Identification of the Multiple β-Thalassemia Mutations by Denaturing Gradient Gel Electrophoresis

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Abstract

We used denaturing gradient gel electrophoresis to detect the β-thalassemia mutations in the Chinese population. By amplifying the β-globin gene in four separate fragments and electrophoresing the amplified DNA in two gels, we were able to distinguish all the 12 known mutations on the basis of the mobility of the homoduplexes and heteroduplexes. We conclude that denaturing gradient gel electrophoresis offers a nonradioactive means of detecting multiple mutations in genetic disorders. (*J. Clin. Invest.* 1990. 85:550–553.) Chinese β-thalassemia • denaturing gradient gel electrophoresis • GC clamp • polymerase chain reaction

Introduction

Thalassemia is a heterogenous group of diseases caused by a variety of mutations that interfere with globin gene expression. While deletion is the most common cause of α-thalassemia, point mutations affecting one or a few nucleotides account for most of the clinically important β-thalassemia. Over 70 different point mutations that interfere with β-globin gene transcription, processing, or translation have been found to produce β-thalassemia (1).

During the past few years, in addition to DNA sequencing, a number of powerful techniques have been devised to detect point mutations. These include hybridization with allelic-specific oligonucleotide probes (2), ribonuclease or chemical cleavage (3, 4), denaturing gradient gel electrophoresis (5), and restriction endonuclease analysis (6). The introduction of DNA amplification by the polymerase chain reaction (PCR)[1] (7) has greatly facilitated implementation of all these detection methods, and made them more adaptable to the clinical diagnostic setting. When many mutations account for the phenotype of a genetic disease, such as in the case of β-thalassemia, detection may be more difficult. Previously, we reported the use of nonradioactive oligonucleotide probes for detecting the multiple β-thalassemia mutations commonly found in southern China (8). In this study, we explore denaturing gradient gel electrophoresis as a means of detecting these mutations.

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1. *Abbreviations used in this paper:* PCR, polymerase chain reaction.

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The gradient gel method is based on the principle that denaturation (or melting) of double stranded DNA retards its mobility on polyacrylamide gel electrophoresis (5). Hence, when double stranded DNA is run on a gel with increasing denaturation conditions, different DNA show different mobilities, depending on the base composition of the first DNA domain that melts. By attaching a GC-rich clamp to the test DNA sample, the test segment becomes relatively GC-poor and will be the first melting domain. Any single basepair change within it will then be detected by the altered mobility of the fragment (9). We amplified four regions of the β-globin gene containing β-thalassemia mutations by PCR and attached GC clamps (10). We were able to resolve and detect all 12 mutations previously reported in the Chinese population.

Methods

DNA was prepared from unaffected as well as individuals with the 12 known Chinese β-thalassemia mutations. These include: three TATA box mutations [position −30 (T → C)], position −29 (A → G), position −28 (A → G)], two nonsense mutations [codon 17 (A → T) and codon 43 (G → T)], three frameshift mutations [position 14/15 (+G), codon 41/42 (−CTTT), codon 71/72 (+A)], four splice mutations [codon 26 (G → A--β^E), IVS-1 position 1 (G → T), IVS-I position 5 (G → C), and IVS-II position 654 (C → T)]. The locations of these mutations on the β-globin gene are shown in Fig. 1.

Four pairs of oligonucleotides, one from each pair with a GC-rich clamp added, were used to amplify regions of the β-globin gene that contain these mutations. The sequences of the oligonucleotides used are shown in Table I, and the fragments of the β-globin gene they amplify are indicated in Fig. 1. The lengths of the amplified fragments I to IV, including the GC clamps, were: 196, 193, 271, and 386 bp, respectively. 0.1–0.5 μg of DNA was amplified in 50 μl containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg/ml gelatin, 200 μmol each dATP, dCTP, dGTP, dTTP, 10 pmol of each primer, 1 U Taq DNA polymerase (Perkin Elmer-Cetus Corp., Norwalk, CT).

The cycling reaction was performed in a programmable heat block (DNA Thermal cycler; Perkin Elmer-Cetus) using the "step-cycle" program. The first cycle denatures the sample at 95°C for 5 min, then cools it to 55°C for 15 s for annealing, and incubates it at 70°C for 30 s for extension. Conditions for the 2nd to 40th cycle were 94°C for 15 s, 55°C for 15 s, and 70°C for 30 s.

Denaturing gradient gel electrophoresis was performed exactly according to the method of Myers et al. (11). The optimal formamide-urea gradient was first determined by running a vertical gel in which the gradient ranged horizontally across the gel from 0 to 80% denaturant concentration and the amplified DNA was applied to a slot that spanned the width of the gel. After electrophoresis at 150 V for 5 h, the gels were stained with ethidium bromide and photographed. The optimal range of denaturant concentrations used to analyze the amplified DNA fragments was ~ 15% above to 15% below the midpoint of the denaturant concentration.

Results

Preliminary determination with a horizontal 0–80% denaturant gel indicated that the mid-denaturant concentrations for

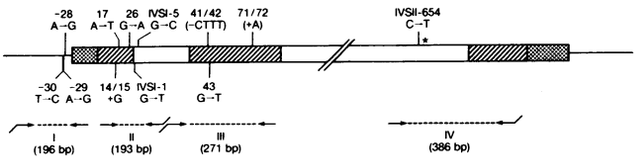

Figure 1. Diagram of the β-globin gene. The positions of the 12 mutations analyzed are shown, with the more common ones on top. The asterisk denotes the C/T polymorphism at IVS-II, position 666. Arrows indicate the position of the primers used to amplify the four fragments. Bent arrows represent primers with GC clamps.

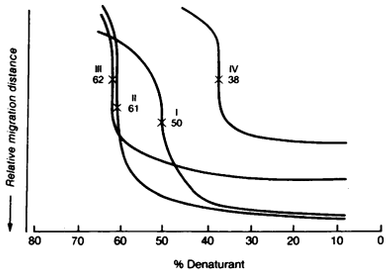

Figure 2. Melting profile of the four amplified fragments with the percent of denaturant at mid-melting point indicated.

fragments I to IV were 50%, 61%, 62%, and 38%, respectively (Fig. 2). The four amplified fragments were then separated on two different gels: I, II, and III in a 40–70% denaturant gel, and IV in a 20–50% denaturant gel.

Fragment I contained three TATA box mutations (Fig. 3). A single band was observed in DNA from the normal, while four bands were seen in each of the three DNA samples heterozygous for mutations at this locus. The more rapidly migrating band found in each of the three mutant samples represents the homoduplex containing the TATA box mutation. The two slower bands observed in each sample are due to heteroduplex formation between the normal and mutant alleles. For example, in the −28 A → G TATA box mutation, the basepairs at position −28 were G:C and A:T for the two homoduplexes, and C:A and G:T for the heteroduplexes. Although the homoduplexes at the −29 and −30 TATA box mutations had similar electrophoretic mobility and migrated faster than normal, the heteroduplexes differed markedly from each other. Thus, the three mutations could be readily distinguished from one another.

The region covered by fragment II contained five different mutations (Fig. 4). Some patients were heterozygous or homozygous for one mutation in this region, while others were doubly heterozygous for two different mutations. The positions of the homoduplex of the normal fragment and of the five different mutations are indicated in this figure. The homoduplexes of the codon 26, IVS-I position 1, codon 17, and codon 14/15 mutations had different migration patterns from normal. However, the homoduplex of the IVS-I position 5 mutant migrated almost exactly as the normal (compare lanes *10* and lane *11*). In fact the two homoduplexes could not be resolved in DNA from an individual heterozygous for the IVS-1 position 5 mutation (lane *9*). However, the heteroduplexes between the normal and mutant alleles indicated that the person must be heterozygous for this mutation. In all samples tested with this fragment, the difference in mobility between the homoduplexes and heteroduplexes was sufficient to permit accurate genotyping.

Fragment III contained three mutations (Fig. 5). The 4-bp deletion at codon 41/42 is the most common cause of β-thalassemia in the Chinese. DNA homozygous and heterozygous for this mutation could be easily separated, as could DNA heterozygous for the nonsense mutation at codons 43 and the frameshift mutation at codon 71/72. One individual with both the 41/42 and 71/72 mutations also exhibited a unique pattern.

Fragment IV contained a splicing mutation at IVS-II position 654 (Fig. 6). A normal polymorphism at position 666 of IVS-II could be either C or T. Thus in a nonthalassemic control, three patterns were seen, depending on whether the two alleles were CC, CT, or TT at position 666. The IVS-II, position 654 mutation is always associated with the nucleotide T at position 666. Hence DNA homozygous for the 654 mutation will show a single band, while heterozygous DNA will have two different patterns, depending on whether nucleotide 666 on the normal allele is C or T.

Discussion

We have used denaturing gradient gel electrophoresis to detect the 12 different mutations that have been described to date in

Table I. Sequence of Oligonucleotide Primers

Fragment	Position	5'-Sequence-3'
I	−123 → −104	*[GC]-CTGTCATCACTTAGACCTCA
	+33 → +14	GCTAGTGAACACAGTTGTGT
II	exon I codon 3 → 9	CTGACTCCTGAGGAGAAGTC
	IVSI-70 → IVSI-51	[GC]-GTCTTCTCTGTCTCCACATG
III	IVSI-101 → IVSI-120	[GC]-CTGCCTATTGGTCTATTTTC
	exon II codon 97 → 89	TGCAGCTTGTCACAGTGCAGCTCACT
IV	IVSII-588 → IVSII-607	ATGATACAATGTATCATGCC
	exon III codon 132 → 125	[GC]-TCTGATAGGCAGCCTGCACT

* [GC] = GC clamp sequence: GCGGGCGGGGCGGGGGCACGGGGGGCGCGGCGGGCGGGGC.

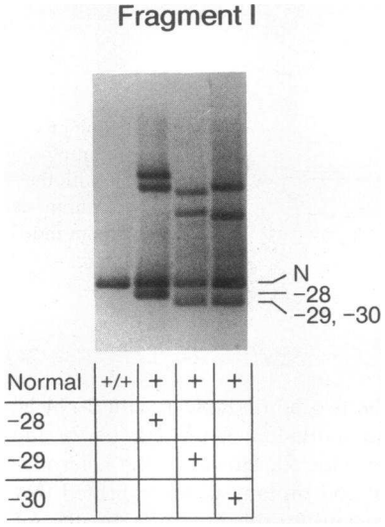

Figure 3. Gradient gel electrophoresis of fragment I showing negative image of ethidium bromide stain. In this and the following three figures, genotypes of the DNA samples are shown below the gel and the positions of the homoduplexes are indicated on the right side.

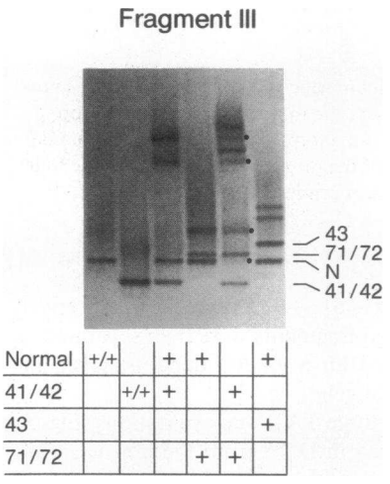

Figure 5. Gradient gel electrophoresis of fragment III. In lane *5*, the DNA from a patient who was doubly heterozygous for the 41/42 and 71/72 frameshift mutations was contaminated with normal DNA. This was the only sample with such a genotype available to us, but we were able to identify the bands that formed homoduplexes and heteroduplexes with contaminated DNA by hybridization with oligonucleotide probes. The contaminated bands are marked with black dots.

β-thalassemia in the Chinese population. This method gives the best resolution when each segment is < 500 nucleotides long. We therefore amplified regions of the β-globin gene that contained these mutations in four separate fragments. Using these four fragments, we were able to differentiate all 12 mutations. In DNA homozygous for a mutation, a single abnormal band was usually seen. When the DNA segment was heterozygous for one mutation or doubly heterozygous for two different ones, two homoduplexes and two heteroduplexes were usually seen. Occasionally the homoduplexes or heteroduplexes overlapped, and only three bands were seen. Even when the differences between the homoduplexes were small or absent, the heteroduplexes were sufficiently characteristic to enable us to differentiate the mutations from each other.

Occasionally a homoduplex of a normal and a mutant migrate so closely that it may be difficult to distinguish the homozygously normal state from the homozygously abnormal one, as occurred in the case of the IVS-I, position 5 mutation (Fig. 4). If this distinction is necessary, an additional reaction could be set up in which equal amounts of normal DNA are added to the test sample before amplification. A single band will be seen in the normal, while the additional heteroduplex bands would appear in a homozygously affected individual.

Once the mobilities of the various mutations have been determined, denaturing gradient gel electrophoresis can be used as a nonradioactive diagnostic method. The common β-thalassemia mutations found in China are the seven shown at the top of Fig. 1. Therefore, fairly simple patterns would be observed for each fragment. Denaturing gradient gel electrophoresis has the added advantage of potentially uncovering new mutations because any bands that have migration patterns different from the ones established for a given population would be seen. The abnormally migrating DNA could be isolated from the gel and the mutation detected by direct sequencing. The PCR segment we used covered only those regions of the β-globin gene containing known mutations in the Chinese population. Additional primers could be generated to cover the entire gene.

This strategy could be applied to detect known and new mutations in other genetic systems. However, it may not be practical for genes much larger than the globin genes. In those cases, one could amplify cDNA reverse transcribed from RNA, instead of amplifying genomic DNA.

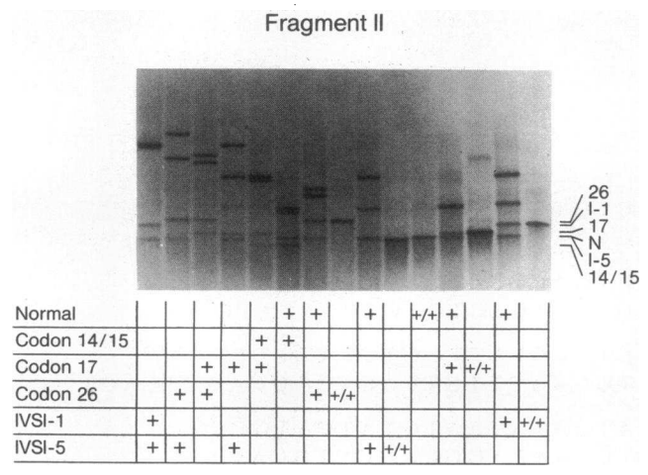

Figure 4. Gradient gel electrophoresis of fragment II.

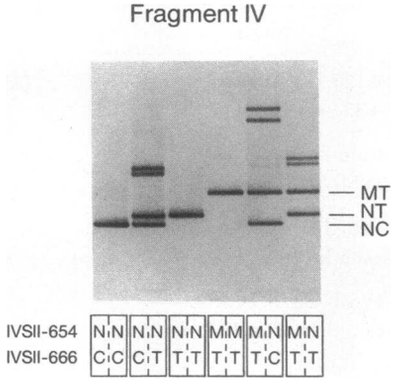

Figure 6. Gradient gel electrophoresis of fragment IV. The sequences of the normal C/T polymorphism at position 666 of the normal (*N*) or mutant (*M*) allele at position 654 are shown.

Acknowledgments

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