



FULL LENGTH ARTICLE

# Genetic investigation of haemoglobinopathies in a large cohort of asymptomatic individuals reveals a higher carrier rate for $\beta$ -thalassaemia in Sichuan Province (Southwestern China)

Xia Yu <sup>a,b</sup>, Min Lin <sup>c</sup>, Chenggui Liu <sup>b</sup>, Zhiyong Liao <sup>b</sup>,  
Yongqiong Wei <sup>b</sup>, Rui Liu <sup>b</sup>, Jing Zhu <sup>a,\*</sup>

<sup>a</sup> *Pediatric Research Institute, Ministry of Education Key Laboratory of Child Development and Disorders, National Clinical Research Center for Child Health and Disorders, China International Science and Technology Cooperation Base of Child Development and Critical Disorders, Chongqing Key Laboratory of Pediatrics, Children's Hospital of Chongqing Medical University, Chongqing, 400014, PR China*

<sup>b</sup> *Chengdu Women's and Children's Central Hospital, School of Medicine, University of Electronic Science and Technology of China, Sichuan Province, 611731, PR China*

<sup>c</sup> *School of Food Engineering and Biotechnology, Hanshan Normal University, Chaozhou, Guangdong Province, 515063, PR China*

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## KEYWORDS

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**Abstract** The incidence of haemoglobinopathy is high in China, especially south of the Yangtze River. However, the exact status of haemoglobinopathy in Sichuan is unknown. To carry out a detailed research of haemoglobinopathy in individuals living in Sichuan, 13,298 subjects without clinical symptoms who were living in Sichuan Province, with an age distribution of 5–73 years, were included in this study. Between March 2014 and July 2017, these subjects received examinations at the Medical Lab of Chengdu Women's & Children's Central Hospital. Mean corpuscular volume (MCV) < 82 fL or mean corpuscular haemoglobin (MCH) < 27 pg was used to indicate haemoglobinopathy carriers. Abnormal haemoglobin was screened by electrophoresis, and genes were sequenced to identify genotypes. Genotype diagnosis of alpha- and beta-thalassaemia was carried out by using PCR and shunt hybridization. There were 638 suspected haemoglobinopathy carriers (4.80%, 638/13,298). DNA sequencing identified 6 subjects with abnormal haemoglobin genotypes and 15 subjects with Hb E. The frequency of heterozygosity for thalassaemia

\* Corresponding author.

E-mail address: [zhujingchcmu@163.com](mailto:zhujingchcmu@163.com) (J. Zhu).

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was 4.12% (1.48% for  $\alpha$ -thalassaemia and 2.61% for  $\beta$ -thalassaemia) in Sichuan Province. The mutation spectrum of  $\alpha$ -thalassaemia consisted of the five most common mutations:  $^{-SEA}$ ,  $^{-\alpha^{3.7}}$ ,  $^{-\alpha^{4.2}}$ ,  $\alpha^{CS}$ , and  $\alpha^{QS}$ . Seven types of  $\beta$ -thalassaemia mutation were found in this study: CD41-42 (-TTCT) was the most frequent (28.47%), followed by 17 (A > T), -28 (A > G), and IVS-II-654 (C > T). The main abnormal haemoglobin genotype (HbE) and thalassaemia genotype ( $^{-SEA}$ , CD41-42 (-TTCT)) were consistent with those in other regions of China, but the carrier rate of  $\beta$ -thalassaemia in Sichuan was higher than that of  $\alpha$ -thalassaemia.

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## Introduction

Haemoglobinopathy is a family of hereditary haematological diseases caused by abnormal haemoglobin with regard to molecular structure and quantity (globin peptide chain synthesis rate). Haemoglobinopathies can be divided into two categories according to the pathogenic mechanism involved: haemoglobin variant and thalassaemia.<sup>1,2</sup> The substitution, mutation, and fusion of genes lead to changes in the primary structure of the globin gene, resulting in altered biological properties of haemoglobin, such as solubility, oxygen affinity, molecular configuration, and stability.<sup>3</sup> Clinical manifestations may include haemolytic anaemia and methemoglobinemia, such as the most common abnormal haemoglobin E.<sup>4</sup> Thalassaemia, also known as Mediterranean anaemia, is characterized by the lack or decrease in one or more of the globin peptide chain of haemoglobin, causing changes in the haemoglobin composition. The clinical symptoms of thalassaemia vary from mild to severe, with chronic progressive haemolytic anaemia being the most common.<sup>5</sup> Additionally, thalassaemia can be divided into  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ - $\beta$ - and  $\gamma$ - $\delta$ - $\beta$ -thalassaemia based on the type of abnormal globin, and the most common types are  $\alpha$ - and  $\beta$ -thalassaemia.<sup>6</sup>

According to the database of human haemoglobin variation and thalassaemia (<http://globin.bx.psu.edu>), researchers around the world have found 1799 globin gene variants, of which 1338 result in abnormal haemoglobin and 500 in thalassaemia (some variants cause both).<sup>7</sup> Haemoglobinopathy is the most common single-gene hereditary disease in the world,<sup>8</sup> but the study of this disease in China began relatively late. Nonetheless, the incidence of haemoglobinopathy in China is very high, especially south of the Yangtze River.<sup>9</sup> Indeed, haemoglobinopathy is common in tropical regions but is now found in most countries due to population migration.<sup>10</sup> Sichuan is an important province in southwest China with a registered population of 83.41 million by the end of 2018 (<http://www.sc.gov.cn>). Furthermore, Sichuan was the main destination of the immigrants from Huguang during the Qing Dynasty.<sup>11</sup> The exact status of haemoglobinopathy in Sichuan is unclear.

Therefore, the aim of the present study was to carry out a detailed screening and gene analysis of haemoglobinopathy in individuals living in Sichuan. The results should provide valuable information regarding genetic counselling

that might be used to decrease the rate of infants born with serious thalassaemia.

## Materials and methods

### Study design and subjects

A total of 13,298 subjects without clinical symptoms living in Sichuan Province were included, with an age distribution of 5–73 years. Between March 2014 and July 2017, these subjects received examinations at the Medical Lab of Chengdu Women's & Children's Central Hospital in Sichuan Province. The study was approved by the ethics committee of the Chengdu Women's & Children's Central Hospital. All subjects provided written informed consent for participation in this study.

The inclusion criteria were as follows: 1) Sichuan residents; 2) Chinese Han; 3) no history of pregnancy; and 4) no known blood disease. All patients with a known primary or secondary cause of anaemia were excluded.

### Haematological parameters and red blood cell indices

Peripheral blood (2 mL) anticoagulated with 4.0 mg of K<sub>2</sub>-EDTA was collected from each subject. The amount of red blood cells (RBCs) was determined using an XN-1000 Blood Analyser (Sysmex Corporation, Kobe, Japan) in accordance with standard laboratory procedures. Quality control data for complete blood counts (CBCs) were collected according to the China National External Quality Assurance Mechanism. Subjects with a mean corpuscular volume (MCV) value (<82.0 fL) or mean corpuscular haemoglobin (MCH) value (<27 pg) were considered to be thalassaemia carriers.<sup>12</sup> Haemoglobin electrophoresis was performed using the Capillarys 2 Flex-piercing automatic capillary electrophoresis apparatus (Sebia, Lisses, France). The main indicators were haemoglobin A (HbA), haemoglobin A<sub>2</sub> (HbA<sub>2</sub>), haemoglobin F (HbF), and abnormal haemoglobin content determination (Capillary 2 Flex Piercing, Sebia, Paris, France).

### Abnormal haemoglobin with DNA sequence

Blood DNA was extracted using a blood genomic DNA extraction kit (Beijing Tiangen Biotechnology Co., Ltd.,

Beijing, China). The HbE variation was identified by PCR and flow-through hybridization.

DNA sequencing of the  $\alpha$ 1- and  $\alpha$ 2-globin genes was performed using the following primers:  $\alpha$ 1-F (5'-CTCCGCGCCAGCCAATGAG-3'),  $\alpha$ 1-R (5'-AGCTGCAGAGAGGTTCTAGCCAT-3');  $\alpha$ 2-F (5'-CTCCGCGCCAGCCAATGAG-3'),  $\alpha$ 2-R (5'-CAGCTGCAGAGAGGTCCTTGGTC-3'). DNA was amplified using a gene amplification kit (Qiagen, GmbH, Hiden, Germany). Reactions were carried out using an MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). After initial denaturation at 95 °C for 15 min, 35 cycles at 97 °C for 50 s, 60 °C for 1 min, and 72 °C for 2 min were performed, followed by a final extension at 72 °C for 10 min. The  $\alpha$ 1- and  $\alpha$ 2-globin gene fragments were sequenced using an ABI 3700 automated sequencer (Applied Biosystems, Foster City, CA, USA).

DNA sequencing of the  $\beta$ -globin gene was performed using three pairs of primers:

$\beta$ 1-F (5'-CGGTGTCATCACTTAGACCT-3'),  $\beta$ 1-R (5'-CAGCTCACTCAGTGTGGCAAA-3');  $\beta$ 2-F (5'-GCTGTTATGGGCAACCT-3'),  $\beta$ 2-R (5'-TTGCTATTGCCTTAACCCAGA-3');  $\beta$ 3-F (5'-ATGTATCATGCCTCTTTGCAC-3'),  $\beta$ 3-R (5'-ATGTATCATGCCTCTTTGCAC-3'). The DNA was amplified using a gene amplification kit (Qiagen, Germany) and MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). The reactions were carried out with incubation at 37 °C for 5 min, followed by an initial denaturation at 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min. Sequencing of the DNA fragments was performed using an ABI 3700 automated sequencer.

### Molecular diagnosis of $\alpha$ - and $\beta$ -thalassaemia

The  $\alpha/\beta$ -thalassaemia gene detection kit (PCR and flow-through hybridization method; Guangdong Kaipu Biotechnology Co., Ltd., Guangdong, China, Reg. No: SFDA (P) 20153401664) was used to detect three known  $\alpha$ -thalassaemia deletions (i.e.,  $-\alpha^{SEA}$ ,  $-\alpha^{3.7}$ , and  $-\alpha^{4.2}$ ), three non-deletional  $\alpha$ -thalassaemia mutations ( $\alpha^{CS}$ ,  $\alpha^{QS}$ , and  $\alpha^{WS}$ ) and the 19  $\beta$ -thalassaemia mutations most commonly seen in the Chinese population [-30 (T > C), -32 (C > A), -28 (A > G), -29 (A > G), Cap+40-43 (-AAAC), Cap+1 (A > C), Int (T > G), CD14/15 (+G), CD17 (A > T), CD27/28 (+C),  $\beta$ EM (G > A), CD31 (-C), CD41/42 (-TCTT), CD43 (G > T), CD71/72 (+A), IVS-I-1 (G > T, G > A), IVS-I-5 (G > C), and IVS-II-654 (C > T)]. The assay was performed according to the manufacturer's instructions.

### Statistical analysis

SPSS23.0 software (IBM, Armonk, NY, USA) was used for the statistical analysis. The Hardy-Weinberg formula was used to calculate the haemoglobin allele frequencies and heterozygous frequencies and determine whether they are representative of the population. Otherwise, descriptive statistics were used. Two-sided  $P < 0.05$  were considered statistically significant.

## Results

### Characteristics of the subjects

The age of the subjects ranged from 5 to 73 years old. There were 7454 males and 5844 females. All subjects were Chinese Han living in Sichuan.

### Haemoglobin variants in Sichuan Province

Among the 13,298 subjects, there were 638 cases of MCV <82 fL or MCH <27 pg, for a phenotypic positivity rate of 4.8% (638/13,298). There were 21 cases of 4 major abnormal haemoglobin bands found by capillary electrophoresis, including one case of Z13, three cases of Z12, two cases of Z (D), and 15 cases of Z (E) (Fig. 1).

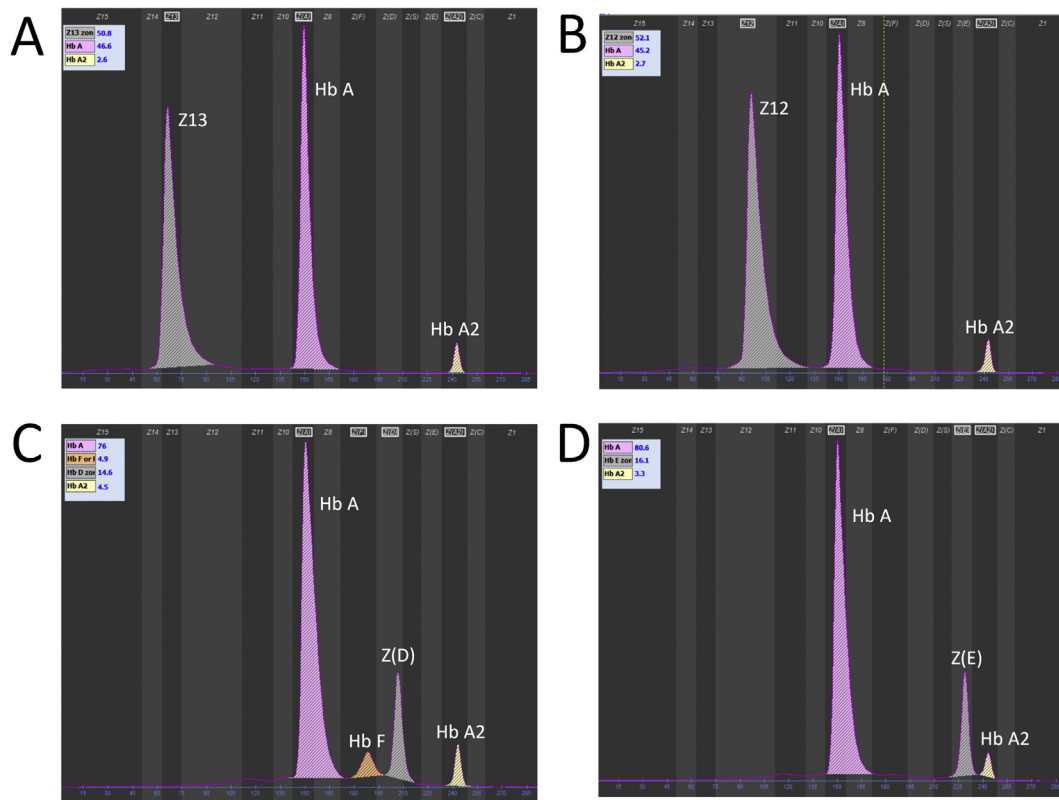
### Globin gene mutations

Data for 21 subjects (six with abnormal  $\alpha$ -chains and 15 with abnormal  $\beta$ -chains) were collected (Table 1). DNA sequencing identified six variants and 15 subjects with HbE, including two HbG-Chinese, one HbJ-Shenyang, two HbJ-Bangkok, and one HbJ-Lome (Fig. 2A-D). Moreover, 15 HbE [ $\beta$ 26 (B8) Glu → Lvs] variants were revealed (Fig. 2E). Most were found to be silent  $\alpha$ -thalassaemia gene variations that would not influence the function or stability of haemoglobin, with no clinical effects. Table 2 shows that the variants were in Hardy-Weinberg equilibrium.

### Prevalence and $\alpha/\beta$ -thalassaemia mutation spectrum

Among the 13,298 subjects, 638 (4.26%) showed microcytosis (MCV <82.0 fL or MCH <27 pg). The thalassaemia genetic diagnostic kit combining GAP-PCR and hybridization technology was applied to analyse the thalassaemia genotypes (Fig. 3A) and detect the full spectrum of mutations; several representative results were found (Fig. 3B). As shown in Table 2, 548 mutant chromosomes, consisting of 197  $\alpha$ -thalassaemia, 347  $\beta$ -thalassaemia, and four both  $\alpha$ - and  $\beta$ -thalassaemia, were identified, indicating a frequency of heterozygosity for thalassaemia of 4.12% ( $\alpha$ -thalassaemia of 1.48% and  $\beta$ -thalassaemia of 2.61%) in Sichuan Province. In addition, four carriers with both  $\alpha$ - and  $\beta$ -thalassaemia mutations were detected (0.03%, 4/13,298).

The mutation spectrum of  $\alpha$ -thalassaemia consisted of the five most common mutations:  $-\alpha^{SEA}$ ,  $-\alpha^{3.7}$ ,  $-\alpha^{4.2}$ ,  $\alpha^{CS}$ , and  $\alpha^{QS}$ . Among them,  $-\alpha^{SEA}$  (20.44%) and  $-\alpha^{3.7}$  (11.86%) were the most common  $\alpha$ -thalassaemia types among all  $\alpha$  mutations (including  $\alpha$ -thalassaemia carriers and carriers with both  $\alpha$ - and  $\beta$ -thalassaemia). Seven types of  $\beta$ -thalassaemia mutation were found in this study; CD41-42 (-TTCT) was the most frequent (28.47%), followed by 17 (A > T), -28 (A > G), and IVS-II-654 (C > T), with allele frequencies of 18.61%, 8.76%, and 6.20%, respectively (Table 3). All mutations were in Hardy-Weinberg equilibrium (Table 4 and 5).



**Figure 1** Peak diagram of abnormal haemoglobin electrophoresis. (A) Anomaly zone Z13. (B) Anomaly zone Z12. (C) Anomaly zone Z(D). (D) Anomaly zone Z(E).

**Table 1** Molecular findings of five types of  $\alpha$ - and  $\beta$ -chain variants in Sichuan province.

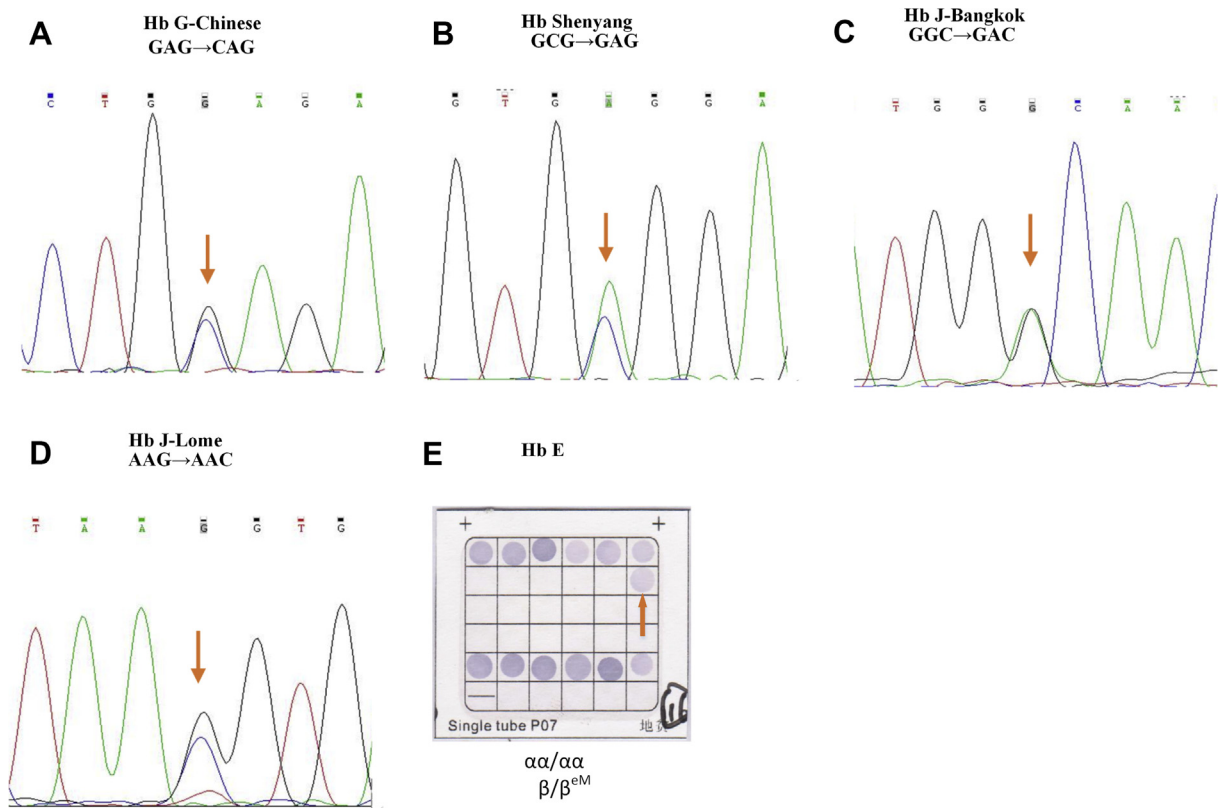
Globin variants	Gene mutants	Mutation site	Amino acid changes	Cases	Percentage (%)
$\alpha$ -globin gene mutation					
Hb G-Chinese	HBA2: c.91G > C	30 (B11)	Glu > Gln	2	9.52
HbJ-Shenyang	HBA2: c.80C > A	27 (B7)	Ala > Glu	1	4.76
$\beta$ -globin gene mutation					
Hb J-Bangkok	HBB: c.170G > A	56 (D7)	Gly > Asp	2	9.52
Hb J-Lome	HBB: c.180G > C	59 (E3)	Lys > Asn	1	4.76
Hb E	HBB: c.79G > A	26 (B8)	Glu > Lys	15	71.44
Total				21	100

## Discussion

Haemoglobinopathy is the most common hereditary genetic blood disorder that can result in clinical phenotypes varying from almost asymptomatic to lethal haemolytic anaemia. As a result of the protection against malaria conferred to haemoglobinopathy carriers, hereditary Hb disorders are common in tropical regions. Although these diseases show typical regional and ethnic differences,<sup>10</sup> they can be found in most countries due to population migration.<sup>13</sup> As investigating the prevalence of haemoglobinopathy in different areas may help in prevention, the purpose of this study was to screen and analyse haemoglobinopathy in the Sichuan Han population. The results suggest that the main abnormal haemoglobin genotype (HbE) and thalassaemia genotype

( $-\text{SEA}$ , CD41-42 (-TTCT)) were consistent with other regions of China, though the carrier rate of  $\beta$ -thalassaemia in Sichuan was higher than that of  $\alpha$ -thalassaemia.

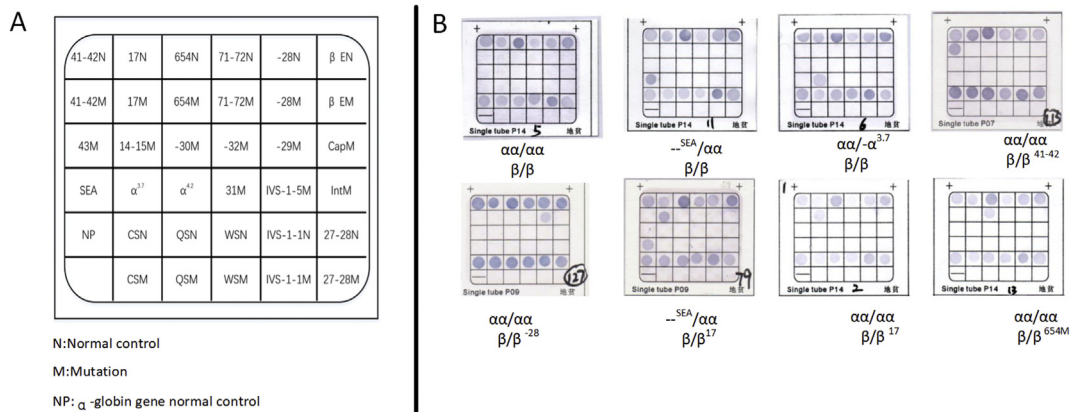
Studies have reported that the rate of those carrying abnormal haemoglobin genes in Sichuan is 0.158%, which is significantly lower than that in high-incidence areas such as Guangdong, Guangxi and Yunnan.<sup>14–16</sup> The rate in Yunnan, the province with the highest rate in China, is as high as 6.06%. Genetic analysis of abnormal haemoglobin genes found the highest incidence for an abnormal  $\beta$ -chain, accounting for 85.71%, of which HbE was the most common, which is consistent with previous reports from southern provinces such as Guangdong and Guangxi. The rate of  $\beta$ -chain variation is also the most common type in Southeast Asia, and it is thought to have spread northward from southern regions.<sup>17</sup> HbE is due to



**Figure 2** Sequencing maps of four abnormal haemoglobin and PCR and conduction hybridization results of HbE. (A) Hb G-Chinese (GAG→CAG at CD 30 of  $\alpha 2$ -globin). (B) Hb Shenyang (GCG→GAG at CD 26 of  $\alpha 2$ -globin). (C) Hb J-Bangkok (GGC→GAC at CD 56 of  $\beta$ -globin). (D) Hb J-Lome (AAG→AAC at CD 59 of  $\beta$ -globin). (E) PCR and conduction hybridization results of HbE ( $\beta^{EM}$ ).

**Table 2** Hardy–Weinberg equilibrium analysis of abnormal hemoglobins in Sichuan area.

	A/a	A/A	a/a	Total
Actual frequency	21	0	13,277	13,298
Expected frequency	20.980,964	0.008289	13,277.01	
Gene frequency	0.001579	0	0.998,421	
$\chi^2$ value	0.008304			
P value	0.927,393			



**Figure 3** Spectrum in the thalassemia gene diagnostic kit (A). Representative of mutations detected (B).

**Table 3** Population prevalence and genotypes of thalassaemia among 548 blood samples from Sichuan Province.

	Mutations	Type	<i>n</i>	Frequency (%)
$\alpha$ -thalassaemia	$^{-SEA}/\alpha\alpha$	alpha-1	112	20.44
	$^{-\alpha^{3.7}}/\alpha\alpha$	alpha-2	65	11.86
	$^{-\alpha^{4.2}}/\alpha\alpha$	alpha-2	15	2.74
	$^{-\alpha^{QS}}/\alpha\alpha$	HbVar	4	0.73
	$^{-\alpha^{CS}}/\alpha\alpha$	HbVar	1	0.18
	Total		197	35.95
$\beta$ -thalassaemia	41/42 (-TCTT)/N	beta0	156	28.47
	17 (A-T)/N	beta+	102	18.61
	-28 (A-G)/N	beta+	48	8.76
	IVS-II-654 (C-T)/N	beta+	34	6.20
	-29 (A-G)/N	beta+	3	0.55
	27/28(+C)/N	beta0	3	0.55
	43(G-T)/N	beta0	1	0.18
	Total		347	63.32
$\alpha$ -thal & $\beta$ -thal	$^{-SEA}/\alpha\alpha&41/42(-TCTT)/N$		2	0.37
	$^{-SEA}/\alpha\alpha&17(A-T)/N$		1	0.18
	$^{-\alpha^{3.7}}/\alpha\alpha&17(A-T)/N$		1	0.18
Total		548	100	

**Table 4** Hardy–Weinberg equilibrium analysis of alpha-thalassaemia gene in Sichuan.

	A/a	A/A	a/a	Total
Actual frequency	197	0	13,101	13,298
Expected frequency	195.5408	0.729,602	13,101.73	
Gene frequency	0.014,814	0	0.985,186	
$\chi^2$ value	0.740,532			
<i>p</i> value	0.389,491			

**Table 5** Hardy–Weinberg equilibrium analysis of  $\beta$  thalassaemia genes in Sichuan area.

	B/b	B/B	b/b	Total
Actual frequency	347	0	12,951	13,298
Expected frequency	342.4727	2.263,667	12,953.26	
Gene frequency	0.026,094	0	0.973,906	
$\chi^2$ Value	2.323,912			
<i>p</i> Value	0.127,399			

a mutation in the 26th codon of the  $\beta$ -globin gene (GAG  $\rightarrow$  AAG), resulting in decreased synthesis. Simple HbE heterozygotes and homozygotes generally do not exhibit anaemia symptoms or only exhibit a decrease in MCV and MCH among haematological parameters; very few homozygotes exhibit manifestations of thalassaemia, such as moderate anaemia. Nevertheless, severe anaemia can result when HbE is combined with  $\beta$ -thalassaemia, especially with  $\beta^0$  thalassaemia.<sup>18</sup> Accordingly, this should be considered in prenatal diagnosis. The abnormal haemoglobin variants HbE, Hb J-Bangkok, and Hb G-Chinese, which are more common in southern China,<sup>19,20</sup> were also found in the present study. The typically geographically diverse nature of these variants is also reflected in the genetics perspective that the main source of the population in Sichuan is from the southern region of China. The present study also found a  $\beta$ -globin variant, Hb J-Lome,

which is a fast haemoglobin variant. It was first discovered in 1997 by Wajcman et al in a Black family in Lomé, the capital of Togo<sup>21</sup>; it has mainly been reported among the populations of Africa, Japan, and Vietnam. In contrast, Hb J-Lome has rarely been reported in China,<sup>22,23</sup> and the present study constitutes the third report. The presence of this rare mutation in Sichuan may also be caused by marriage or population migration.

In this study, a total of 548 thalassaemia-positive subjects were detected among 13,298 cases in Sichuan, with a carry rate of 4.12%, with the rate of  $\alpha$ -thalassaemia being 1.48%, far lower than that of 23.9% in the Sichuan area reported by He et al.<sup>24</sup> The reason may be due to the different research populations. The subjects in He's and other studies were outpatients and hospitalized patients with suspected thalassaemia, whereas our research subjects were from the medical examination population in

various regions of the province and can more objectively reflect the rate among the general population.

The clinical manifestation of SEA homozygous patients with severe thalassaemia is Bart's Hydrops Syndrome, usually causing suffocation death in the third trimester of pregnancy or within several hours after birth.<sup>25</sup> In the present study, the <sup>-SEA</sup> genotype was most common, suggesting that the probability of Bart's Hydrops Syndrome in Sichuan is relatively high. Therefore, for married carriers, prenatal diagnosis should focus on the detection of Bart's Hydrops Syndrome.<sup>26</sup>

The carry rate of  $\beta$ -thalassaemia was 2.61%, and the main gene types were CD41-42 (-TTCT), 17 (A-T), -28 (A-G), and IVS-II-654 (C-T), accounting for 98% of the total genotypes of  $\beta$ -thalassaemia, which was consistent with the major genotypes reported in the southern provinces of Guangdong and Guangxi.<sup>27,28</sup> It has also been confirmed that the population of Sichuan is mainly from the southern part of China. Nevertheless, thalassaemia in Sichuan developed its own characteristics over time. Compared with other provinces in southern China, the carry rate of  $\beta$ -thalassaemia in Sichuan was higher than that of  $\alpha$ -thalassaemia. According to our data, 2.61% of the population harbours mutations in the  $\beta$ -globin gene; there were approximately 920,000 births in Sichuan province in 2018. Thus, it is estimated that the number of children with moderate-to-severe  $\beta$ -thalassaemia to be born each year is approximately 157. Children with severe  $\beta$ -thalassaemia have no clinical symptoms at birth, though symptoms usually begin to appear within 3–6 months.<sup>29</sup> The current routine treatment of this disease is regular blood transfusion, which brings economic burden to the family and consumes a certain amount of social resources. Therefore, our results indicate that severe  $\beta$ -thalassaemia is a public health issue in Sichuan.

This is the latest study to examine the molecular characteristics of haemoglobinopathies in Sichuan Province. On the basis of our results, local government and medical staff should pay more attention to increasing knowledge about haemoglobinopathy and screen the population, particularly for  $\beta$ -thalassaemia. This may decrease the rate of infants born with haemoglobinopathy and significantly improve public health.

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## Conflict of Interests

The authors declare no conflict of interests.

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