RESEARCH ARTICLE

The prevalence and spectrum of thalassemia in Changsha, Hunan province, China: discussion of an innovative screening strategy

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Running title: Thalassaemia in Changsha of China

Abstract

Hunan province located in the south of China has a high incidence of hemoglobinopathies. In the present study, we surveyed the accurate population frequency data of the local population in Changsha City of Hunan Province in China. The data include the carrying rate, gene mutation types, and their distribution features for thalassemia. In total, 7,500 consecutive samples from five geographical areas of Changsha were analyzed for both hematological and molecular parameters. There was a high prevalence of carriers of α-thalassemia (2.57%), β-thalassemia (1.9%), and both α- and β-thalassemia (0.08%). Overall, 4.54% of the population in this area represented heterozygous carriers of α- and β-thalassemia. The mutation spectrum of α- and β-thalassemia and its hematological characterization were fully described for this area. The present study is the first to report the prevalence of thalassemia in Hunan province population. Both α- and β-thalassemia carriers are widely distributed in Changsha. The knowledge gained from the present study will allow for an estimation of the

projected number of pregnant women at risk for thalassemia, and the design of a screening strategy for the control of thalassemia in Changsha.

Keywords

Thalassaemia; Changsha; Hunan province

Introduction

Thalassemia is the most common autosomal recessive disorder worldwide (Joly *et al*. 2014; Sabath *et al*. 2015). Thalassemia has two main forms, alpha [α]- and beta [β]-thalassemia (Bank 2015). The phenotype of α-thalassemia depends upon the degree of α-globin chain deficiency relative to β-globin production (Liebhaber and Kan 1983; Piel and Weatherall 2014). The Bart's hydrops fetalis syndrome is characterized by severe intrauterine anemia, and is lethal *in utero* or soon after birth, due to a homozygous α^0 thalassemia mutation that results in the loss of all four globins (Chen *et al*. 2015). Hemoglobin H disease is an intermediate form, having moderately severe but variable anemia that is caused by the loss of three functional a genes that mostly combine to form an α+ thalassemia (2α/ or $\alpha^T\alpha$/) and an α^0 thalassemia defect (Chen *et al*. 2000). β-thalassemia is caused by the absence (β^0) or ineffective (β^+) synthesis of β-globin chains (Joly *et al*. 2014). Thalassemia major involves the inheritance of two mutant β-globin alleles and is a severe transfusion-dependent anemia. If left untreated with regular blood transfusions, Thalassemia major can lead to death within the first year of life (Van de Velde *et al*. 2004). To date, Over 200 different β-globin mutations have been identified, with the majority being single nucleotide substitutions, deletions, or insertions of nucleotides leading to a frame-shift; rarely does β-thalassemia results from a gross gene deletion (Bank 2015; Colah *et al*. 2010; Higgs 1993).

Thalassemia is most prevalent in developing countries. Approximately 5% of the global populations are carriers of thalassemia, and more than half of these cases occur in South East Asia (Colah *et al*. 2010). In China, there is a high population frequency of thalassemia in the southern regions of the Yangtze River, particularly in the three most southerly provinces of Guangdong, Guangxi, and Hainan (Shi *et al*. 2011). Hunan province is located at the south bank of the middle reaches of the Yangtze River, adjacent to the Guangdong and Guangxi province. In a previous report, the incidence of thalassemia in Shenzhen inhabitants who migrated from Hunan Province was 4.18% (α-thalassemia: 2.15%; β-thalassemia: 2.03%), suggesting that thalassemia is highly prevalent in the Hunan populations (Huang *et al*. 2006; Li *et al*. 2005). However, little is known about the prevalence of thalassemia or the molecular characteristics of the inhabitants of Changsha in Hunan province.

In the present study, we performed a large-scale prenatal screening of α-thalassemia and β-thalassemia in 7,500 samples from Hunan province using hematological and molecular analyses. We aimed to determine the prevalence of thalassemia and the molecular characteristics of the inhabitants of Changsha to provide an innovative strategy for carrier screening of pregnant couples, genetic counseling, and prenatal diagnosis.

Materials and methods

Population samples

The study population included 7,500 individuals (5,584 women and 1,184 men) from five regional maternal and Child Health Hospitals (1,500 specimens were collected in each hospital) who attended a clinic for prenatal testing in Changsha between May 2014 and May

2015. These regions--Liuyang county (east), Changsha county (middle east), the Changsha district (middle), Wangcheng (middle west), and Ningxiang (west)--have the largest populations of Changsha city (Figure 1). All of the samples used in the study were of Han Chinese and Changsha descent. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Changsha Maternal and Child Health Hospital. Written informed consent was obtained from all participants' guardians.

Screening strategy and experimental analysis

The diagnostic flowchart used in this study is illustrated in Figure 2. We designed a strategy that combined phenotypic screening and genotyping (Xiong et al. 2010, Xu et al. 2004, Zeng et al. 2001). All 7,500 participants were screened for the presence of defects using two procedures. We used a hematological screening protocol to detect all of the suspected subjects who had hematological phenotypes based on full blood counts (FBCs) and hemoglobin test as described previously (Daniel 2007). We found 799 positive samples, and all positive samples were further characterized using a molecular diagnosis, as described previously (Chatterjee *et al*. 2014) and guided by blood analysis to identify three different types of hemoglobin disorders. We found 172 α-thalassemia carriers, 143 β-thalassemia carriers, 6 α-thalassemia compound β-thalassemia carriers based on this confirmative test. For those subjects who had no positive hematological phenotypes, we directly used a DNA-based molecular screening protocol to detect α-thalassemia silent carriers (Atanasovska *et al*. 2012). A total of 21 such carriers were detected using this protocol. The criteria that indicated the

possibility of heterozygosity for various thalassemia types were based on previous studies (Jia *et al*. 2004; Yang *et al*. 1989).

Hematological analysis

Hematological screening was performed on all specimens in the Child Health Hospitals of Changsha. A total of 2 ml of peripheral blood was collected into a K3-EDTA tube and used for peripheral blood counts and red blood cell indices according to standard laboratory procedures for the F820 type blood cell analysis system (CIS Company, Chiba, Japan). Subjects with low red blood cell mean corpuscular volume (MCV) values (80 fl) or low mean corpuscular hemoglobin (MCH) values (27 pg) were considered possible thalassemia carriers and deeply analyzed using a high speed automatic electrophoresis analytic system (Sebia, France) to assess the concentration of hemoglobin (Hb) A, A2, and F, and any abnormal hemoglobin. The carrier state for phenotypic β-thalassemia or β-thalassemia compounded with α-thalassemia was detected in samples in which both the concentration of Hb A2 exceeded 3.5%, and the mean corpuscular volume (MCV) was lower than 80 fl. A low concentration of Hb A2 (less than 2.5%) should be present in α-thalassemia carriers.

DNA analysis

The molecular diagnosis of α- and β-thalassemia was performed on specimens that have a high risk of being possible thalassemia carriers. For these samples, genomic DNA was extracted from peripheral blood leukocytes using the DNA Blood Mini Kit (Yi Sheng Tang China Shengzhen Co., Ltd). The three known α-thalassemia deletions –SEA, -a3.7, and -a4.2, the three α-thalassemia mutations [Hb Constant Spring (HBA2: c.427T.C), Hb WS (HBA2:

c.369C>G) and Hb Quong Sze (HBA2: c.377T.C)], and 17 known β-thalassemia mutations that are most commonly observed in Chinese populations (HBB: c.279A.G, HBB: c. 278A.G, HBB: c.250A. C, HBB: c.2T.G, HBB: c.45_46insG, HBB: c.52A.T, HBB: c.79G.A, HBB: c.84_85insC, HBB: c.94delC, HBB: c.92+1G.T HBB: c.92+1G.A, HBB: c.92+5G.C, HBB: c.126_129delCTTT, HBB: c.130G.T, HBB: c.216_217insA, HBB: c.316–197C.T; HBB: c.79G>A) were analyzed using a commercial thalassemia reverse dot blot (RDB) gene chip (Yi Sheng Tang China Shengzhen Co., Ltd). Sequencing was performed for the specimens that were not phenotyped using the reagent kit for detecting an abnormal blood phenotype.

Statistical analysis

Statistical analysis was conducted with SPSS 19.0 statistical software. The prevalence of different thalassemia alleles was calculated using the standard Hardy-Weinberg formula. All statistical tests were two-tailed test, and the data were deemed significantly different when $P < 0.05$.

Results

The details of the survey of population are shown in Table 1. Of the 7,500 participants who received hematological screening, 10.7% (799/7,500) were microcytosis carriers (MCV <80 fl or MCH <27 pg). Of these, 643 carriers exhibited α-thalassemia with a Hb A2 concentration of less than 2.5%, based on hemoglobin electrophoresis. In addition, 156 carriers presented with an Hb A2 concentration that was higher than 3.5%; these were considered as carriers of α-thalassemia or β-thalassemia compounded with α-thalassemia. We also used DNA sequencing to characterize point mutations in the α-, β- or δ-globin gene. We

identified a total of 341 cases as carriers, of which 193 (2.57%) had α-thalassemia, 143 (1.92%) had β-thalassemia, and 6 (0.08%) had both α- and β-thalassemia (Table 1). A total of 193 samples were analyzed for the most common three α-thalassemia deletions (–SEA, a3.7, and a4.2) using Gap PCR. The other three non-deletion types of α-thalassemia mutations (Hb Constant Spring, Hb Quong Sze, and Hb Westmead) were detected using an RDB gene chip (Table 2). A total of 156 microcytosis carriers were deeply genotyped for both α-thalassemia and β-thalassemia, the latter were detected using an RDB gene chip for analysis of 17 common mutations in Chinese populations. If we could identify a mutation, we then characterized the sample using DNA sequencing. We found that the prevalence of thalassemia mutations in the Changsha population is 4.55% (Table 3).

Discussion

Thalassemia is common in southern China, especially in the Guangxi province (Yin *et al*. 2011). The screening of severe determinants of thalassemia is critically important for the management and control of thalassemia. The management and control of thalassemia remain unsatisfactory and suboptimal in China compared to other countries where the disease is prevalent.

No previous study has investigated thalassemia in the city of Changsha in Hunan Province of China. To estimate the future burden of this disease and the requirements for its control in Changsha, the city has begun screening for thalassemia using premarital pregnancy tests and gene screening because epidemiological studies of the disease indicate a high incidence in similar "poor" areas, such as Sichuan and Guizhou (Cai *et al*. 2010; Huang *et al*. 2013). Previous studies have suggested that thalassemia is only highly prevalent in the cities of

Guangdong and Guangxi; however, large-scale thalassemia epidemiological survey data has not been collected in Hunan Changsha regions. Our study reveals the actual rate of the population of gene mutation spectrum carriers in Changsha, and characteristics of the thalassemia gene mutation types. Our results show that the prevalence of thalassemia mutations in the Changsha population is 4.55% (Table 3). From the Table 3, the rate of thalassemia is different between the 5 districts of Changsha, Liuyang and the urban area of the city the highest and the Wangcheng district the lowest. Regional features and population mobility may account for the differences. Liuyang is located at the north-east of the city and neighbors with Jiangxi province, leading to high movement of population, while the urban area of Changsha is where the capital of the province located and the movement of population is naturally high. High population movement results in the increase of thalassemia rate. In contrast, the Wangcheng district is located at the north of the city and its population movement is relatively low.

Based on our results on the epidemiological regularity of thalassemia, a series of effective screening programs for the region can be established. The Chinese Center for Disease Control and Prevention sought to establish the molecular basis of thalassemia to provide first-hand data for its prevention, and determine the prevalence of thalassemia in high-risk areas, such as Changsha. To reduce the frequency of thalassemia in subsequent generations in Changsha, our results support the implementation of programs to screen for carriers of the common severe determinants of thalassemia.

Conflicts of interest

All of the authors declare that they have no conflicts of interest regarding this paper.

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Figure legends

Figure 1. Showed the location of Changsha city in Hunan province and the five study regions including Liuyang county(east), Changsha county(middle east), Changsha distrct (middle), Wangcheng (middle west), Ningxiang (west)

Figure 2. Diagnostic flowchart for the detection of Hb disorders in this study.
The positive sample numbers detected at each step are indicated in the left most position. In the molecular screening step, Gap-PCR was used for genotyping ofthe −α3.7/ and −α4.2/ deletions and the--SEA/ mutation, respectively. In the confirmative test step, Gap-PCR was used to type the common known α-thalassaemia deletions or δβ-thalassemia/HPFH deletions; MLPA method was used to detect unknown gross deletions in the α- or β-globin gene cluster; RDB was used to detect known point mutations causing α- or β-thalassaemia; DNA sequencing was used to identify novel or rare point mutations in the entire α1- and α2- or

β-globin gene. The detailed information on the primer design and PCR/DHPLC conditions is available. In addition, DNA sequencing was used to characterize point mutations in the α-, β- or δ-globin gene, in all samples that contained a Hb band that migrated abnormally. All individuals with β-thalassaemia were analysed to determine whether they had co-inherited one of the six α-thalassaemia defects (– –SEA/, −α3.7/, −α4.2/, αWSα/, αQSα/ and αCSα/) that are common among the Chinese.

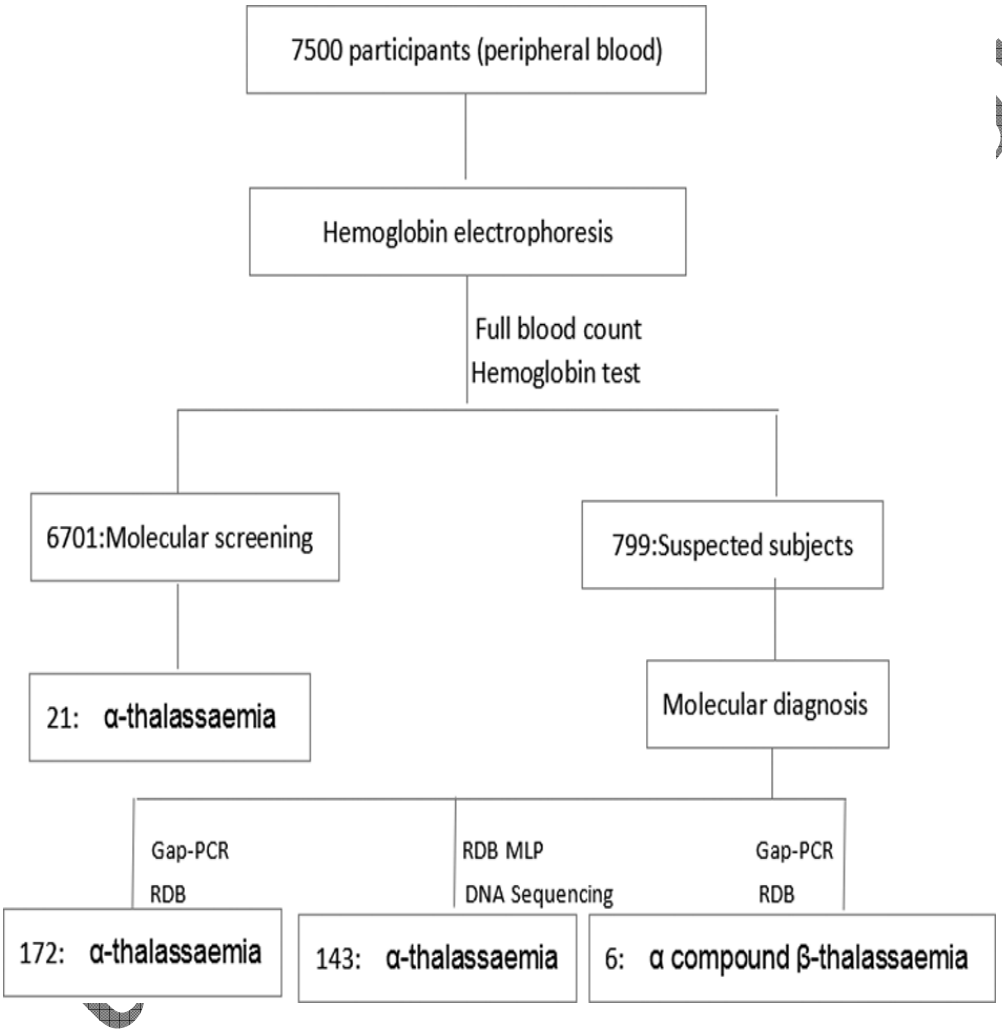

Table 1. Results of Screening of Blood Samples from 7500 Adults for Thalassemia in Changsha, Hunan Province, People's Republic of China

Mutation	n	%
α-Thalassemia	193	2.57
—SEA deletion	121	1.61
–α3.7 deletion	47	0.6
–α4.2 deletion	11	0.14
–αQS mutation	6	0.08
–αCS mutation	4	0.05
–αWS mutation	3	0.04
β-Thalassemia	143	1.92
IVS-II-654 (C>T)	48	0.64
CD 41-42 (–CTTT)	44	0.59
CD 17 (AAG>TAG)	17	0.23
–28 (A>G)	5	0.07
CD 71/72 (+A)	5	0.07
CD 26 (G>A)	5	0.07
CD 43 (GAG>TAG)	5	0.07
–29 (A>G)	4	0.05
CD 27/28 (+C)	3	0.04
IVS-1-1 (G>T)	1	0.01
IVS-II-5(G>C)	1	0.01

CD 30 (AGG>GGG)	1	0.01
CD 95 (+A)	1	0.01
CD 112(TGT>TGA)	1	0.01
CD 54-58165-177del	1	0.01
CD 31 (-C)	1	0.01
α- compound β-Thalassemia	6	0.08
–α3.7 compound CD17	1	0.01
--SEA compound CD41-42	2	0.02
-3.7 compound IVS-II-654	2	0.02
--3.7 compound CD26	1	0.01

Table 2. α-Globin Genotypes and α-Thalassemia Frequency in Changsha, Hunan Province, China.

Genotype	n	%
—SEA/αα	121	62.7
–α3.7/αα	41	21.2
–α4.2/αα	10	5.2
–α3.7/– –SEA	3	1.6
–α4.2/– –SEA	1	0.5
–α3.7/–α3.7	3	1.6
αQSα/αα	6	3.1
αWSα/αα	3	1.6

αCSα/αα	4	2.1
Total	193	100

Table 3. The five regional centers in changsha city that formed the basis of our study.

	α	β	α+β	Total mutation	District samples	Total population (million)	Total carrier rate (%)
Changsha county	42	38	3	83	1500	3.48	5.53
Wangcheng District	21	15	0	36	1500	0.54	2.4
Liuyang city	54	36	1	91	1500	1.22	6.0
Ningxiang county	29	26	1	55	1500	1.21	3.67
Changsha District	47	28	1	76	1500	1.03	5.07
Total	193	143	6	341	7500	7.3	4.55