The Molecular Basis of β-Thalassemia in Lebanon: Application to Prenatal Diagnosis

By F.F. Chehab, V. Der Kaloustian, F.P. Khouri, S.S. Deeb, and Y.W. Kan

A study of the molecular lesions of β-thalassemia in Lebanon revealed the presence of eight different mutations in 25 patients with Cooley’s anemia. The IVS1 position 110 mutation predominated with a frequency of 62% and was almost invariably associated with Mediterranean chromosome haplotype I. Five other mutations commonly found in the Mediterranean area occurred with frequencies of 2% to 8%. In addition a G → C substitution in IVS1 position 5 (a lesion previously found in Chinese and Asian Indians) was demonstrated in a patient with Mediterranean haplotype IX. A new mutation at codon 29 was found in two other patients with haplotype II. The characterization of these β-thalassemia mutations should allow the implementation of a prenatal diagnosis program in that country.

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LEBANON, a small country on the Mediterranean Sea, is known to have interacted with many civilizations over the centuries, and the Lebanese population has resulted from an admixture of various ethnic groups. The incidence of β-thalassemia trait of both the β0 and β+ forms is 2% to 3% in Lebanon.1 Consanguineous marriages within certain ethnic communities having a high incidence of β-thalassemia have led to an increase in homozygous patients within that particular community. Clinically, homozygous β-thalassemia in Lebanon is manifested as the severe transfusion-dependent state, a form of Cooley’s anemia that prevails in the Mediterranean region, throughout the Middle East, the Indian subcontinent, Burma, and southeast Asia.2 The elucidation of the molecular defects of β-thalassemia in Lebanon should be informative on both scientific as well as anthropologic levels, since it would reflect the diversity of populations residing in that country. Whereas therapy for β-thalassemia has been mainly supportive, prevention of the disease by prenatal diagnosis has been a major goal in areas where the incidence is high. The characterization of the molecular defects would allow implementation of a prenatal diagnosis program. In this study, we have delineated the molecular lesions of β-thalassemia in Lebanon. We first studied the linkage of DNA polymorphisms in the β-globin gene cluster to specific β-thalassemia mutations and then defined specific mutations with oligonucleotide probes or by cloning and sequencing. We found eight different DNA haplotypes and eight different mutations among 50 β-thalassemia chromosomes. Six mutations were previously described in the Mediterranean population; one mutation previously found in the Chinese and Asian Indian populations was also found in this Mediterranean population on a new haplotype. In addition, we uncovered a new mutation that has not been previously described.

METHODS

Patients. Twenty-five unrelated homozygous β-thalassemic subjects (three β0 and 22 β+) were studied. All but one Christian patient were either Sunni or Shiite Moslems. Individuals in the present study have typical histories of transfusion-dependent Cooley’s anemia.

DNA extraction and Southern blotting. DNA was extracted from the peripheral blood of affected individuals by either the proteinase K method4 or by the standard procedure of phenol-chloroform extraction.5 Ten micrograms of DNA were digested to completion with the appropriate restriction enzyme, separated according to size on agarose gels, transferred to nitrocellulose filters,4 and hybridized to 32P nick-translated β, γ, Ψβ, and ε globin gene probes. DNA haplotypes were determined using seven common restriction site polymorphisms in the β-globin cluster.7

Oligonucleotide hybridization. Synthetic 19-mer oligonucleotide probes were labeled by phosphorylation of the 5’ ends with γ-32P-ATP (7,000 Ci/mmol, ICN or New England Nuclear, Boston) and T4 polynucleotide kinase. The labeled oligonucleotide was then separated from unincorporated nucleotides by thin layer chromatography on diethyl aminoethyl (DEAE) cellulose plates (Analytech, Newark, DE) in the presence of urea and hydrolysed yeast tRNA.4 Five to ten micrograms of genomic DNA were digested with Bam HI, separated on 0.8% agarose gels, and processed exactly as described,4 except that the hybridization and washing temperatures were varied according to the probe used as shown in Table I. These temperatures were empirically selected according to the formula of Conner et al,8 then experimentally adjusted.

Molecular cloning and DNA sequencing. One hundred fifty to two hundred micrograms of genomic DNA were completely digested with Eco RI or Hind III, fractionated on 10% to 40% sucrose gradients, and the β-globin-rich fraction identified by Southern blotting. Bacteriophage λ Charon 4A and Charon 30 were used for Eco RI and Hind III vectors, respectively. The recombinant bacteriophage clones containing β-globin inserts were generated as described.10 Bacteriophage clones containing the 5.2 kb, 3.6 kb Eco RI fragments and 7.8 kb Hind III β-globin fragments were thus isolated. The 5.2 kb and 7.8 kb fragments were further subcloned in either of the bacterial plasmids pBR322, pUC18, or pBS (Stratagene Cloning Systems, San Diego). DNA sequencing of double-stranded supercoil DNA was essentially as described.11

Prenatal diagnosis. DNA from a Lebanese couple with a child affected by homozygous β-thalassemia was haplotyped for DNA polymorphisms in the β-globin cluster, and the most likely mutation

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linked to this haplotype was identified by oligonucleotide hybridization. After obtaining informed consent from the parents, 35 mL of amniotic fluid was collected in Lebanon and flown to San Francisco within 24 hours. The packed cells were collected by centrifugation, washed, resuspended in 400 μL of 0.1 mol/L NaCl, 0.05 mol/L Tris HCl pH 7.5, 1 mmol/L EDTA, and lysed with 25 μL of 10% SDS. The lysate was digested overnight with 100 μg/mL proteinase K at 55°C, phenol extraction was performed, and the DNA was precipitated. Five micrograms of DNA were digested with Bam HI and were processed as described above for oligonucleotide hybridization.

RESULTS

Haplotypes. One Asian and seven common Mediterranean haplotypes were found in the Lebanese population. Table 2 lists the haplotypes detected and their frequencies. Mediterranean haplotype I predominated over the other haplotypes. Twenty-three of the 25 patients were homozygous for a single haplotype (I, II, III, IV, VI, VII, and IX) and two were doubly heterozygous for two different haplotypes (I/VI and II/Asian H).

Detection of mutations. To elucidate the molecular defects of β-thalassemia in this population, we tested each patient's DNA by either Southern blotting or oligonucleotide hybridization for one or more mutations previously shown to be linked to that haplotype. If the molecular lesions could not be uncovered according to the above strategy, the β-globin genes were cloned and sequenced.

Two mutations were detected by Southern blotting. Patients with haplotype III and haplotype VII were tested for the IVS2 position 1 (G → T) and IVS2 position 745 (C → G) lesions that respectively alter the Hph I and Rsa I restriction endonuclease sites. One patient each was found to be homozygous for these two mutations.

Synthetic oligonucleotide hybridization detected the mutation in 18 other patients who are homozygous for a single haplotype. These include the IVS1 position 110 mutation in 15 β-thalassemia patients homozygous for haplotype I, the IVS1 position 6 mutation in a patient homozygous for haplotype VI, the nonsense mutation at codon 39 in a homozygote for haplotype II, and the dinucleotide deletion at codon 8 in a homozygote for haplotype IV. In addition, a patient who is doubly heterozygous for haplotypes I and VI is also homozygous for the IVS1 position 6 mutation.

The molecular lesions in the β-globin genes from four other patients were not detected by the above strategy. The β-globin genes of a patient homozygous for haplotype IX were cloned in Charon 4A and subcloned in pBR322. Supercil DNA sequencing showed that the mutation was a G → C substitution at IVS1 position 5 (Fig 1). This substitution was subsequently confirmed by oligonucleotide hybridization to be present on both of this patient's chromosomes (Fig 2).

The second patient with an uncharacterized mutation was a double heterozygote for haplotypes II and Asian H. By oligonucleotide analysis, he carried the IVS1 position 110 lesion on one chromosome. His β-globin genes were cloned in Charon 4A followed by subcloning in pBS, and DNA sequencing revealed the IVS1 position 110 mutation on haplotype II. The other chromosome with the Asian H haplotype carried the dinucleotide deletion in codon 8, which has previously been reported to be associated with Mediterranean haplotype IV.

Two other patients homozygous for haplotype II failed to show any of the above mutations. The β-globin genes from one were cloned in Charon 30 and subcloned in pUC18. DNA sequencing revealed a base change (C → T) at codon 29 (Fig 1). Two oligonucleotide probes complementary to the normal and mutated sequence at codon 29 were then synthesized and hybridized to genomic DNA from these two patients.
account for 60% of the \( \beta \)-thalassemia mutations. The other two mutations were found to be strongly linked in this population and thus account for 5% of the \( \beta \)-thalassemia mutations.

The substitution at codon 29 was found in two patients, both proved to be homozygous for the same substitution at codon 29 (Fig 2).

Thus in this sample of 50 \( \beta \)-thalassemia chromosomes from Lebanon, eight different mutations were found to account for \( \beta \)-thalassemia. Haplotype I and the IVS1 position 110 lesion are strongly linked in this population and accounted for 60% of the \( \beta \)-thalassemia mutations. The other seven mutations are distributed at a frequency range of 2% to 8%. The heterogeneous \( \beta \)-thalassemia mutations in Lebanon can thus be detected by the use of six oligonucleotide probes and two restriction enzymes (Table 3).

\textbf{Prenatal diagnosis.} We have performed a prenatal diagnosis for a Lebanese fetus at risk for \( \beta \)-thalassemia by oligonucleotide hybridization of amniotic DNA. Previous screening of the parents and an affected child revealed that the \( \beta \)-thalassemia alleles were on chromosomes haplotype I, and hybridization with oligonucleotide probes showed that the mutation was the IVS1 position 110 (G \( \rightarrow \) A). The fetal DNA when tested with oligonucleotide probes for this mutation hybridized only to the normal probe. Thus the fetus did not carry this mutant \( \beta \)-globin allele.

\section*{DISCUSSION}

The clinical manifestation of \( \beta \)-thalassemia in Lebanon is severe, and homozygous patients have \( \beta / \alpha \) globin synthetic ratios of 0 to 0.34.\(^1\) In this study we have delineated the molecular defects in 25 transfusion-dependent \( \beta \)-thalassemia Lebanese patients by Southern blotting, synthetic oligonucleotide probing of genomic DNA, and molecular cloning followed by DNA sequencing. The Lebanese population carries eight different \( \beta \)-thalassemia mutations, six of which have been previously reported in the Mediterranean area. The IVS1 position 110, IVS1 position 6, IVS2 position 1, IVS2 position 745, \( \beta^5 \) nonsense mutation, and frameshift mutation at codon 8 have all been shown to significantly alter the normal expression of the \( \beta \)-globin gene.\(^{12,14}\) The linkage of \( \beta \)-thalassemia mutations in Lebanon to DNA haplotypes in the \( \beta \)-globin cluster was similar to that described\(^2\) for haplotypes I, II, III, IV, V, and VII. In addition we found four new linkages in this population: the mutation in IVS1 position 6 to haplotype I, the mutation at IVS1 position 5 (G \( \rightarrow \) C) to haplotype IX, the dinucleotide deletion at codon 8 to the Asian H haplotype new to the Mediterranean, and the new mutation at codon 29 to haplotype II.

All of these mutations except the one at codon 29 have been previously described. The \( \beta^5 \) mutation\(^{13,14}\) has also been found in Sardinia,\(^{15}\) Morocco, Algeria,\(^{13}\) Greece, and Turkey.\(^{16}\) The nucleotide substitution at the 5' splice site of IVS2 has been described in an Italian and in some Iranian patients.\(^{16}\) The dinucleotide deletion at codon 8 was originally detected in a Turkish patient.\(^{7}\) The IVS1 position 110 mutation\(^{17,18}\) is the most common cause of \( \beta \)-thalassemia in Mediterranean countries.\(^7\)

The IVS1 position 6 mutation was originally found in a Portuguese patient\(^{19}\) and subsequently in Greek Cypriots.\(^{20}\) Typically the clinical course of patients with this mutation is mild in nature. However, the two Lebanese patients with this mutation have a more severe clinical disease as they both showed typical thalassemic facies, had \( \beta / \alpha \) globin synthetic ratios of 0.34 and 0.20, and required regular blood transfusions when their hemoglobin levels fell below 6 to 8 g/dL. Although the reason for the more severe clinical course in the Lebanese patients is not known at this time, Lebanese parents at risk for this mutation should also receive genetic counseling for prenatal diagnosis.

The substitution (G \( \rightarrow \) C) at IVS1 position 5 is interesting because it was previously found in the Chinese\(^{21}\) and Asian Indian\(^{22}\) populations. With its occurrence also in the Mediterranean region, it has now been found in three different populations. The fact that this mutation lies on a different haplotype in each population suggests that it arose independently in each and that this region of the \( \beta \)-globin gene is a hot spot for mutation.

The substitution at codon 29 was found in two patients, and while silent at the amino acid level, it creates a GT dinucleotide at this position 2 bp 5' to the normal GT donor
splice site of IVS1. The new GT dinucleotide may act as a donor splice site, thus causing a frameshift. Alternatively, as in the case of hemoglobin E,\textsuperscript{23} Knossos,\textsuperscript{24} and a \( \beta \)-thalassemia found in a black patient,\textsuperscript{25} this mutation may activate cryptic splice sites present in this region of the \( \beta \)-globin gene. We are currently investigating the precise splice point of the mutant is \( \beta^+ \), some normal splicing of this mRNA must remain.

Until recently, prenatal diagnosis of \( \beta \)-thalassemia using DNA analysis was usually performed by establishing linkage of polymorphic restriction sites along the \( \beta \)-globin cluster.\textsuperscript{26,27} Currently synthetic oligonucleotide probes provide a more direct method for detecting the mutation in prenatal diagnosis.\textsuperscript{9,28} The delineation of the molecular lesion would allow a rational approach to prenatal diagnosis in the Lebanese population. While haplotype I and the IVS1 position 110 mutation are predominant, parents and any affected children should be screened for specific \( \beta \)-thalassemia lesions and DNA polymorphisms in the \( \beta \)-globin cluster prior to an attempted pregnancy. This approach has been successful in the case reported herein.

The heterogeneity of \( \beta \)-thalassemia mutations in Lebanon may be explained by the history of this country, which has long been a crossroads of several civilizations. The ancient Phoenician culture has been well documented as a migratory civilization from Lebanon to various regions of the Mediterranean Sea and Africa and could have thus served as a genetic vehicle across populations contributing to the widespread nature of these mutations. Lebanon has witnessed the passage of many different populations in the last 500 years, from the Crusades to the Ottoman empire, and for the last 200 years has seen the convergence of various Moslem sects from neighboring Arab countries. This admixture of ethnicities may well be reflected by the genetic diversity of \( \beta \)-thalassemia mutations resulting from population migration and gene flow.

Table 3. Linkage of Haplotypes to \( \beta \)-Thalassemia Mutations in the Lebanese Population

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Type</th>
<th>No. of Chrom</th>
<th>Mutation</th>
<th>Frequency</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>( \beta^+ )</td>
<td>30</td>
<td>IVS1 position 110 ((G \rightarrow A))</td>
<td>0.60</td>
<td>Oligo 1</td>
</tr>
<tr>
<td>I</td>
<td>( \beta^+ )</td>
<td>1</td>
<td>IVS1 position 6 ((T \rightarrow C))</td>
<td>0.02</td>
<td>Oligo 2</td>
</tr>
<tr>
<td>II</td>
<td>( \beta^0 )</td>
<td>2</td>
<td>Codon 39 ((C \rightarrow T))</td>
<td>0.04</td>
<td>Oligo 3</td>
</tr>
<tr>
<td>II</td>
<td>( \beta^+ )</td>
<td>4</td>
<td>Codon 29 ((C \rightarrow T))</td>
<td>0.08</td>
<td>Oligo 5*</td>
</tr>
<tr>
<td>II</td>
<td>( \beta^+ )</td>
<td>1</td>
<td>IVS1 position 110 ((G \rightarrow A))</td>
<td>0.02</td>
<td>Oligo 1</td>
</tr>
<tr>
<td>III</td>
<td>( \beta^0 )</td>
<td>2</td>
<td>IVS2 position 1 ((G \rightarrow T))</td>
<td>0.04</td>
<td>Hph1</td>
</tr>
<tr>
<td>IV</td>
<td>( \beta^0 )</td>
<td>2</td>
<td>Codon 8 ((-2, AA))</td>
<td>0.04</td>
<td>Oligo 4</td>
</tr>
<tr>
<td>VI</td>
<td>( \beta^+ )</td>
<td>3</td>
<td>IVS1 position 6 ((T \rightarrow C))</td>
<td>0.06</td>
<td>Oligo 2</td>
</tr>
<tr>
<td>VII</td>
<td>( \beta^+ )</td>
<td>2</td>
<td>IVS2 position 745 ((C \rightarrow G))</td>
<td>0.04</td>
<td>Rsa 1</td>
</tr>
<tr>
<td>IX</td>
<td>( \beta^0 )</td>
<td>2</td>
<td>IVS1 position 5 ((G \rightarrow C))</td>
<td>0.04</td>
<td>Oligo 6*</td>
</tr>
<tr>
<td>Asian H</td>
<td>( \beta^0 )</td>
<td>2</td>
<td>Codon 8 ((-2, AA))</td>
<td>0.02</td>
<td>Oligo 4*</td>
</tr>
</tbody>
</table>

*These mutations were initially detected by cloning and sequencing.
REFERENCES


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