Null Retinoschisin-Protein Expression from an RS1 c354del1-ins18 Mutation Causing Progressive and Severe XLRS in a Cross-Sectional Family Study

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PURPOSE. To explore the retinoschisin (RS1) protein biochemical phenotype from an RS1 exon-5 deletion/insertion frameshift mutation in a family with X-linked retinoschisis (XLRS) and describe the clinical and electrophysiological features.

METHODS. Six XLRS males underwent ophthalmic examination and electroretinogram (ERG) recording. The RS1 gene was sequenced. Mutant RS1-RNA and protein expression were assessed by transfecting COS-7 cells with minigene constructs.

RESULTS. All six males carried the RS1 c354del1-ins18 mutation in which an 18-bp insertion replaced nucleotide 354, duplicating the adjacent upstream intron 4-to-exon 5 junction and creating a premature termination codon downstream. Analysis indicated normal pre-mRNA splicing producing mRNA transcripts. Truncated RS1 protein was expressed transiently but was degraded rapidly by a proteasomal pathway rather than by nonsense-mediated mRNA decay. Two boys, 1.5 and 5 years of age, had foveal cysts and minimal peripheral schisis, and retained near-normal scotopic b-wave amplitude and normal ERG waveforms. The 5-year-old’s ERG was diminished when repeated 3 years later. Four older XLRS relatives had substantial b-wave loss and strongly electronegative ERGs; three had overt macular atrophy. Cross-sectional family analysis showed the b/a-wave amplitude ratio as inversely related to age in the six males.

CONCLUSIONS. The c354del1-ins18 mutation caused an RS1-null biochemical phenotype and a progressive clinical phenotype in a 5-year-old boy, whereas the older XLRS relatives had macular atrophy and marked ERG changes. The phenotypic heterogeneity with age by cross-sectional study of this family mutation argues that XLRS disease is not stationary and raises questions regarding factors involved in progression. (Invest Ophthalmol Vis Sci. 2009;50:5375–5383) DOI:10.1167/iovs.09-3839

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perimetry (Haag-Streit, Bern, Switzerland) and optical coherence tomography (Stratus OCT 3; Carl Zeiss Meditec, Dublin, CA) were performed. The central visual absolute luminance threshold was measured after 30 minutes of dark adaptation with a Goldmann-Weekers adaptometer (Haag-Streit).

**ERG Recording**

ERG responses were elicited by full-field flash stimuli (UTAS 2000; LKC Technologies, Gaithersburg, MD, and Espion 1, Diagnosys Inc., Lowell, MA) after pupil dilation (phenylephrine hydrochloride 2.5% and tropicamide 1%) and 30 minutes of dark adaptation. Burian-Allen bipolar corneal ERG electrodes (Hansen Ophthalmic instruments, Iowa City, IA) were placed after topical corneal anesthesia (proparacaine hydrochloride 0.5%). Dark-adapted, rod-mediated, and combined rod-plus-cone ERG responses were recorded. Cone-mediated responses were recorded by DNA sequencing. The creation of the EcoRI site altered neither the RS1-protein sequence nor the expression and localization.

**RS1 Minigene Expression Vectors**

The RS1 minigene included RS1 genomic introns 4 and 5 in frame with exons 1–6 of RS1 cDNA in the pCMV Tag 4A expression vector. Genomic DNA from normal control males and XLRS-affected males of this family was PCR-amplified to generate fragments encoding intron-4 and -5 sequences, and the corresponding flanking exon sequences. Primers sequences were: forward: 5'CAGTATCTACAGCTTGTTGAGCAGG-3' and reverse: 5'CTGATTCTACAGCTTGTTGAGCAGG-3'. The PCR product was digested by BglII and XhoI and subcloned in pCMV-Tag4A mammalian expression vector (Stratagene, La Jolla, CA) between the NotI/XhoI enzyme sites (Tag4A-RS1). The sense primer design included the Kozak sequence (GCCACC) immediately upstream of the AUG start codon for efficient initiation of translation.

**Site-Directed Mutagenesis**

For downstream cloning, the EcoRI site was introduced by four silent point mutations at nucleotides (NT)-309–314 in exon 4 of RS1 cDNA. Sense and antisense oligomers that differed from the wild-type sequence at four sites (C309G, C312T, A313T, and G314C) were chemically synthesized. These synthesized fragments were introduced into a Tag4A-RS1 vector (Quick Change Site-Directed Mutagenesis kit; Stratagene). The clones were verified by DNA sequencing. The creation of the EcoRI site altered neither the RS1-protein sequence nor the expression and localization.

**Cell Culture and Transfection**

Functionality of the wild-type (WT) and mutant (MUT) [c354del1-ins18] RS1 minigenes was studied. COS-7 cells were grown in DMEM with 10% FBS. A day before transfection, the cells were plated at 400,000 cells per 10-cm culture dish. These were transiently transfection the next day using 18 μL of transfection reagent (FuGene 6; Roche Applied Science, Indianapolis, IN) and 6 μg of pCMV-Tag 4A control plasmid (without insert) or with minigenes expressing RS1-WT or RS1-MUT. Whole-cell lysates were prepared 72 hours later by the freeze-thaw method in a lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, and 0.5% Igepal CA 630 plus protease inhibitor cocktail). Transfected cell-culture medium was also collected and analyzed for RS1 protein. To investigate
the protein degradation pathway, proteasome MG132 (Z-Leu-Leu-Leu-Val, Sigma Aldrich Corp, St. Louis, MO) was added to cultures at 48 hours after minigene transfections. After 6 hours, the cells were collected by harvesting for downstream applications.

**RT-PCR and Subcloning**

Total RNA was isolated from COS-7 cells expressing the RS1-WT and RS1-MUT minigenes (TRIZol; Invitrogen). DNA contamination was removed (DNA-free kit; ABI). RT-PCR was performed in one step (SuperScript One Step RT-PCR with Platinum Taq; Invitrogen) with the following primers: (1) RS1 exon 4-forward: NT-206–227: 5′-aggatgcgaagttccatgggcttggc-3′; (2) RS1 exon 5-reverse: NT-463–441: 5′-acttgcatctgtgtctcgttacgctc-3′; (3) RS1 exon 1-forward: NT-1-23: 5′-atgtcacgctgttggtttc-3′; and (4) RS1 exon 6-reverse: NT-675–654: 5′-tatgcaacctgtgacgacac-3′. The absence of genomic DNA in RNA was also verified by omitting the enzyme mix and substituting 2 units of high fidelity Taq DNA polymerase (Platinum; Invitrogen) in the reaction. The RT-PCR products were separated on a 1% agarose gel and visualized with ethidium bromide.

The intronless cDNA derived from RT-PCR analysis of transcripts encoded by the minigenes were cloned in pcDNA3 Blunt II TOPO vector (Zero Blunt TOPO PCR cloning kit; Invitrogen), and DNA was isolated. After confirming the authenticity of DNA sequences, the WT and MUT coding sequences were subcloned into pCMV Tag 4A expression vector (Zero Blunt TOPO PCR cloning kit; Invitrogen), and DNA was isolated.

**Real-Time RT-PCR**

Real-time RT-PCR was performed using DNase treated total RNA, RS1 RT1 qPCR primer set (SA Biosciences, Frederick, MD), the housekeeping GAPDH gene primer set and qPCR master mix according to the manufacturer’s protocol (SYBR GreenER Reagent System; Invitrogen). The PCR amplifications were then performed (I-cycler; Bio-Rad Laboratories, Hercules, CA).

**RESULTS**

**Ophthalmic Examination**

The six XLRS males showed heterogeneity and clinical and electroretinographic severity (Table 1). The two young boys had a cartwheel pattern of microcystic macular lesions typical of classic XLRS24 (Figs. 2, 3). The four older relatives all had some degree of foveomacular atrophy in one or both eyes and showed an advanced and typical electronegative ERG waveform. In contrast, the b-wave amplitude was normal for the two younger subjects (Fig. 4). The younger affected family members (VI.5 and VI.6) had nearly normal b/a-wave ratios (respectively, 1.43 and 1.35), indicating that the synaptic connections between photoreceptors and bipolar cells were preserved across much of the retina. By contrast, the four older relatives all had a quite substantially lower b/a-wave ratio, indicating widespread retinal disturbances in synaptic and/or bipolar cell function. The difference with age is readily observed on the plot of age versus b/a-wave ratio (Fig. 5).

**Table 1.** ERG and Visual Function Test Results

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (y)</th>
<th>Eye</th>
<th>Visual Acuity</th>
<th>Spherical Equivalent</th>
<th>Visual Field (Both Eyes Similar)</th>
<th>a-Wave Amplitude (µV)*</th>
<th>ERG b-/a-Wave Ratio†</th>
<th>Threshold Luminance (log cd/m²)‡</th>
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</thead>
<tbody>
<tr>
<td>V.1.5</td>
<td>5</td>
<td>OD</td>
<td>20/80</td>
<td>+0.25</td>
<td>Normal V4 isopter other isopters constricted central scotoma</td>
<td>212</td>
<td>1.45</td>
<td>Not measured</td>
</tr>
<tr>
<td>V.1.6</td>
<td>1.5</td>
<td>OD</td>
<td>20/80</td>
<td>+0.25</td>
<td>Normal V4 isopter other isopters constricted central scotoma</td>
<td>Not recorded</td>
<td>Not recorded</td>
<td>Not measured</td>
</tr>
<tr>
<td>V.3</td>
<td>45</td>
<td>OD</td>
<td>20/250</td>
<td>+6</td>
<td>Normal V4 isopter other isopters constricted central scotoma</td>
<td>235</td>
<td>0.26</td>
<td>3.9</td>
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<tr>
<td>V.10</td>
<td>32</td>
<td>OD</td>
<td>20/250</td>
<td>+6.25</td>
<td>Normal V4 isopter other isopters constricted central scotoma</td>
<td>248</td>
<td>0.42</td>
<td>3.9</td>
</tr>
<tr>
<td>V.24</td>
<td>45</td>
<td>OD</td>
<td>20/520</td>
<td>+2</td>
<td>Normal V4 isopter other isopters constricted central scotoma</td>
<td>158</td>
<td>0.68</td>
<td>3.6</td>
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<tr>
<td>V.28</td>
<td>45</td>
<td>OD</td>
<td>20/322</td>
<td>+2</td>
<td>Normal V4 isopter other isopters constricted central scotoma</td>
<td>188</td>
<td>0.33</td>
<td>4.2</td>
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</tbody>
</table>

* Nonparametric reference values for a-wave amplitude (obtained from 96 normal volunteers); median, 297 µV; lower limit, 185 µV.
† Nonparametric reference values for b-wave/a-wave amplitude ratio (obtained from 96 normal volunteers); median, 1.89; lower limit, 1.42.
‡ Reference values for absolute luminance threshold (obtained from 20 normal volunteers): mean, −4.89 log cd/m²; upper limit, −4.13 log cd/m².
Following is a brief description of the findings (see Table 1): The 5 year-old proband (VI.5) had macular radial cysts typical for XLRS in both eyes, confirmed by OCT (Fig. 3). The peripheral RPE had grayish pigmentation in the right eye inferior quadrant and left eye temporal quadrant. Vitreous veils were detected bilaterally in the posterior vitreous, but no retinal

**FIGURE 2.** Fundus photographs of the right eye of all subjects (FF 450 Fundus Camera; Carl Zeiss Meditec, Dublin, CA). VI.5 and VI.6 had classic foveal schisis; V.3 had a macular hole, and abnormal RPE pigmentation was associated with atrophic macular changes in V.10, V.24, and V.28.

More severe abnormalities were seen in V.3 (pseudomacular hole) and in V.10, V.24, and V.28 (atrophic macular thinning).

**FIGURE 3.** Optical coherence tomograms through a horizontal section of the right eye of all subjects. VI.5 and VI.6 showed classic foveal schisis. More severe abnormalities were seen in V.3 (pseudomacular hole) and in V.10, V.24, and V.28 (atrophic macular thinning).

**FIGURE 4.** Combined (rod and cone mediated) ERG response of the right eye of all subjects and of a representative normal control subject. Note the relative preservation of b-waves in VI.5 and VI.6, but the marked reduction in b-wave amplitude in V.3, V.10, V.24, and V.28, which gives these responses an electronegative configuration that is prototypical of XLRS disease.
bullous schisis was seen. Follow-up 3 years later at 8 years of age showed no new fundus changes, but the scotopic ERG a- and b-wave amplitudes were both reduced 25% compared with the first recording at 5 years of age.

The 1.5-year-old brother (VI.6) had esotropia. His retinal examination under general anesthesia showed foveal cysts and parafoveal radial cysts in both eyes, with grayish RPE pigmentation of flat schisis in the temporal periphery. Vitreous veils were present in the superior temporal quadrant of both eyes but no bullous schisis. The ERG recording showed only a minimal technical reduction of a- and b-wave amplitudes. Clinical examination 3 years later showed no overt progression.

Subject V.3, a 43-year-old man, was a maternal uncle of the proband. He recalled central visual loss by 6 to 7 years of age. At 43 years, his right eye had a foveal pseudohole documented by OCT (Fig. 3). Parafoveal granular RPE pigmentation and mid peripheral white reticular areas and linear hyperpigmentation in the inferior temporal quadrant were seen. OCT of the left eye showed foveal atrophy. The scotopic ERG of both eyes was quite electronegative, although the photoreceptor a-wave remained normal.

XLRS was diagnosed in subject V.10 at 8 years of age and had bilateral poor vision since childhood. At 32 years, both maculas had granular pigmentation and fibrotic scarring. The OCT showed foveal atrophy in the right eye. Bullous schisis was present in the inferotemporal periphery of the right eye. Flat schisis was observed in the inferotemporal periphery of the left eye. The scotopic ERG was electronegative.

Subject V.24 had exotropia and macular dystrophy since childhood. At 45 years, he had bilateral macular atrophy confirmed by OCT (Fig. 3). A vitreous band spanned the superotemporal periphery. The scotopic ERG was grossly electronegative.

Subject V.28 reported poor vision since childhood. At 43 years, the right eye fovea was atrophic, and the left eye fovea had multiple pigmented lesions. XLRS cysts were not detectable clinically, but were evident on OCT. The retinal periphery of both eyes had inferotemporal whitish deposits, but no overt schisis. The scotopic ERG was grossly electronegative.

**Molecular Genetic Findings: Exon-5 Deletion/Insertion Mutation**

Sequencing RS1 along both strands showed a deletion/insertion mutation in all six affected subjects, with a 1-bp deletion replaced by an 18-bp insertion at the same nucleotide (354 del C, 354–371 Ins GGTGTGCCTGGCTCTCCA; Fig. 6). The resulting frame shift creates a UAG termination signal six codons downstream. The inserted sequence duplicates the adjacent upstream sequence of both strands. The duplicated sequence spanned an intron–exon junction, from the last nucleotide of intron 4 through the first 17 nucleotides of exon 5. This duplication involves the RNA splice-donor and splice-acceptor sites surrounding the splice-donor site of intron 4 and generates an additional intron–exon junction 27 bp downstream of the original intron–exon junction. Consequently, we examined whether this mutation affects pre-RNA splicing.

**FIGURE 5.** The b-/a-wave amplitude ratio (right eye) plotted as a function of age in the six subjects with XLRS. Pearson correlation coefficient, $R = -0.979; \ P = 0.0006$.

**FIGURE 6.** RS1 genomic sequence encompassing the c354del1-ins18 mutation in exon 5. The normal control and XLRS subject genomic sequences that are shown include a 3-bp upstream intron-4 sequence and downstream exon-5 sequence. The XLRS mutation is a 1-bp deletion and an 18-bp insertion at NT-354. Dashed lines: the inserted sequence at NT-354–371, a direct copy of the upstream sequence (NT-327–343). Bold lines: the intron sequence. The first G nucleotide of the inserted sequence probably originated from the terminal nucleotide of intron 4. The coding sequence derived from Homo sapiens retinoschisin (RS1), mRNA (NM_000330) is numbered from 1–675.
More than one mutant clone was tested. 

RS1-WT and RS1-MUT minigenes were constructed by PCR amplification of normal genomic DNA and the XLRs subject’s genomic DNA (c354del1-ins18; Fig. 7), and were transiently transfected into COS-7 cells to monitor the mutational effect on pre-mRNA splicing and RS1-protein expression. Total RNA isolated from COS-7 cells expressing the minigenes was used as templates for the RT-PCR with primers flanking the mutation (NT-206–463, exon 4–5 of RS1 cDNA). The RS1-WT minigene cells yielded the predicted 257-bp RT-PCR product. Cells expressing the RS1-MUT minigene produced a longer PCR product (Fig. 8A, top) that corresponded to the size of the 18-bp insertion. RT-PCR with primers flanking the CDS of the RS1 gene (NT-1–675) produced the similar result, with longer PCR product in cells with the RS1-MUT minigene (Fig. 8A, bottom). No aberrantly spliced products were observed for the mutant mRNA, indicating that the duplication of the intron–exon junction 27 bp 3’ downstream of the original splice junction did not affect pre-RNA splicing. This finding was confirmed by subcloning the full length cDNA derived from RNA isolated from COS-7 cells expressing the minigenes. DNA sequence analysis of several of these subclones confirmed that the wild-type and mutant RNAs are processed similarly.

Western-blot analysis of the medium of cells transfected with the RS1-WT minigene showed a single ~22-kDa RS1-protein band (Fig. 8B), with very little detected in the cell lysates, consistent with the secreted nature of RS1 protein. However, mutant RS1 protein was not detected either in the medium or in the cellular fractions of RS1-MUT minigene cells. The 18-bp insertion introduces an amber stop codon, UAG, that prematurely terminates translation—that is, a frame-shift change with Asp 118 as the first affected amino acid changing to Glu and creating a new reading frame ending in a UAG stop codon at position 14 (pAsp118Glu fsX14). We were unable to detect any truncated RS1 protein in the Western blot analyses. It is unlikely from loss of the antibody epitope, as the anti-RS1 polyclonal antibody against the N terminus amino acids 24–37 of RS1 would recognize the N terminus epitope if a truncated 131-amino-acid protein were present. The absence of any truncated RS1 mutant is consistent with rapid degradation of the protein. The loss of mutant RS1 could also be due to loss of mRNA triggered by NMD. Nonsense-mediated mRNA decay (NMD) in mammalian cells is a well-characterized mRNA surveillance mechanism by which aberrant mRNAs harboring premature translation termination codons (PTCs) are rapidly degraded—an intron-dependent regulatory mechanism that eliminates abnormal transcripts. To analyze whether the premature termination codon introduced by the c354del1-ins18-bp mutation interferes with RS1 mRNA expression levels, we performed real-time RT-PCR on total RNA isolated from COS-7 cells expressing the RS1-WT and RS1-MUT minigenes. Control and mutant cells showed no difference of RNA levels, which indicate that the loss of mutant protein was not due to NMD. We then used an intronless mutant cDNA construct, which escapes NMD-dependent RNA degradation, to transfect COS-7 cells (FuGene; Roche) to amplify the mutant protein. However, the truncated RS1-protein product was never observed in cells expressing the intronless mutant minigene. As this absence of mutant protein could result from reduced synthesis or accelerated degradation, we investigated whether the proteasome pathway is involved in degradation of the mutant protein. Cells expressing the intronless WT- and MUT-cDNA constructs were exposed to 50 μM MG 132 proteasome that splicing was not disrupted in the mutant. However, the insertion caused a frame shift and introduced premature termination codons in the RNA. This finding was confirmed on repetition. (B) RS1-protein expression. Whole cell lysates derived from cells transfected with WT or MUT minigene were subjected to SDS-PAGE followed by immunoblot analysis with RS1 antibody. Cells transfected with WT minigene express RS1, which is predominantly detected in the secreted fraction. No RS1 expression was seen with the MUT minigene possibly because of premature termination of translation and rapid degradation of the truncated product. The findings were confirmed on repetition. (C) At 48hours after transfection, cells expressing the intronless WT- and MUT-cDNA constructs were exposed to a proteasome inhibitor MG132 (50 μM for 6 hours) followed by SDS-PAGE and immunoblot analysis. Two protein bands (14 and 28 kDa) immunoreactive to RS1 antibody were detected in MG132 treated cells expressing the intronless MUT. Presumably, they represent the monomer and dimer of the truncated RS1 species. RS1 was not detected in the medium. Cells with the intronless WT-cDNA construct expressed both the precursor (24 kDa) and mature (22 kDa) forms of RS1. More than one mutant clone was tested.
inhibitor for 6 hours and subjected to immunoblot analysis. Protein bands immunoreactive to RS1 antibody were detected in MG132-treated cells with the intronless MUT (Fig. 8C). A protein band of ~14-kDa approximate mass was seen that corresponds to the size of the truncated RS1, along with a 28-kDa band presumed to be the dimer of the truncated species. However, no truncated RS1 was found in the medium. Untreated control cells did not exhibit these bands. The finding that truncated RS1 accumulates only in the presence of proteasome inhibitor demonstrates that the mechanism underlying the rapid degradation of the mutant protein involves the proteasomal pathway and that the c354del1-ins18 mutation gives a RS1-protein null phenotype.

**DISCUSSION**

**Molecular Biology of the Mutation**

The findings imply that there is a putative mechanism that rapidly degrades truncated RS1 protein with premature termination codons (PTCs). Although the c354del1-ins18 mutation resulted in a PTC, we found no evidence of nonsense mediated decay of mRNA (NMD) as the underlying cause of the RS1-protein deficiency. Short deletion-insertion mutations with duplication of adjacent sequence have been described in other human diseases involving α2 globin and factor VIII genes, and in some of these mutations, the inserted nucleotides duplicate the neighboring sequence on the same DNA strand, as we found in the XLRS family of this study. A combination of slipped mispairing during replication and intragenic recombination has been suggested as the mechanism for the deletion-insertion-duplication process. In our c354del1-ins18 XLRS family the inserted sequence duplicated the upstream intron-exon junction, but this had no effect on pre-RNA splicing. Nevertheless, the deletion-duplication mutation within the exon-5 coding sequence terminated the expression of RS1 protein.

The 1-bp deletion and 18-bp insertion creates a new reading frame that ends in a premature termination codon (UAG) at position 14. Eukaryotic mRNAs that contain PTCs are rapidly degraded by NMD. This conserved eukaryotic mRNA quality control system thereby prevents the accumulation of potentially detrimental truncated proteins. We investigated whether NMD is responsible for the deficiency of RS1 protein in the cells expressing the mutant minigene. Although to what NMD would predict, real-time RT-PCR analysis revealed no significant decrease in c354del1-ins18 mRNA levels in the cells expressing the RS1-MUT minigene. Although the stability of RS1 mRNA with PTC was surprising, several other reports have also indicated that not all mRNAs that contain PTCs are targeted for destruction by NMD, and that a proteasome or proteases-dependent mechanism causes rapid degradation of truncated protein in some cases. This is consistent with the "rescue" of truncated RS1 we observed using the proteasome inhibitor MG132, confirming that rapid degradation of c354del1-ins18 mutant RS1 involves the proteasomal pathway.

**Clinical Considerations**

Because clinical changes from XLRS occur at most very slowly, it is difficult to document disease progression in single affected individuals, and XLRS has historically been described as a stationary condition. The present study, however, indicates that the condition is progressive in this RS1 mutation. The proband showed a 25% ERG reduction over a 3-year interval, between 5 and 8 years of age. Furthermore, the disease in this family varied considerably across 1.5 to 45 years and was substantially more severe in the older members. The four older men had major b-wave loss leading to classic XLRS electronegative ERGs, and three of them exhibited overt foveal atrophy. The two youngest had not progressed to that state and had more limited macular schisis and a nearly normal scotopic ERG. As all six affected males carried the same RS1 mutation, the cross-sectional analysis suggests progression with age.

These findings are consistent with the progression documented in natural history studies of the RS1 knockout-mouse model. Kjellstrom et al. found that the retinal cavities were greatest at 4 months of age in the RS1-KO mouse and then collapsed and were progressively reduced at later ages. Progressive photoreceptor loss with age was also found, and by 16 months the outer nuclear layer showed major loss of cells in the mouse XLRS model. Both the RS1 mutation in this human family and the RS1-KO mouse model lack retinoschisin protein expression, indicating that the absence of retinoschisin is deleterious and can cause progressive disease.

Some XLRS-affected males exhibit severe disease at very early ages, including bilateral bulbar schisis, vitreous hemorrhages, and retinal detachment. By contrast, the two affected young boys in our family, despite the absence of RS1 protein with this mutation, had only macular schisis and retained nearly normal ERG response. The normal b-/a-wave ratios indicate appropriate transmission through the rod synapse onto rod bipolar cells. The clinical findings in this family suggest that the absence of RS1 may initially cause less severe disease but that the retinopathy apparently is not stable, as the same mutation and absence of RS1 protein in the 32- to 45-year-old men caused considerably more dysfunction and vision loss. The central atrophy in the three older males implies a progressive death of the foveal cones. The atrophic maculopathy noted in three of the 32- to 45-year-olds is not exclusive to a loss of RS1 protein, as macular atrophy has also been found in relatively young adults associated with RS1-missense mutations.

**Questions Raised**

Environmental and genetic factors, including modifier genes, are predicted to account for some of the phenotypic variation in an XLRS mouse model and hence, are the possible contributors to the wide phenotypic heterogeneity found across the ages of members of this XLRS family. However, modifier genes have not been described for human XLRS. RS1 mutations can give rise to functional null biochemical phenotypes in at least two ways. Several discoidin domain missense mutations in exons 4 to 6 perturb RS1-protein folding and prevent secretion across the plasma membrane. Other missense mutations, including M1L in exon 1 and the deletion-insertion exon-5 mutation in this study, either abolish or severely reduce retinoschisin protein expression. Whether these two biochemical phenotypes affect the clinical course of XLRS disease in the same or in different ways remains unclear.

Retinoschisin has been described as a signaling protein, secreted by photoreceptor cells. Reid and Farber have suggested that retinoschisin interacts with bipolar and Müller cells and thereby normally contributes to the development of the cellular and synaptic architecture of the retina. Loss of retinoschisin disturbs retinal structure and causes loss of synaptic transmission to the retinal bipolar cells, as reflected in the reduction of the scotopic ERG b-wave. Evidently, beyond the period of retinal development, RS1 is required for structural maintenance, and some XLRS subjects may have normal retinal development with substantial bipolar function.
at an early age but subsequently exhibit a failure of maintenance.24

These clinical and mutational considerations give rise to three questions:

1. Is the apparent progression of XLRS dysfunction due exclusively to age?
2. How is it that the retina is organized in the youngest two males in the absence of the RS1 protein such that synaptic transmission can support a normal b/a-wave ratio?
3. Does the degree of ERG dysfunction and b/a-wave loss correlate with the extent of fundus involvement? The pathology in the youngest two males appeared relatively limited, whereas the older four males had considerable peripheral involvement in addition, suggesting that synaptic integrity may be impaired secondarily over a greater extent of the fundus.

We are currently exploring the possibility that the absence of RS1 protein results in upregulation and overexpression of other adhesion proteins in the extracellular matrix and thereby provides a substitute that initially supports development and preserves synaptic integrity and retinal structure during the early stage of disease.

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