X-linked Retinitis Pigmentosa: RPGR Mutations in Most Families with Definite X Linkage and Clustering of Mutations in a Short Sequence Stretch of Exon ORF15

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PURPOSE. A comprehensive screening was conducted for RP2 and retinitis pigmentosa GTPase regulator (RPGR) gene mutations including RPGR exon ORF15 in 58 index patients. The frequency of RPGR mutations was assessed in families with definite X-linked recessive disease (xLRP), and a strategy for analyzing the highly repetitive mutational hot spot in exon ORF15 is provided.

METHODS. Fifty-eight apparently unrelated index-patients were screened for mutations in all coding exons of the RP2 and the RPGR genes, including splice-sites, by single-strand conformation polymorphism (SSCP) analysis, except for RPGR exon ORF15. A strategy for directly sequencing the large repetitive stretch of exon ORF15 from a 1.6-kb PCR-product was developed. According to pedigree size and evidence for X linkage, families were subdivided into three categories.

RESULTS. Screening of 58 xLRP families revealed RP2 mutations in 8% and RPGR mutations in 71% of families with definite X-linked inheritance. Mutations clustered within a ~500-bp stretch in exon ORF15. In-frame sequence alterations in exon ORF15 ranged from the deletion of 36 bp to the insertion of 500 bp.

CONCLUSIONS. Mutations in the RPGR gene are estimated to cause 15% to 20% of all cases of RP, higher than any other single RP locus. This report provides a detailed strategy to analyze the mutational hot spot in RPGR exon ORF15, which cannot be screened by standard procedures. The discrepancy of the proportion of families linked to the RP3 locus and those having RPGR mutations is resolved in a subset of families with definite X linkage. (Invest Ophthalmol Vis Sci. 2003;44:1458–1465) DOI:10.1167/iovs.02-0605

Retinitis pigmentosa (RP) is a genetically and clinically heterogeneous group of progressive photoreceptor degenerations with an overall incidence of 1 in 4000 in the general population. X-linked forms (xLRP, online Mendelian inheritance in man [OMIM 268000]) account for approximately 15% of RP cases and represent the most severe subtypes of this disease. They manifest themselves typically within the first two decades of life with night blindness and constriction of visual fields, progressing to severe visual loss or complete blindness by the third or fourth decade in affected men.1,2 Five distinct RP loci on the X chromosome have been identified by linkage analyses predicting the loci RP3 (OMIM 312619; Xp21.1) and RP2 (OMIM 312600; Xp11.3) to account for 70% to 75% and 11% to 25% of all xLRP cases, respectively.3 The RP2 gene consists of five exons and codes for a predicted protein of 350 amino acids with domains homologous to cofactor C, to a porcine microtubule-associated protein (g-subunit of T-complex) and to a member of the nucleoside diphosphate kinase family.4,5 It is mutated in 7% to 18% of different xLRP patient families. Mutations in the N terminus of the protein interfere with normal targeting to the plasma membrane.3,6–8 Positional cloning of the RPGR gene (RP3) originally revealed a 2784-nucleotide (nt) ubiquitously expressed transcript that is organized in 19 exons coding for a predicted protein of 815 amino acids.9

Originally, this gene was found to be mutated in only 11% to 26% of patients with xLRP, with mutations being restricted to exons 1 to 15, which harbor a domain homologous to RCC1 (regulator of chromosome condensation).10–14 Using the RCC1 homologous RPGR domain as a bait in yeast two-hybrid screens revealed two interacting proteins: the delta subunit of rod cyclic GMP phosphodiesterase (PDE-δ)11 and the RPGR-interacting protein (RPGRIP) which localizes to the connecting cilium and is mutated in patients with Leber congenital amaurosis, a retinal dystrophy clinically related to RP.12–15 Ectopic localization of cone opsins with subsequent cone and rod degeneration was observed in an RPGR knockout mouse.16 Multiple 3′ splice variants were discovered in different tissues in humans, mice, cattle, and dogs, and two different mutations in exon ORF15 were detected in two distinct mutant dog strains with phenotypically distinguishable X-linked progressive retinal atrophy.17–20 Recently, an alternatively spliced transcript containing a novel 1.7-kb 5′ terminal exon (ORF15) was identified. It is predominantly expressed in retina and harbors a mutational hot spot in patients with xLRP. This exon results from the retention of 1554 nt of the previously defined intron 15, includes a purine-rich repetitive region, and
codes for 567 C-terminal amino acids rich in glutamic acid and glycine residues. Exon ORF15 was mutated in 60% of xIRP families of mainly British and Irish descent, 18 in 18% to 30% of North American xIRP families, 19 in 32% of an unselected European xIRP population, 20 and in 15% of sporadic male patients with RP who have early onset of disease. 21 These data indicate that mutations in the RPGR gene may account for 15% to 20% of all cases of RP. 22,23 Mutations in exon ORF15 were also identified in patients with X-linked dominant RP 22 and in males with an X-linked cone–rod dystrophy phenotype. 24,25 In this report, we present the results of a comprehensive screening for RPGR and RP2 gene mutations in 58 xIRP-affected families and provide a strategy to screen the diagnostically most relevant RPGR exon ORF15.

**METHODS**

**Patients**

The study, which involved human subjects, conformed to the tenets of the Declaration of Helsinki. Fifty-eight apparently unrelated index cases in members of clinically diagnosed xIRP families were screened for mutations in the RP2 and the RPGR gene. Ophthalmic examination, including visual acuity, fundoscopy, Goldmann perimetry, dark-adaptation studies, and electoretinography (ERG), was performed in the affected males and in probable carrier females. The patients came from Germany (n = 54), Croatia (n = 1), Luxembourg (n = 1), Switzerland (n = 1), and Spain (n = 1). According to the size of the families, the number of affected males, and the number of carriers, three groups with variable probability of an X-linked recessive trait were defined: group I, families with at least two affected males in two generations connected by at least two obligate carrier females (n = 24); group II, families with affected males in two generations connected by a single carrier female (n = 18); group III, families with only two affected brothers or one affected male and a mother showing clinical signs confirming her carrier status (n = 16). Male-to-male transmission was absent in all pedigrees.

**Mutation Screens in the RPGR Gene in Exons 1 to 19 and in the RP2 Gene in Exons 1 to 5**

The originally reported exons of the RPGR (exons 1–19) and the RP2 gene (exons 1–5) were amplified from leukocyte DNA samples, as described by Meindl et al. 9 and Schwahn et al., 4 and analyzed by single-strand conformation polymorphism (SSCP) analysis, using acrylamide gels (0.6% Sdergel; Serva, Heidelberg, Germany) running at 20°C with or without 10% glycerol in the gel matrix. Gels were stained with fluorescent dye (VistraGreen; Amersham, Freiburg, Germany), and bands were visualized with a fluorescence imager (FluorImager; Molecular Dynamics, Sunnyvale, CA). Products with aberrant SSCP patterns were column purified with a PCR purification kit (QiAquick; Qiagen, Hilden, Germany), sequenced using one of the SSCP PCR primers in a sequencing kit reaction (BigDye Terminator Cycle Sequencing Kit; Applied Biosystems, Foster City, CA) and analyzed on a DNA sequencer (Prism 377 DNA Sequencer; Applied Biosystems). All mutations were confirmed by sequencing a second PCR reaction.

**Analysis of Exon ORF15 of the RPGR Gene**

A strategy for directly sequencing the repetitive part of the RPGR exon ORF15 with nested sequencing primers (Table 1) from a 1.6-kb fragment with nested primers.

**Table 1. Primer Sequences and Conditions for Analyzing RPGR Exon ORF15**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Annealing °C (Cycles)</th>
<th>Length (Name)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15F</td>
<td>5'-CGAGATCCCTATCAGATGACC-3'</td>
<td>60 (10); 58 (30)</td>
<td>1.6 kb</td>
</tr>
<tr>
<td>15aR3</td>
<td>5'-TGTCGTACGTGGCATAATCG-3'</td>
<td>58 (35)</td>
<td>237 bp</td>
</tr>
<tr>
<td>15F</td>
<td>5'-CGAGATCCCTATCAGATGACC-3'</td>
<td>58 (35)</td>
<td>271 bp</td>
</tr>
</tbody>
</table>

*Primers 15F and 15aR3 were used to amplify the 1.6-kb fragment which includes the repetitive stretch. PCR products a and b were screened by SSCP-analysis.

†Conditions for sequencing the repetitive stretch from the 1.6-kb fragment with nested primers.
manufacturer’s protocol. PCR-products were analyzed by SSCP and aberrantly migrating deletions were sequenced according to standard methods, using one of the PCR-primers (Fig. 1D, Table 1). The sequence of primer 15aF5 (Fig. 1E) is 5′-GTAGAGGCAGGAGGAAGTAG-3′.

RESULTS

Thirty-six patients with xLRP from the present patient collective had been screened for mutations in *RPGR* exons 1 to 19 resulting in the detection of four different mutations.† Six additional mutations affecting exons 2, 5, 6, 10, 15, and 11 to 19 have now been found after screening exons 1 to 19 in a further 25 xLRP-affected families. None of these mutations has been reported before. Mutations affecting exons 16, 17, 18, or 19 exclusively were not detected. Altogether, mutations were found in 10 of the 58 xLRP-affected families by screening the originally reported *RPGR* gene exons (Table 2). Screening of the *RP2* gene revealed mutations in three of the 58 families, including a deletion mutation encompassing exon 1 that has not been reported so far (Table 2). Screening the large novel *RPGR* exon ORF15 in the remaining families revealed another 17 different mutations in 24 (41%) of the 58 families (Fig. 1B).

Segregation analysis of ORF15 mutations was performed in 14 independent families. The identified mutations were not present in 100 control chromosomes. Predominant mutations were frameshifts caused by 2-, 1-, and 4-bp deletions. Less common were 1-bp insertions and a single larger deletion (22 bp). Only two point mutations were detected, causing protein truncation by premature stop codons (Fig. 1C). Frameshift mutations clustered within a 448-bp stretch in exon ORF15 between g.ORF15+402-849, which contained 83% (20/24) of all mutations detected in exon ORF15. In-frame alterations which were all located within the most repetitive stretch of ORF15 ranged from the loss of 36 bp in chromosomes in which two different deletions coincided (g.ORF15+788_808del121 and g.ORF15+853_867del15) to the gain of 75 bp (Fig. 1E); They were detected in 34% of all the analyzed chromosomes, and most of them were present in 100 unaffected control chromosomes (Fig. 1C). Two in-frame alterations were found in patients only: First, the deletion g.ORF15+694_708del15 (p.ORF15+231_235delGGEVE) was present in two families that additionally harbored a disease causing frameshift mutation (g.ORF15+504_507delGGAG), indicating that those families may be related. Second, the in-frame duplication g.ORF15+917_991dup, which reduplicates three of four completely identical 24-bp repeat units, did not segregate with the disease in family RP15 (Fig. 1E).

DISCUSSION

Mutations in the *RPGR* gene are estimated to be responsible for 15% to 20% of all cases of RP, higher than any other single locus.‡ Two comprehensive mutational screens in xLRP-affected patients have been published to date with various results regarding the proportion of *RPGR* mutations detected in families with an X-linked recessive trait. Vervoort et al. found *RPGR* gene mutations in 72% of a patient collective of British and Irish descent, which is in accordance with linkage analyses. Of the mutations in xLRP families, 60% were found in exon ORF15.† In the first confirmatory screen *RPGR* mutations were found in 55% (exon ORF15: 30%) of families of North American decent with definite X linkage.‡ In a recent study a similar mutation detection rate (exon ORF15: 32%) was observed in an unselected European xLRP-affected patient collective.†

In the present study *RPGR* mutations were found in 71% and exon ORF15 mutations were present in 63% of families.

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**Table 2. RP2 and RPGR mutations**

<table>
<thead>
<tr>
<th>Individual</th>
<th>Exon</th>
<th>DNA Mutation</th>
<th>Predicted Effect on Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP04/1759</td>
<td>1</td>
<td>Deletion of exon1</td>
<td>Truncated protein</td>
</tr>
<tr>
<td>RP28/1125</td>
<td>2</td>
<td>353G→A</td>
<td>R118H</td>
</tr>
<tr>
<td>XRP3/8800</td>
<td>2</td>
<td>552C→T</td>
<td>R118C</td>
</tr>
<tr>
<td><em>RPGR</em> gene mutations in exons 1–15*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP07/5619</td>
<td>2</td>
<td>72Ains</td>
<td></td>
</tr>
<tr>
<td>XRP2/8800</td>
<td>5</td>
<td>514C→T</td>
<td></td>
</tr>
<tr>
<td>RP09/13552</td>
<td>6</td>
<td>677_680delAACA</td>
<td></td>
</tr>
<tr>
<td>RP87/11893</td>
<td>10</td>
<td>1275_1276delCT</td>
<td></td>
</tr>
<tr>
<td>XRP3/8800†</td>
<td>15</td>
<td>1928_1929delAG</td>
<td></td>
</tr>
<tr>
<td>RP07/1221†</td>
<td>2</td>
<td>213G→T</td>
<td></td>
</tr>
<tr>
<td>RP49/5474†</td>
<td>7</td>
<td>705G→T</td>
<td></td>
</tr>
<tr>
<td>RP02/59†</td>
<td>8</td>
<td>945_959del15bp</td>
<td></td>
</tr>
<tr>
<td>RP22/4094</td>
<td>11-19</td>
<td>Deletion of exons 11-19</td>
<td>Truncated protein</td>
</tr>
<tr>
<td>RP16/489‡</td>
<td>14,15</td>
<td>Deletion of exons 14-15†</td>
<td>Truncated protein</td>
</tr>
</tbody>
</table>

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* Nomenclature according to Meindl et al.9
† Annotation of this mutation according to Vervoort et al. is: g.ORF15+116_117delAG (p.ORF15E39fsX43).19
‡ The mutations in these patients have been reported previously.9

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**Figure 1.** (A) Three mutations in *RPGR* exon ORF15. (B) Distribution of 17 different mutations in *RPGR* exon ORF15, detected in 24 families. Deletions (△), insertions (∇), point mutations (●). Two parts of exon ORF15 are indicated: exon 15 of the originally reported *RPGR* transcript (g.ORF15+1-152 □), and the repetitive purine-rich stretch (g.ORF15+402-1406 p) consisting of imperfect direct repeats of 6 to 35 bp. (C) Distribution and frequency (in parentheses) of sequence variations detected in 100 male control chromosomes. Two sequence variations (○) were present only in patients with xLRP patients. (D) Outline of the strategy for screening exon ORF15. PCR products a and b were screened using SSCP analysis, and the large 1.6-kb PCR product (bold line) was sequenced using the primers depicted (dashed arrows). (E) Segregation analysis in family RP15. The reduplication g.ORF15+917_991dup does not segregate with the disease. The insertion is present in the affected male II:2 and in his heterozygote mother, but not in the affected son II:1. A 447-bp PCR product generated with the primers 15aF5 and TER3 (Table 1) was separated on a 1.5% agarose gel. The reduplication is predicted to insert 16 glutamic acid and 9 glycine residues into the glutamic-acid-rich stretch.
with definite X linkage (Table III). In the present study, the inclusion criteria for group I families (definite X linkage) differed from the criteria applied in the first confirmatory screen.3 There had to be at least two obligate carrier females per pedigree (see the Methods section) to include only families with a pedigree structure that is comparable to those pedigrees generally used in linkage analyses (large multiplex families providing multiple informative meioses). In this subset of families, the discrepancy of the proportion of xlrP families linked to the RP3 locus and those having RPGR mutations is resolved. The missing mutations reside in exon ORF15. We conclude from our data that not yet identified exons at the RP3 locus are not likely to play a major role in xlrP.

Screening of exon ORF15 is technically challenging25,26 because of its repetitive nature and because of the presence of in-frame deletions or duplications in 34% of the general population, the majority of which delete or duplicate one or more tandem repeats. The strategy provided in the present study allows sequencing of both strands of the repetitive stretch with nested primers from a single PCR product. This approach avoids mispriming and PCR artifacts, which may easily occur with the use of PCR primers situated within the repetitive stretch.

The substantial decrease of mutation detection rate in the RPGR and the RP2 gene in families with smaller pedigrees (groups II and III, Table 3) may be due to unrecognized autosomal disease with reduced penetrance, as the possibility of an autosomal disease with reduced penetrance, as the possibility of an autosomal mode of inheritance cannot be ruled out with certainty. Unrecognized autosomal traits in smaller families may also be the reason for the lower mutation detection rate in the study screening families of North American descent with definite X linkage (definite X linkage: at least two generations of affected males that were related through an unaffected or carrier female)3 and in a screening of unselected European xlrP families.21

In families with fewer carrier females (groups II and III) a low rate of exon ORF15 mutations contrasted with the relative high frequency of mutations in RPGR exons 1 to 14 in the present study. An increased mutability of exon ORF15 in the male germline resulting preferentially in pedigrees with multiple obligate carrier females would be a possible explanation for this bias. However, because our pedigree data do not clearly support this hypothesis and because DNA samples from the earlier family generations have not been collected, this could not be investigated further.

It is interesting that 83% of the pathogenic mutations (mainly frameshifts) in exon ORF15 were detected within a 447-bp stretch at the 5’ end of the most repetitive sequence. A similar clustering was observed in two previous comprehensive studies.5,19 Mutation analysis therefore should be primarily focused on this 447-bp stretch.

Frameshifts occurring at the beginning of the purine-rich stretch (mutations: g.ORF15+652_653delAG, g.ORF15+742_763del22bp, g.ORF15+748delA, g.ORF15+801_802insG, g.ORF15+848_849delGG) create long open reading frames, due to the absence of T-nucleotides in the following 0.6 kb. The shifted open reading frames are predicted to code for 232-298 amino acid stretches rich in alkaline residues (arginine and/or lysine), reversing the charge of the polypeptide-stretch. Detailed genotype-phenotype analysis in these patients will be of use, as a mutant dog strain carrying a frameshift mutation that leads to 34 additional alkaline residues before protein truncation displays a severe phenotype that becomes manifest during retinal development with mutant protein aggregating in the endoplasmic reticulum.20

No mutations in the RPGR or RP2 gene were detected in 20% of group I families. In these patients, undiscovered mutations may reside either in regulatory sequences of the RPGR or RP2 gene or in one of the three additional xlrP loci (RP6, RP23, RP24) described to date.27–29 SSCP may not identify all mutations. The average mutation detection rate of SSCP analysis using acrylamide gels without glycerol is reported to be in the range of 70% to 90%; with glycerol, it is reported to be approximately 68%.30,31 Combination of the two conditions dramatically increases the sensitivity and produces detection frequencies above 90%.30,31 In the present study, gels were examined under both conditions.

Recently the in-frame deletion g.ORF15+694_708del15 has been proposed to cause X-linked cone dystrophy in affected males of a single family where no other RPGR mutation was found.33 It originally was supposed to be a benign sequence alteration because of the additional presence of a protein-truncating mutation (g.ORF15+897G→T) in one patient with xlrP.34 The present study identified this in-frame deletion in two patients from different families that have, in addition, a protein-truncating mutation (g.ORF15+504_507delGGAG) distinct from the mutation found in the patient in the study by Vervoort et al.19 This provides further evidence that the 15 bp in-frame deletion may instead be a rare sequence variant that does not cause XIRP or X-linked cone dystrophy. The in-frame duplication g.ORF15+917_991dup is the largest in-frame alteration detected in exon ORF15 to date. This duplication is not the cause of xlrP in the described family (Fig. 1E) and is predicted to leave intact the acidic character of the glutamic-acid-rich domain. It is therefore interpreted as a rare benign sequence alteration, because it was not present in 100 control chromosomes.

In conclusion, the results of the present study demonstrate that the majority of RPGR mutations reside in exon ORF15 in families with definite X linkage. Together with previous studies,3,19 they confirm the RPGR gene as the diagnostically most important single genetic locus in RP. Our data point to the high degree of sequence variation in RPGR exon ORF15 in the general population and indicate that the pathogenicity of rare in-frame sequence variations must be cautiously interpreted.

Acknowledgments

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References