Identification of Four Novel δ-Globin Gene Mutations in Greek Cypriots Using Polymerase Chain Reaction and Automated Fluorescence-Based DNA Sequence Analysis

By P. Trifillis, P. Ioannou, E. Schwartz, and S. Surrey

The molecular basis of most β-thalassemia syndromes has been defined, while the spectrum of mutations causing δ-thalassemia is not well characterized. In an attempt to identify such mutations, the region encompassing the δ-globin gene from three Greek Cypriot families suspected of having δ-thalassemia was amplified by polymerase chain reaction (PCR), and DNA sequence determined using an automated fluorescence-based sequencer. Four novel mutations were identified: a G → T change at codon 27 that results in an alanine to serine change; a C → T change at codon 116 converting arginine to cysteine; a T → C change at codon 141 converting leucine to proline; and an AG → GG change at the consensus 3'-acceptor site in IVS-2. While the latter is clearly a thalassemia mutation, the low hemoglobin A₂ in the first three may be due to either decreased production or instability of the altered δ-globin chain. All four mutations may be detected by PCR amplification of genomic DNA followed by restriction enzyme digestion. Two mutations abolish restriction sites while two create new cleavage sites. Screening for molecular defects that cause δ-thalassemia or unstable δ-globin by PCR amplification and restriction enzyme digestion will lead to correct diagnosis of β/δ-thalassemia compound heterozygotes and improved genetic counseling.

FURTHER RESEARCH

Materials and Methods

Hematologic studies, DNA extraction, and α-globin gene analysis. Hematologic studies, DNA isolation, and Hb A₂ determinations were performed in Cyprus, using standard protocols, while Southern blots were performed at the Children’s Hospital of Philadelphia, as previously described. The α-globin gene analysis included gene blotting after digestion of genomic DNA with BamHI and BglII, and subsequent hybridization with an α-gene cDNA probe.

Oligonucleotide primers. Oligonucleotide primers were prepared using phosphoramidite chemistry on a 380B DNA synthesizer (Applied Biosystems, Inc, Foster City, CA). Primers 1 to 8 were synthesized with the trityl group off, while primers 9 to 14 were synthesized with the trityl group on, and were subsequently purified on OPC columns (Applied Biosystems). Primer 15 was supplied with the Taq Dye Primer Cycle Sequencing Core kit (Applied Biosystems). The primers used are listed in Table 1.

Amplification of δ-globin gene and fluorescent DNA sequence analysis using cycle sequencing. Portions of the δ-globin gene were amplified from DNA by PCR in the presence of specific primers and primed with dye-labeled primer using the Dyedeoxy PCR kit (Perkin Elmer). The PCR products were separated by electrophoresis on an automated DNA sequencer (model 373A, Applied Biosystems, Foster City, CA). The fluorescent products were detected on a 373A DNA sequencer calibrated with a dye-labeled molecular weight ladder (3600 and 4000 bases). The sequence of the amplification products were determined by automated fluorescence-based DNA sequencing.
amplified from genomic DNA using four sets of primers (primer pairs 1/3, 2/6, 4/7, and 5/8; see Fig 1). First round PCR amplification reactions for the four primer pairs contained the following in 100 μL: 1 μg of genomic DNA, 10 μL of 10X PCR buffer (100 mmol/L Tris buffer, pH 8.3, 500 mmol/L KCl, 15 mmol/L MgCl₂, and 0.1% [wt/vol] gelatin), 8 μL of 1.25 mmol/L dNTPs, 5 pmol of the 5'-limiting primer, and 20 pmol of the 3'-excess primer. Samples were heated to 95°C for 5 minutes, centrifuged briefly in a microfuge, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) was added. Samples were covered with 100 μL of paraffin oil and incubated in a DNA thermal cycler (Perkin-Elmer Cetus) at 96°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute for a total of 30 cycles. Samples were then extracted with 100 μL of chloroform, and 10 μL was electrophoresed on a 1.8% (wt/vol) agarose gel to monitor the size of the PCR products. A second round amplification for each of the initial four reactions included 1 μL of the first PCR product as template (Fig 1C). Reactions were as described above except that 1.6 μL of dNTPs was used, 1.5 pmol of a different 5'-limiting primer, and 50 pmol of the same 3' primer as in the initial PCR were used. The 5'-limiting primer used in the second amplification is a nested primer to which the M13 universal primer sequence has been attached (primers 9 through 14 in Table 1). This serves to incorporate the M13 universal primer into the second round PCR products, enabling the complementary strand to serve as template for DNA sequence analysis using dye-tagged M13 universal primer added later. The 5'-nested products used in the second amplifications were as follows: primer 9 for amplification of (1/3) PCR products; primers 10 and 11 for (2/6) products; primer 12 for (4/7) products, and primers 13 and 14 for (5/8) first round PCR products. Second round amplification cycles (denaturation/annealing/extension) were at 96°C for 30 seconds, 50°C for 1 minute, and 72°C for 2 minutes with each additional cycle increased by a 5-second polymerase extension for a total of 20 cycles. After the second PCR, the products were extracted and subjected to electrophoresis, as described above. The products of the second PCR were used as template for fluorescence-based DNA sequence analysis with Taq polymerase using the Taq Dye Primer Cycle Sequencing Core kit supplied by Applied Biosystems. Four reactions were run per sample, each corresponding to terminations with one of the four ddNTPs. One microliter of template was mixed with 1 μL of appropriate d/ddNTP mix. 1 μL of 5X cycle sequencing buffer, 1 μL of Taq polymerase (0.6 U), and 1 μL of the appropriate dye primer (0.4 pmol/μL) for the A and C terminations. Volumes were doubled for G and T terminations. Each of the four reactions were overlaid with 20 μL of paraffin oil, and placed in a thermal cycler preheated to 95°C. Thermal cycling was performed at 95°C for 30 seconds, 55°C for 30 seconds, and 70°C for 1 minute for 15 cycles, followed by another 15 cycles at 95°C for 30 seconds and 70°C for 1 minute. The four reactions were then extracted from underneath the oil and pooled. Fluorescent-tagged extension products were precipitated by addition of 80 μL of 95% ethanol and 1.5 μL of 3 mol/L NaOAc, pH 5.2. The tube was
incubated on ice for 15 minutes, microfuged for 15 minutes, and the pellet dried and resuspended in 6 μL of loading buffer (5/1 [vol/vol] deionized formamide/50 mmol/L EDTA, pH 8.0). The tube was then heated at 95°C for 3 minutes before loading on a sequencing gel for analysis in the Applied Biosystems Automated DNA Sequencer, Model 373A.

Sequence alignment and comparison. The sequence obtained from each run was compared with the known sequence of the δ-globin gene11 with the help of SeqEd, a software program (Applied Biosystems).

PCR-based identification of mutations after restriction enzyme digestion. Digests contained 20 U of a restriction enzyme (EcoO 109 I, SacI II, Hpa II, or Sac II), 15 μL of a first round PCR product, and buffer conditions as described by the manufacturer (Bethesda Research Laboratories, Gaithersburg, MD, or New England Biolabs, Beverly, MA). Digests were incubated at 37°C overnight, and then subjected to electrophoresis on a 1.8% (wt/vol) agarose gel in 1X TBE buffer (89 mmol/L Tris-borate, 89 mmol/L boric acid, and 2 mmol/L EDTA) containing ethidium bromide. Bands were visualized by transillumination with a UV light source (Hoefer Scientific Instruments, San Francisco, CA).

RESULTS

Hematologic data from the three Cypriot families studied are summarized in Table 2. Family A consists of an extended pedigree of 25 individuals, seven of whom are listed in Table 2. Individual II-3 of family A has no detectable Hb A₂, suggesting the presence of homozygous δ-thalassemia. Blotting studies for α-genes showed an α²/α² genotype in II-3 (data not shown). Her husband (II-4) is a classic δ-thalassemia carrier with high Hb A₂ levels (1.4% to 1.6%), consistent with heterozygosity for δ-thalassemia.

Table 2. Hematologic Data

<table>
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<tr>
<th>Family</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>Hb A₂ (%)</th>
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<td>31.7</td>
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</table>

Abbreviations: MCH, mean corpuscular Hb; MCV, mean corpuscular volume. *Hb A₂ variant.

Their son (II-3) is suspected of inheriting a δ-thalassemia gene from his mother (II-3) and a β-thalassemia gene from his father (II-4). Inheritance of the maternal δ-thalassemia gene masks proper diagnosis of β-thalassemia because this individual has a normal Hb A₂ level (2.5%) with a low MCV (66.0). Individual III-1 is a suspected compound heterozygote for δ-thalassemia, inheriting an Hb A₂-electrophoretic variant with decreased production from his mother (II-1) and a δ-thalassemia gene from his father (II-2). This electrophoretic variant migrates between Hbs S and Hb F on cellulose acetate, pH 8.9.

In family B, II-3 has a low Hb A₂ (0.2%) and is presumed to be homozygous for δ-thalassemia. All other members of this family, with the exception of II-1, were suspected δ-thalassemia heterozygotes due to their low Hb A₂ levels (1.5% to 1.7%).

In family C, II-1 has no detectable Hb A₂, suggesting homozygosity for δ-thalassemia. Two of her sisters (II-2 and II-4), as well as her parents (I-1 and I-2), have decreased Hb A₂ levels (1.4% to 1.6%), consistent with homozygosity for δ-thalassemia.

δ-Globin genes from patients suspected of having a δ-thalassemia syndrome (eg, II-3 and III-1 in family A, II-3 in family B, and II-1 in family C) were amplified using PCR and DNA sequence determined from −220 nucleotides 5' to the cap site to +50 nucleotides 3' to the poly A signal site. DNA sequence analysis (Fig 1E). The cycle sequencing strategy16 used for fluorescence-based DNA sequence analysis is outlined in Fig 1. This strategy incorporates the M13 universal primer sequence into the second round-seminested PCR product for subsequent use as a template using a dye-tagged M13 universal primer with fluorescence-based DNA sequence analysis (Fig 1E).

Portions of the sequence printouts in which mutations were found are shown in Fig 2. Individual II-3 from family A, who has no detectable Hb A₂, is homozygous for a T→C change at the second position of codon 141 that, if expressed, would result in a leucine to proline substitution (Fig 2C). Individual III-1 from family A, who expresses low levels of an Hb A₂ variant (0.6%), is a compound heterozygote for δ-thalassemia containing two different δ-globin gene mutations. In addition to the same T→C change at codon 141 in one of his δ-globin genes, a C→T change was found in the other gene at the first position of codon 116 that converts arginine to cysteine (Fig 2B). Individual III-1 from family B, who expresses low levels of Hb A₂ (0.2%), is also a compound heterozygote for δ-thalassemia. He has a G→T change at the first position of codon 27 that converts alanine to serine in one δ-gene (Fig 2A); in addition, he has an A→G change in his other δ-gene at the 3′-splice acceptor site of IVS-2 (Fig 2D). This individual is also homozygous for a C→T change at position −199 nucleotide 5′ to the cap site of the δ-globin gene (sequence printout not shown) that is most likely a neutral sequence changes.
Fig 2. δ-Globin gene mutations in Greek Cypriots. Portions of sequence printout showing location of four different δ-globin gene mutations (arrow) observed in four heterozygotes (A through D) and in two homozygotes (C and D) with family and pedigree designation indicated in parentheses. Normal nucleotide and amino acid sequence for region are shown in the upper panels with corresponding codon numbers. The letter “N” in the sequence printout denotes the presence of two different nucleotides at the same position in heterozygotes.
polymorphism. Individual II-1 from family C is homozygous for both the -199 polymorphism and for an A → G change at the 3′-splice acceptor site of IVS-2 (Fig 2D).

Four of the five observed changes within the δ-globin gene result in either the generation or loss of a restriction endonuclease cleavage site that is readily detected on sizing gels after digestion of the appropriate PCR products. The G → T change at codon 27 (ala → ser) and the C → T change at codon 116 (arg → cys) abolish EcoO 109 I (AGGCCCT → AGTCCCT) and Sau96 I (G’GCCCT → GGCT) sites, respectively; while the T → C change at codon 141 (leu → pro) and the AG → GG change at the 3′-acceptor site of IVS-2 create Hpa II (CTGG → C’CGG) and Sac II (CCGCAG → CCGC’GG) sites, respectively. EcoO 109 I was used for digestion of first round PCR products using primers 1 and 6; Sau96 I, Hpa II, and Sac II were used for digestion of first round PCR products using primers 5 and 8 (see Table 1). Representative digest patterns of PCR products are shown in Fig 3.

The δ 27 (ala → ser) change results in loss of an EcoO 109 I site (Fig 3A). PCR digests from unaffected individuals (open square) show 733- and 428-bp bands, while heterozygotes for the change (half-shaded circle) show, in addition to the 733- and 428-bp bands, a third band of 1,161 bp caused by loss of an EcoO 109 I site in one of the δ-globin genes. The δ 116 (arg → cys) change results in loss of a Sau96 I site (Fig 3B). Similarly, digestion with Sau96 I generates two bands in normals, 664- and 283-bp, and three in heterozygotes, a unique 947-bp band in addition to the normal 664- and 283-bp bands. The δ 141 (leu → pro) change creates a Hpa II site, resulting in digestion of the normal 947-bp band into 743- and 204-bp bands (Fig 3C); while the A → G mutation in the 3′-acceptor site of IVS-2 generates a new Sac II site resulting in digestion of the normal 947-bp band into 632- and 315-bp bands (Fig 3D).

Inheritance patterns for the various δ-globin gene mutations and relevant hematologic data for the three Cypriot families are summarized in Fig 4. Of the 25 members in family A, three are homozygous for the δ 141 (leu → pro) change with no detectable Hb A₂, (eg, II-3), six are heterozygotes for the δ 141 (leu → pro) change and express 0.8% to 1.2% Hb A₂ (eg, I-2), and two are compound

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**Fig 3.** Identification of δ-globin gene mutations by selective cleavage of PCR products. Sizing of PCR products before (bold B) and after restriction enzyme digestion with EcoO 109 I (A), Sau96 I (B), Hpa II (C), and Sac II (D). Solid and half-shaded circles and squares above lanes indicate family members in three pedigrees who are either homozygous or heterozygous, respectively, for different δ-globin gene mutations. (○, □) Unaffected controls.
heterozygotes for the $\delta 141$ (leu $\rightarrow$ pro) change and a $\beta$-thalassemia allele resulting in 2.1% and 2.5% Hb $A_2$ (eg, III-2). In a branch of this family a $\delta 116$ (arg $\rightarrow$ cys) variant is seen, so two individuals are compound heterozygotes for the $\delta 141$ (leu $\rightarrow$ pro) change and $\delta 116$ (arg $\rightarrow$ cys) variant with 0.5% and 0.6% Hb $A_2$ variant and no Hb $A_2$ (eg, III-1), and one is a heterozygote for the $\delta 116$ (arg $\rightarrow$ cys) variant expressing 1.4% Hb $A_2$ and 0.5% Hb $A_2$ variant (eg, II-1). Nine individuals in this family are normal and two are deceased. In family B, three individuals are heterozygotes for the $A \rightarrow G$ mutation in the $3'$-acceptor site of IVS-2 and express 1.5% to 1.7% Hb $A_2$; one is a heterozygote for the $\delta 27$ (ala $\rightarrow$ ser) variant with 1.7% Hb $A_2$, one is a heterozygote for the $\delta 27$ (ala $\rightarrow$ ser) variant and the $A \rightarrow G$ mutation in the $3'$-acceptor site of IVS-2, with 0.2% Hb $A_2$, and one is normal. In family C, four individuals are heterozygotes for the $A \rightarrow G$ mutation in the $3'$-acceptor site of IVS-2 with 1.4% to 1.6% Hb $A_2$, one is a homozygote for this mutation with no detectable Hb $A_2$, and one is normal. Because homozygosity for the splice defect results in no Hb $A_2$ (family C, II-1), the 0.2% Hb $A_2$ detected in individual II-3 of family B probably represents expression from the $\delta 27$ (ala $\rightarrow$ ser) gene.

**DISCUSSION**

In this study we describe the characterization of four novel mutations causing $\delta$-thalassemia or unstable $\delta$-globin variants in Greek Cypriots. One structural variant ($\delta 141$ [leu $\rightarrow$ pro]) leads to absence of $\delta$-chains while two others ($\delta 27$ [ala $\rightarrow$ ser] and $\delta 116$ [arg $\rightarrow$ cys]) cause a $\delta$-thalassemia phenotype. We propose to name the three variants after the villages in Cyprus where these patients reside: $\delta 27$ (ala $\rightarrow$ ser), Hb $A_2$-Ta-Li; $\delta 116$ (arg $\rightarrow$ cys), Hb $A_2$-Troodos; and $\delta 141$ (leu $\rightarrow$ pro), Hb $A_2$-Pelendri. The fourth mutation described here abolishes the $3'$-acceptor site in IVS-2 and leads to a $\delta$-thalassemia phenotype. While this report was in review, the identical $\delta$-globin 116 variant was described in a Greek family (Hb $A_2$-Corfu). In that instance, the relative amount of the variant was not reduced in the simple heterozygote. Hb $A_2$ was 1.3% and Hb $A_2$-Corfu 1.5%, while in our family A Hb $A_2$ was 1.4% and the variant 0.5% (Fig 4, family A, II-1). The decreased MCV in the Hb $A_2$-Corfu heterozygote (75 fl) is unexplained; in our family A, II-1, MCV was 93.7 fl (Table 2).

The amino acid change from leucine to proline at codon 141 is a nonconservative substitution. Proline interferes with $\alpha$-helix formation. Thus far, 24 $\beta$-chain variants have been reported that change an amino acid to proline. Twenty-one of these variants result in unstable Hbs. In addition, position 141 in the $\beta$-chain of Hb is in the interior of the molecule and its hydrophobic side chain is in direct contact with the heme group. In $\beta$-globin, a change to arginine at codon 141 results in marked instability of Hb $A_2$ tetramers, while an alanine to proline change at $\beta$ 142 (H20) results in instability and heme loss. The substitution of $\delta 141$ (H19) leu $\rightarrow$ pro observed here is at a homologous site with the $\alpha$-chain variant Hb Bibba $\alpha$ 136 (H19) leu $\rightarrow$ pro, which is also a very unstable variant. From these data and our observation that no Hb $A_2$ was detected in two siblings homozygous for this mutation, it appears likely that the change from leucine to proline at this position in $\delta$-globin results in marked instability of the variant Hb $A_2$.

The $\delta 116$ (G18) arg $\rightarrow$ cys change results in an Hb $A_2$ structural variant with altered electrophoretic mobility. No comparable $\beta$-chain variant has been reported so far. The presence of the cysteinyl residue at codon 116 could cause formation of intermolecular disulfide bonds. In two $\beta$-chain variants, Hb Porto Alegre $\beta$ 9 (A6) ser $\rightarrow$ cys and Hb Ta-Li $\beta$ 83 (EF7) gly $\rightarrow$ cys, the change occurs at a position on the outside surface of the molecule that allows intermolecular disulfide bonding. In the case of Hb Rainer $\beta$ 145 (tyr $\rightarrow$ cys), the change occurs at a position where the active cysteinyl residue can hydrogen bond intramolecularly with the naturally occurring cysteinyl residue at $\beta$ 93 (F9). We have no information on disulfide bonding in this new variant. The $\delta$-thalassemia phenotype of the $\delta 116$ (arg $\rightarrow$ cys) mutation is probably due to instability of the variant. Two $\beta$-chain variants, Hb Saïtama $\beta$ 115 (ala $\rightarrow$ pro) and Hb Madrid $\beta$ 117 (his $\rightarrow$ pro), occurring at positions...
The 3'-acceptor site mutation and position 579 of IVS-2 are used. This alternate splicing results in a 6'-globin gene expressing very low levels of Hb A, suggesting that the occurrence of α-thalassemia or unstable 6-globin variants in Cyprus is quite high. Characterization of compound heterozygotes for β/δ-thalassemia will help improve genetic counseling in the Cypriot population and in other areas where β-thalassemia syndromes are prevalent.

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