A Novel Locus for Leber Congenital Amaurosis (LCA4) with Anterior Keratoconus Mapping to Chromosome 17p13

Abdul Hameed, 1 Shagufta Khaliq, 1 Muhammed Ismail, 1 Kbalid Anwar, 1 Neil D. Ebenezer, 2 Tim Jordan, 2 S. Qasim Mehdí, 1 Annette M. Payne, 2 and Shomi S. Bhattacharya 2

PURPOSE. A two-generation consanguineous Pakistani family with autosomal recessive Leber congenital amaurosis (LCA, MIM 204,000) and keratoconus was identified. All affected individuals have bilateral keratoconus and congenital pigmentary retinopathy. The goal of this study was to link the disease phenotype in this family.

METHODS. Genomic DNA was amplified across the polymorphic microsatellite poly-CA regions identified by markers. Polymerase chain reaction (PCR) products were separated by nondenaturing polyacrylamide gel electrophoresis. Alleles were assigned to individuals, which allowed calculation of LOD scores using the Cyrillic and MLINK software program. The retinal guanylate cyclase (RETGC-1, GDB symbol GUC2D) and pigment epithelium-derived factor (PEDF) genes were analyzed by heteroduplex analysis and direct sequencing for mutations in diseased individuals.

RESULTS. Based on a whole genome linkage analysis the first locus for this combined phenotype has been mapped to chromosome 17p13. Linkage analysis gave a two point LOD score of 3.21 for marker D17S829. Surrounding this marker is a region of homozygosity of 15.77 cM, between the markers D17S1866 and D17S960; however, the crossover for the marker D17S1529 refines the region to 10.77 cM within which the disease gene is predicted to lie. Mutation screening of the nearby RETGC-1 gene, which has been shown to be associated with LCA1, revealed no mutations in the affected individuals of this family. Similarly, another prime candidate in the region PEDF was also screened for mutations. The factor has been shown to be involved in the photoreceptor differentiation and neuronal survival. No mutations were found in this gene either. Furthermore, RETGC-1 was physically excluded from the critical disease region based on the existing physical map.

CONCLUSIONS. It is therefore suggested that this combined phenotype maps to a new locus and is due to an as yet uncharacterized gene within the 17p13 chromosomal region. (Invest Ophthalmol Vis Sci. 2000;41:629–633)

Leber congenital amaurosis (LCA) is a clinically heterogeneous group of childhood retinal degenerations inherited in an autosomal recessive manner. It is diagnosed at birth or during the first few months of life. Infants are totally blind or have greatly impaired vision, a fundal appearance ranging from normal to pigmented, and an extinguished electroretinogram (ERG). 1 Hypermetropia and keratoconus frequently develop in the course of the disease. Until now very little has been known about the pathophysiology of the disease, but LCA is usually regarded as the consequence of either impaired development of photoreceptors or extremely early degeneration of cells that have developed normally. LCA is genetically heterogeneous; it has recently been shown to be associated with mutations in the RETGC-1 (17p13.1), RPE65 (1p31), and CRX (19q13.3) genes. 2–5 Further heterogeneity has been revealed by the demonstration of linkage to a novel locus on chromosome 14q24 by Stockton et al. 6 Anterior keratoconus is a bilateral noninflammatory progressive corneal ectasia with an incidence of between 50 and 230 per 100,000 in the general population, dependent on the clinical detection method used and diagnostic criteria applied. 7–8 There are a number of well-described clinical signs, including stromal thinning, conical protrusion, a ring-like deposition of iron around the base of the cone (Fleischer’s ring), fine vertical lines in the deep stroma and Descemet’s membrane (Vogt’s striae), anterior stromal scarring, and enlarged corneal nerves. Gross alterations in the corneal curvature can be detected by retroillumination techniques; however, early or
mild forms of the disease may go undetected unless the anterior corneal topography is studied with computer-assisted videokeratography. The disease is rarely congenital but usually appears at puberty, and although one eye may be affected first, the condition is always eventually bilateral. Corneal distortion and scarring lead to irregular astigmatism, myopia, and mild-to-marked impairment of vision. The occurrence of breaks in Descemet’s membrane can lead to acute hydrops, swelling of the adjacent cornea due to localized edema, and acute ectasia, resulting in a sudden marked decrease in visual acuity. Electron microscopy suggests that the earliest histopathologic changes may occur in the corneal epithelium. The basal cells appear to degenerate and release proteolytic enzymes that destroy underlying stromal tissue. Stromal keratocytes near the cone also show degenerative changes, as does the endothelium, the latter leading to localized overproduction of Descemet’s membrane.9,10

Keratoconus is most commonly an isolated disorder, although it has been described in association with a large number of ocular and systemic disorders, and there is an established association with Down’s syndrome, mitral valve prolapse, and LCA.9,10 The study of families of keratoconus patients provides evidence for a genetic role in the etiology of keratoconus.8 Although there is an established association between Down’s syndrome and keratoconus, the karyogram of the patients of this family did not have trisomy 21. In the family presented the keratoconus is associated with LCA.

Here we provide data supporting the first genetic linkage of LCA with keratoconus to chromosome 17p13.1. RETGC-1 gene has been physically mapped in this region and is known to be associated with LCA1 therefore; it was the possible candidate for this phenotype. RETGC-1 is predominantly found in the cone outer segments of the retina, suggesting that the high RETGC-1 activity in cones may be responsible for the rapid recovery of cGMP concentration to the dark level.11 Although RETGC-1 maps just outside our critical region, mutation screening for this gene was done to exclude the possibility of this gene being responsible for this phenotype.

The pigment epithelium-derived factor (PEDF) gene has also been mapped to the short arm of chromosome 17, distal to the recoverin gene locus and very close to the RP13 locus in a large South African family.12 PEDF, a 50-kDa protein secreted by human fetal retinal pigment epithelial cells, is a neurotrophic factor that may aid the development, differentiation, and survival of the adjacent neural retina. The wider distribution of PEDF mRNA in the central nervous system suggests that this factor could have pleiotropic, neurotrophic, and neuroprotective effects on nonretinal neurons as well.13 The gene for PEDF is highly expressed by both fetal human and young adult retinal pigment epithelium cells. No mutation has yet been reported in PEDF gene in any of the eye disorder except few polymorphic variations.14 However, because this gene lies within our critical region, mutation screening for this gene has also been carried out.

**METHODS**

A two-generation consanguineous Pakistani family in which LCA and keratoconus affected four living members was ascertained (Fig. 1). Ophthalmic examination of the unaffected parents revealed no evidence of keratoconus or LCA. In addition, nine siblings were examined and found to be unaffected. Clinical examination of the affected individuals revealed bilateral ectasia with central thinning of the cornea, before they reach their 20s. The central cornea has a pronounced cone shape with severe corneal clouding (Fig. 2). All affected individuals were blind from birth, with absence of rod and cone function as demonstrated by ERG. Patients also showed bone spicule pigmentation, atrophic aspect of the retina, especially in the macular area, narrow blood vessels, waxy optic disc, and a generalized grayish sheen of the retina.

![Figure 1](image-url)
Microsatellite and Linkage Analysis

Genomic DNA was amplified using primers that specifically amplify the polymorphic microsatellite poly-CA regions identified by markers. PCR products were separated by nondenaturing polyacrylamide gel electrophoresis (Protogel; National Diagnostics) and visualized under UV illumination after staining with ethidium bromide. Alleles were assigned to individuals, and genotypic data were used to calculate the LOD scores using the Cyrillic and MLINK software program (Cherwell Scientific Publishing Ltd, Oxford, UK). Allele frequencies were calculated from the spouses in this family and a control ethnically matched population. The phenotype was analyzed as an autosomal recessive trait, with complete penetrance, and a frequency of 0.0001 for the affected allele.

Mutation Screening

The 18 exons of RETGC-1, which are expressed as protein, were amplified using the intronic primers and annealing temperatures essentially as described by Perrault et al.2 The exceptions were as follows: (1) The 5' portion of exon 2 was amplified with (forward/reverse, 5'9-3') primers TTACGGGGAGAACCCTAGGGGAGGCCG/AGAGAAGATGGGGTCGAAAG at an annealing temperature of 68°C; (2) middle portion of exon 2 CTCTCCGCCGTGTTCACGGT/GCGATCCCGGTCTTCGGC at 60°C; (3) 3' portion of exon 2 TCCGGTGAACCCCTGCGGCCT/TGCCGGCAGGACCAGCCGAC at 68°C; (4) a different forward primer was used for exon 8, GCATTCTGG-

GACAGTGAGCC; and (5) exons 6 and 7 were amplified with the same published primers but at lower annealing temperatures of 55°C, exon 11 at 68°C, exon 15 at 58°C, and exon 17 at 68°C.

New intronic forward and reverse primers were designed for the eight exons of the PEDF gene (Table 1) and were used, at 55°C annealing temperature, for mutation screening in this family. Twenty-five or 50 μl PCRs were performed, each containing 1.5 mM MgCl₂, 0.4 mM each primer, 200 mM each dNTP, 16 mM (NH₄)₂SO₄, 67 mM Tris–HCl (pH 8.8), 0.01% Tween-20, and 1 U of Taq DNA polymerase (Bioline). After an initial denaturation for 3 minutes at 94°C, 30 cycles of denaturation at 94°C for 1 minute, annealing at the exon-specific temperature for 1 minute, and extension at 72°C for 1 minute were performed, with a final extension at 72°C for 3 minutes. The amplified exons were analyzed by electrophoresis using MDE gel (National Diagnostics, Atlanta, GA) run at 180 V overnight using Hoeffer 600S apparatus.15

Direct Sequencing

Products of PCR amplification were sequenced using the PRISM Ready Reaction Sequencing Kit (Perkin–Elmer, PE Biosystems), and the products were analyzed on an ABI 373A XL automated sequencer. All PCR products were sequenced in the forward and reverse directions.

Table 1. PEDF Gene Primers User for Mutation Screening

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
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<tr>
<td>1</td>
<td>GCG CCT TAG GCC TAA TGG ATG</td>
<td>CTG CTC CCT GGA GTG CCC CTG</td>
</tr>
<tr>
<td>2</td>
<td>GTC CTG GCT GGG GTG GCC AGG</td>
<td>GGG TCC GGG TCC CCT GTC C</td>
</tr>
<tr>
<td>3</td>
<td>GGA GAC AGT CCC TGT GCA TCT C</td>
<td>AAC TCA GCC AGG TTA CGC AG</td>
</tr>
<tr>
<td>4</td>
<td>GTA TAG TGT CTG TGT TCT GGG</td>
<td>CTT CAC TCC AAC CTG GGC AAC</td>
</tr>
<tr>
<td>5</td>
<td>CCA GAA CCC GAG CCT GGC AGG</td>
<td>AGG GCT ACA GAG TAA GAC TCC</td>
</tr>
<tr>
<td>6</td>
<td>CCC CTG ACA GTA CTC CC</td>
<td>GGT AAC GAT CCC CTG CCC CAT C</td>
</tr>
<tr>
<td>7</td>
<td>GGC AGC TCC TGG CTG CTG CTG</td>
<td>GCC TTC TGC TTA GCA CAG TGG</td>
</tr>
<tr>
<td>8</td>
<td>GCG CCA TCC CAG CCT GTC GC</td>
<td>CAG GGG CAG CCT TG TGT CTT G</td>
</tr>
</tbody>
</table>

FIGURE 2. Pedigree of consanguineous Pakistani family with keratoconus and LCA showing homozygosity of the disease haplotype in affected individuals. Shaded bars denote the disease haplotype and, therefore, indicate the critical disease region on chromosome 17p13.
RESULTS

Twelve members of the family were typed for over 150 microsatellite markers. Significant exclusion was obtained for all markers except those located on chromosome 17p13. Haplotypes for these markers are shown in Figure 1. Two point LOD scores between LCA/keratoconus and the markers in this region (D17S1866, D17S849, D17S1529, D17S829, D17S796, D17S960, D17S796, D17S1353, and D17S1789) are summarized in Table 2. The maximum LOD score of 3.21 was obtained for the marker D17S829 (Table 2). Recombination events involving markers D17S1529 and D17S960 define the telomeric and centromeric boundaries, respectively, of the disease region. The critical region of this disease is, therefore, 10.77 cM, between these two markers (Fig. 2).

The RETGC-1 gene has been physically mapped just outside the critical interval using a YAC contig of the region. However, the gene has not been genetically positioned, and because it is known to be associated with LCA1,2 it is a possible candidate for this phenotype. Therefore, although RETGC-1 maps just outside this region, and because this family has LCA cosegregating with the keratoconus, we decided to screen the family for mutations in this gene. The coding exons of this gene were screened by heteroduplex and direct sequencing, in affected and nonaffected members of the family. Sequencing of the 18 exons of RETGC-1 revealed only previously reported polymorphisms and no disease-associated mutations.

The PEDF gene has been physically mapped to the 17p13.3 between markers D17S849 and D17S938, which lies within our critical region (Fig. 3). Because it was the possible candidate gene for the disease, the gene was also screened for mutations in this family. Heteroduplex and direct sequencing of the eight exons of RETGC-1 revealed only previously reported polymorphisms and no disease-associated mutations.

In summary, homozygosity mapping in an inbred pedigree has permitted us to identify a novel locus for LCA associated with autosomal recessive keratoconus at chromosome 17p13. This chromosomal region is near the locus for the Leber congenital amaurosis (LCA1), and overlaps with the loci for other types of retinal degenerations, namely cone-rod dystrophy (CORD6), cone dystrophy (CORD5), autosomal dominant retinitis pigmentosa (RP13), and central areolar choroidal dystrophy (CACD).17,18 The region has also been linked with the anterior polar cataract.19 This fifth novel autosomal locus illustrates further genetic heterogeneity of the LCA phenotype.

The family investigated in the present study presented with LCA and early onset keratoconus, cosegregating in an autosomal recessive manner. The incidence of keratoconus associated with LCA is between 30% and 50% above the age of 15 years.20 In a study by Elder20 of children with LCA, he demonstrated that keratoconus was specifically linked to LCA due to genetic factors rather than poor visual acuity per se. It is possible that the close association of keratoconus with LCA in this pedigree could be due to mutations in two genes in very close proximity to each other. Alternatively, the gene defect causing the LCA could predispose the formation of the keratoconus phenotype in this family.

### Table 2. LOD Scores Calculated for the Microsatellite Markers Analyzed in this Family

<table>
<thead>
<tr>
<th>Marker</th>
<th>0.0</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
<th>Z max</th>
<th>θ max</th>
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<tr>
<td>D17S1866</td>
<td>-0.21</td>
<td>-0.15</td>
<td>0.00</td>
<td>0.10</td>
<td>0.14</td>
<td>0.09</td>
<td>0.05</td>
<td>0.14</td>
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</tr>
<tr>
<td>D17S849</td>
<td>2.63</td>
<td>2.56</td>
<td>2.31</td>
<td>2.00</td>
<td>1.35</td>
<td>0.74</td>
<td>0.24</td>
<td>2.63</td>
<td>0.0</td>
</tr>
<tr>
<td>D17S1529</td>
<td>1.55</td>
<td>1.90</td>
<td>1.93</td>
<td>1.51</td>
<td>0.95</td>
<td>0.35</td>
<td>1.98</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>D17S829</td>
<td>3.21</td>
<td>3.14</td>
<td>2.84</td>
<td>2.46</td>
<td>1.70</td>
<td>0.94</td>
<td>0.29</td>
<td>3.21</td>
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</tr>
<tr>
<td>D17S796</td>
<td>3.20</td>
<td>3.11</td>
<td>2.78</td>
<td>2.36</td>
<td>1.55</td>
<td>0.80</td>
<td>0.25</td>
<td>3.20</td>
<td>0.0</td>
</tr>
<tr>
<td>D17S960</td>
<td>1.18</td>
<td>1.32</td>
<td>1.42</td>
<td>1.29</td>
<td>0.87</td>
<td>0.43</td>
<td>0.11</td>
<td>1.42</td>
<td>0.05</td>
</tr>
<tr>
<td>D17S1796</td>
<td>1.42</td>
<td>1.07</td>
<td>-0.58</td>
<td>-0.34</td>
<td>-0.13</td>
<td>-0.04</td>
<td>0.01</td>
<td>0.01</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Values represent recombination fraction.
The RETGC-1 gene, which is associated with LCA1 and CORD6, has been excluded as a candidate in this family, both by mutation screening and by physical exclusion indicated by mapping in YACs based on the physical mapping of the gene by Perrault et al. PEDF, another candidate in the region, has also been excluded as the disease gene. The genes associated with CORD5, RP13, and CACD are yet to be identified, however when they are they will provide additional candidates for this phenotype.

Heredity plays a significant role in at least a portion of keratoconus patients. Von Ammon first described the familial incidence of keratoconus in 1830; it has since been found that familial incidence varies from less than 5% to as high as 20%. Topographic studies of the corneal surface to evaluate 28 family members of 5 unrelated patients with keratoconus found abnormalities in 50% of the family members, which was significantly higher than in the controls. In another study of the 12 patients with clinical disease, 7 of the patients had at least one parent with evidence of subclinical keratoconus. These observations were mainly of dominant inheritance of the keratoconus phenotype, recessive inheritance being much more difficult to identify in an outbred population. Interestingly, a recent report by Rabinowitz et al. suggests a keratoconus gene locus near the centromere of chromosome 21 based on nonparametric linkage analysis. The identification of this locus in an inbred family will have a bearing on future genetic studies of keratoconus in the general population.

The region to which this family maps is known to be one of the most gene-rich regions of the genome due to the greatest concentration of mapped expressed sequence tags (ESTs). The 15.77-cM interval between these markers contains no well-characterized candidate genes; however, 4 ESTs identified from the human genome transcript map (http://www.ncbi.nlm.nih.gov/SCIENCE96/) that are expressed in the retina, 6 in epithelial or keratinocyte cells, and 19 in nervous tissue. These represent the best candidates available at this time. Further analysis of these cDNA clones will be needed before mutation screening in this family can be undertaken.

References