Rapid Diagnosis of β-Thalassemia Mutations in Chinese by Naturally and Amplified Created Restriction Sites

By Jan-Gowth Chang, Pao-Huei Chen, Shyh-Shin Chiou, Long-Shyong Lee, Liuh-I Perng, and Ta-Chih Liu

We developed a rapid and simple method to diagnose the molecular defects of β-thalassemia in Chinese patients. This method involves the selective amplification of a DNA fragment from human β globin gene with specific oligonucleotide primers, followed by digestion with restriction enzymes that recognize artificially created or naturally occurring restriction sites. To detect the 4-nucleotide deletion of codon 41-42, we introduced a single mismatch nucleotide into the 3' end of the upstream primer to create an artificial Taq I restriction site. With a similar approach, an artificial Rsa I site was generated to detect the nucleotide 654 mutation (C → T) of IVS-2, an Alu I restriction site was created to detect the codon 17 mutation (A → T), and EcoRI restriction site was created for the -28 mutation (A → G). A Rsa I restriction site was created for the nucleotide 5 mutation (G → C) of IVS-1, and a Spe I restriction site was created to distinguish the codon 71 (+T) and codon 71/72 (+A) mutations from a normal sequence. The other eight rare mutations that occur in the genes of the Chinese people naturally create or abolish restriction sites. Using this kind of approach, we are able to provide a simple, rapid, accurate, and nonradioactive method to detect the genetic defects of β-thalassemia in the Chinese population. It should be used not only for routine screening but also for prenatal diagnosis.

BETA-THALASSEMIA is a heterogeneous group of an inherited disease characterized by an abnormality of β globin production. More than 90 types of mutation have been reported. In general, each population has a different group of mutations, consisting of a few very common ones and a variable number of rare ones. In the Chinese, the spectra of mutation are: (1) frameshift codon 41 and 42 (−TCTT); (2) the C → T substitution at position 654 of intron 2 (IVS-2); (3) the nonsense mutation A → T at codon 17; (4) the mutation at position -28 (A → G), position -29 (A → G), and position -30 (T → C) of the promoter region; (5) the G → T mutation at position 1 of intron 1 (IVS-1); (6) the frameshift codon 14 and 15 (+G); (7) the G → C mutation at position 5 of IVS-1; (8) the frameshift codon 71 and 72 (+A) and the frameshift codon 71 (+T); (9) the frameshift codon 27 and 28 (+C); (10) the nonsense mutation G → T at codon 43; (11) a 4-nucleotide (nt) deletion (−AAAC) at position +40 from the cap site; and (12) the initial codon mutation (ATG→AGG). The first four mutations account for 91% of β-thalassemia in South China.

The study of these genetic mutations has been based on polymerase chain reaction (PCR) and allele-specific oligonucleotide hybridization patterns. These methods require radioisotope or immunochemical detection, which is inconvenient for clinical use. Another approach is the use of simple PCR followed by the restrictive enzyme digestion and visualization of the fragments after electrophoresis in agarose gel. However, this process has its difficulties because not all the mutations have created or abolished an enzyme cutting site. To overcome this problem, we used artificial base substitution to create restriction sites after PCR. The created restriction sites are immediately adjacent to loci for point mutations and enable us to distinguish the normal from mutated genes on the basis of restriction analysis of the appropriate enzyme.

MATERIALS AND METHODS

DNA isolated from peripheral blood samples of 60 Chinese patients with β-thalassemia minor or major was analyzed. Of those patients, 45 patients with β-thalassemia minor had been previously analyzed by allele-specific oligonucleotide hybridization or direct sequencing.

The strategy of the characterization of the mutations is shown in Table 1. The PCR products with artificial base substitutions near position -28, codon 17, codon 41-42, and nt 654 of IVS-2 are generated by the means of a terminal 3' base alteration (Figs. 1A, 2A, 3A, and 4A).

For the A → G mutation at position -28, the mutant G at position -28 together with the artificial T at position -25 create a GAATTC site for the EcoRI restriction enzyme, while the normal AAATTC sequence disrupts this target sequence.

The A → T substitution at codon 17 creates a new Mae I restriction site, but the enzyme (Mae I) is expensive and inconvenient to use. For this mutation, we introduce an artificial A to codon 16 of the upstream primer, with the mutant base of T at codon 17, so that an AGCT site for the restriction enzyme of Alu I is created.

For the 4 nt deletion of codon 41-42, the artificial C at codon 41, together with the 4-base deletion, creates a TCGA site for the restriction enzyme Taq I, while the normal TCTT site is destabilized.

For the nt 654 mutation (C → T) of IVS-2, the mutant T at position 654, together with the artificial C at position 656, creates a GTAC site for the restriction enzyme of Rsa I, while the normal GCAC sequence disrupts this target sequence.

For the nt 5 mutation (G → C) of IVS-1, the normal G at position 5, together with the artificial G at position 8 (antisense...
Table 1. List of Restriction Enzymes That Distinguish Between Normal and All Reported Mutants in Chinese

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Position</th>
<th>Restriction Enzyme</th>
<th>Natural Created (for mutant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A → G</td>
<td>-28</td>
<td>EcoRI</td>
<td>-</td>
</tr>
<tr>
<td>A → G</td>
<td>-29</td>
<td>Nla III, Rsa I, Taq I</td>
<td>-</td>
</tr>
<tr>
<td>T → A</td>
<td>-30</td>
<td>Bsp I, Mse I</td>
<td>-</td>
</tr>
<tr>
<td>−AAAC</td>
<td>+40 +43</td>
<td>EcoRI</td>
<td>-</td>
</tr>
<tr>
<td>ATG → AGG</td>
<td>Initial codon</td>
<td>EcoRI</td>
<td>-</td>
</tr>
<tr>
<td>+G</td>
<td>Codon 14/15</td>
<td>EcoRI</td>
<td>-</td>
</tr>
<tr>
<td>AAG → TAG</td>
<td>Codon 17</td>
<td>Mael, Alu I</td>
<td>-</td>
</tr>
<tr>
<td>+C</td>
<td>Codon 27/28</td>
<td>Nla IV, Bsp I</td>
<td>-</td>
</tr>
<tr>
<td>G → T</td>
<td>IVS-1 nt 1</td>
<td>Bsp I, Mse I</td>
<td>-</td>
</tr>
<tr>
<td>G → C</td>
<td>IVS-1 nt 5</td>
<td>Rsa I (for normal)</td>
<td>-</td>
</tr>
<tr>
<td>−TCTT</td>
<td>Codon 41/42</td>
<td>Taq I</td>
<td>-</td>
</tr>
<tr>
<td>GAG → TAG</td>
<td>Codon 43</td>
<td>Abolish Hinfl</td>
<td>-</td>
</tr>
<tr>
<td>+T</td>
<td>Codon 71</td>
<td>Mse I, Spe I (for normal)</td>
<td>-</td>
</tr>
<tr>
<td>+A</td>
<td>Codon 71/72</td>
<td>Mse I, Spe I (for normal)</td>
<td>-</td>
</tr>
<tr>
<td>C → T</td>
<td>IVS-2 nt 654</td>
<td>Rsa I</td>
<td>-</td>
</tr>
</tbody>
</table>

strand), creates a GTAC site (downstream primer, 5'-CTAAAC-CTGTCTTTGTAACCTTGGTA-3') for the restriction enzyme of Rsa I, while the mutant CTAG disrupts this target sequence. The upstream primer for this mutation is 5'-GCAACCTCAACAGACACCA-3'.

![Fig 1](image1)

Fig 1. (A) The strategy for diagnosis of the position -28 mutation (A → G) of the promoter area. The normal DNA sequences adjacent to position -28, the mutagenesis primer (downstream primer), and the upstream primer are shown. The sequences of amplified product and the site for the restriction enzyme EcoRI are indicated. The theory of the strategy is the same in each of the following figures. (B) The results of PCR product digested by EcoRI. The molecular weight marker in lane M is the pGem marker. Lane 1 contains undigested amplified DNA. Lane 6 is a heterozygote mutation, whereas lanes 2, 3, 4, and 5 are normal cases. In each of the figures, M represents the pGem marker. Similarly, lane 1 represents the undigested PCR product. (C) The results of direct sequencing of heterozygote (case 6, left panel) and normal cases (case 5, right panel). Position -28 is denoted by an arrow.

For the mutations of codon 71 (+T) and codon 71/72 (+A), we substitute the TTT sequences of codon 71 to ACT artificially, which, together with the normal sequence of codon 72 (AGT), will create a ACTAGT site for Spe I. The mutant ACTAAGT or ACTTAGT disrupts this target sequence (Fig 5A). These two mutations are further distinguished by Mse I digestion of the regular PCR product.

For the remaining eight rare genetic mutations of the Chinese, which naturally create or abolish a specific restriction site, three pairs of primers were used for PCR. For position -29 (A → G)
mutation of the initial codon (ATG → AGG), the addition of G at codon 14/15 (+G), the addition of C at codon 27/28 (C), and the mutation of nt 1 of IVS-1 (G → T), the primer sets are: upstream primer, 5'-CTGGGCTATAAAAGTCAAGG-3'; and downstream primer, 5'-GGGAGAGAGGTCACTGCT-3'. For the mutation of codon 43 (GAG → TAG) and codon 71/72 (+A), the primer sets are: upstream primer, 5'-CATGGGAGAGGAGGAC-3'; and downstream primer, 5'-TCATTCGTCTGTTCCATCTAAAC-3' (Table 1).

All the mutants can be distinguished from the normal allele on the basis of enzyme digestion of the PCR product, with changes in fragment sizes detected in the 3% agarose gel electrophoresis.

The DNA amplification was performed as described,15 with some modification. Genomic DNA (0.5 μg) was mixed with 100 ng of each primer and 200 μmol/L of each dNTP in 100 μL reaction buffer containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, and 0.01% gelatin. The mixture was heated to 94°C for 10 minutes for strand separation and then 2.5 U of Taq polymerase (Promega, Madison, WI) was added. The PCR programs were the same for all primer pairs; the procedure was denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes.

RESULTS

The results of the restriction map change of β-thalassemia after restriction enzyme digestion are shown in Figs 1B, 2B, 3B, 4B, and 5B. For the position -30 mutation (T → A), the upstream primer is 5'-ATCACATTGACCTCACCCTGTGGAGCCA-3', and the downstream primer is 5'-GACCTCACCCTGTGGAGCCA-3'. For the 4-base deletion (-AAAC) of position +40 to +43 from the cap sites, the

and position -30 (T → A), the upstream primer is 5'-ATCACCTTA-GACCTCACCCTGTGGAGCCA-3', and the downstream primer is 5'-GACCTCACCCTGTGGAGCCA-3'. For the 4-base deletion (-AAAC) of position +40 to +43 from the cap sites, the

For the codon 17 mutation (A → T), a 170-bp fragment was amplified by the PCR. After digestion of the PCR products by restriction enzyme Alu I, a 170-bp fragment identical to the undigested product was noted in the normal chromosome. However, there was a 267-bp band for the mutant case (Fig 1B). The normal and mutant cases were also confirmed by direct sequencing (Fig 1C).

For the codon 17 mutation (A → T), a 170-bp fragment was amplified by the PCR. After digestion of the PCR products by restriction enzyme Alu I, a 170-bp fragment identical to the undigested product was shown in the normal allele, but a 147-bp band was found in the mutant case (Fig 2B). Direct sequencing of normal, heterozygote, and homozygote is shown in Fig 2C.

For nt 654 deletion of codon 41-42, the size of the PCR product was 334 bp. The fragment was 334 bp in the undigested product and normal allele digested by Taq I, but 306 bp in the mutant digested with Taq I (Fig 3B). The cases of normal, heterozygote, and homozygote were confirmed by direct sequencing (Fig 3C).

For nt 654 mutation (C → T) of IVS-2, digestion of PCR product by Rsa I showed a 233-bp fragment in the normal allele and a 198-bp fragment in the mutant allele (Fig 4B). Lanes 2, 5, and 6 were heterozygote cases and lane 8 was a homozygote case, as confirmed by direct sequencing (Fig 4C).

For the mutations of codon 71 (+T) and codon 71/72 (+A), the PCR product was 241 bp in length. The PCR products were digested by Spe I. A 241-bp fragment
RAPID DIAGNOSIS OF β-THALASSEMIA IN MUTATIONS

![Diagram](Image)

Fig 6. (A) The results of PCR product digested by *Mae*I for diagnosis of codon 71/72 (+A) mutation. Lane 2 is a heterozygote mutation, the 418-bp PCR product was digested to 354-bp, 204-bp, 150-bp, and 64-bp fragments. Lane 3 is a normal case, the PCR product was digested to 354-bp and 64-bp fragments. (B) The results of PCR product digested by *Nla*IV for diagnosis of the codon 27/28 (+C) mutation. Lane 2 is a heterozygote mutation; 170-bp and 114-bp fragments were noted. Lane 3 is a normal case.

identical to the uncut product was noted in the mutant and a 216-bp fragment was found in the normal allele (Fig 5B). The normal and mutant cases were confirmed by direct sequencing (Fig 5C).

In the nt 5 mutation of IVS-1 (G → C), the PCR product was 142 bp in length. After digestion of the PCR product by *Rsa*I, a 142-bp fragment, the same length as the undigested product, was noted in the mutant and the normal showed a 116-bp band (data not shown). For the other eight rare mutations of the Chinese, their PCR products were digested by their respective specific restriction enzymes and analyzed in comparison with the normal restriction map. The results of two of these eight mutations are shown in Fig 6. Comparing the data with our previous studies,13,14 we found this study was in agreement with previous data (data not shown).

DISCUSSION

In the genes of the Chinese, there are 15 or more mutations for β-thalassemia. Nine of these mutations create new restriction sites of various restriction enzymes and one mutation abolishes a normal restriction site; another five mutations neither create nor abolish restriction sites (Table 1). Traditionally, detection of these mutations is dependent on allele-specific hybridization or direct sequencing.4-14 In this study, we devised a nonradioactive method by using selective amplification of a DNA fragment of β-globin gene with specific oligonucleotide primers, followed by digestion of the amplified product by restriction enzymes that recognize artificially created or naturally occurring restriction sites. In comparison with our previous studies,15,14 we find this method provides a rapid and simple procedure for identifying all the mutations found in the genes of the Chinese. The method is not only very useful for detecting mutations of β-thalassemia and prenatal diagnosis in Chinese, but also for further investigation of new mutations.

The same strategy has been used to diagnose phenylketonuria (PKU),16 β-thalassemia in Mediterranean,17 ras oncogene mutation,18 G-6-PD deficiency,19 cystic fibrosis, and retinitis pigmentosa.20 All the studies show that this kind of approach provides a simple, rapid, and accurate screening method and should be used routinely.

ACKNOWLEDGMENT

We thank L.H. Yang, W.D. Su, and J.S. Yang for their excellent technical assistance.

REFERENCES


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