A Novel β-Globin Mutation, βDurham-NC (β114 Leu → Pro), Produces a Dominant Thalassemia-Like Phenotype

By Carlos M. de Castro, Blythe Devlin, Don E. Fleenor, Michael E. Lee, and Russel E. Kaufman

Mutations within exon 3 of the β-globin gene are relatively uncommon, and many of these mutations produce a dominant thalassemia-like phenotype. We describe a novel thalassemic hemoglobinopathy caused by a single nucleotide substitution (CTG → CCG) at codon 114 resulting in a leucine to proline substitution and designate it βDurham-NC (β114 Leu → Pro). The mutation producing this thalassemic hemoglobinopathy is located near to the β-savaya-Yakushiji mutation (β110 Leu → Pro). Both of these hemoglobinopathies share similar phenotypic features with moderately severe microcytic anemia. Using computer imaging of the hemoglobin molecule, we examined several reported point mutations within exon 3 of the β-globin gene. These point mutations cause a single amino acid substitution in the G helix, and result in a thalassemic and/or hemolytic phenotype. Computer imaging of nine separate examples suggests that amino acid substitutions affecting side chains that project into the heme pocket may destabilize the heme moiety within the β-globin chain, resulting in a thalassemic phenotype. Hemolytic phenotypes may be the result of decreased αβ interactions. The βDurham-NC mutation further characterizes a novel group of thalassemias/hemoglobinopathies that are clinically difficult to identify and require accessory laboratory testing.

© 1994 by The American Society of Hematology.

MUTATIONS WITHIN the β-globin gene have been among the most widely studied mutations. Deletions, substitutions, and frameshift mutations have been described within the three exons of the β-globin gene, as have mutations affecting both the promoter region and the splicing sites of the gene. These mutations lead to a variety of phenotypes, as has been recently reviewed.

In general, mutations that produce a quantitative defect by decreasing or eliminating β-globin chain synthesis have been labeled as thalassemias, whereas those mutations producing a qualitative defect such as a structurally abnormal globin are referred to as hemoglobinopathies. Historically, the hemoglobinopathies and the thalassemias have been considered as distinct clinical disorders. However, there are mutations that result in a structurally abnormal hemoglobin that produces the phenotype of thalassemia, and these disorders have been termed thalassemic hemoglobinopathies. Mutations within exon 3 of the β-globin gene are relatively uncommon, and several of these exon 3 mutations produce a dominant thalassemia-like phenotype. We report here a novel exon 3 mutation that creates a leucine to proline substitution at codon 114, and results in a β-thalassemia phenotype. Computer imaging of exon 3 mutations suggests that the thalassemic phenotype may be caused by disruption of the G helix and changes in the interaction with the heme pocket. The diagnosis requires a recognition of the syndrome and the use of nonstandard laboratory testing.

MATERIALS AND METHODS

Hematologic parameters and hemoglobin (Hb) procedures. Standard hematologic measurements were performed at Duke University Medical Center (DUMC) Clinical Laboratories. Peripheral blood counts and erythrocyte indices were determined using a Sysmex NE8000 electronic cell counter (Toa, Kobe, Japan). Hb electrophoresis was performed on cellulose acetate membrane at pH 8.6. HbF levels were determined by alkali denaturation, and HbA2 in incubated with L-3H-leucine (40 to 60 Ci/mmol; NEN Research Products, Boston, MA) for 2.5, 15, 60, and 120 minutes at 37°C. The α- and β-globin chains were separated and collected by reverse-phase high-performance liquid chromatography (HPLC) and incorporation of radioactive label was quantified using a Beckman LS6800 scintillation counter (Beckman Instruments, Palo Alto, CA).

DNA isolation and gene mapping. Genomic DNA was isolated from peripheral blood leukocytes by standard methods. Ten micrograms of DNA was digested with restriction endonucleases, separated by gel electrophoresis through a 1.0% agarose slab gel, and transferred to nylon membranes by standard methods. Probes specific for the γ-, δ-, and β-globin gene regions were labeled with 32P and hybridized to the membranes at 42°C overnight. After stringent washing, autoradiography was performed as previously described.

Polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) analysis. Exon 3 of the β-globin gene was amplified by PCR using 200 to 500 ng of genomic DNA as the template and using primers: sense 5'-CTGATCAGCTAAGCTAGGCC-3', and antisense 5'-TATCCCCAGTTTAGTAGTTGGA'-3'. These primers lie in the intervening sequences 60 bp upstream and 74 bp downstream of the coding sequence of exon 3, and amplify a 263-bp fragment containing the coding region of exon 3. PCR was performed using the hot start technique followed by 94°C for 1 minute, 50°C for 1 minute, and 72°C for 30 seconds for 30 cycles. The PCR product and the product digested withMspI were electrophoresed on 2% agarose and stained with ethidium bromide.

SSCP analysis was performed using a modification of the methods of Orita et al. Briefly, 0.5 µl of α32P-dCTP (3,000 Ci/mmol; NEN) was added to the PCR reaction to label the amplified product.

From the Department of Medicine, Division of Hematology and Oncology, Duke University Medical Center, Durham, NC.


Presented in part at the 1992 American Society of Hematology Meeting (Blood 80:6a, 1992 [abstr, suppl I]).

Address reprint requests to Russel E. Kaufman, MD, Box 3250, Duke University Medical Center, Durham, NC 27710.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1994 by The American Society of Hematology.
Southern blot analysis of β-globin genes. Southern blot analysis was performed on the patient’s genomic DNA to detect any structural lesions in the β-globin gene that might be producing the thalassemia phenotype. The genomic DNA was digested with restriction endonucleases, electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and hybridized with three distinct probes specific for the γ-, β-, and β-globin regions. Southern analysis of the γ-, β-, and β-globin regions failed to show any structural abnormalities (data not shown).

Analysis of globin chain synthesis using reverse-phase HPLC. To investigate globin chain synthesis rates, we incubated a peripheral blood sample from the patient and from a normal control with L-3H-leucine for 2.5, 15, 60, and 120 minutes at 37°C. The labeled globin chains were then analyzed with reverse-phase HPLC. No abnormally migrating globin chains were noted at 2.5 minutes or at any of the later timepoints, and an analysis of the kinetics of β-Durham-NC was therefore not possible. The α/β and β-globin chain fractions were collected, and the incorporation of the L-3H-leucine into newly synthesized globin chains was then measured in six separate measurements. The α/β ratio was calculated as the ratio of the mean counts per minute (cpm) from the respective fraction. At 2.5 minutes, the α/β ratio was 0.64 for the proband and 0.68 for the control, consistent with the results of previous studies showing an increase of labeled β globin over α globin at incubation times less than 5 minutes. At the later time points measured (15, 60, and 120 minutes), the α/β ratio remained constant around unity for both the patient and the normal control.

PCR-SSCP analysis of β-globin gene exon 3. Based on the globin synthetic rates and the thalassemic phenotype, we reasoned that the patient might have a dominantly inherited thalassemic hemoglobinopathy. Many of these dominant thalassemic hemoglobinopathies have been caused by mutations within the third exon of the β-globin gene. To screen for a possible mutation within this exon, we performed PCR-SSCP analysis. Oligonucleotide primers lying within the flanking introns were synthesized and used to PCR-amplify exon 3 from genomic DNA samples from both the patient and a normal control. ω-3P-dCTP was used to label the PCR product, and the denatured product was then electrophoresed on a polyacrylamide gel at 6 W at room temperature. Autoradiography (Fig 3) showed an abnormal migration of a band when compared with a normal control, thus suggesting a conformational polymorphism within exon 3.

Sequence analysis of β-globin exon 3. The PCR-amplified exon 3 fragment was cloned into the TA cloning vector (Invitrogen). DNA from two separate PCR reactions was cloned to confirm that any sequence difference was not due to Taq misincorporation. Cloned exon 3 fragments were then directly sequenced, as shown in Fig 4. Sequence analysis showed a T to C nucleotide substitution in the second position of codon 114, which results in a Leucine to a Proline substitution.
site. In normal controls, no Msp I site is located within the 263-bp PCR product containing the coding region of β-globin exon 3. With the βDurham-NC mutation creating an Msp I site, digestion should result in two fragments of 174 bp and 93 bp from the mutant allele, in addition to the 263-bp band from the normal allele. We therefore subjected the PCR-amplified exon 3 product of a normal control, the propositus, and the proband’s mother, who also carries a thalassemia phenotype, to Msp I digestion. As shown in Fig 5, both the proband and the proband’s mother show heterozygosity for the mutant Msp I site, whereas the normal control shows only the 263-bp band.

Table 1. Hematologic Parameters and Other Laboratory Values of the Proband and Her Mother

<table>
<thead>
<tr>
<th></th>
<th>Proband</th>
<th>Mother</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>8.6</td>
<td>9.5</td>
</tr>
<tr>
<td>RBC (10⁹/µL)</td>
<td>3.55</td>
<td>4.53</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>27</td>
<td>32</td>
</tr>
<tr>
<td>MCV (µL)</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>Reticulocytes (mol/L/µL)</td>
<td>94</td>
<td>(3.6%)</td>
</tr>
<tr>
<td>Total bilirubin (mg%)</td>
<td>2.5</td>
<td>(1.3)</td>
</tr>
<tr>
<td>LDH (IU)</td>
<td>432</td>
<td>ND</td>
</tr>
<tr>
<td>Ferritin</td>
<td>478</td>
<td>ND</td>
</tr>
<tr>
<td>HbF (%)</td>
<td>6.1</td>
<td>7.5</td>
</tr>
<tr>
<td>HbA2</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>G6PD (U/mL)</td>
<td>5.5</td>
<td>ND</td>
</tr>
<tr>
<td>Osmotic fragility</td>
<td>Neg</td>
<td>ND</td>
</tr>
<tr>
<td>Autohemolysis</td>
<td>Neg</td>
<td>ND</td>
</tr>
<tr>
<td>Hb electrophoresis</td>
<td>No abnormal bands</td>
<td></td>
</tr>
</tbody>
</table>

All values except those shown in parentheses were performed at the clinical laboratories at DUMC.

Abbreviations: ND, not determined; Neg, negative; LDH, lactate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase.

Computer imaging of Hb. To study the structural effects of this novel mutation, computer-generated graphic images of the Hb molecule were examined (Fig 6). Review of the literature found 12 other point mutations located within the G helix near to the βDurham-NC mutation (Table 2). These mutations show a spectrum of phenotypes ranging from thalassemia to chronic hemolytic anemia, and were therefore divided into two groups based on either a predominantly thalassemic phenotype (Fig 6A and B) or a predominantly hemolytic phenotype (Fig 6C and D). Several different views of the G helix were generated and scrutinized. The four point mutations that result in a thalassemia phenotype seem to affect amino acids whose side chains (shown in yellow) are pointed inward toward the heme pocket, whereas the five point mutations resulting in a hemolytic phenotype seem to alter amino acids with side chains (shown in magenta) aimed toward the αβ contact regions.

Mutations affecting the H helix were also examined on the computer images (data not shown). Two point mutations, Hb Houston (β127 Gln → Pro)¹ and Hb Gunma (β127-128 Gln-Ala → Pro),⁶ lead to a thalassemia phenotype. The effect of mutations at codon 127/128 is not apparent from the computer image, and therefore neither supports nor invalidates the hypothesis.

DISCUSSION

In the heterozygous state, β-thalassemia usually produces a mild anemia. RBCs with a reduced MCV, a reduced mean corpuscular Hb (MCH), and an increased HbA2 level. A unique class of mutations in β-globin genes produces a structurally altered globin chain that results in a thalassemia intermedia phenotype and is inherited in a dominant manner. These conditions have been termed “thalassemic he-
Fig 3. PCR-SSCP analysis of $\beta$-globin gene exon 3. Oligonucleotide primers from the flanking introns were used to PCR-amplify $\beta$-globin exon 3 from genomic samples from both a normal control (lane 1) and the patient (lane 2). $\alpha$-P-dCTP was used to label the PCR product, and the denatured product was electrophoresed on a polyacrylamide gel at 5.5 W at room temperature. Autoradiography showed a shift in the migration of a band (at the arrow) when compared with the normal control.

Fig 4. DNA sequence analysis of the abnormal $\beta$-globin allele from the propositus, demonstrating a T to C nucleotide substitution at the second position of codon 114, which results in the substitution of a proline for a leucine.
BDURHAM-NC, A THALASSEMIC HEMOGLOBINOPATHY

Fig 5. MspI digestion of exon 3. Exon 3 was amplified from peripheral blood DNA samples from a normal control (lane 1), the proband (lane 2), and the proband’s mother (lane 3). The base change at codon 114 converts the DNA sequence from CTGG to CCGG, thus creating a novel MspI restriction enzyme recognition site. In normal controls, no MspI site is located within the 263-bp PCR product containing the coding region of β-globin exon 3. With the βDurham-NC mutation, digestion results in two fragments of 174 bp and 89 bp, in addition to the 263-bp band from the normal allele.

It has been postulated that mutations within exon 3 of the β-globin gene lead to a thalassemia phenotype by disrupting the formation of αβ dimers.\(^1\),\(^3\),\(^10\) Exon 3 encodes most of the G helix and all of the H helix of the β-globin chain, and both helices contain several αβ contact points. Within the G helix of the β-globin chain are four αβ contact points at codons 108, 112, 115, and 116.\(^3\) The substitution of a proline for a leucine at codon 114 in Hb Durham-NC would disrupt the G helix and interfere with these contact points, thus interfering with the αβ dimerization. The catabolism of the free globin chains might lead to the thalassemia phenotype. However, this does not explain the difference in the resulting thalassemic versus hemolytic phenotypes seen with various exon 3 mutations.

To examine the basis for the phenotypic differences caused by mutations in the G helix, we have reviewed the known exon 3 mutations and examined the region by computer imaging. Thirteen point mutations within the portion of exon 3 that encodes the G helix are summarized (Table 2), of which nine result in a single amino acid substitution. Four of these point mutations occur within the same codon, yet result in different amino acid substitutions with different resultant phenotypes. Hb Southampton (β106 Leu → Pro) results in a severe hemolytic phenotype,\(^4\) Hb Tubingen (β106 Leu → Gin) results in a methemoglobinemia,\(^5\) whereas Hb Terre Haute (β106 Leu → Arg) results in a thalassemic phenotype.\(^6\) Another mutation at this codon results in a +1 frameshift, and also leads to a thalassemia phenotype.\(^7\) Using computer-generated images of the Hb molecule, we have examined the relative position of the exon 3-G helix substitutions that produce the different phenotypes (Fig 6). Those mutations that result in a predominantly thalassemic phenotype are shown in Fig 6A and B, whereas those that result in a hemolytic phenotype are depicted in Fig 6C and D. A view down the barrel and a lateral view of the G helix shows that all the mutations that produce the thalassemic phenotype involve amino acids with side chains that project into the heme pocket, whereas the mutations that produce a hemolytic phenotype involve amino acids with side chains that project outside the heme pocket, usually towards the αβ contact region. Substitutions at codon 106 that produce a side chain that projects into the pocket are associated with a thalassemic phenotype.

The location of the amino acids affected by the various mutations suggests an alternative explanation, ie, that the thalassemia phenotypes are caused by alterations that affect the ability of the β-globin chain to bind heme or to alterations that destabilize the heme pocket. Although the β-globin B, G, and H helices are extensively involved in αβ contact and dimerization, these helices also form the floor of the hydrophobic pocket where the heme moiety resides. Disruption of this pocket by amino acid substitutions might lead to the synthesis of a β-globin molecule with a decreased or absent heme binding ability. Such a globin molecule would be unstable and subsequently catabolized before dimer formation. The preferential catabolism of β-globin over α-globin may result in a thalassemia phenotype. In contrast, those mutations that disrupt αβ dimerization might allow some degree of dimer formation but lead to an equal rate of catabolism of both globin chains, thus producing a chronic hemolytic picture.

Support of this hypothesis is seen by again examining and contrasting Hb Durham-NC [β114 Leu → Pro], which results in a thalassemic phenotype, and Hb Madrid [β115 Ala → Pro], which results in a hemolytic phenotype. The computer images in Fig 6 show that the side chain of the amino acid at codon 114 projects into the heme pocket, whereas the side chain of the amino acid at codon 115 projects towards the αβ contact region. Other exon 3 mutations with a dominant thalassemia phenotype include Hb Terre Haute,\(^16\) Hb Houston,\(^1\) Hb Kohn Kaen,\(^18\) Hb New York,\(^19\),\(^20\) Hb Gunma,\(^6\) Hb Chesterfield,\(^21\) and a frameshift mutation reported at codon 106.\(^17\) Some of these mutations also produce a variable degree of hemolysis. In all the thalassemic mutations that were examined, the side chain of the affected amino acid is pointed into the heme pocket.

Although the computer images support the hypothesis, the mechanism underlying thalassemic versus hemolytic phenotypes is still not completely explained. There are several unstable Hb variants caused by amino acid substitutions in the vicinity of the heme pocket.\(^22\) With the exception of Hb Southampton, as discussed below, none of these mutations occur in exon 3. Two exceptions to the hypothesis we have espoused are also apparent from the computer images. The first is Hb Southampton [β106 Leu → Pro], which results in a hemolytic phenotype,\(^4\) whereas an arginine substitution (Hb Terre Haute) at this location results in a thalassemic phenotype.\(^6\) Perhaps the proline substitution at this location causes a severe disruption of the G helix...
and therefore has its strongest effect on $\alpha_1\beta_1$ contacts. The arginine substitution may have a predominant effect on the heme moiety with a lesser effect on $\alpha_1\beta_1$ dimerization. The second apparent exception may be Hb Saitama ($\beta117$ His $\rightarrow$ Pro), in which the side chain of the histidine appears to be inward towards the heme pocket. However, this residue is at the terminus of the G helix and is well removed from the heme moiety. It is likely that the proline substitution at this location has a more disruptive effect on the H helix, and may therefore interfere with the $\alpha_1\beta_1$ dimerization. We have not attempted to determine the detailed conformational changes that are produced by this class of variants because the rules for computer modeling are not fully developed and would be highly speculative.
Previously, investigation and characterization of atypical thalassemia cases has used various methods of isolotopic labeling of globin chains, including synthesis analysis by incubation of RBC’s with ¹³C-leucine using various incubation times, HPLC analysis, and tryptic digestion followed by HPLC analysis. Specific globin gene mutations could also be identified by cloning of the β-globin gene and DNA sequence analysis, or more recently by PCR amplification followed by DNA sequence analysis. Because most of the mutations that result in a dominant thalassemia phenotype are located within exon 3, we approached this case with the PCR-SSCP technique to directly screen for a possible mutation within exon 3. The PCR-SSCP technique was initially developed as a method for screening for sαt mutations in various cancers⁴ and has been extended to screen for p53 mutations in cancers.²⁴ This method is based on a shift in migration caused by alterations in the conformation of single-stranded DNA dependent on the nucleotide sequence. The method has been shown to be easily performed and to be extremely sensitive for detecting changes in nucleotide sequence. Our results suggest that the PCR-SSCP technique can be extended to screening of possible point mutations within the β-globin gene, which in this case produced a dominant thalassemia-like phenotype.

ACKNOWLEDGMENT

The authors are grateful to Dr David Richardson (Department of Biochemistry, Duke University, Durham, NC) for his technical assistance with computer imaging. We thank Beth Maynard and Bobbie Reeves for assistance with the manuscript preparation.

REFERENCES

23. Ohba Y, Hasegawa Y, Amato H, Miwa S, Nakatsuji T, Hat-


A novel beta-globin mutation, beta Durham-NC [beta 114 Leu-->Pro], produces a dominant thalassemia-like phenotype

CM de Castro, B Devlin, DE Fleenor, ME Lee and RE Kaufman

Updated information and services can be found at:
http://www.bloodjournal.org/content/83/4/1109.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml