CONCISE REPORT

Prenatal Diagnosis of β-Thalassemias by Amniocentesis: Linkage Analysis Using Multiple Polymorphic Restriction Endonuclease Sites

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In order to assess the applicability of multiple restriction endonuclease analyses of amniocyte DNA to the prenatal diagnosis of β-thalassemias in general, we studied 12 consecutive couples at risk. DNA of both members of the 12 couples and a previous offspring of each was analyzed for the presence of 4 polymorphic restriction endonuclease sites: the Hpa I site 3' to the β-globin gene, the Hind III site in the β-globin gene, the Hind III site in the β-globin gene, and the Bam HI site 3' to the β-gene. Linkage disequilibrium between these sites and βA or βthal genes was not found, presumably due to the heterogeneity of βthal genes. However, the high frequency of polymorphism at these sites allowed differentiation of βA-bearing chromosomes from βand βthal-bearing chromosomes in both members of 6 couples. In these couples, complete prenatal diagnosis by linkage analysis of amniocyte DNA would be possible. In the remaining 6 couples, βA and βthal chromosomes could be discriminated in one member. In about 50% of the pregnancies of these couples, exclusion of β-thalassemia was possible by this analysis. These data suggest that when linkage analysis of polymorphic restriction endonuclease sites is carried out, prenatal diagnosis of β-thalassemia states can be accomplished by amniocentesis alone in 75% of pregnancies at risk.

PRENATAL DIAGNOSIS of β-thalassemia by globin synthetic studies of fetal red cells obtained by fetoscopy or placental aspiration has been carried out worldwide in over 500 couples at risk. Although the diagnoses obtained are quite accurate (error rate, 1.5%), the procedure is limited by (1) an associated 7% risk of fetal mortality and (2) the inability to obtain sufficient fetal blood samples in 8% of cases. In addition, the procedure has a limited availability because of the specialized skill required to obtain fetal blood samples.

In 1978, Kan and Dozy reported that a polymorphic Hpa I site 3' to the β-globin locus was in linkage disequilibrium with βA and βthal genes, and that prenatal diagnosis of sickle cell anemia by amniocentesis was possible. Using this linkage disequilibrium and that between polymorphic Hind III sites in the intervening sequences of the β-globin gene and the combined β-globin–Hpa I site haplotype, one can now carry out complete prenatal diagnosis of sickle cell anemia by amniocentesis in 80%-90% of couples at risk. Investigators have begun to apply a similar approach to the prenatal diagnosis of β-thalassemia. Kan et al. found that in Sardinians a Bam HI restriction site was absent 3' to 30% of βA genes, but was present 3' to all βthal genes. This linkage disequilibrium should be useful for prenatal diagnosis in about 30% of pregnancies at risk for βthal in Sardinia. Little et al. have demonstrated that in individuals with β-thalassemia trait, polymorphic Hind III sites in the βA and βthal genes have a high incidence similar to that reported in nonthalassemic whites and American blacks. However, these investigators did not provide data on the presence or absence of linkage disequilibrium between these sites and βA or βthal genes. Even so, they did take advantage of the high frequency of these Hind III sites to exclude β-thalassemia prenatally in an Asian-Indian family by linkage analysis of amniocyte DNA.

The present study was carried out in order (1) to determine the applicability of multiple restriction endonuclease analyses to prenatal diagnosis of β-thalassemia in general and (2) to provide information concerning the existence of linkage disequilibrium between polymorphic sites and βA or βthal genes.

MATERIALS AND METHODS

A probe for β-globin gene sequences was made from an Mbo II plus Hind III fragment (1.2 kb) of the recombinant plasmid J W 102.10.11 A 1.1-kb fragment containing γ-globin sequences was obtained by digestion of the recombinant plasmid J W 151 with Taq I.10 The β and γ-containing fragments were radiolabeled with [3P]-dCTP and [3P]-dTTP by the nick translation function of Escherichia coli DNA polymerase I. A quantity of 10–15 ml of whole blood was obtained from all subjects, and genomic DNA was isolated from leukocytes. Ten micrograms of DNA was digested with Bam HI, Hpa I, or Hind III, under conditions recommended by the commer-
cial supplier. The DNA was then subjected to electrophoresis in 0.8% agarose gels, transferred to nitrocellulose filters, and hybridized with either the \( \beta\)-globin probe (Bam HI or Hpa I digests) or the \( \gamma\)-globin probe (Hind III digests). DNA transfer, prehybridization of filters, hybridization with probe, washing of filters, and autoradiography were carried out as described.\(^{1,14,15}\) Experiments involving recombinant DNA were conducted at P2-EK-2 containment in accordance with the National Institutes of Health Guidelines.

**RESULTS**

Twelve consecutive couples at risk for \( \beta\)-thalassemia states with a previous child or an affected fetus were studied (Table 1) for the presence of four polymorphic restriction sites (Fig. 1A). Five couples were at risk for \( \beta^+\)-thalassemia in their children. Three had a probable risk of \( \beta^2\)-thalassemia, and although none of them had borne affected children, all three had had fetuses with no \( \beta\)-chain synthesis on prenatal diagnostic studies performed at Yale University. Two couples were at risk for HbS-\( \beta^+\)-thalassemia, and two couples were at risk for either \( \beta^+\) or \( \beta^2\)-thalassemia. Of the latter two couples, one did not have an affected child or fetus but did have a normal child, while the other had a transfusion-dependent affected child in whom globin synthetic studies were not carried out.

DNA fragments containing portions of the \( \gamma\)-globin genes derived from Hind III digestion and the \( \beta\)-bearing fragments obtained by Bam HI digestion of couple no. 1 and their thalassemic offspring are shown to illustrate the technique (Fig. 1B and C). The \( \beta\)-globin gene region on each chromosome 11 of this family are diagrammed in Fig. 2. \( \beta^{thal} \) and \( \beta^A \) genes of each parent can be differentiated by analysis of polymorphic Hind III sites in \( \gamma^A \) and \( \gamma^\alpha \) genes, thereby allowing complete prenatal diagnosis of \( \beta\)-thalassemia status by amniocentesis. If we designate the presence of Hind III sites in \( \gamma^A \) and \( \gamma^\alpha \) genes as \( G^+ \gamma^A \gamma^/G^\gamma^\alpha \gamma^ \), then the \( \beta^{thal}\beta^A \) child is \( G^+\gamma^A\gamma^/G^\gamma^\alpha \gamma^ \), while \( \beta^A\beta^A \) children will be \( G^\gamma^A\gamma^/G^\gamma^\alpha \gamma^ \) or \( G^\gamma^\alpha \gamma^/G^\gamma^\alpha \gamma^ \). In addition, one parent also lacks a Bam HI site 3' to his \( \beta^A \) gene, allowing partial confirmation of the Hind III data. The presence of a \( G^\gamma^\alpha \gamma^ \) chromosome in the father was confirmed by analysis of their \( \beta^A\beta^A \) child. It is worth stating that we have not yet observed an \( \alpha^+\) gene linked to a \( G^\gamma \) gene (\( G^\gamma^\alpha \gamma^ \) chromosome), nor has this been reported by others.\(^{1,14,15}\)

Of 24 individuals (2 members each of 12 couples) analyzed with 3 restriction endonuclease and \( \gamma \) or \( \beta \) probe combinations, 6 individuals had polymorphic restriction sites detected with 2 endonucleases (Table 1). Twelve individuals had a polymorphic restriction endonuclease pattern detected with one enzyme, which allowed differentiation of their \( \beta^A \) chromosome from their \( \beta^{thal} \) or \( \beta^A \) chromosome. In only 6 of 24 individuals was no informative polymorphic restriction pattern detected.

In 3 of the 12 couples, although each parent had one or more polymorphic restriction sites, analyses with two restriction endonucleases were necessary to discern the fetal genotype. For example, in couple no. 5, both members were heterozygous for a chromosome containing polymorphic Hind III sites in both \( \gamma^A \) and \( \gamma^\alpha \) genes (\( G^+\gamma^A\gamma^/G^\gamma^\alpha \gamma^ \) (Fig. 2). Their \( \beta\)-thalassemic child was also heterozygous (\( G^+\gamma^A\gamma^/G^\gamma^\alpha \gamma^ \)) and another child with \( \beta\)-thal trait lacked these sites (\( G^\gamma^\alpha \gamma^/G^\gamma^\alpha \gamma^ \)). Thus, one member of the couple must have both polymorphic Hind III sites syntenic with his/her \( \beta^A \) gene, while the other must have both of these Hind III sites adjacent to his/her \( \beta^{thal} \) gene. Offspring containing either \( \beta^A\beta^A \) or \( \beta^{thal}\beta^{thal} \) genotypes would be heterozygous for the Hind III sites (\( G^+\gamma^A\gamma^/G^\gamma^\alpha \gamma^ \)) and could not be differentiated by this analysis alone. The fact that one parent also lacked a Bam HI site 3' to her \( \beta^A \) gene allows the distinction between \( \beta^A\beta^A \) and \( \beta^{thal}\beta^{thal} \) offspring. A \( \beta^{thal}\beta^{thal} \) offspring is homozygous for the polymorphic Bam HI site (as shown in Fig. 2), while a \( \beta^A\beta^A \) individual will be heterozygous for this site. \( \beta^A\beta^{thal} \) offspring of this mating are either (\( G^+\gamma^A\gamma^/G^\gamma^\alpha \gamma^ \)) or (\( G^\gamma^A\gamma^/G^\gamma^\alpha \gamma^ \)). Since the \( \beta^{thal} \) trait offspring studied lacked \( \gamma \) Hind III sites and was homozygous for the polymorphic Bam HI site, the polymorphic Hind III sites may be assigned to the \( \gamma \) genes of the

**Table 1. Prenatal Diagnosis of \( \beta\)-Thalassemias by Linkage Analysis**

<table>
<thead>
<tr>
<th>Couple</th>
<th>Ethnic Origin</th>
<th>Thalassemia</th>
<th>Father</th>
<th>Mother</th>
<th>Possible Diagnosis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Greek</td>
<td>( \beta^+ )</td>
<td>Hind III</td>
<td>Hind III</td>
<td>Complete</td>
</tr>
<tr>
<td>2</td>
<td>Greek</td>
<td>( \beta )</td>
<td>None</td>
<td>Bam HI</td>
<td>Exclusion</td>
</tr>
<tr>
<td>3</td>
<td>Greek</td>
<td>( \beta^+ )</td>
<td>Hind III</td>
<td>Hind III</td>
<td>Complete</td>
</tr>
<tr>
<td>4</td>
<td>Greek</td>
<td>( \beta^+ )</td>
<td>None</td>
<td>Bam HI</td>
<td>Exclusion</td>
</tr>
<tr>
<td>5</td>
<td>Greek</td>
<td>( \beta^+ )</td>
<td>Hind III</td>
<td>Hind III</td>
<td>Complete</td>
</tr>
<tr>
<td>6</td>
<td>Italian</td>
<td>( \beta^+ )</td>
<td>None</td>
<td>Bam HI</td>
<td>Exclusion</td>
</tr>
<tr>
<td>7</td>
<td>Italian</td>
<td>( \beta^+ )</td>
<td>Hind III</td>
<td>Hind III</td>
<td>Complete</td>
</tr>
<tr>
<td>8</td>
<td>Italian-Greek</td>
<td>S-( \beta^+ )</td>
<td>Hind III</td>
<td>Hpa I</td>
<td>Complete</td>
</tr>
<tr>
<td>9</td>
<td>Italian</td>
<td>S-( \beta^+ )</td>
<td>Hind III</td>
<td>Hpa I</td>
<td>Complete</td>
</tr>
<tr>
<td>10</td>
<td>Italian</td>
<td>( \beta^+ )</td>
<td>Hind III</td>
<td>None</td>
<td>Exclusion</td>
</tr>
<tr>
<td>11</td>
<td>Black</td>
<td>( \beta^+ )</td>
<td>None</td>
<td>Hind III</td>
<td>Exclusion</td>
</tr>
<tr>
<td>12</td>
<td>West Punjabi</td>
<td>( \beta )</td>
<td>None</td>
<td>Hind III</td>
<td>Exclusion</td>
</tr>
</tbody>
</table>

*In couples in whom only exclusion diagnoses are possible, the absence of \( \beta\)-thalassemia can be documented by chance in 50% of pregnancies. In the other 50% of pregnancies of these couples, the absence of one \( \beta\)-thalassemia gene is documented and the risk of disease becomes 50%.
Fig. 1. (A) Polymorphic restriction endonuclease sites in the \(\alpha\gamma-\delta\gamma-\beta\) globin gene region studied. In this report numbers given below indicate the length of DNA in kilobase pairs (kb). Restriction endonuclease patterns obtained for couple no. 1 after (B) digestion of DNA with Hind III and hybridization with a \(\gamma\) probe and (C) digestion of DNA with Bam HI and hybridization with a \(\beta\) probe. From left to right in both (B) and (C), DNA is derived from father, mother, and affected child. Fragment sizes in kb are on the left and identity of each fragment is on the right. When a Hind III site is present in the gene, the 7.8-kb fragment is cleaved into 7.1- and 0.7-kb fragments. When a Hind III site is present in the \(\gamma\) gene, the 3.4-kb fragment is cleaved into 2.7- and 0.7-kb fragments. The 0.7-kb fragments found in association with \(\gamma\) and \(\delta\gamma\) Hind III sites were seen on other autoradiograms, but were not seen on those presented because these fragments were run off the gels during electrophoresis. In the figure \(\gamma\) and \(\delta\gamma\) represent fragments derived by cleavage at the polymorphic Hind III sites in the \(\gamma\) and \(\delta\gamma\) genes. In the text \(\delta\gamma\) and \(\gamma\) are used to designate the presence of these sites. In (C), the presence of a 22-kb fragment in father indicates the absence of the Bam HI site 3’ to one of father’s two \(\beta\)-globin genes.

\(\beta^{thal}\) chromosome of father and the \(\beta^A\) chromosome of mother.

In the 12 couples, polymorphic Hind III sites were found in 8 of 24 (33%) \(\beta^A\) chromosomes and 8 of 22 (36%) \(\beta^{thal}\) chromosomes. In the 10 couples of Greek and Italian origin, these sites were present in 6 of 20 \(\beta^A\) chromosomes and 5 of 18 \(\beta^{thal}\) chromosomes. In addition, the distribution of polymorphic Hind III sites was nearly identical in \(\beta^A\) and \(\beta^{thal}\) chromosomes, indicating linkage equilibrium of these \(\beta\) genes and Hind III
Results. Shown in Fig. 1 these or and no. 5. Linkage of polymorphic HindII site and the polymorphic restriction sites should be.

ethnic groups, polymorphic restriction sites should be heterogeneous both between and within genes are obtained. Six of 24 (25%) $A$ and 2 of 22 (9%) $\beta$thal chromosomes lacked 3' Bam HI sites. Moreover, no evidence of linkage disequilibrium between the polymorphic Bam HI site and the $\beta^A$ or $\beta$thal genes was obtained. Six of 24 (25%) $A$ and 2 of 22 (9%) $\beta$thal chromosomes lacked 3' Bam HI sites. ($\chi^2 = 2.9, p > 0.1$). In the 10 couples of Greek and Italian origin these sites were absent in 6 of 20 (30%) $A$ and 2 of 18 (11%) $\beta$thal chromosomes. ($\chi^2 = 2.2, p > 0.1$).

The two $\beta^A$ chromosomes observed in Italians were similar to those seen in blacks in that they lacked the polymorphic Hpa I site, resulting in a 13 kb $\beta$-containing fragment. Thus, this site may be useful in marking $\beta^A$ chromosomes in Italians as well as in blacks.

**DISCUSSION**

Prenatal diagnosis of sickle cell anemia depends on linkage disequilibrium between (1) a polymorphic Hpa I restriction site and the $\beta^A$ gene and/or (2) polymorphic Hind III sites and the combined $\beta$-globin–Hpa I site haplotype. Since $\beta$-thalassemia genes are heterogeneous both between and within ethnic groups, polymorphic restriction sites should be randomly associated with $\beta^A$ and $\beta$thal genes except in genetic isolates. Sardinians are an example of such an isolate, and linkage disequilibrium exists between their polymorphic Bam HI site and $\beta$thal genes. This site is absent 3' to 30% of $\beta^A$ genes, but is present adjacent to all $\beta$thal genes in that isolated population. In contrast, in our families who originated from various regions of Italy and Greece, both $\beta^A$ chromosomes (6 of 20) and $\beta$thal chromosomes (2 of 18) lacked the polymorphic Bam HI site. Likewise, in our Italian and Greek couples, the distribution of polymorphic Hind III sites on $\beta^A$ and $\beta$thal chromosomes was nearly equal; they were present on 6 of 20 $\beta^A$ and 5 of 18 $\beta$thal chromosomes. Thus, the usefulness of a particular polymorphic site to the prenatal diagnosis of most $\beta$-thalassemias depends on its frequency in the target population and not on its linkage disequilibrium with either $\beta^A$ or $\beta$thal genes.

This lack of linkage disequilibrium limits the applicability of restriction analysis to prenatal detection of this disease. Only couples in whom linkage analysis is feasible are eligible. Yet when linkage analysis can be carried out, our study suggests that prenatal diagnosis of $\beta$-thalassemia states may be accomplished by amniocentesis alone in 75% of pregnancies at risk. It is important to note, however, that couples at risk who desire prenatal diagnosis should be studied either prior to or early in pregnancy to determine their eligibility for the restriction endonuclease test.

Amniocentesis is a relatively safe procedure with a less than 0.5% risk of fetal loss, and it can be carried out by a large number of obstetricians. In contrast, fetoscopy still carries a 7% risk of fetal mortality, an 8% chance of failure to obtain an adequate fetal blood sample, and a limited availability. Fetoscopy should be reserved for couples in whom (1) linkage analysis is not possible or (2) $\beta$-thalassemia cannot be excluded by restriction endonuclease analysis of amniocyte DNA.

If one assumes that the frequencies of polymorphic restriction sites obtained in this study are representative of Greek and Italian populations, one can calculate the fraction of such couples at risk in whom the $\beta^A$ and $\beta$thal chromosomes can be differentiated in both members, one member, or no member after linkage analysis. These fractions are 0.46, 0.40, and 0.14, respectively, and are similar to the observed fractions of 0.50, 0.50, and 0. One can also calculate that discovery of one more polymorphic site in the $\beta$-globin gene region whose presence or absence has a frequency of 0.2 in Greeks and Italians should make possible differentiation of $\beta^A$ and $\beta$thal chromosomes in both members of 80% of such couples at risk. We have examined the polymorphic Kpn I site in the $\beta^A$ gene and the polymorphic Pst I site in the $\beta$ gene in about half of our couples. No polymorphic Kpn I site was
found (0 of 20 chromosomes studied), but the mother in couple no. 4 was heterozygous for the polymorphic \textit{Pst} I site (1 in 18 chromosomes studied).

The polymorphic $^{G_\gamma}$ \textit{Hind} III and \textit{Bam} HI sites are separated by 35 kb. Kurnit has estimated a recombination frequency between sites separated by 7 kb, the distance between the $^{\beta_\delta}$ mutation site and the polymorphic \textit{Hpa} I site, of one in 14,000 gametes. This estimate assumes that recombination occurs in this region of the genome at a frequency similar to the average frequency of recombination in the entire genome. An extrapolation from this estimate places the potential error rate in diagnosis in our couples due to recombination between polymorphic $^{G_\gamma}$ \textit{Hind} III and \textit{Bam} HI sites at 1 in 2800 gametes. However, the exact recombination frequency specific for the $^{\beta_\delta}$-globin gene region remains to be elucidated.

Although the basic defects in each of the $^{\beta}$-thalassemia families studied here are unknown, the mutations involved are allelic with the $^{\beta}$-globin structural gene allowing their detection because of their close linkage with the restriction sites studied. Analysis of polymorphic restriction sites may have a general usefulness in the prenatal diagnosis of other single gene disorders. For such other disorders, one must have a probe for DNA sequences that are so closely linked to the defective gene as to make recombination unlikely, the presence of high-frequency polymorphic sites, and sufficient family members to establish coupling phase.

REFERENCES

18. NICHD National Registry for Amniocentesis Group: Mid trimester amniocentesis for prenatal diagnosis. JAMA 236:1471, 1976

NOTE ADDED IN PROOF

We have now successfully carried out prenatal diagnosis by amniocentesis in three couples at risk for $^{\beta}$-Thalassemia in their offspring. In addition, expanded data on Greeks and Italians suggest that linkage disequilibrium exists between both \textit{Hind} III and \textit{Bam} HI sites and the $^{\beta}$-globin genes in Greeks, but not in Italians.
Prenatal diagnosis of beta-thalassemias by amniocentesis: linkage analysis using multiple polymorphic restriction endonuclease sites

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