95)	4 (40)	1 (50)	0	1 (100)	
18-35 years	56 (34.15)	23 (38.98)	0	45 (13.50)	3 (15.79)	4 (40)	1 (50)	1 (50)	0	
> 35 years	14 (8.54)	4 (6.78)	0	8 (2.40)	1 (5.26)	2 (20)	0	1 (50)	0	
Gender, female / male, N	79 / 85	28 / 31	0 / 1	175 / 158	12 / 7	5 / 5	1 / 1	0 / 2	0 / 1	0.598
Ethnic group, N (%)										<0.001
Han	134 (81.71)	46 (77.97)	0	193 (57.96)	15 (78.95)	10 (100)	1 (50)	2 (100)	1 (100)	
Zhuang	26 (15.85)	11 (18.64)	1 (100)	133 (39.94)	4 (21.05)	0	1 (50)	0	0	
Yao	3 (1.83)	2 (3.39)	0	4 (1.20)	0	0	0	0	0	
Buyi	1 (0.61)	0	0	0	0	0	0	0	0	
She	0	0	0	1 (0.30)	0	0	0	0	0	
Li	0	0	0	1 (0.30)	0	0	0	0	0	
Miao	0	0	0	1 (0.30)	0	0	0	0	0	
HBB genotype, N										0.031
β0/β	2	2	0	2	0	1	0	0	0	
β+/β	2	0	0	4	0	0	0	0	0	
Age at thalassemia presentation in months, mean±SD	128.15±142.54	117.55±132.80	-	36.72±55.45	55.84±82.12	159.6±158.9	14.00±14.14	270.00±296.98	60	<0.001
Age at first blood transfusion in months, mean±SD	101.82±128.45	86.06±110.63	-	44.36±54.70	76.94±116.76	-	14.00±14.14	60	72	0.38
Growth retardation, N (%)	34 (20.73)	12 (20.34)	0	111 (33.33)	4 (21.05)	1 (10)	0	1 (50)	0	0.052
Laboratory values, mean±SD										
Hemoglobin, g/L	99.23±10.95	98.64±9.58	95	93.65±12.37	90.68±8.54	120.90±13.03	83.50±2.12	89.50±13.44	101	<0.001
Serum ferritin, ng/mL	266.93±369.25	266.40±306.25	42.5	1,002.69±989.76	557.28±522.43	133.02±171.53	652.50±762.97	1,689.89±291.96	1,104.5	<0.001
< 800, N (%)	149 (90.85)	55 (93.22)	1 (100)	185 (55.56)	15 (78.95)	10 (100)	1 (50)	0	0	<0.001
800-2,500, N (%)	15 (9.15)	4 (6.78)	0	126 (37.84)	4 (21.05)	0	1 (50)	2 (100)	1 (100)	
> 2,500, N (%)	0	0	0	22 (6.60)	0	0	0	0	0	
Total bilirubin, μmol/L	21.32±12.67	24.89±12.94	16	42.85±21.47	41.88±19.81	12.80±5.67	38.70±17.11	56.45±2.47	20.6	<0.001
RBC, $\times10^6$/mm^3	5.69±0.71	5.63±0.66	6.07	4.44±0.55	4.86±0.55	5.88±0.53	4.01±0.18	4.35±0.97	4.55	<0.001
MCV,[b] μm^3	57.34±5.36	57.96±6.30	50.5	75.01±5.91	65.74±5.56	65.90±4.24	78.90±2.40	75.15±3.61	75.4	<0.001
MCH,[c] pg	17.55±1.51	17.63±1.81	15.6	21.34±3.60	18.77±1.63	20.57±1.11	20.80±0.42	20.75±1.49	22.3	<0.001
MCHC,[d] g/dL	306.34±9.18	304.71±10.58	310	282.35±16.07	285.42±10.23	312.40±4.40	263.50±2.12	276.50±6.36	295	<0.001

Continued on following page.

Parameter	Deletional Hb H disease (N=224)			Non-deletional Hb H disease (N=367)						
	$--^{SEA}/-\alpha^{3.7}$	$--^{SEA}/-\alpha^{4.2}$	$--^{THAI}/-\alpha^{3.7}$	$--^{SEA}/\alpha^{CS}\alpha$	$--^{SEA}/\alpha^{QS}\alpha$	$--^{SEA}/\alpha^{WS}\alpha$	$--^{THAI}/\alpha^{CS}\alpha$	$--^{SEA}/\alpha^{CD30}\alpha$	$--^{SEA}/\alpha^{intA-G}\alpha$	*P*[a]
Platelets, x10^9/L	329.93±113.08	319.39±103.43	363	322.90±145.22	349.68±129.11	301.00±64.09	474.00±268.70	152.50±34.65	390	0.060
Reticulocytes, x10^{12}/L	0.24±0.08	0.25±0.09	0.2	0.41±0.17	0.41±0.11	0.10±0.03	0.68±0.20	0.36±0.14	0.28	<0.001
Fetal Hb, %	0.88±0.41	0.86±0.22	0.89	0.82±0.31	0.69±0.18	0.82±0.48	0.72±0.04	0.75±0.19	0.88	0.039
Hemoglobin A2, %	2.09±1.18	2.02±0.96	2.35	1.26±0.74	1.36±0.34	3.05±1.83	0.67±0.14	1.09±0.16	2.2	<0.001
Hemoglobin CS, %	0	0	0	2.05±1.53	0	0	2.63±1.39	0	0	<0.001
Hemoglobin H, %	3.00±3.13	4.39±3.98	0	7.17±4.92	20.78±5.36	0	11.61±7.88	18.65±5.18	8.79	<0.001
Hemoglobin Bart's, %	1.36±2.23	2.33±3.27	0.73	5.86±3.78	3.04±4.36	0	4.51±6.39	3.25±4.60	11.47	<0.001
Transferrin, g/L	2.01±0.31	1.94±0.38	2.66	1.55±0.35	1.83±0.34	2.29±0.30	1.48±0.23	1.59±0.23	2.06	<0.001
STfR,[e] mg/L	3.84±0.99	4.10±1.05	4.35	9.47±3.13	6.36±1.83	1.66±0.59	7.10±3.08	5.78±2.07	3.93	<0.001
Abdominal ultrasonography, mean±SD										
Spleen size, cm	9.86±3.47	10.47±3.74	7.40	11.29±6.77	10.30±5.05	8.80±1.32	5.34±7.55	17.7±4.95	14.4	0.081
Subcostal spleen size, cm	0.84±1.58	1.18±1.91	0	3.00±2.19	2.86±3.20	0	1.75±2.47	5.73±2.02	3.6	<0.001
Liver size, cm	6.83±2.13	6.92±2.06	6.1	7.50±1.45	7.07±1.30	7.42±1.69	8.01±0.70	8.75±0.73	9.20	0.0046
Subcostal liver size, cm	1.73±1.75	1.58±1.91	1.1	3.28±1.63	2.96±1.78	0.68±1.45	5.03±0.24	3.37±3.80	3.5	<0.001
Cholelithiasis, N (/%)	11 (6.71)	0	0	30 (9.01)	1 (5.26)	0	0	1(50)	0	0.011
Treatment,[f] N (%)										
None/Rare transfusion	141 (89.24)	51 (87.93)	1 (100)	70 (23.89)	13 (68.42)	10 (100)	1 (50)	1 (50)	0	<0.001
Occasional transfusion	7 (4.43)	4 (6.90)	0	45 (15.36)	2 (10.53)	0	1 (50)	0	0	
Regular transfusion	10 (6.33)	3 (5.17)	0	178 (60.75)	4 (21.05)	0	0	1 (50)	1 (100)	
Splenectomy	5 (3.05)	1 (1.69)	0	39 (11.71)	2 (15.53)	0	0	0	0	0.035
Iron chelation	40 (24.4)	17 (28.81)	1	99 (29.73)	10 (52.63)	0	1 (50)	1 (50)	0	0.059
Other drugs	4 (2.44)	0	0	7 (2.10)	0	0	0	0	0	0.988

N: number; Hb: hemoglobin; RBC: red blood cells; SD: standard deviation; SEA: Southeast Asia; THAI: Thailand; CS: Constant Spring; QS: Quong Sze; WS: Westmead; $-\alpha^{3.7}$: Single-gene deletion; $-\alpha^{4.2}$: Single-gene deletion; $\alpha^{CD30(-GAG)}$: HBA2:c.91_93del; α^{intA-G}: initiation codon(ATG>GTG): HBA2:c.1A>G. [a]*P*: χ^2 test for categorical variables and one-way ANOVA for continuous variables or discrete variables. [b]MCV: mean corpuscular volume. [c]MCH: mean corpuscular Hb. [d]MCHC: mean corpuscular Hb concentration. [e]sTfR: serum transferrin receptor. [f]Regular transfusion: >4 times/year; occasional transfusion: 1-3 times/year; None/Rare transfusion: blood transfusion therapy was never given, or transfusions were given every few years. Patients with splenectomy were excluded from blood transfusion events. Since this was a cross-sectional observation study, the transfusion treatment events of patients with splenectomy before surgery could not be fully counted.

estimating Odds Ratios (OR) with 95% Confidence Intervals (CI) for the training cohort (*Online Supplementary Table S1*). We selected variables with *P* values <0.1 in the univariate regression analysis and used an ordinal logistic regression model to determine the extent to which independent variables affected the dependent variable. *P*<0.05 was considered statistically significant. The results of the multivariate logistic regression analysis showed that the best model combined seven parameters: Hb level, age at thalassemia presentation, age at first blood transfusion, transfusion frequency, subcostal spleen size, growth and development, and soluble transferrin receptor (sTfR) level (*Online Supplementary Table S2*). There was no evidence of multicollinearity among these parameters, suggesting that each criterion was independently associated with disease severity. To simplify the scoring process and avoid using complex mathematical formulae, we classified each of the seven significant criteria into three levels based on the severity of the phenotype. We then assigned a score of 0, 1, or 2 points to each criterion to reflect increasing levels of severity (Table 2). This allowed us to establish a final clinical scoring system for assessing disease severity in patients with Hb H disease, with total scores ranging from 0-14 (Table 2). To determine the relevant total score thresholds, we tested various combinations of cut-off values, including 4, 5, 6, 8, 9, and 10. We found that the patients' disease severity was best distinguished by a severity cut-off score of 8. Based on these results, we assigned the following Hb H disease severity categories: mild (0-5), moderate (6-8), and severe (9-14) (Table 2). We then used the validation cohort to test the performance of the scoring system. The scoring system correctly identified 114 mild cases, 101 moderate cases, and 78 severe cases, and the consistency rates according to the 6 objective parameters were 89.47% (mild), 85.15% (moderate), and 87.18% (severe), respectively (Figure 1A-C), suggesting the practicability of this scoring system for the clinical assessment of Hb H diseases. In addition, 130 out of the 591 patients had blood transfusions more than 8 times a year, of which 95 (73%) were classified in the severe group, indicating that our proposed model could sensitively distinguish those patients who needed regular transfusions as having "severe" conditions. Our results support the use of this newly established scoring system for the clinical assessment and management of Hb H disease.

Based on our scoring system, we categorized 219 of 591 (37%) Hb H patients as mildly affected, 210 (36%) as moderately affected, and 162 (27%) as severely affected. Our results indicate that most of the deletional Hb H disease patients had a relatively mild phenotype, with scores ranging from 0-9 and a mean value of 3.5. In comparison, the mean value for non-deletional Hb H patients was 8.1, significantly higher than for the former group (*P*<0.0001) (Figure 1D). Notably, 13 of the 591 Hb H patients were also β-thalassemia carriers (Table 1). Coinheritance of β-thalassemia mutations seemingly reduced the severity scores, especially among the HCS patients with critical *P* values (*P*=0.054) (*Online Supplementary Figure S1C*).

Of the 224 deletional Hb H patients, 185 cases (83%) were classified as mild, whereas only 3 cases (1%) were classified as severe (*Online Supplementary Figure S1D*). We found no difference in disease severity between - $-^{SEA}/-\alpha^{3.7}$ patients and - $-^{SEA}/-\alpha^{4.2}$ patients in the deletional Hb H disease group (*P*=0.42), with mean scores of 3.4 and 3.7, respectively (Figure 1E). However, all 3 cases of deletional Hb H patients classified as severe were - $-^{SEA}/-\alpha^{3.7}$ patients, with

Table 2. Scoring criteria and weighted effects of these on the severity outcome according to the regression model.

Clinical criteria	Total severity score	Points scored[a]		
		0	1	2
Age at thalassemia presentation in months	-	>120	60-120	<60
Age at receiving first blood transfusion in months		>120	36-120	<36
Transfusion frequency, times/year		0-3	4-8	>8
Growth and development[b]		>25th	3rd–25th	<3rd
Subcostal spleen size, cm		<3	03/05/23	>5 or splenectomized
Hemoglobin, g/L		>90	75-90	<75
sTfRc, mg/L		0-4.32	4.32-4.92	>4.92
Severity category Mild Moderate Severe	 0-5 6-8 9-14	-		

[a]The weighted-score was obtained by dividing the Odds Ratio of the criteria by the smallest significant Odds Ratio obtained from the multivariable logistic regression model and rounding the resulting number to the 1 or 2. [b]Percentile of growth development was assessed based on weight and height measurements plotted on a China standard growth chart. [c]sTfR: soluble transferrin receptor.

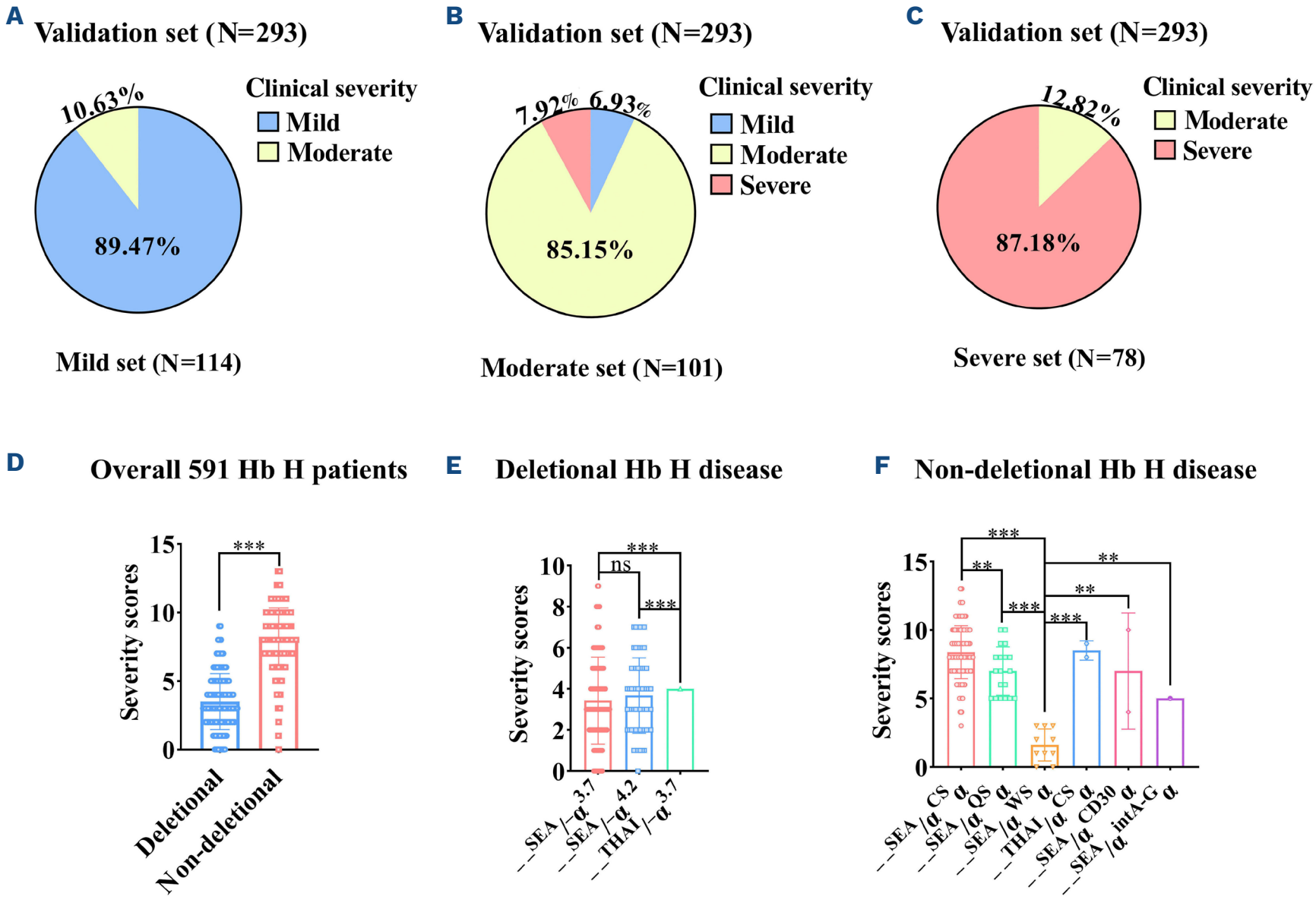

Figure 1. Distribution of disease severity in 591 hemoglobin H patients with different HBA genotypes. (A-C) Pie chart showing the distribution of clinical disease severity for patient classification by hematologists into mild, moderate, and severe groups using a severity scoring system, respectively. (D) Bar-scatter plot showing the severity score comparison between the deletional hemoglobin (Hb) H disease group and non-deletional Hb H disease group. (E) Bar-scatter plot showing the distribution of severity score for each genotype in the deletional Hb H disease cohort. (F) Bar-scatter plot showing the distribution of severity score for each genotype in the non-deletional Hb H disease cohort. (Mann-Whitney U test: $P<0.05$).

a score of 9. These patients, aged between 4 and 5 years old, exhibited severe delays in growth and development. In contrast, non-deletional Hb H disease patients generally had a more severe phenotype, with 159 out of 367 cases (43%) classified as severe (*Online Supplementary Figure S1D*). In the non-deletional Hb H disease group, HCS patients displayed the most severe symptoms and HWS patients the mildest ($P<0.0001$) (Figure 1F). Interestingly, we identified a subset of HCS patients (N=19, 6%) with mild symptoms and a score range of 3-5. None of these patients had severe growth retardation, and their Hb levels ranged from 71-122 g/L (93g/L on average). Notably, none of these patients had received regular blood transfusions in the past year, and 6 had never received blood transfusions. These findings suggest that other potential genetic factors may contribute to these unusually mild symptoms.

The heterogeneity and phenotype predictions of Hb H patients have not been comprehensively documented. To address this gap, we first established a clinical scoring system for Hb H disease severity that considered various aspects of the disease.[8] Through these efforts, we confirmed less severe clinical symptoms in the deletional than in the non-deletional Hb H patients. Meanwhile, the clinical severity of these patients' conditions was highly heterogeneous, including some cases that defied the conventional understanding of the disease (*Online Supplementary Figure S1E, F*). Our study highlights the need to consider the complexity of Hb H disease when investigating its genetic modifiers.

Despite these findings, our study has some limitations, such as the lack of a long-term patient follow-up, and the limited genotypes of Hb H patients due to the sampling including only Chinese ethnic groups. Nevertheless, we were able to leverage the available clinical records to extract valuable information on patients' growth, development, and treatment history, and to reflect on the understanding of disease progression and clinical outcomes. For example, we identified a group of HCS patients (N=57) with marginal values between the "severe" and "moderate" groups. Their scores were among the highest in the moderate group, but

few of them received regular transfusions (score = 8). They were assigned "warning" scores due to their low pre-transfusion Hb levels, high levels of serum ferritin representing iron overload, bilirubin (an indicator of hemolysis), and hepatosplenomegaly. The scoring system for Hb H disease proposed in this paper can serve as an effective tool for accurate classification and phenotype prediction of Hb H diseases, which may facilitate better treatment decisions and prognostic predictions. In addition, the scoring system can be used as a tool for prenatal diagnosis and genetic counseling, enabling families with a history of Hb H disease to make more informed decisions.

In summary, our study contributes to the understanding of Hb H disease phenotypes and provides a framework for further genetic and clinical investigations. By addressing the limitations of previous research, we hope to promote more comprehensive and personalized approaches to Hb H disease diagnosis, treatment, and prevention.

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Disclosures

No conflicts of interest to disclose.

Contributions

YL, YY, YZ and XX designed the study. YL and YZ organized the clinical data and conducted the analyses. XZ, JC and JF assessed the patients' clinical symptoms. BL and ZT conducted genetic testing. YL, PiL, XW, MS, HuL, LQ and LH collected the clinical and genetic data, and prepared the blood DNA samples. ZZ, XL, BX, PeL, JT, HaL, TZ, LY, ZL and LH performed clinical hematologic and ultrasound examinations. YL, YY and XX drafted the paper. XX supervised the study.

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Data-sharing statement

The data that support the findings of this study are available on request from the corresponding author.

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