A Simple Approach to Prenatal Diagnosis of \( \beta \)-Thalassemia in a Geographic Area Where Multiple Mutations Occur

By Shi-Ping Cai, Ji-Zeng Zhang, Dao-Hua Huang, Zhong-Xiang Wang, and Yuet Wai Kan

We describe a simple approach for detecting \( \beta \)-thalassemia mutations in geographic areas such as southern China where multiple mutations are known to occur. Segments of the \( \beta \)-globin gene were amplified in vitro by using the polymerase chain reaction. Dot blot hybridization of the amplified DNA with oligonucleotide probes corresponding to the six mutations found in southern China could directly identify the mutations causing \( \beta \)-thalassemia in the affected families. The increased number of target sequences after amplification allows the use of \( \beta \)-labeled probes, which are reusable for up to 3 months. The mutations can be determined in two days.

\[ \text{DNA ANALYSIS has become the method of choice for the diagnosis of the many genetic disorders. Among the major hemoglobin disorders, sickle cell anemia and } \alpha \text{-thalassemia can now readily be detected in this manner. The } A \rightarrow T \text{ mutation at the } \beta \text{ codon in sickle cell anemia is detectable by digestion with the restriction enzyme } MstI \text{ II or one of its isoschizomers.} \]

Because most of the severe \( \alpha \)-thalassemias are caused by gene deletion, the absence of \( \alpha \)-globin genes is diagnostic for homozygous \( \alpha \)-thalassemia. Recently, nonradioactive methods for detecting these two disorders have been devised. The diagnosis of \( \beta \)-thalassemia has remained a complicated and involved procedure because most of the clinically important \( \beta \)-thalassemias are not due to gene deletion but to point mutations that affect mRNA transcription, processing, or translation. Some 40 such mutations have been described worldwide, although within a given population there appear to be only a few characteristic mutations.

We determined the mutations in 80 chromosomes from the Guangdong province in southern China and found six different \( \beta \)-thalassemic mutations in addition to the \( \beta \) mutation. In order of decreasing frequency, these mutations are the 4-base pair (bp) (−CTTT) deletion at codon 41-42, the C → T mutation at IVS-2 position 654, the A → T \( \beta \) nonsense mutation, the A → G mutation at position −28, the 1-bp insertion (+A) at codon 71-72, and the G → C mutation at IVS-1, position 5. (In this report, these mutations are referred to as 41/42, II-654, \( \beta \) −28, 71/72 and I-5, respectively.) Their locations in the \( \beta \)-globin gene are depicted in Fig 1. These mutations have been detected with the oligonucleotide probe technique that, until now, involved enzyme digestion, gel electrophoresis, and hybridization.

The introduction of the in vitro gene amplification method by Saiki et al has greatly simplified the DNA diagnostic procedure. Because of the increased sensitivity of detection after amplification, dot blot hybridization can be used in place of enzyme digestion and gel electrophoresis. On the basis of this technique and the prior characterization of the thalassemia mutations, we have devised a simple approach for prenatal diagnosis that is applicable to any region where multiple mutations occur.

MATERIALS AND METHODS

DNA was obtained from southern Chinese \( \beta \)-thalassemia patients with previously characterized mutations. We also studied three families who requested prenatal diagnosis for \( \beta \)-thalassemia. In these three families, DNA was extracted from the parents' white cells and from the amniocytes at Nan Fang Hospital in Guangzhou, China, and sent to San Francisco for analysis.

In vitro DNA amplification was performed as previously described with the DNA polymerase (New England Biolabs, Beverly, MA) from the thermophilic organism Thermus aquaticus (Taq). The oligonucleotide primers used are indicated in Table 1, and the regions of homology to the \( \beta \)-globin gene and the direction of amplification are shown in Fig 1. Regions of the \( \beta \)-globin gene were amplified in two segments by using primers A plus B and primers C or D in one segment by using primers A plus D. Fifty promoles of each pair of oligonucleotide primers was mixed with 1 \( \mu \)g of genomic DNA in a 100-\( \mu \)L reaction containing 67 mmol/L Tris-HCl (pH 8.8), 6.7 mmol/L MgCl\(_2\), 16.6 mmol/L (NH\(_4\))\(_2\)SO\(_4\), 10 mmol/L \( \beta \)-mercaptoethanol, 10% (vol/vol) dimethylsulfoxide, 6.7 \( \mu \)mol/L EDTA, 170 \( \mu \)g/mL bovine serum albumin, and 1.5 mmol/L each of dATP, dCTP, dGTP and TTP. The mixture was sealed with 33 \( \mu \)L of mineral oil and incubated at 95°C for five minutes to separate the DNA strands, and 2 to 3 units of Taq polymerase was added. The mixture was incubated through three temperature cycles: first at 65°C for DNA synthesis, then at 93°C for 30 seconds for denaturation, and then at 45°C for 30 seconds for cooling. The incubation time for extension of DNA synthesis at 65°C was 90 seconds for primer A plus B, 60 seconds for primers C plus D, and 210 seconds for primers A plus D. The 65°-93°-45°C cycle was repeated 30 times, the reaction terminated after the last 65°C cycle, and the reaction mixture stored at 4°C.

A 10-\( \mu \)L aliquot was loaded on a 10 \( \times \) 10 \( \times \) 0.1-cm 5% polyacrylamide gel in a buffer (pH 8.0) containing 89 mmol/L Tris, 89 mmol/L boric acid, and 9 mmol/L EDTA and electrophoresed at 250 V for 40 minutes. The gel was stained with ethidium bromide and visualized under UV light.

For hybridization, approximately 10 \( \mu \)L of the mixture containing the amplified DNA was applied to nitrocellulose filters by using a dot blot apparatus (Schleicher and Schuell, Keene, NH). The applied volumes were adjusted according to the amount of amplified DNA as determined visually by the ethidium bromide stain. We

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Submitted October 5, 1987; accepted December 30, 1987.

Supported in part by Grants DK 16666 and HL 20985 from the National Institutes of Health. Dr Zhang was a recipient of a fellowship from the World Health Organization. Dr. Kan is an Investigator of the Howard Hughes Medical Institute.

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0006-4971/88/7105-0024$3.00/0
hybridized the DNA with six pairs of oligonucleotide probes (each 19 nucleotides long) corresponding to the normal and mutated sequence of the six mutations previously described in southern China. The probes were labeled with T4 polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis) by using [γ-32P]ATP (Amersham Corp., Arlington Heights, IL) to a specific activity of 2.5 to 5 × 10^7 cpm/μg. Each filter strip was prehybridized for one hour at 42°C in a buffer containing 5 × SSC (1 × SSC: 0.15 mol/L sodium chloride and 0.015 mol/L sodium citrate), 5 × Denhardt’s, and 0.1% sodium dodecyl sulfate. They were then hybridized for 2 hours at 42°C to 5 × 10^6 cpm/mL of 32P-labeled oligonucleotide in the same buffer to which 10 mmol/L of dithiothreitol was added. The filters were washed twice with 6 × SSC at room temperature, once at 48°C to 50°C for 15 minutes for the II-654 probe, and at 53°C to 55°C for the other five probes. They were then dried and autoradiographed for 16 hours. The hybridization mixtures containing the 32P-labeled probes were stored at −80°C and thawed immediately before use.

RESULTS

The high reaction temperatures of the Taq polymerase favor specific hybridization of the primers for DNA synthesis and enable visualization of discrete bands of amplified globin gene fragments on electrophoresis. Primers A plus B yielded a 601-bp fragment that contains five of the six mutations, whereas primers C plus D gave a 422-bp fragment containing the II-654 mutation. All six mutations were contained within the 1,502-bp fragment amplified with primers A and D (Fig 2).

To test the feasibility of detecting all six mutations, we applied dot blot analysis to six strips of DNA, each containing the following: a control known to harbor a defined β-thalassemia mutation (a different mutation was used for each strip) and amplified DNA samples from five patients homozygous for the β-thalassemia phenotype. Each of the strips was then hybridized with the “mutant” oligonucleotide probe appropriate for the control mutation. As expected, all the amplified control DNA samples containing known mutations hybridized with the appropriate mutant probes (Fig 3, first horizontal row). DNA samples from patient 1 hybridized with the mutant probes for the 41/42 and 71/72 mutations, and this patient is therefore doubly heterozygous for these two mutations. Similarly, patient 2 is doubly heterozygous for the II-654 and the -28, whereas patient 3 is doubly heterozygous for the 41/42 and the β7 mutations. DNA from patients 4 and 5 hybridized only with the probes for mutations II-654 and 41/42, respectively, which is compatible with the fact that each is homozygous for their respective mutation.

We used this method to study three pregnancies at risk for β-thalassemia. Each of these families had a previous child affected with homozygous β-thalassemia. We screened the parents with oligonucleotide probes and found that both parents in the first family are heterozygous for the 41/42 mutation and both in the second family for the β7 mutation. We amplified the DNA obtained from amniotic fluid cells with primers A plus B and by dot blot hybridization showed that the fetus in family 1 was homozygous for the 41/42 mutation whereas the DNA from the fetus in family 2 hybridized with both the normal and mutant β7 mutation probe and was therefore heterozygous for this mutation (Fig 4). The parents in a third family were both found to carry the 41/42 mutation. The amniotic DNA hybridized with both the normal and mutant 41/42 probe, thus indicating that the fetus was heterozygous for this mutation (data not shown). The parents of the first family chose to terminate the pregnancy, and DNA from fetal liver hybridized only to the mutant 41/42 probe, thus confirming the diagnosis of homozygous β-thalassemia resulting from homozygosity for this mutation.

![Diagram of the β-globin gene and the four oligonucleotide primers (A, B, C, D) used to amplify the DNA segments. Arrows indicate the location of these primers and the direction of amplification. The numbers depict the positions of the six mutations as follows: 1, -28; 2, β7; 3, I-5; 4, 41/42; 5, 71/72 and 6, II-654.](image)

![Polyacrylamide gel electrophoresis of the amplified DNA fragments. The primers used and the fragment lengths (in base pairs) are indicated. The markers (M) are a mixture of HindIII-digested λ DNA and Haelll-digested φX174 RF DNA.](image)

Table 1. Primers Used to Amplify Regions of the β-Globin Gene

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ → 3’)</th>
<th>Position</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>GTACGGCTGTCACTACATAGACCTCA</td>
<td>−129 → −104</td>
</tr>
<tr>
<td>B</td>
<td>TGGACAGTTTGACAGTCACT</td>
<td>Codon 97 → 89</td>
</tr>
<tr>
<td>C</td>
<td>GTGTACACATATTGACAA</td>
<td>IVS-2 457 → 476</td>
</tr>
<tr>
<td>D</td>
<td>AGCACACAGACACGAGTT</td>
<td>Codon 114 → 108</td>
</tr>
</tbody>
</table>

Fig 1. Diagram of the β-globin gene and the four oligonucleotide primers (A, B, C, D) used to amplify the DNA segments. Arrows indicate the location of these primers and the direction of amplification. The numbers depict the positions of the six mutations as follows: 1, -28; 2, β7; 3, I-5; 4, 41/42; 5, 71/72; and 6, II-654.
Prenatal Diagnosis of β Thalassemia

Fig 3. Autoradiograph of dot blot hybridization of amplified DNA samples. Controls in each of the six strips are DNA from patients known to carry the mutation indicated. In each strip, five other DNA samples from patients homozygous for the β-thalassemia phenotype were dotted. Each strip was hybridized with the mutant probe as indicated.

DISCUSSION

Prenatal diagnosis of the hemoglobinopathies and thalassemias has evolved considerably since it was first introduced in the 1970s. Fetal blood sampling, at first the only analytic method available, has largely been replaced by analysis of amniocyte or chorionic villus DNA. The methods for diagnosis of α-thalassemia and sickle cell anemia are straightforward, and the availability of nonradioactive techniques is expected to further extend the utilization of these tests. Because of the multiplicity of mutations known to cause the β-thalassemia phenotype, DNA analysis of this condition is more difficult and is currently used primarily in geographic areas such as Sardinia where one or a few mutations predominate. Linkage analysis with polymorphic restriction endonuclease sites is too complicated a procedure for routine application for most laboratories. Thus, there is clearly a need for a simple approach to the diagnosis of β-thalassemia in those parts of the world where multiple mutations occur. This study describes such an approach that makes use of improved technology together with the knowledge of the specific mutations present in a particular geographic area.

The in vitro gene amplification method could increase the amount of target sequences by as much as several million fold, and as a result, as little as 100 ng of genomic DNA can yield a discrete band of amplified DNA on electrophoresis. We amplified segments up to 1.5 kb in length to cover the six mutations in southern China. The choice of which segment to amplify depends on the location of the mutation. If the mutation has not been defined, then the entire 1.5-kb fragment could be amplified. If the mutation is known, as in the three cases of prenatal diagnosis reported here, only the shorter segments containing the mutations need to be amplified. The shorter amplification times needed for short segments reduce the total time required for analysis.

The amplification of specific target sequences also allows dot blot hybridization and the use of 35S-labeled probes, which, although less sensitive than 32P-labeled probes, provide adequate signal on an overnight exposure of the film. We have stored the 35S-labeled probes at -80°C and reused them for up to 3 months with no discernable loss of hybridization signal.

The delineation of the β-thalassemia mutations is a prerequisite for setting up a prenatal diagnosis program. Previously, it was necessary to analyze the β-globin haplotypes in family members at risk to choose the appropriate oligonucleotide probes; however, this approach is not only cumbersome, it is also not helpful for probe selection in areas such as southern China where a single mutation often occurs in two haplotypes, and one haplotype can harbor from two to four mutations. Thus, to determine the thalassemia mutation in a homozygote by using the new strategy, one would amplify the DNA for dot blot hybridization with the six oligonucleotide probes corresponding to the six mutant sequences present in southern China. If only one probe hybridizes with the patient’s DNA, then the patient is either homozygous for the mutation or doubly heterozygous for the mutation and a rare one not included in the panel. Homozygosity for a single mutation can be confirmed by demonstrating the absence of hybridization to the oligonucleotide probe corresponding to that normal sequence. If the DNA hybridizes to two of the six mutant probes, then the patient is doubly heterozygous for those two mutations.

Two recent innovations will further simplify the analysis procedure. First, it is now possible to initiate the polymerase chain reaction on lysed cells without prior DNA extraction. Second, a newly developed nonradioactive method of tagging the oligonucleotide probes by using horseradish peroxidase is sufficiently sensitive to detect mutations. Applying these advances to the strategy described here will permit rapid delineation of mutations and prenatal diagnosis of β-thalassemia in those parts of the world where multiple mutations occur.

ACKNOWLEDGMENT

We thank Dr Farid Chehab for his advice on DNA amplification of the β-globin gene, Marie Doherty for synthesizing the oligonucleotide probes, and Jennifer Gampell for editorial assistance.

REFERENCES


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