Mutational Screening of the *RP2* and *RPGR* Genes in Spanish Families with X-Linked Retinitis Pigmentosa

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Purpose. The X-linked form of retinitis pigmentosa (XLRP) is the most severe type because of its early onset and rapid progression. Five XLRP loci have been mapped, although only two genes, *RPGR* (for RP3) and *RP2*, have been cloned. In this study, 30 unrelated XLRP Spanish families were screened to determine the molecular cause of the disease.

Methods. Haplotype analysis was performed, to determine whether the disease is linked to the RP3 or RP2 region. In those families in which the disease cosegregates with either locus, mutational screening was performed. The *RP2* gene, the first 15 exons of *RPGR* at the cDNA level, and the open reading frame (ORF) 14 and 15 exons were screened at the genomic DNA level.

Results. Haplotype analysis ruled out the implication in the disease of *RP2* in six families and of *RPGR* in four families. Among the 30 unrelated XLRP families, there 4 mutations were identified in *RP2* (13%), 3 of which are novel, and 16 mutations in *RPGR* (53.3%), 7 of which are novel.

Conclusions. In this cohort of XLRP families, as has happened in previous studies, RP3 also seems to be the most prevalent form of XLRP, and, based on the results, the authors propose a four-step protocol for molecular diagnosis of XLRP families. (*Invest Ophthalmol Vis Sci.* 2006;47:3777–3782) DOI: 10.1167/iovs.06-0323

Retinitis pigmentosa (RP; MIM 268000; Mendelian Inheritance in Man; National Center for Biotechnology Information, Bethesda, MD) reflects a heterogeneous group of inherited ocular diseases representing the most recurrent retinal dystrophy. This disease is clinically and genetically heterogeneous, with wide variation in severity, mode of inheritance, age of onset, progression, and phenotype. One hundred twelve causative genes have been identified for RP, and 49 additional genes have been mapped, but not yet identified (www.sph.uth.tmc.edu/RetNet/disease.htm; provided in the public domain by the University of Texas Houston Health Science Center, Houston, TX). The diagnosis criteria for RP were established by Marmor et al.[1] and are characterized by night blindness, progressive constriction of the visual field, and pigmentary retinopathy, that result from the gradual degeneration of photoreceptor cells, which begins in the periphery and progresses to the central region of the retina.

The X-linked form of RP accounts for approximately 12% of cases in Spain.[2] It is, on average, the most severe form because of its early onset and rapid progression, typically presenting in the first decade of life and progressing to partial or complete blindness by the beginning of the fourth decade. Five XLRP loci have been mapped: RP23,[3] RP6,[4] RP3,[5] RP2,[6] and RP24.[7] So far, only two XLRP genes have been cloned, *RP2* and *RPGR* (for the RP3 locus), which account for approximately 10% and 70% of XLRP families respectively.[8]

The *RP2* gene (RP2; MIM 312600) was localized by linkage mapping on Xp11.3-11.23,[9] and it was isolated by positional cloning in Xp11.3.[10] The five exons identified in this gene encode an ubiquitously expressed protein of 350 amino acids. The function of the RP2 protein is not completely known,[11–13] but it shows homology to human cofactor C (residues 42-192), which is involved in β-tubulin folding. Human RP2 has been shown to interact with the Arl3 protein, and the data suggest that both proteins work together in photoreceptors with a linkage function between the cytoskeleton and cell membrane as part of the cell signaling or vesicular transport machinery.[14]

Linkage analysis and deletion mapping have been used to localize the *RPGR* gene (RPGR; MIM 312610) in the interval between the *CYBB* and *OTC* genes in Xp21.1.[4] The genomic structure shows a 19-exon gene, spanning 60 kb of genomic DNA.[15,16] *RPGR* transcription studies in mouse and human tissues have revealed at least 12 different alternatively spliced isoforms, some of which are tissue specific and contain new exons.[17] In the photoreceptors, RPGR is concentrated in the connecting cilium linked to an RPGR interacting protein (RPGRIP).[16,18–20] It has been proposed that it plays a role in the maintenance of the polarized protein distribution across the connecting cilium by regulating directional transport. The RPGR isoform, which contains the new exon open reading frame (ORF) 15, is prominently expressed in the retina.[21] It contains exon 15 and part of intron 15, and it is mutated in most patients with XLRP.[22] No mutation has been identified in exons 16 to 19 of the *RPGR* gene in XLRP.

We screened 30 unrelated XLRP Spanish families to determine the molecular cause of the disease and to give them appropriate genetic counseling.

Patients and Methods

Ascertainment of Patients

We studied 30 unrelated XLRP Spanish families, including 70 affected male patients, 43 unaffected male family members, and 106 women at

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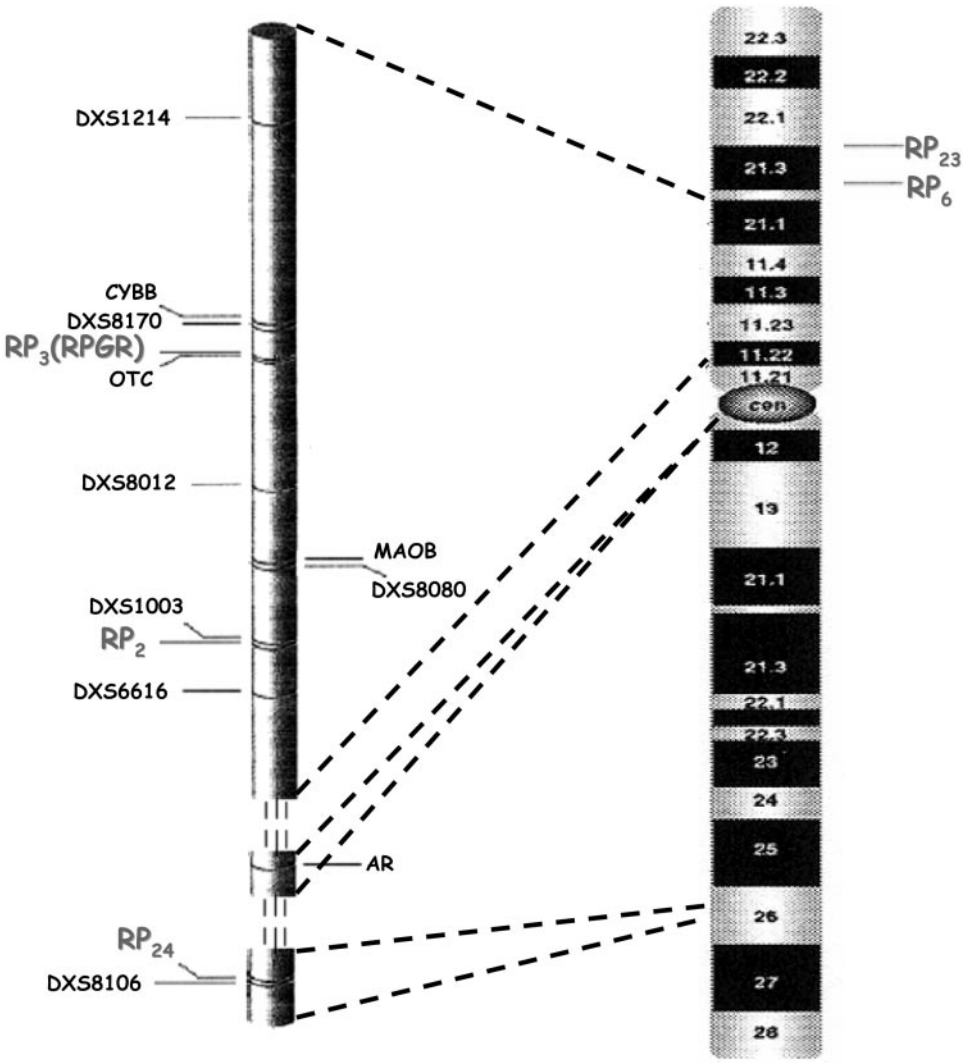

FIGURE 1. Localization on the X chromosome of the microsatellite markers used to perform the haplotype analysis in each XLRP Spanish family.

risk of being carriers. We also performed early molecular diagnosis in three boys at risk who were younger than 5 years.

A complete ophthalmic examination was performed, including medical and ophthalmic history, measurements of best corrected visual acuity and intraocular pressure, slit lamp and fundus examinations, and electroretinography (ERG).

The diagnosis of XLRP was made according to the RP criteria established by Marmor et al.,[1] provided that pedigree data were compatible with XL segregation.

Informed consent was obtained from patients participating in the study. The research protocols were approved by the hospital's bioethical committee and were in accordance with the Declaration of Helsinki.

DNA was extracted from 15 mL of peripheral blood samples by a standard salting out method. Total RNA was extracted from 2.5 mL of peripheral blood (PAXgene Kit; Qiagen, Valencia, CA) and reverse-transcribed to cDNA (ImPromt Kit; Promega, Madison, WI), using the Oligo-p$(dT)_{15}$ primer according to the manufacturer's instructions.

Haplotype Analysis

Haplotype analysis was performed in 26 XLRP families to assign the locus responsible for the disease in each family. In the remaining four families, haplotype analysis was not possible, because only the proband's sample was available.

Six dinucleotide repeat microsatellite markers (*DXS1214*, *CYBB*, *DXS8170*, *DXS8012*, *DXS1003*, and *DXS6616*) spanning the RP3–RP2 region, plus an additional microsatellite marker strongly linked to RP24 (*DXS8106*) were used (Fig. 1).

Each forward primer was fluorescently labeled, and two multiplex PCR assays, A and B, were performed in a total volume of 15 μL containing 120 ng of genomic DNA, 150 μM dNTPs (Invitrogen Corp., Carlsbad, CA), 1.26 to 37.5 picomoles of each primer, 1× polymerase buffer (500 mM Tris/HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, and 20 mM $MgCl_2$) and 1 U of *Taq* DNA polymerase (FastStart; Roche, Indianapolis, IN). After denaturation at 95°C for 5 minutes, PCR was performed (GeneAmp PCR System 2700; Applied Biosystems, Inc. [ABI]) for 10 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds; 15 cycles at 89°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds; with a final extension time of 30 minutes at 72°C. For the genotyping process, PCR products were electrophoresed and analyzed (Prism 3100 Genetic Analyzer; with the GeneMapper 3.5 software package; ABI). For uninformative results or to refine the recombination events, additional markers (*MAO-B*, *DXS8080*, and *AR*) were separately PCR amplified in a total volume of 15 μL containing genomic DNA, 125 μM dNTPs, 10 picomole of each primer, 1× *Taq* DNA polymerase buffer (500 mM Tris/HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, and 20 mM $MgCl_2$), and 0.6 U of *Taq* DNA polymerase (FastStart; Roche). PCR cycles were the same as those for the A and B PCR assays.

TABLE 1. Mutations Identified in the ORF15 Exon

Family	Mutation
XLRP-1	g.ORF15 + 651_652 del AG
XLRP-37	g.ORF15 + 651_652 del AG
XLRP-179	g.ORF15 + 1140 del G
XLRP-264	g.ORF15 + 651_652 del AG
XLRP-408	g.ORF15 + 481_482 del AG
XLRP-562	g.ORF15 + 1211_1212 del GG
XLRP-599	g.ORF15 + 481_482 del AG
XLRP-616	g.ORF15 + 566_567 del AG
XLRP-655	g.ORF15 + 855_857 del GAAG
XLRP-681	g.ORF15 + 720_721 del AG
XLRP-760	g.ORF15 + 481_482 del AG
XLRP-761	g.ORF15 + 481_482 del AG
XLRP-824	g.ORF15 + 1422_1423 del AG

According to GenBank accession no. AF86472.

Screening for Mutations

The *RPGR* gene (NM_000328) was screened in 30 families using the single-strand conformation polymorphism (SSCP) technique. We analyzed the ORF14 and -15 exons at the genomic DNA level, and we studied the first exons (1-15) of *RPGR* at the cDNA level. Fourteen primer sets for ORF14 and -15 and four primer sets for RPGR exons (1-15) were used (available on request).

The *RP2* gene (NM_006915) was analyzed in those families (14) where no mutation in RPGR was found. PCR-SSCP analysis of all 5 exons of the *RP2* gene was performed using previously described intragenic primers.[10] To detect mutations that cause alternative splicing we also analyzed *RP2* at the cDNA level with two specific PCRs (available on request).

PCR amplifications were performed in 50-μL reactions containing template (200 ng of genomic DNA or 2 μL of cDNA; 1× polymerase buffer (500 mM Tris/HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, and 20 mM $MgCl_2$); 200 μM each of dATP, dTTP, and dGTP and 20 μM of dCTP; 0.2 μCi α-^{32}P-dCTP; 20 picomoles of each primer; and 2.5 U of DNA polymerase (FastStart *Taq*; Roche). After denaturation at 95°C for 5 minutes, PCR was performed (GeneAmp PCR System2700; ABI) for 35 to 40 cycles at 94°C for 90 seconds, annealing for 90 seconds, and 72°C for 90 seconds with a final extension time of 30 minutes at 72°C. Amplification products were denatured for 5 minutes and loaded into different SSCP gels.

Each fragment displaying an abnormal pattern in SSCP was automatically sequenced and analyzed (Sequence Analysis program, Prism 3100 Genetic Analyzer; ABI), to identify the mutation.

RESULTS

Haplotype Analysis

Haplotype analysis enabled us to rule out the involvement in the disease of *RP2* in six families and of *RPGR* in four families. We found no informative meiosis for RP2/RP3 in most cases (16 families).

According to haplotype results, 74 of 106 women at risk were found to be carriers of the disease, 32 were noncarriers, and 3 were noninformative. We also confirmed the molecular diagnosis of three presymptomatic affected males.

Mutation Screening

In 20 (66.7%) of the 30 families with XLRP that were included in this study we characterized mutations that cosegregate with the disease. In these families, 43.33% of the mutations were localized in the ORF15 exon of *RPGR*, whereas no mutations were found in the ORF14 exon.

TABLE 2. Mutations Identified in the *RPGR* Gene

Family	Mutation
XLRP-762	c.482 del T*
XLRP-53	c.1117 A→T, (p.Lys373X)
XLRP-192	IVS 13-2 G→A*

According to GenBank accession no. NM_000328.
* Mutations described by Miano et al.[26]

In our study, all mutations detected in the *RPGR* gene were frameshift, nonsense, or splice-site, all of which have the potential of producing a severely altered protein.

Mutations in ORF15 have been shown to cosegregate with the disease in 13 families. We identified eight different frameshift mutations caused by 1-, 2-, or 4-bp deletions. Six of the mutations have not been previously reported (Table 1). Two further mutations, g.ORF15+481_482delAG and g.ORF15+651_652delAG, which in our population account for four and three unrelated Spanish XLRP families respectively, have already been observed in previous studies.[23-25]

Affected males of these families did not share a common haplotype, and so apparently there was not an ancestral origin. Thus the mutations appeared to be de novo in each family.

Analysis of RPGR exons 1 to 15 revealed three different mutations in 3 of the 30 unrelated Spanish XLRP families. In two previously reported Spanish families, XLRP-192 and XLRP-762,[26] the disease was found to be associated with a frameshift and a splice-site mutation, respectively. In the third family, we identified a nonsense mutation that cosegregates with the disease (Table 2).

In 14 families which had been excluded as having RPGR mutations, the study of the *RP2* gene led us to identify four mutations: two novel nonsense mutations (Glu18Stop and Gln134Stop), one novel missense mutation (Ser140Phe) and a previously reported[27] frameshift mutation (c.303 InsT) (Table 3). The Ser140Phe missense change was not detected in 100 control subjects and cosegregates with the disease in the affected family (XLRP-737).

Ophthalmologic Features of Patients with *RPGR* and *RP2* Mutations

Results are summarized in (Table 4). The male patients with mutations in *RP2* were characterized by early onset, and all had myopia and nystagmus, where all male patients with mutations in RPGR showed a later onset. The clinically affected men were 31 to 59 years old, and at those ages their visual acuity was <0.1 in both eyes, their visual fields were constricted to the central 5° on Goldmann perimetery, and they had characteristic findings of RP on fundus examination.

DISCUSSION

The X-linked form of RP accounts for only approximately 12% of RP cases in Spain.[2] However, the severe course of the

TABLE 3. Mutations Identified in the *RP2* Gene

Family	Mutation
XLRP-200	299 Ins T*
XLRP-677	c.419 C→T (p.Ser 140 Phe)
XLRP-720	c.52 G→T (p.Glu 18 Stop)
XLRP-737	c.400 C→T (p.Gln 134 Stop)

According to GenBank accession no. NM_006915.
* Mutations described by Miano et al.[27]

Table 4. Clinical Features of the Families with Characterized Mutations in *RPGR* or *RP2*

Family	Number Examined		Early Onset of Night Blindness in Affected Males (≤5 y)	Early Onset of Field Constriction in Affected Males (≤15 y)	Myopia in Affected Males	Subcapsular Cataracts in Affected Males
	Male	Female				
ORF15						
XLRP-1	1	2	Yes	Yes (5 y)	No	Yes
XLRP-37	1	3	No	No	Low*	Yes
XLRP-179	2	3	No	Yes (14 y)	High†	No
XLRP-264	1	3	No	No	Low	No
XLRP-408	1	—	Yes	Yes (5 y)	No	Yes
XLRP-562	1	1	No	Yes (11 y)	No	Yes
XLRP-599	5	4	No	No	Low	Yes
XLRP-616	2	1	No	No	High	No
XLRP-655	1	3	No	Yes (10 y)	No	No
XLRP-681	1	1	No	Yes (8 y)	High	Yes
XLRP-760	1	1	—	No	Low	Yes
XLRP-761	—	—	—	—	—	—
XLRP-824	3	—	No	No	High	No
RPGR						
XLRP-53	1	1	Yes	Yes (12 y)	Low	No
XLRP-192	1	—	Yes	Yes (15 y)	Low	No
XLRP-762	2	—	—	—	Low	No
RP2						
XLRP-200	2	2	Yes	Yes (6 y)	Low	Yes
XLRP-677	3	—	Yes	Yes (4 y)	High	Yes
XLRP-720	4	—	Yes	Yes (10 y)	No	Yes
XLRP-737	1	1	Yes	—	Low	Yes

* Less than 6 D.
† 6 D or more.

disease and the high recurrence risk within the XLRP families make the molecular characterization of the disorder in each family very relevant for genetic counseling.

Haplotype analysis does not appear to be a particularly informative method for distinguishing between the *RP3* (for *RPGR*) and *RP2* loci, because of the scarcity of recombination events between these two loci. However, the rapid and effective detection of female carriers and presymptomatic males make it a very useful tool as a first approach to genetic counseling in this form of RP.

Herein, we report 15 different mutations: 8 in ORF15 (responsible for the disease in 13 families), 3 in *RPGR* (present in 3 families) and 4 in *RP2* (present in 4 families).

Mutations in *RPGR* seem to be responsible for most of the cases of XLRP. The percentage of *RPGR*-related cases varies in different populations ranging from 36.32%[8] to 59.36%[28] (Table 5), and in the present study it was 53.33%. From this, 43.3% were found in the ORF15 and 10% in exons 1 to 15. Of interest, we detected a lower proportion of mutations in exons 1 to 15 of RPGR gene than have other researchers. Although we have not included in this series three mutations previously reported[26] in other XLRP Spanish families, it is very tempting to speculate about the possibility of a different distribution of XLRP mutations in our families. In fact, we detected 13% with mutations in *RP2*, almost double the percentages detected in other studies (Table 5).

All the identified mutations in both the *RPGR* and *RP2* genes in our Spanish XLRP cases, except two mutations located in the ORF15 exon (g.ORF15+481_482delAG and g.ORF15+651_652delAG) have not been described in any other XLRP family.

Finally, in more than one third of the families, the mutation responsible for the disease remained unidentified. At least two possibilities can be put forward to explain why no mutation was detected. In these families, the disease may be associated with either mutations in other studied regions of the genes that

Table 5. Mutation Detection Rates in the *RPGR* and *RP2* Genes in Previous Reports and in Our Cohort of XLRP Families

Population	*RP2* Mutations	*RPGR* Mutations			Without Mutation
		ORF15	Exon 1–15	Total	
Sweden:	1/17	4/17	4/17	8/17	9/17
Andreasson et al.[24]	(5.88%)	(23.58%)	(23.58%)	(47%)	(52.94%)
North America	15/234	51/234	34/234	85/234	100/234
Breuer et al.[8]	(6.41%)	(21.79%)	(14.53%)	(36.32%)	(42.73%)
North America	11/187	80/187	31/187	111/187	65/187
Sharon et al.[28]	(5.88%)	(42.78%)	(16.57%)	(59.36%)	(34.76%)
Germany	3/58	24/58	10/58	34/58	21/58
Bader et al.[29]	(5.17%)	(41.37%)	(17.24%)	(59%)	(36.2%)
Spain	4/30	13/30	3/30	16/30	10/30
Present study	(13.33%)	(43.33%)	(10%)	(53.33%)	(33.33%)

Data are the number cases/total group (percentage of total).

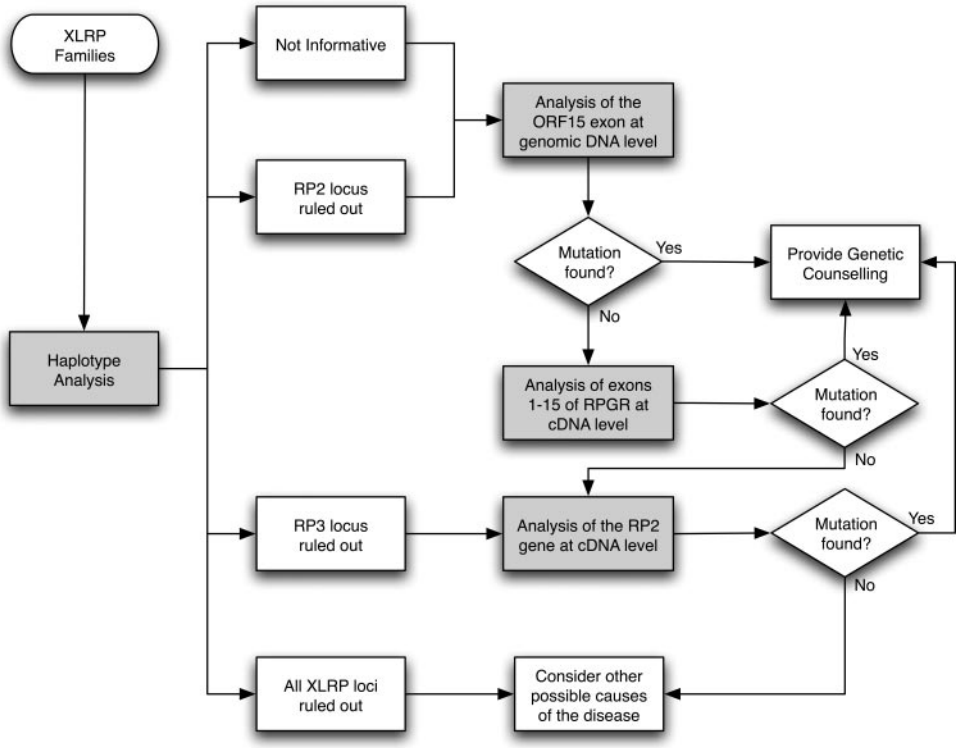

FIGURE 2. Proposed protocol for molecular diagnosis of XLRP families.

we did not study (possible additional undiscovered exons or noncoding regions) or other XLRP loci localized in the X chromosome.

As has been mentioned, more than 43.3% of the mutations in our series were found in ORF15. This high number of mutations may indicate that it is a mutation hot spot.[29] The ORF15 contains a repetitive domain.[30] It has also been suggested that sequences rich in pyrimidines, such as the one present in ORF15, may adopt unusual non B-DNA conformations which are associated with reduced fidelity of replication.[31]

All the previously reported ORF15 mutations are frame-shift mutations comprising mostly small out-of-frame deletion mutations, although it has been suggested that ORF15 can accommodate insertions and in-frame deletions and still be nonpathogenic.[30] No missense mutation in this exon has been identified so far.

Two mutations, g.ORF15+481_482delAG and g.ORF15+651_652delAG, were detected in 4 and 3 unrelated Spanish XLRP families, respectively, and they were published in 10 and 13 XLRP families.[23–25]

Moreover, by haplotype analysis of the Spanish series, these mutations appear to have arisen apparently de novo in each family. Therefore, based on previous reports and our results, we conclude that there are two mutation hot spots within this exon, corresponding to the sites of these mutations.

Three of the four identified mutations in *RP2* truncate the protein, and the remaining mutation (Ser140Phe) is localized in the cofactor-C homologous domain of the protein and presumably affects its function.

Several studies have compared the ophthalmic features of patients with *RPGR* versus those with *RP2* mutations. No clear phenotypic differences were found between the two subtypes of XLRP in two different studies (Bailey CC, et al. *IOVS* 2001; 42;ARVO Abstract 417).[32] In contrast, Kaplan et al.[33] observed that patients with mutations in *RP2* are characterized by early onset of the disease and more severe myopia, while patients with *RPGR* mutations are associated with a later onset, beginning with night blindness. However, other investigators[34] have found that patients with *RPGR* mutations usually have smaller visual fields and more severely reduced full field ERG amplitudes. Although we did not perform an extensive genotype–phenotype correlation in our cohort of XLRP families, it seems that male patients with mutations in *RP2* were characterized by early onset (4–5 years) and myopia and nystagmus, whereas male patients with mutations in *RPGR* showed a later onset (8–9 years) of the disease. This type of severe clinical features is comparable to that seen in previously reported XLRP families.[28,32]

Genetic studies of families with retinal dystrophies are important in determining the status of female carriers and affected males. Carrier detection and prenatal diagnosis are crucial for the prevention of this type of progressive and untreatable disease. Moreover, the knowledge of the molecular mechanism of these diseases will allow the development of new rational potential therapeutic tools such as gene therapy.

Based on our results, we propose a protocol for the molecular diagnosis of XLRP families in four consecutive steps (Fig. 2). After haplotype analysis and in case it is not informative, we propose to analyze the ORF15 exon of *RPGR*. Then, the first 15 exons of the RPGR gene should be studied, and finally the 5 exons of the *RP2* gene (by using mRNA in both cases). All mutations detected at the cDNA level must be additionally characterized at the genomic DNA level.

We consider that this four-step approach, combining the analysis of RNA and DNA is the most effective method (rapid and accurate) for mutation screening in XLRP cases.

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