Identification of Novel \textit{RPGR} ORF15 Mutations in X-linked Progressive Cone-Rod Dystrophy (XLCORD) Families

Neil D. Ebenezer, Michel Michaelides, Sharon A. Jenkins, Isabelle Audo, Andrew R. Webster, Michael E. Cheetham, Andrew Stockman, Eamonn R. Maber, John R. Ainsworth, John R. Yates, Keith Bradshaw, Graham E. Holder, Anthony T. Moore and Alison J. Hardcastle

\textbf{PURPOSE.} To test the incidence of mutations in \textit{RPGR} ORF15 in six families with X-linked progressive retinal degeneration (cone-rod dystrophy [XLCORD], macular or cone dystrophy) and to undertake a detailed phenotypic assessment of families in whom ORF15 mutations were identified.

\textbf{METHODS.} To amplify and sequence ORF15 in its entirety, a cloning strategy was developed. Families with mutations in ORF15 underwent electrophysiological testing, color vision assessment, color fundus photography, and fundus autofluorescence (AF) imaging.

\textbf{RESULTS.} Novel protein truncation mutations were identified in two families. In family A, a 2-bp mutation was identified in ORF15 +1 A1094C G1095T, predicted to result in a truncated protein (E364D/E365X). In family B, a G-to-T transversion (ORF15 +1176G>T) resulted in a nonsense mutation (G392X). Characteristics of phenotype in both families included progressive deterioration of central vision and subsequently night vision, mild photophobia, and moderate to high myopia. Ophthalmoscopic abnormalities were generally confined to the macula. A parafoveal ring of increased AF was observed, and electrophysiological evidence of a greater generalized abnormality in cone than rod responses were consistent with a cone-rod dystrophy phenotype.

\textbf{CONCLUSIONS.} The cloning strategy for ORF15 facilitated comprehensive sequence analysis in patients. Two families were identified with nonsense mutations, and clinical evaluation revealed them both to have a similar phenotype. The presence of a parafoveal ring of increased AF was an early indicator of affected status in these families. No disease-causing mutations in ORF15 were detected in four other families, suggesting that ORF15 mutations may not be the most common cause of XLCORD. (\textit{Invest Ophthalmol Vis Sci.} 2005;46:1891-1898) DOI:10.1167/iovs.04-1482

Mutations in the retinitis pigmentosa GTPase regulator (\textit{RPGR}) gene are a major cause of X-linked retinitis pigmentosa (XL RP). \textit{SLRP} is a severe form of RP in which affected males are symptomatic from early childhood. Most affected males are myopic, having night blindness in the first decade followed by peripheral field loss, and finally central vision loss. Most patients are blind by the end of the third decade. Female carriers display a broad spectrum of fundus appearances, ranging from normal to extensive retinal degeneration.

X-linked cone–rod dystrophy (XLCORD) is a progressive disorder in which cone degeneration is greater than rod degeneration and usually involves the macula. Three chromosomal loci for XLCORD have been identified to date: \textit{CORDX1} (Xp21.1-11.4; OMIM 300420), \textit{CORDX2} (Xq27.2-28; OMIM 300085), and \textit{CORDX3} (Xp 11.4-q13.1; OMIM 300476). Affected males have reduced visual acuity, variable photophobia, and color vision disturbance. Myopic refractive errors are common. In contrast to \textit{XL RP}, full-field electroretinography (ffERG) shows a greater correlation of cone responses than rod in the early stages of the disease.

A new exon of \textit{RPGR}, ORF15, has been identified as a mutation hotspot for \textit{XLRP}. More recently, mutations in ORF15 have also been identified in subjects with XLCORD and in a pedigree with X-linked recessive atrophic macular degeneration. Four different ORF15 frameshift insertions and deletions, and one nonsense mutation have been described as a cause of \textit{XLCORD}. \textit{ORF15} nonsense mutation was described as a cause of X-linked macular degeneration (although several affected males in this pedigree had abnormal cone and rod \textit{ffERGs}).

The purpose of this study was to identify families with X-linked cone, cone-rod, or macular dystrophy; determine the contribution and distribution of \textit{ORF15} mutations; and characterize the phenotype in those patients in whom an \textit{ORF15} mutation was identified.

\textbf{PATIENTS AND METHODS}

\textbf{Families}

Six British families with X-linked retinal dystrophy primarily affecting cone photoreceptors (cone, cone–rod, and macular dystrophy pheno-
The coding region of \textit{RPGR} ORF15 was sequenced in its entirety by PCR amplification, cloning, and subsequent sequencing of the cloned product. Genomic DNA was extracted from peripheral blood leukocytes (Nucleon BAC2 kit; Tepnel Life Sciences, Manchester, UK), and a single PCR product of 1786 bp was amplified with forward and reverse primers (ORF15F 5'-GTAATGATTTAAATGTGATCGCTTGTCAGAG-3' and ORF15R 5'-AAGGATTTAAATGTCGATCTGCATCCATAC-3') encompassing the entire open reading frame of 1706 bp. The following conditions were used: a 50 \mu L PCR reaction containing 100 ng of DNA, 1 \mu M of forward and reverse primers, 25 \mu L PCR mix (ReddyMix; AB0795; ABgene, Epsom, UK); PCR amplification was performed with 1 cycle of 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute 30 seconds; and 1 cycle of 72°C for 5 minutes. The resultant amplifier was purified through a column (Qiagen, Valencia, CA) and then cloned into the pGEM-Teasy vector, according to the manufacturer's recommendations (Promega, Madison, WI). The plasmid DNA was isolated (GenElute mini prep kit; Sigma-Aldrich, Poole, UK) and sequenced (model 3100; Applied Biosystems, Inc., [ABI], Foster City, CA) with M13 vector primers and ORF15-specific primers, to provide full bidirectional coverage of ORF15 (details of all primers used for sequencing are available on request). The sequencing reactions were performed with dye terminator chemistry (BigDye Terminator Cycle Sequencing, for the pyrimidine-rich strand and dGTP BigDye Terminator Cycle Sequencing, for the purine-rich strand; ABI).

Mutations identified were confirmed by a combination of independent cloning experiments, specific PCR amplification and direct sequencing of the mutated ORF15 fragment, and segregation with disease.

**Results**

**RPGR ORF15 Genotype**

A bidirectional sequence contig was created for each sequence, by using M13 forward and reverse primers and internal sequencing primers within \textit{RPGR} ORF15.

In family A, two consecutive substitutions were identified in ORF15+A1094C G1095T (Fig. 2A) that created a premature stop codon that was predicted to result in a truncated protein (E364D/E365X). In family B, a G-to-T transversion (ORF15+1176G>T; Fig. 2B) resulted in a nonsense mutation (G392X). Both of the changes identified are novel and are likely to result in a truncated protein. These sequence variations were therefore considered to be pathogenic mutations.

The mutations were confirmed by PCR amplification and direct sequencing of the mutated ORF15 fragment in other individuals from each family to demonstrate segregation with disease. The ORF15 mutations identified segregated uniquely in all affected subjects and carrier females tested in each family. These mutations were not detected in unaffected control chromosomes.

**Clinical Assessment**

**Family A.** Five affected males, three obligate carriers, and two nonobligate female members of a three-generation British family with X-linked retinal dystrophy were assessed (Fig. 1). Two affected males, III:7 and III:9, aged 40 and 37 years, respectively; admitted to mild photophobia and had myopia, but were otherwise asymptomatic. The remaining three affected males reported mild to moderate photophobia and reduced central and color vision. Onset of symptoms was in the fourth decade. All three reported that vision was better under mesopic conditions and that they experienced slow visual adaptation when moving from a dark to a bright environment. Nyctalopia was noted 5 to 10 years after the onset of reduced central visual function; nevertheless, all three symptomatic males reported that their night vision was better than that in daylight. All five affected males had moderate to high myopia with variable astigmatism. Visual acuity ranged between 6/5 and counting fingers. Color vision was absent in all the affected males. There was no evidence of nystagmus. Dilated fundoscopy revealed a range of age-dependent macular phenotypes, from mild changes at the level of the retinal pigment epithe-
A bilateral disturbance of the RPE located temporal to the macula (Fig. 3). These four subjects were found to carry the RPGR ORF15 mutation segregating in family A, suggesting that the ophthalmoscopically identified RPE mottling temporal to the macula signifies carrier status. Unlike the affected male subjects, the carrier females in this family displayed no abnormality on AF imaging. Individual III:8 does not carry the gene mutation. Peripheral retinal examination was normal in all individuals. Electrophysiological testing revealed variable retinal responses. III:1, III:2, and III:8 had normal PERGs and fERGs. However, the two older individuals II:3 and III:5 were found to have abnormal retinal electrophysiology. Both II:3 (73 years of age), and III:5 (50 years of age) had bilaterally undetectable pattern and focal ERGs; the fERG showed reduced responses with greater reduction in cone ERGs than rod-specific responses (Fig. 4). Clinical findings for this family are summarized in Table 1.

**Family B.** Five affected males and four obligate carriers of a five-generation British family with X-linked retinal dystrophy were assessed, and retrospective data were examined (Fig. 1). The five affected males had bilateral reduced central vision, with three of these subjects (IV:13, IV:11, and V:10) complaining of photophobia. Onset was between the second and fourth decades, with subsequent gradual deterioration of visual acuity and color vision. Two of the affected males (IV:11 and V:10) also reported nyctalopia, although individual V:10 (22 years of age) at the time of the study had normal rod responses. All the affected males had myopia with variable astigmatism, with the exception of V:10, who had hyperopia and astigmatism. Visual acuities ranged from 6/6 to counting fingers. None of the subjects had nystagmus, and color vision was absent in all affected male subjects. On fundus examination, the older affected males (IV:3, V:1, IV:13, and IV:11) showed variable degrees of macular RPE atrophy and chorioretinal degeneration, which was most prominent in the inferior retina (Fig. 3). The youngest affected male patient (V:10) had subtle bilateral foveal RPE mottling, with AF imaging revealing bilateral parafoveal rings of increased AF (Fig. 3). V:10 also had a bilateral inferior retinal sheen or tapetal reflex. IV:11 had also been noted to have a retinal sheen in the third decade of life. Recent AF imaging of IV:11 showed reduced AF corresponding to the macular atrophy seen ophthalmoscopically, with a surrounding ring of increased AF (Fig. 3).

Electrophysiological testing performed in three of the affected males (IV:3, V:1, and IV:13) revealed data consistent with a cone–rod dystrophy phenotype (Fig. 5). IV:11 (44 years of age) was severely affected and found to have only residual cone and rod responses on fERG testing. His nephew, V:10 (22 years of age), had bilateral markedly reduced pattern ERGs, cone and rod responses on fERG testing. His nephew, V:10 (22 years of age), had bilateral markedly reduced pattern ERGs, suggestive of bilateral severe macular dysfunction, with fERG recordings demonstrated generalized loss of cone function with minimal evidence of rod system involvement, consistent with a diagnosis of cone dystrophy. Representative electrophysiological traces are shown in Figure 5.

Visual acuity of obligate female carriers ranged from 6/5 to counting fingers (obligate carrier III:3 age 70 years). One female carrier, V:14, had poor vision secondary to bilateral congenital cataracts. Female carriers in this family had been noted to have a golden tapetal reflex. Subject IV:9 had macular RPE atrophy in the left eye, associated with reduced vision. The eldest obligate carrier, III:3 (70 years of age), had more extensive macular, peripapillary, and peripheral retinal atrophic changes (Fig. 3), and AF imaging showed decreased macular AF. Electrophysiological testing was performed for individual III:3, which showed unilateral abnormal pattern and full-field responses (Fig. 5). Clinical findings in this family are summarized in Table 2.
DISCUSSION

RPGR mutations, and in particular RPGR ORF15 mutations, are a major cause of retinal degeneration,\(^1\,\text{-}^{12}\,\text{and}^{21\,\text{-}^{25}}\) and molecular diagnosis greatly facilitates genetic counseling and advice on prognosis. However, screening ORF15 presents a major technical challenge because of the repetitive and purine-rich nature of the DNA sequence. We have used a cloning strategy to obtain accurate bidirectional full sequence of ORF15 reproducibly which was both cost and time effective. The high level of polymorphism within ORF15, which can often lead to primer-specific amplification failure, is overcome with this method. Once the mutation had been identified within a family, specific primer pairs were then used to amplify and sequence the mutation in other family members. In this study, six unrelated affected males were initially screened for ORF15 mutations, and two of these families were found to harbor ORF15 mutations that segregated with disease. These two families can therefore be described as CORDX1 families. The RPGR ORF15 mutations identified in both families were protein-truncating.

**FIGURE 3.** Fundus photographs and AF images of affected males and carrier females from families A and B. *Family A, patient III*: (A) Fundus photographs showing bilateral macular atrophy and pigmentation. Myopic chorioretinal degeneration with peripapillary atrophy can also be seen. (B) Fundus AF imaging showing bilateral markedly reduced macular AF, corresponding to the atrophy seen on ophthalmoscopy, with a surrounding ring of increased AF. *Family A, patient III*: (C) Fundus photographs showing bilateral areas of macular RPE atrophy. (D) Fundus AF imaging showing bilateral areas of decreased macular AF corresponding to atrophy seen on ophthalmoscopy, with a surrounding ring of increased AF. *Family A, patient III*: (E) Fundus photographs showing bilateral RPE mottling temporal to the macula. *Family B, patient IV*: (F) Fundus photographs showing bilateral RPE atrophy at the macular and peripapillary region, extending mainly into the inferior retina. (G) Fundus AF imaging showing bilateral reduced regions of AF of the RPE. *Family B, patient V*: (H) Fundus photographs showing bilateral foveal RPE mottling. (I) Fundus AF showing bilateral macular rings of increased AF, more prominent in the right eye than in the left. *Family B, patient III*: (J) Fundus photographs from a female carrier showing an asymmetrical bilateral macular, peripapillary, and peripheral RPE atrophy, most prominent inferiorly.
nonsense mutations located 3' to the highly repetitive region of ORF15. It is worth noting that ORF15 mutations reported as a cause of CORDX1 are also at the 3'-end of ORF15; four of the mutations previously described were insertions and deletions resulting in frame shifts, and one was a nonsense mutation. Similarly, an ORF15 nonsense mutation 3' to the highly repetitive region was described as a cause of atrophic macular degeneration (although several affected male subjects in that pedigree had electrophysiological data consistent with a cone-rod dystrophy phenotype). Our data strengthen the hypothesis that mutations 3' to the highly repetitive region cause predominant cone dysfunction. However, exceptions exist to this rule, for example a frame-shift insertion detected 5' to the repetitive region of ORF15 has been reported in a cone-rod dystrophy pedigree. The significance of the distribution and mutation type is difficult to assess, since the numbers of families with CORDX1 are small in comparison with the large number of XLRP families with ORF15 mutations, and currently there are no functional tests for RPGR. Recently, we and others have described RPGR mutations causing an XLRP syndrome associated with hearing loss and recurrent respiratory tract infections, highlighting the expanding clinical spectrum of disease associated with RPGR mutations. Although none of the mutations described occur in ORF15, there was notable inter- and intrafamilial phenotypic variation, with no clear correlation with mutation type. Questions are raised about the potential function of RPGR isoforms in different tissues and the consequence of different mutations, and it is hoped that future functional evaluation of this important protein will extend our understanding of mutation hotspots, environmental interplay, and genetic background.

An important finding of this study was the absence of RPGR ORF15 mutations in four families with X-linked predominantly cone photoreceptor dysfunction. Two other loci have been reported on the X-chromosome, CORDX2 on Xq27.2-q28 and CORDX3 spanning Xp11.4-q13.1, for which the causative genes remain to be identified. We are currently performing linkage analysis and additional clinical evaluation of the remaining pedigrees to identify the X-linked genes that cause cone and cone-rod dystrophies.

The detailed phenotypes described in two unrelated families in which the affected males have premature termination mutations in RPGR are consistent with X-linked cone-rod dystrophy (XLCORD). Affected males experience onset of visual symptoms from the second to fourth decade and show progressive deterioration in central vision and subsequently night vision, mild photophobia, and moderate to high myopia. A variation in the severity of the phenotype was seen in family A, with two males being only mildly affected (III:7 and III:9). This is to be expected because of their younger age compared with the other affected males in this family, although it is also possible that this intrafamilial variability of phenotype is due to genetic or environmental modifying factors. The fundoscopic abnormalities were generally restricted to the macular region and ranged from mild RPE disturbance in the early stages to extensive chorioretinal atrophy and pigmentations.

AF imaging allows the visualization of the RPE by taking advantage of the fluorescent properties of lipofuscin. To our knowledge, there are no previous data on AF imaging in XLCORD. Older affected male subjects showed decreased AF corresponding to areas of atrophy seen ophthalmoscopically, whereas in family A all five affected males had a ring of increased AF encircling the fovea. This was also clearly observed in the youngest affected male in family B (V:10). One of the five male subjects in family A was asymptomatic with no definite macular disturbance detected on fundoscopy, thereby suggesting that the presence of a parfoveal ring may be used as an early indicator of affected status. This high-density annulus has
FIGURE 5: Representative electrophysiological traces for family B. III:3 showed severe generalized loss of retinal function, with the rod system more affected. The PERG was undetectable, in keeping with severe macular involvement. IV:2 showed no detectable ERG responses. V:10 showed normal rod and maximum responses. Cone ERGs were markedly reduced and mildly delayed, with S-cone ERGs showing loss of the early L/M-cone component but preservation of the later, S-cone specific component. PERG showed some residual activity but are markedly abnormal in keeping with moderate to severe macular involvement.

TABLE 1. Summary of Clinical Findings for Family A

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Visual Acuity (OD-OS)</th>
<th>Fundus</th>
<th>AF Imaging</th>
<th>ERG</th>
<th>PERG</th>
<th>Color Vision</th>
</tr>
</thead>
<tbody>
<tr>
<td>II:1</td>
<td>M</td>
<td>74</td>
<td>CF-CF</td>
<td>Bilateral macular atrophy and peripheral retinal degeneration</td>
<td>Bilateral decreased AF corresponding to atrophy seen on ophthalmoscopy with surrounding ring of increased AF</td>
<td>Cone responses more markedly reduced than rod</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>II:3</td>
<td>F</td>
<td>73</td>
<td>6/9-6/36</td>
<td>Bilateral RPE mottling temporal to the macula; areas of myopic chorioretinal atrophy</td>
<td>Normal</td>
<td>Cone responses more markedly reduced than rod</td>
<td>Absent</td>
<td>Normal</td>
</tr>
<tr>
<td>III:1</td>
<td>F</td>
<td>41</td>
<td>6/4-6/4</td>
<td>Bilateral RPE mottling temporal to the macula</td>
<td>Normal</td>
<td>Cone responses more markedly reduced than rod</td>
<td>Absent</td>
<td>Normal</td>
</tr>
<tr>
<td>III:2</td>
<td>F</td>
<td>38</td>
<td>6/4-6/4</td>
<td>Bilateral RPE mottling temporal to the macula</td>
<td>Normal</td>
<td>Cone responses more markedly reduced than rod</td>
<td>Absent</td>
<td>Normal</td>
</tr>
<tr>
<td>III:3</td>
<td>M</td>
<td>51</td>
<td>CF-CF</td>
<td>Bilateral macular atrophy with surrounding RPE changes</td>
<td>Bilateral decreased AF corresponding to atrophy, with surrounding ring of increased AF (Fig. 3A)</td>
<td>Cone responses more markedly reduced than rod</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>III:4</td>
<td>M</td>
<td>48</td>
<td>CF-CF</td>
<td>Bilateral macular atrophy and pigmentation (Fig. 3A)</td>
<td>Bilateral decreased AF corresponding to atrophy, with surrounding ring of increased AF (Fig. 3B)</td>
<td>Cone responses more markedly reduced than rod</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>III:5</td>
<td>F</td>
<td>50</td>
<td>6/9-6/9</td>
<td>Bilateral RPE mottling temporal to the macula (Fig. 3E)</td>
<td>Normal</td>
<td>Cone responses more markedly reduced than rod</td>
<td>Reduced</td>
<td>Normal</td>
</tr>
<tr>
<td>III:7</td>
<td>M</td>
<td>40</td>
<td>6/5-6/5</td>
<td>Bilateral macular RPE atrophy (Fig. 3C)</td>
<td>Bilateral decreased AF corresponding to notable areas of atrophy with a surrounding ring of increased AF (Fig. 3D)</td>
<td>Cone responses more markedly reduced than rod</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>III:8</td>
<td>F</td>
<td>38</td>
<td>6/5-6/5</td>
<td>Normal</td>
<td>Normal</td>
<td>Cone responses more markedly reduced than rod</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>III:9</td>
<td>M</td>
<td>37</td>
<td>6/6-6/6</td>
<td>No definite abnormality</td>
<td>Normal</td>
<td>Cone responses more markedly reduced than rod</td>
<td>Absent</td>
<td>Absent</td>
</tr>
</tbody>
</table>

CF, counting fingers; NP, not performed.
also been reported in the autosomal dominant cone–rod dystrophy, CORD7.54 It has been proposed that there is an accumulation of autofluorescent material before photoreceptor cell death. This is supported by the histologic demonstration of a reduction in the number of photoreceptors in the presence of and increased quantity of lipofuscin in the RPE in age-related macular disease.35,36 These findings suggest that the ring of increased AF that can occur in retinitis pigmentosa, and a similar mechanism has been proposed.37 It has also been suggested that this hyperfluorescent annulus may correspond to the “rod ring” that surrounds the fovea; an area of high rod photoreceptor density.38 Previous studies of XLCORD pedigrees have reported variable clinical, electrophysiologic, and psychophysical findings in carrier females, with a lack of consistent abnormalities between subjects.15,18–20 In our family A, the younger carrier females were asymptomatic and had normal electrophysiological and psychophysical testing. The two older carrier females had reduced central and peripheral retinal function, consistent with a cone–rod pattern of dysfunction with marked bilateral macular involvement. Another consistent finding in all carrier females in family A was a mottling of the RPE temporal to the macula, suggesting that this may be an indicator of carrier status in this family. Unlike these cone–rod dystrophy carrier females, who were found to have normal AF imaging, a recent study of carrier females of rod–cone dystrophy associated with RPGR mutations has described an abnormal AF pattern in 9 of 11 carriers investigated,39 suggesting further phenotypic differences between XLCORD and XLRP carriers. In family B, obligate female carriers and two affected males had a golden tapetal retinal reflex. No tapetal sheen was observed in individuals from family A. A tapetal-like retinal sheen has been variously described in affected male and carrier females of XLCORD.15–20 One of the two carrier females tested in family B had electrophysiologic evidence of bilateral generalized retinal dysfunction affecting both cone and rod photoreceptors.

We have described the detailed phenotype of two XLCORD families with novel disease causing RPGR ORF15 mutations. The clinical findings are consistent with previous reports of CORDX1 phenotypes associated with mutations in RPGR ORF15. Important novel AF data are described that suggest that, in addition to electrophysiology, AF imaging may be helpful in establishing affected status at an early asymptomatic stage. We have also identified a consistent retinal phenotype in both of our families that will assist in clinically identifying carrier females. Our cloning strategy for ORF15 facilitated comprehensive sequence analysis of ORF15 in patients with X-linked retinal degeneration. This method is recommended as a potential initial strategy for diagnostics. The data add to the genotype and phenotype descriptions for CORDX1 and highlight the lack of ORF15 mutations in other families, suggesting that ORF15 may not be the most common cause of X-linked cone–rod dystrophy.

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References


