Genotype-Phenotype Correlation for Leber Congenital Amaurosis in Northern Pakistan

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**Objectives:** To report the genetic basis of Leber congenital amaurosis (LCA) in northern Pakistan and to describe the phenotype.

**Methods:** DNA from 14 families was analyzed using single-nucleotide polymorphism and microsatellite genotyping and direct sequencing to determine the genes and mutations involved. The history and examination findings from 64 affected individuals were analyzed to show genotype-phenotype correlation and phenotypic progression.

**Results:** Homozygous mutations were found in RPRGRIP1 (4 families), AIPL1 and LCA5 (3 families each), and RPE65, CRB1, and TULP1 (1 family each). Six of the mutations are novel. An additional family demonstrated linkage to the LCA9 locus. Visual acuity, severe keratoconus, cataract, and macular atrophy were the most helpful features in predicting the genotype. Many of the phenotypic variables became more prevalent with increasing age.

**Conclusions:** Leber congenital amaurosis in northern Pakistan is genetically heterogeneous. Mutations in RPRGRIP1, AIPL1, and LCA5 accounted for disease in 10 of the 14 families. This study illustrates the differences in phenotype, for both the anterior and posterior segments, seen between patients with identical or different mutations in the LCA genes and also suggests that at least some of the phenotypic variation is age dependent.

**Clinical Relevance:** The LCA phenotype, especially one including different generations in the same family, may be used to refine a molecular diagnostic strategy.

Arch Ophthalmol. 2010;128(1):107-113

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**METHODS**

We examined 55 families with a variety of inherited eye diseases in northern Pakistan. After the initial examination, members of each family were invited to participate in research aiming to identify novel genes implicated in inherited retinal degeneration. Informed consent was obtained from participants and from the elders in each household. This study was performed according to the principles of the Declaration of Helsinki, using a process approved by a UK Research Ethics Committee.

A clinical diagnosis of LCA was made in 23 families, based on the clinical history, recessive inheritance, and examination findings. These included visual acuity testing and anterior and posterior segment examination using direct and indirect ophthalmoscopy. Cases were examined in their own community and in a nonmedical setting. As a result, neither ocular electrophysiology nor corneal topography were available.
Affected and unaffected cases were examined by 2 ophthalmologists (M.M. and either A.P.B. or M.D.M.). Visual acuity was recorded together with a history of nyctalopia or photosensitivity. The presence or absence of 5 key phenotypic variables was then noted. Each variable was felt to be present when noted in at least 1 eye. Severe keratoconus was considered to be present when apical scarring, an “oil droplet” reflex, or a positive Munson sign was noted on anterior segment examination. No patient underwent corneal topography and so less severe cases may have been overlooked. Significant cataract was considered present when posterior subcapsular or cortical cataract was noted or cataract surgery had been performed previously. Subjects with lens dislocation and/or aphakia were not included in the analysis. Macular atrophy was recorded as present when there was evidence of either atrophy or a staphyloma, as have previously been noted in LCA. Nummular or coarse pigment clumping in the macula was recorded separately from peripheral intraretinal pigment migration or bone spicule pigmentation (Figure 1). If an adequate examination of the anterior or posterior segment was rendered impossible by media opacity, then the presence or absence of that finding was recorded as not known. The overall prevalence of each of the 5 key phenotypic features was compared for each of the genes and loci implicated in the pathogenesis. Each feature was noted to be either universal, common (present in 50%-99% of subjects), rare (present in 1%-49%), or absent. Evidence of phenotypic progression with age was provided by recording the cumulative prevalence of these clinical features for all individuals younger than a certain age, measured in decades, when known.

DNA was extracted from peripheral blood leukocytes. Three different approaches were used to obtain a molecular diagnosis. In 3 families identified initially, a whole-genome, microsatellite-based linkage search revealed linkage to the LCA3 locus, leading to the identification of the gene involved. For families sampled more recently, single affected DNA from each family was sent to Asper Biotech for microarray screening on the LCA mutation chip available at the time (www.asperbio.com). Finally, for those families in whom no mutation was found using this microarray, DNA from affected individuals was mixed in equimolar amounts then screened in a single hybridization using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, California) to generate more than 1 million single-nucleotide polymorphism genotypes. This analysis was performed by AROS Applied Biotechnology (www.arosab.com). The resultant genotypes were examined using the program IBD-finder (http://dna.leeds.ac.uk/ibdfinder) to identify regions of homozygosity shared by all affected family members.

Homozygosity at a given locus was confirmed by polymerase chain reaction amplifying microsatellite marker alleles from affected and unaffected family members. Products were resolved by electrophoresis on a 3130xl Genetic Analyzer (Applied Biosystems, Warrington, England). The results were analyzed using Genemapper version 4.0 software (Applied Biosystems).

Any known LCA genes within regions of shared homozygosity were sequenced to search for mutations. Primer pairs encompassing the exons of each gene were used in a polymerase chain reaction to amplify products that were initially digested with ExoSAP-IT (GE Healthcare, Chalfont St Giles, England). This DNA was then sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on a 3130xl Genetic Analyzer.

RESULTS

GENETIC ANALYSIS

The molecular diagnostic strategy has identified disease-causing mutations or linkage to known loci in 14 of the 23 LCA families (61%) to date. These involve AIPL1 (3 families), RGRIP1 (4 families), LCA5 (3 families), CRB1 (1 family), TULP1 (1 family), RPE65 (1 family), and the LCA9 locus (1 family). All cases were homozygous for the relevant mutation, consistent with autozygosity at these loci (Table 1). Six of these mutations are new, namely the c.587+1G>C, c.1180C>T, and c.3620T>G mutations in RGRIP1, the c.107C>G mutation in CRB1, the c.1138A>G mutation in TULP1, and the c.751G>T mutation in RPE65. In each case, the relevant alleles segregated with disease. Mutations not previously reported in the literature were tested for frequency in ethnically matched controls. The c.587+1G>C mutation in RGRIP1, the c.1138A>G mutation in TULP1, and the c.751G>T mutation in RPE65 were excluded from DNA of 171 controls. The second and third of these cause nonconservative change in evolutionary conserved residues, while the
first almost certainly ablates the splice donor site for exon 4. The premature stop codon mutations, c.107C>G in CRB1 and c.3620T>G and c.1180C>T in RPGRIP1, were excluded from DNA of 48 controls, which was felt to be sufficient given that these mutations almost certainly represent complete null alleles.

The AIPL1 c.834 G>A and LCA5 c.1151delC mutations, each of which accounted for LCA in 3 of the 14 families, are almost certainly founder mutations shared between distantly related families. Affected members of each family shared a common haplotype across the linked chromosomal region.

**PHENOTYPIC ANALYSIS BY GENE**

For the 14 families in whom a disease-causing mutation or linkage was identified, the number of affected cases examined, the mean age, and visual acuity at the time of examination are given in Table 2, together with the prevalence of nyctalopia and photoaversion. For the 5 key phenotypic variables, the overall prevalence for each gene/locus is given in Figure 2A, C, E, G, and I. Table 2 gives the frequency of each variable, when known, by gene or locus.

**PHENOTYPE-GENOTYPE CORRELATION AND PROGRESSION**

Visual acuity ranged from 6/60 to no perception of light in all subjects. A recordable Snellen acuity of either 6/60 or 6/76 was obtained only for the RPGRIP1, LCA5, TULP1, and RPE65 phenotypes. Nyctalopia was a universal finding in the families with RPE65- and TULP1-related disease. It was common in LCA5-linked disease (92% of subjects), rare in AIPL1-, RPGRIP1-, and CRB1-related disease, and absent in all cases with LCA9-linked disease. Photoaversion was a common finding in LCA5- (77% of subjects), LCA9-(83%), and RPE65-related (50%) disease. It was rare in AIPL1-, RPGRIP1-, and CRB1-related disease and absent in TULP1-related disease (Table 2).

Severe keratoconus was common in cases with mutations in CRB1 (70%) and AIPL1 (60%) but was not seen in cases with TULP1 mutations (Table 3). It was not seen before the second decade, except in the families with AIPL1 mutations. Keratoconus became more common with increasing age in families with mutations in AIPL1, CRB1, RPGRIP1, LCA5, or RPE65. The cumulative prevalence of keratoconus is shown in Figure 2B.

Cataract was a universal finding in cases with LCA9-linked disease and was common with AIPL1 (70%), TULP1 (67%), and RPGRIP1 (54%) mutations (Table 3). It was never seen before the second decade and was a late feature in LCA5- and CRB1-related LCA, not seen before the fifth and sixth decades, respectively. Cataract was absent from the RPE65 series. It became more prevalent with increasing age in the RPGRIP1, AIPL1, LCA5, CRB1, and TULP1 series (Figure 2D).

Macular atrophy was a universal finding in the LCA9-linked family and in those with mutations in AIPL1 and

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**Table 1. Mutations and Linkage Identified in 14 Families With Leber Congenital Amaurosis From Northern Pakistan**

<table>
<thead>
<tr>
<th>Gene or Locus</th>
<th>Mutation Type</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIPL1</td>
<td>MEP20</td>
<td>c.834 G&gt;A</td>
<td>p.Try278X</td>
</tr>
<tr>
<td></td>
<td>MEP21</td>
<td>c.834 G&gt;A</td>
<td>p.Try278X</td>
</tr>
<tr>
<td></td>
<td>MEP29</td>
<td>c.834 G&gt;A</td>
<td>p.Try278X</td>
</tr>
<tr>
<td>RPGRIP1</td>
<td>MEP1</td>
<td>c.587 +1G&gt;C</td>
<td>Unclear</td>
</tr>
<tr>
<td>LCA5</td>
<td>MEP2</td>
<td>c.1151delC</td>
<td>p.Pro384GlnfsX17</td>
</tr>
<tr>
<td></td>
<td>MEP4</td>
<td>c.1151delC</td>
<td>p.Pro384GlnfsX17</td>
</tr>
<tr>
<td></td>
<td>MEP50</td>
<td>c.2480 G&gt;T</td>
<td>p.Arg827Leu</td>
</tr>
<tr>
<td>CRB1</td>
<td>MEP53</td>
<td>c.107C&gt;G</td>
<td>p.Ser36X</td>
</tr>
<tr>
<td>TULP1</td>
<td>MEP51</td>
<td>c.1138A&gt;G</td>
<td>p.Thr380Ala</td>
</tr>
<tr>
<td>RPE65</td>
<td>MEP55</td>
<td>c.751G&gt;T</td>
<td>p.Val251Phe</td>
</tr>
<tr>
<td>LCA9-linked</td>
<td>MEP34</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. Age, Visual Acuity, and Prevalence of Nyctalopia and Photoaversion for Each Gene or Locus**

<table>
<thead>
<tr>
<th>Gene or Locus</th>
<th>No. of Cases Examined</th>
<th>Age, y, Mean (Range)</th>
<th>Visual Acuity</th>
<th>Prevalence of Nyctalopia (%)</th>
<th>Prevalence of Photoaversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIPL1</td>
<td>10</td>
<td>21.7 (7-43)</td>
<td>HM to PL</td>
<td>Rare (20)</td>
<td>Rare (10)</td>
</tr>
<tr>
<td>RPGRIP1</td>
<td>16</td>
<td>23.5 (8-67)</td>
<td>6/60 to PL</td>
<td>Rare (38)</td>
<td>Rare (31)</td>
</tr>
<tr>
<td>LCA5</td>
<td>13</td>
<td>22.2 (2-43)</td>
<td>6/60 to NPL</td>
<td>Common (92)</td>
<td>Common (77)</td>
</tr>
<tr>
<td>CRB1</td>
<td>10</td>
<td>28.6 (9-65)</td>
<td>HM to PL</td>
<td>Rare (10)</td>
<td>Rare (10)</td>
</tr>
<tr>
<td>TULP1</td>
<td>3</td>
<td>27.7 (19-40)</td>
<td>6/76 to CF</td>
<td>Universal (100)</td>
<td>Absent</td>
</tr>
<tr>
<td>RPE65</td>
<td>6</td>
<td>26 (17-30)</td>
<td>6/76 to CF</td>
<td>Universal (100)</td>
<td>Common (50)</td>
</tr>
<tr>
<td>LCA9-linked</td>
<td>6</td>
<td>18 (13-21)</td>
<td>PL</td>
<td>Absent</td>
<td>Common (83)</td>
</tr>
</tbody>
</table>

**Abbreviations:** CF, counting fingers; HM, hand movements; NPL, no perception of light; PL, perception of light.
TULP1. It was a common finding with CRB1 (88%) and LCA5 (64%) mutations. It was often seen in the first decade, except in families with RPGRIP1 mutations, when it was not seen before the third decade. It was absent from the family with RPE65 mutations (Figure 2F).

Nummular pigmentation was a common finding with RPE65- (83%), LCA5- (64%), CRB1- (71%), TULP1- (50%), and LCA9-linked (50%) disease. It was seen during the first decade only in the family with mutations in CRB1. It was a late finding with RPGRIP1 and TULP1 mutations, not seen until the fourth and fifth decades, respectively (Figure 2H).

A pigmentary retinopathy was a universal finding with RPE65- and LCA9-linked disease. It was a common finding with mutations in all the other genes. It was never seen before the second decade and became increasingly

Figure 2. Total and cumulative prevalence of severe keratoconus (A and B, respectively), cataract (C and D, respectively), macular atrophy (E and F, respectively), nummular pigmentation (G and H, respectively), and peripheral bone spiculation (I and J, respectively).
prevalent with increasing age in all the families in which it was not universal (Figure 2J).

**COMMENT**

This study confirms that LCA is genetically heterogeneous, even within a population from a defined geographical area and in which consanguineous marriage is common. The genes most commonly involved in this LCA series from northern Pakistan are RPGRIP1 (29% of families with molecular confirmation), AIPL1 (21% of families), and LCA5 (21% of families). This contrasts with pooled data from other series that identified the 3 most common genes to be CEP290 (15% of cases), GUCY2D (12% of cases), and CRB1 (10% of cases) whereas the RPGRIP1, AIPL1, and LCA5 genes accounted for only 4.2%, 5.3%, and 1.8% of cases, respectively.1 This probably reflects the different ethnic origins of the series studied and the presence of founder mutations within these populations. It also suggests that different screening strategies may be required to obtain a molecular diagnosis in different ethnic groups, particularly when microarrays are used.2,6

The Asper Biotech LCA microarray (www.asperbio.com) detected only 2 of the 9 mutations identified, accounting for 4 of the 14 families. These were the AIP1 c.834G>A and RPGRIP1 c.2480G>T mutations, both published previously.7,8 This contrasts with our findings in white cases previously. For all the genes in this series, the most striking phenotype was the presence of macular atrophy were less frequent and not seen before the second decade.8,10-12 The pooled phenotype for all 4 families was different from the AIPL1-related phenotype. Both nystagmus and photoaversion were more frequent. Some patients had a recordable Snellen acuity of 6/60. The anterior segment findings and the presence of macular atrophy were less frequent and were not seen before the second decade.

This series of 16 RPGRIP1-related cases is the largest published to date, to our knowledge, and a comparison with other RPGRIP1 mutations is difficult given the small numbers in most other series.10-12 The pooled phenotype for all 4 families was different from the AIPL1-related phenotype. Both nystagmus and photoaversion were more frequent. Some patients had a recordable Snellen acuity of 6/60. The anterior segment findings and the presence of macular atrophy were less frequent and were not seen before the second decade.

For all the genes in this series, the most striking phenotypic difference was between the RPE65S-related and the other phenotypes. All 6 cases with the RPE65 phenotype had poor visual function with nystagmus identified shortly after birth, but only 1 had nystagmus at the time of examination. This was followed by photoaversion from the third decade in 3 cases, at which point acuity and reading ability deteriorated rapidly. This evolution of symptoms and the absence of many other examination findings are in keeping with most other series with RPE65 phenotypic data.12,13,19-21

Having identified the genes most often implicated in causing LCA in a given ethnic group, the phenotypic data may help to refine the choice of genes screened. Hanein et al10 have previously suggested a strategy, based on a combination of physical signs and symptoms, to identify the likely causative gene. This strategy would have been partially successful in our families. For example, nystagmus as a common finding would have implicated CRB1-related disease, but this was not universal with increasing age in all the families in which it was not universal (Figure 2J).

**Table 3. Prevalence of the Anterior and Posterior Segment Signs for Each Gene or Locus**

<table>
<thead>
<tr>
<th>Gene or Locus</th>
<th>Severe Keratoconus</th>
<th>Cataract</th>
<th>Macular Atrophy</th>
<th>Nummular Pigmentation</th>
<th>Bone Spicule Pigmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIP1</td>
<td>Common (60)</td>
<td>Common (70)</td>
<td>Universal (100)</td>
<td>Rare (11)</td>
<td>Common (80)</td>
</tr>
<tr>
<td>RPGRIP1</td>
<td>Rare (38)</td>
<td>Common (54)</td>
<td>Rare (31)</td>
<td>Common (64)</td>
<td>Common (64)</td>
</tr>
<tr>
<td>LCA5</td>
<td>Rare (15)</td>
<td>Rare (8)</td>
<td>Common (64)</td>
<td>Common (64)</td>
<td>Common (64)</td>
</tr>
<tr>
<td>CRB1</td>
<td>Common (70)</td>
<td>Rare (22)</td>
<td>Common (88)</td>
<td>Common (71)</td>
<td>Common (57)</td>
</tr>
<tr>
<td>TULP1</td>
<td>Absent</td>
<td>Common (67)</td>
<td>Universal (100)</td>
<td>Common (50)</td>
<td>Common (67)</td>
</tr>
<tr>
<td>RPE65</td>
<td>Rare (17)</td>
<td>Absent</td>
<td>Absent</td>
<td>Common (83)</td>
<td>Universal (100)</td>
</tr>
<tr>
<td>LCA5-linked</td>
<td>Rare (17)</td>
<td>Universal (100)</td>
<td>Universal (100)</td>
<td>Common (50)</td>
<td>Universal (100)</td>
</tr>
</tbody>
</table>

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not the case in this series. Our experience is that nyctalopia and light sensitivity do not accurately help predict the genes responsible, even when a reliable history of function from birth is available, and this has been the experience of others.12,18,22 Instead, we would suggest that the use of objective data relating to the presence of key physical signs may be the most helpful way to implicate certain genes and to render others as less likely to be involved in the pathogenesis.10 Given the progression of many phenotypic features, the age of an individual subject must be considered. In a family in which several family members are affected, the use of pooled phenotypic data from individuals across the age spectrum will probably carry the greatest predictive value. This value would be made even greater if the novel databases for inherited retinal disease provided accurate and continuous rather than categorical phenotype data for a larger number of cases than are found in this article.

Mutations in 4 of the genes in this series, namely RPGRIP1, CRB1, TULP1, and RPE65, have been reported to cause either LCA or an acquired, later-onset rod-cone dystrophy. A more severe retinal dystrophy such as LCA may result from an excess of null alleles when compared with other less severe dystrophies such as retinitis pigmentosa in which visual function may be normal initially.14,23 In this series, the family with CRB1-related disease and 2 of the families with RPGRIP1-related disease were homozygous for nonsense mutations. Four other families had missense mutations, 2 in RPGRIP1 and 1 in each of TULP1 and RPE65. Based on the available histologic results from 13 probable LCA cases, LCA may be the clinical appearance of either an aplasia in which there is abnormal formation of key elements within the retina, a degeneration in which the retina develops correctly but there is early and progressive photoreceptor death, or a dysfunction in which the retina is structurally normal initially but a key biochemical message is missing.4 This dysfunction may be followed by a secondary degeneration. However, only 1 of these cases, with RPE65-related disease, was genotyped. For the genes in this series, it seems possible that mutations in CRB1 might cause an aplasia, given the role in photoreceptor development and structure, and that mutations in all the other genes might cause a dysfunction with secondary retinal degeneration, given the various roles in vitamin A metabolism (RPE65), transduction (AIPL1), and innerphotoreceptor ciliary transport (TULP1, RPGRIP1, and LCA5). However, all 3 retinal signs in this study were common but not universal for CRB1-related disease and the frequency of each sign was similar to the cases with TULP1- and LCA5-related disease. Many retinal signs also became more prevalent with increasing age, suggesting that a primary or secondary degenerative process is common to all LCA genes. This concept is supported by the evidence that microscopic features precede macroscopic changes.24

In this LCA series from northern Pakistan, mutations were identified in 6 different genes and the disease in another family was linked to the LCA9 locus. The genes most commonly involved in this population differ from other series published to date. Visual acuity and signs in the anterior and posterior segments may be helpful in selecting the genes or loci most likely to be implicated in the pathogenesis. However, the prevalence of many signs varies with age and this must be considered.

Submitted for Publication: January 31, 2009; final revision received May 5, 2009; accepted May 10, 2009.

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Financial Disclosure: None reported.

Funding/Support: This work was funded by Yorkshire Eye Research and grant 073477 from the Wellcome Trust. Dr Ali is funded through a Medical Research Council New Investigator Award.

REFERENCES


congratulations to the winner of our August quiz, Veeral S. Sheth, MD, Vitreoretinal Fellow, Section of Ophthalmology and Visual Science, Department of Surgery, University of Chicago, Chicago, Illinois. The correct answer to our August challenge was age-related hyperplasia of the nonpigmented ciliary body epithelium (Fuchs adenoma). For a complete discussion of this case, see the Letters: Research Letters section in the September Archives (Shields JA, Shields CL, Eagle RC Jr, Friedman ES, Wheatley HM. Age-related hyperplasia of the nonpigmented ciliary body epithelium [Fuchs adenoma] simulating a ciliary body malignant neoplasm. Arch Ophthalmol. 2009;127(9):1224).

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