The studies of hemoglobinopathies and thalassemia in China—the experiences in Shanghai Institute of Medical Genetics

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Abstract

Background: In the past two decades, a large-scale survey of hemoglobinopathies and thalassemia was carried out in mainland China, involving nearly one million people in 28 provinces. The incidences of hemoglobin (Hb) variants, α-thalassemia and β-thalassemia were 0.33%, 2.64% and 0.66%, respectively. The chemical structural analysis identified 67 Hb variants. Among them, 20 are new variants. The analysis of the α-globin gene organization in 111 HbH patients showed 76 cases (68.5%) were of the deletion type, 8 had Hb Constant Spring and the other cases were of non-deletion type. The results of the molecular characterization of more than 200 β-thalassemia alleles showed that the most common types of β-thalassemia mutations in China are CD 41/42 (– 4 bp), IVS-II-nt.654 C → T, CD 17 A → T, CD 71/72 (+ A) and −28 A → G. Methods: To explore the simple method for molecular diagnosis of β-thalassemia, multiplex allele-specific amplification (MAS-PCR) was used that could simultaneously detect the above five common types of β-thalassemia mutations. Results: Based on the molecular analysis of β-thalassemia intermedia, β-thalassemia homozygotes or compound heterozygotes combined with α-thalassemia, as well as the conjunctive abnormalities of β-thalassemia heterozygote with triplicated haplotype of α-globin genes, were the most common cause of thalassemia intermedia in China. We also used the RT-PCR quantitation method to show that the most common β-thalassemia allele, IVS-II-nt.654 C → T, still produced a small amount (about 15%) of normally spliced β-globin mRNA, therefore, causing β⁺-thalassemia. In clinical trials of hydroxyurea (HU) treatment for β-thalassemia patients, we found that HU may enhance the expression of the β-globin gene in some patients, leading to an alleviation of clinical symptoms. In the studies of the reversal of aberrant splicing of IVS-II-nt.654 C → T allele by the antisense approach, we constructed a mammalian expression vector that can produce an antisense RNA targeting against the aberrant splice sites of IVS-II-nt.654 C → T pre-mRNA. Conclusions: The results indicated that the antisense RNA produced from the vector could efficiently suppress the aberrant splicing pattern and restore the correct splicing pathway in vitro and in vivo, leading to the improvement of globin chain biosynthesis in thalassemia cells. © 2001 Published by Elsevier Science B.V.

Keywords: Hemoglobin; Disease; Screening; Diagnosis; Treatment

1. Introduction

Hemoglobinopathies are relatively common in China. In the past 20 years, studies of hemoglobin variants and thalassemia, including screening, chemi-
cal structural analysis of hemoglobin variants, molecular and prenatal diagnosis of thalassemia as well as the treatment of hemoglobin disorders, have been conducted in the authors’ laboratory. In this report, we summarize the data of the studies.

2. Survey of hemoglobinopathies and thalassemia in China

In the past two decades, a large-scale survey of hemoglobinopathies and thalassemia has been carried out in mainland China, involving nearly one million people in 28 provinces [1]. It has resulted in the finding of many new hemoglobin (Hb) variants and some interesting cases of thalassemia syndrome. Using electrophoresis, blood samples from 902,204 individuals were screened for Hb variants. A total of 2936 families with Hb variants were found incidence of 0.33%. The data indicated that the Yunnan, Fujian, Xinjiang, Guangxi, Guangdong and Jiangxi provinces and autonomous regions had the highest incidences of Hb variants. The HbE variant, in particular, was very prevalent in Yunnan province.

Alpha-thalassemia was screened from cord blood samples of newborn babies by electrophoresis in seven provinces including autonomous regions and municipalities of China. Among 12,821 cord blood samples, 339 cases of $\alpha$-thalassemia were found with increased levels of HbBarts. The mean incidence of $\alpha$-thalassemia was calculated to be 2.64%. Using cellulose acetate electrophoretic quantification of HbA2 as well as an alkali denaturation method for determination of HbF, more than 360,000 Chinese were screened for $\beta$-thalassemia and about 2400 cases of this disorder were found. The mean incidence of $\beta$-thalassemia in China was 0.66%. The data showed that both $\alpha$- and $\beta$-thalassemias had higher incidences in the south than in the north of China.

During an analysis of the $\gamma$-globin composition of over 1100 Chinese newborns by HPLC, we found 25 babies who were heterozygotes for $\gamma$-thalassemia, while one infant was a homozygote for this condition. DNA analysis with gene mapping of this baby and his parents showed the baby as a homozygote for −$G\gamma A\gamma$-thalassemia, caused by a deletion of about 5 kb due to an unequal crossing-over between the $G\gamma$ and $A\gamma$ genes. The resulting −$G\gamma A\gamma$-hybrid gene produced $A\gamma$ chain only in this baby whose HbF, accordingly, consists of $\alpha$-globin chains and $A\gamma$-chains only. The frequency of the $G\gamma A\gamma$-gene among babies in the Shanghai area may be as high as 0.012% [2].

3. Studies of hemoglobin variants

By using fingerprinting or HPL chromatography as well as amino acid sequencing of the abnormal globin chains, the chemical structure of 67 Hb variants were identified in over 700 families in China. Among them, 20 were new variants listed in Table 1. The geographical distribution of Hb variants in China showed that HbE, New York, G Chinese, Q Thailand and J Bangkok were mainly distributed over the provinces of southern China. These Hb variants were also very common, adjoining southeast Asia. In contrast, Hb D Punjab was principally found in northern China. This variant was more common in India and southwest Asia. Hb D Punjab in China and India may have the same origin. It was possible that as early as the Han Dynasty (2000 years ago), this

<table>
<thead>
<tr>
<th>Hb Chongqing</th>
<th>Hb Wuning–Wenchang</th>
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<tbody>
<tr>
<td>(a2 Leu → Arg)</td>
<td>(a11 Lys → Gin)</td>
</tr>
<tr>
<td>Hb Beijing</td>
<td>Hb Herbin</td>
</tr>
<tr>
<td>(a16 Lys → Asn)</td>
<td>(a16 Lys → Met)</td>
</tr>
<tr>
<td>Hb Tashikurgeran</td>
<td>Hb Shenyang</td>
</tr>
<tr>
<td>(a19 Ala → Glu)</td>
<td>(a26 Ala → Glu)</td>
</tr>
<tr>
<td>Hb Shunfeng</td>
<td>Hb Xuchang</td>
</tr>
<tr>
<td>(a27 Glu → Lys)</td>
<td>(a27 Glu → Ala)</td>
</tr>
<tr>
<td>Hb Huaxi</td>
<td>Hb Guangzhou</td>
</tr>
<tr>
<td>(a42 Tyr → Asp)</td>
<td>(a64 Asp → Gly)</td>
</tr>
<tr>
<td>Hb Duan</td>
<td>Hb Guangzhou</td>
</tr>
<tr>
<td>(a75 Asp → Ala)</td>
<td>(a77 Pro → Arg)</td>
</tr>
<tr>
<td>Hb J luhe</td>
<td>Hb Quinhai</td>
</tr>
<tr>
<td>(b8 Lys → Gin)</td>
<td>(b78 Leu → Arg)</td>
</tr>
<tr>
<td>Hb Jianghua</td>
<td>Hb Shanghai</td>
</tr>
<tr>
<td>(b120 Lys → Ile)</td>
<td>(b131 Gin → Pro)</td>
</tr>
<tr>
<td>Hb F Urumqi</td>
<td>Hb F Xinjiang</td>
</tr>
<tr>
<td>(Gy1 22 Asp → Gly)</td>
<td>(Gy1 25 Gly → Arg)</td>
</tr>
<tr>
<td>Hb F Shanghai</td>
<td>Hb F Xin-Su</td>
</tr>
<tr>
<td>(Gy1 66 Lys → Arg)</td>
<td>(Aγ1 73 Asp → His)</td>
</tr>
</tbody>
</table>
variant gene was introduced along the “Silk Road” between India and China.

4. Molecular diagnosis of thalassemia and hemoglobinopathies

Since we successfully applied DNA dot-blot hybridization for prenatal diagnosis of α-thalassemia in early 1980s [3], molecular biological methods have been widely used in the diagnosis and management of hemoglobin disorders in the authors’ laboratory. By using restriction endonuclease (BamHI and Bg/II) mapping, we analyzed the α-globin gene organization in 111 cases of HbH disease from different families. The results showed that 76 cases (68.5%) were of the deletion type in their α-globin genes, 8 cases (7.2%) had Constant Spring (Hb CS), and 27 cases (24.3%) were of the non-deletion and Hb CS types (α^3) [4]. The distribution of α-thalassemia-2 gene varies in different regions of China. The rightward deletion (α^-3.7) was found chiefly in the Guangdong Province, the leftward deletion (α^-4.5) mostly in Jiangxi Province, and the non-deletion type in Guangxi Province where the Han nationality was the most prominent. We studied the non-deletion Hb H type by DNA gene mapping, digestion with MspI and hybridization with a ^32P-α probe for the presence of the Hb Quong-Sze [α125(H8)Leu→Pro] mutation. It appeared that none of these α-thal-2 genes contain the Hb Quong-Sze mutation.

By using the method of micro-DNA sampling with PCR from dried blood specimens and allele-specific oligonucleotide (ASO) probe techniques, more than 200 β-thalassemia alleles originating from eastern China, southwestern China and southern China were molecularly characterized [5]. The results showed that different types of β-thalassemia alleles prevail in different regions. The most common types in southern China are frameshift at CD 41/42 and C→T transition at IVS-II-nt.654. The most frequent types in southwestern China are a nonsense mutation at CD 17 and IVS-II-nt.654 mutation; while in eastern China, the predominant mutant alleles are frameshifts at CD 41/42 and CD 71/72. Molecular diagnosis of Hb D Punjab and HbE was also performed using either PCR/EcoRI or PCR/MnlI restriction mapping in Uygur race, Xinjiang as well as in Achang race, Yunnan, respectively [6]. To explore the new method for prenatal diagnosis of β-thalassemia, multiplex allele-specific amplification (MAS-PCR), which could simultaneously detect five common types of β-thalassemia mutations: −28 A→G, CD 17 A→T, CD 41/42 (−4 bp) and IVS-II-nt.654 C→T in one PCR system, was developed in our laboratory [7]. Nearly 50 fetuses at risk for severe β-thalassemia were prenatally diagnosed using this method.

Beta-thalassemia intermedia is another relatively common blood disease in China. To analyze the possible molecular defects and to contribute to the knowledge of molecular diagnosis of this disease, the hematology, α-, β- and γ-globin gene organization and structure as well as globin chain biosynthesis in 14 patients with β-thalassemia intermedia were studied [8]. The results showed that: four cases were found to be β-thalassemia heterozygotes combined with rightward cross-over or/and leftward cross-over-triplicated haplotype of α-globin genes (α^±α^±γ^±) or α^±α^±γ^±; three were compound heterozygotes for β-thalassemia combined with α-thalassemia 1 or 2; and one was identified as a compound heterozygote for β-thalassemia combined with Gγ promoter −158 (C→T) mutation. We also analyzed α-globin genes in 250 cord blood specimens. The data showed that 8 out of 500 tested chromosomes (1.6%) were abnormal: three were α^±α^±γ^±, four were α^-3.7 and one was α^−SEA. Thus, we conclude that β-thalassemia homozygotes or compound heterozygotes combined with α-thalassemia as well as the conjunctive abnormalities of β-thalassemia heterozygote with triplicated haplotype of α-globin genes were the common causes of β-thalassemia intermedia.

In the early 1990s, we developed a technique to diagnose the α- and β-thalassemia syndromes using PCR to amplify cDNA copies of circulating erythroid cell mRNA so as to quantitate the amounts of α-, β- and γ-globin mRNA contained therein in relative and absolute terms [9]. This methodology of ascertaining the ratios of globin mRNA species provided a new simplified approach towards the diagnosis of thalassemia syndrome and was of value in other studies of globin gene expression at the transcriptional level. Using this method, we analyzed the
glo\-bin transcripts in the common Chinese β-thalas-
semia gene—IVS-II-nt.654—and found that this β-
thalasemia mutant still produced a small amount (about 15\%) of normal β-glo\-bin mRNA, therefore, leading to β⁺-thalasemia [10]. We also quantitated the δ-glo\-bin mRNA levels in peripheral blood-en-
riched reticulocytes and characterized the variation of β-
levels in 30 β-thalasemia heterozygotes who indi-
vidually carried one of the four common Chinese 
β-thalasemia alleles (frameshift at CD 41/42, CD 17 A \rightarrow T, IVS-II-nt.654 C \rightarrow T and −28 A \rightarrow G) using the above RT-PCR quantitative method [11]. The results showed a large increase in δ-glo\-bin mRNA amounts in all the carriers examined (72.3 \pm 9.0 mol globin RNA/μg total RNA) as compared with those in 12 controls (1.2 \pm 0.2 mol/μg RNA). There was a direct correlation between the δ-mRNA levels and types of β-thalasemia alleles; generally, the δ-mRNA levels were higher in heterozygotes for β⁺-thalasemia mutations than for β⁻-thalasemia mutations. The δ-mRNA levels correlated inversely with the hemoglobin and red cell indices but directly with HbA₂ levels in heterozygotes of each of the group of β-thalasemia mutations. These results sug-
ggested that a greater impairment in the β-glo\-bin gene expression resulted in an increased transcription of the δ-glo\-bin gene and in a higher level of HbA₂.

5. Treatment of β-thalasemia with hydroxyurea (HU)

Increased levels of HbF by pharmacological agents have been proposed to ameliorate the severity of the disease by improving the balance in globin chain synthesis. HU, as an effective agent with low toxic-
ity for activating the γ-glo\-bin gene, has been shown to enhance HbF synthesis in experimental animals and in patients with sickle cell anemia. However, previous trials of HU in β-thalasemia patients are am-
biguous, with a small number having increased the HbF synthesis. In a study of HU effects in Chinese 
β-thalasemia patients, we found that two unrelated patients with β-thalasemia intermedia, who carried IVS-I-654 splicing mutation, demonstrated an im-
provement in the effectiveness of erythropoiesis, which is reflected by an increase in the hemoglobin concentration (from 4.1 to 6.3 g/dl in one patient and 6.5–9.7 g/dl in another) and in red cell volume (from 68 to 104 fl and 68–85 fl, respectively) after a period in excess of 300 days of low dosage HU treatment. These effects, however, appear to be due to the increased β-glo\-bin biosynthesis because the percentage of HbF decreased in each patient as total Hb increased. This was reflected by changes in the β/α ratio (from 0.301 to 0.581 in one patient and 0.348–0.487 in the other) with minimal changes in γ-glo\-bin biosynthesis [12]. Using an RT-PCR/competi-
tive PCR approach for measuring the relative and absolute contents of globin mRNA, we also studied the changes in globin transcripts in these two pa-
tients before and during HU treatment. The data showed that HU could increase the β-glo\-bin gene transcription. We concluded that in addition to its known effects in stimulating γ-glo\-bin production, HU may have a more general role in augmenting glo\-bin gene expression, including β-glo\-bin gene in some thalassemia patients who maintained the capac-
ity to produce normal β-glo\-bin chains. A marked increase in the β-glo\-bin chain synthesis resulted in more effective erythropoiesis and in the alleviation of clinical symptoms in these representative thalassemia patients.

6. Reversal of aberrant splicing of βIVS-II-nt.654 splicing defect by antisense RNA expression vector

Studies on the glo\-bin gene regulatory mechanism in thalassemia as well as gene therapy of genetic diseases by antisense oligonucleotides have been ex-
tensively developed in recent years. We realized from these studies that antisense oligonucleotide ap-
proach may not only inhibit gene expression but may also reverse incorrect splicing of the pre-transcript. However, a significant drawback of the antisense oligonucleotide approach is the fact that the oligo-
nucleotides do not remove the mutations, and therefore, would require periodic administration. In an attempt to overcome this problem, we recently con-
structed a mammalian expression vector that could produce an antisense fragment targeting against the aberrant splice sites of β-thalassemia allele IVS-II-
nt.654 C \rightarrow T (β⁶⁵⁴) pre-transcript. After efficient restoration of the correct splicing pattern in an in
vitro transcription/splicing system [13] as well as in cultured HeLa654 cells [14] transfected with this vector, we further tested whether it is possible to use this antisense vector to repair the splicing defect of the mutant pre-transcript in cultured \( \beta^{654} \) erythroid cells by lipofectin-mediated DNA transfection method [15]. The total RNA was extracted at a given time point after transfection, and the effect of antisense RNA was studied by RT-PCR-mediated mRNA quantitative assay as well as globin chain micro-biosynthesis. The results showed that the antisense fragment transcribed from the vector effectively improved the \( \beta^{654} \) splicing pattern in cultured erythroid cells. The level of correctly spliced transcript increased from 0.19 (day 0 after transfection) to 0.58 (day 8) in \( \beta^{654}/\beta^{654} \) homozygous erythroid cells, and from 0.45 (day 0) to 0.83 (day 8) in \( \beta^{654}/\beta^{A} \) heterozygous erythroid cells, as determined by the ratio of normally spliced \( \beta \)-globin transcript over the total \( \beta \)-globin transcript. Correspondingly, the ratios of globin chain biosynthesis (\( \beta/\alpha \)) increased from 0.16 (day 0) to 0.52 (day 8) in \( \beta^{654}/\beta^{654} \) erythroid cells, and from 0.39 (day 0) to 0.84 (day 8) in \( \beta^{654}/\beta^{A} \) erythroid cells. Antisense RNA had no significant effect on the splicing pattern in \( \beta^{A}/\beta^{A} \) erythroid cells. The splicing pattern in transfected cells with the antisense vector showed significant changes compared to that in untransfected cells and that in transfected cells with control antisense fragment (human SRY gene sequence). In addition, we did not observe side effects on cytological features after the introduction of the antisense vector. All these results indicated that the antisense RNA transcribed from the vector could efficiently and specifically suppress the aberrant splicing pattern of the \( \beta^{654} \) mutant pre-transcript and restore the correct splicing pathway in vivo, leading to the improvement of globin chain biosynthesis in thalassemic cells.

Acknowledgements

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References