Genetics

Mutations in *RPGR* and *RP2* Account for 15% of Males with Simplex Retinal Degenerative Disease

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Purpose. To determine the proportion of male patients presenting simplex retinal degenerative disease (RD: retinitis pigmentosa [RP] or cone/cone-rod dystrophy [COD/CORD]) with mutations in the X-linked retinal degeneration genes *RPGR* and *RP2*.

Methods. Simplex males were defined as patients with no known affected family members. Patients were excluded if they had a family history of parental consanguinity. Blood samples from a total of 214 simplex males with a diagnosis of retinal degeneration were collected for genetic analysis. The patients were screened for mutations in *RPGR* and *RP2* by direct sequencing of PCR-amplified genomic DNA.

Results. We identified pathogenic mutations in 32 of the 214 patients screened (15%). Of the 29 patients with a diagnosis of COD/CORD, four mutations were identified in the ORF15 mutational hotspot of the *RPGR* gene. Of the 185 RP patients, three patients had mutations in *RP2* and 25 had *RPGR* mutations (including 12 in the ORF15 region).

Conclusions. This study represents mutation screening of *RPGR* and *RP2* in the largest cohort, to date, of simplex males affected with RP or COD/CORD. Our results demonstrate a substantial contribution of *RPGR* mutations to retinal degenerations, and in particular, to simplex RP. Based on our findings, we suggest that *RPGR* should be considered as a first tier gene for screening isolated males with retinal degeneration. (*Invest Ophthalmol Vis Sci.* 2012;53:8232–8237) DOI: 10.1167/iovs.12-11025

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Retinitis pigmentosa (RP) is the clinical diagnosis for a large group of inherited retinal disorders, characterized by progressive photoreceptor degenerative disease leading to vision loss.[1] RP affects approximately 1 in 4000 individuals in the United States and other developed countries.[1–3] Autosomal dominant, autosomal recessive, and X-linked forms of RP exist, and more than 60 genes have been identified as the cause of nonsyndromic disease (https://sph.uth.tmc.edu/retnet/disease.htm). Clinical and population-based studies have shown that a substantial portion of RP patients are isolates or "simplex,"[1,3–6] that is, patients with no family history of disease. These simplex cases pose a dilemma for clinicians who are ordering genetic testing for diagnosis and inheritance counseling or for clinical management of patients and respective families. Prior to the era of molecular genetic testing, isolated cases were considered by default to be of autosomal recessive inheritance, and the families were counseled that there was a negligible recurrence risk for future generations to be affected. However, such cases might represent de novo autosomal dominant or X-linked disease. Furthermore, as gene-based therapies are being translated to treatment, determining the genetic basis of a patient's clinical phenotype is expected to become an important standard of care for disease management.[7]

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X-linked RP (XLRP) is estimated to comprise 6% to 20% of all RP.[3,5,6,8,9] XLRP males typically show a rapid course of vision loss, with a significant proportion progressing to legal blindness by 40 years of age. Affected males usually present with night blindness and decreased or nonrecordable ERG responses in the first or second decade. Some XLRP patients exhibit high myopia and decreased visual acuity even at an early age.[10] Heterozygous carrier females also manifest a range of phenotypes, varying from asymptomatic to mild fundus changes and pigment migration to some females showing a severe phenotype.[8,11] Although the presence of a tapetal-like reflex has been described as indicative of carrier status, this is not universally detected.[8]

Two principal genes, *RP2* and *RPGR*, have been identified as the primary causative genes in XLRP. One additional gene, *OFD1*, has been identified recently to have changes associated with XLRP in a single family.[12] Two additional chromosomal loci, *RP24*[13] and *RP6*,[14] have also been associated with XLRP. *RP2* is mutated in 7% to 18% of XLRP,[15–18] whereas *RPGR* mutations are observed in 56% to 90% of patients with X-linked disease.[14,19–22] Though a substantial fraction of *RPGR* mutations are detected in the RCC1 homology domain, the fraction of XLRP patients carrying identifiable mutations was lower than expected before the discovery of the mutation hotspot region ORF15. This 15-exon transcript is highly expressed in the retina and is mutated in 30% to 63% of males with XLRP.[15,23–30] Mutations in *RPGR* are associated with XLRP, as well as with X-linked cone-dystrophy (COD),[29,31] cone rod dystrophy (CORD),[32,33] and an atrophic form of macular degeneration.[34] Patients with COD or CORD may present with decreased visual acuity, central vision loss, and decreased color vision. ERG responses are abnormal; the photopic system is more severely affected than the scotopic ERG responses.[35] Patients with *RPGR* mutations have also been reported to have nonocular phenotypes, such as hearing loss, recurrent respiratory infections, and primary ciliary dyskensis.[30,36,37] Simplex patients comprise a substantial proportion, ranging from 41% to 63%, of all RP patients.[1,4,5,38,39] In two of the reported cohorts, males comprised 51% to 59% of the simplex patients.[3,4] Previous mutation screening studies included 5 to 55 simplex males and identified XLRP mutation rates of 0% to 32%.[15,26,28,40,41] However, there was much variation in the ascertainment for these samples, and some of the cohorts included only those with presumed X-linked mutations based on clinical presentation of the patients. We examined a large cohort of simplex males for mutations in the X-linked retinal dystrophy (RD) genes *RP2* and *RPGR*. Our genetic analyses argue in favor of including *RPGR* as a candidate gene for initial mutation screening of male patients with isolated RP.

Methods

Subjects

A cohort of simplex males diagnosed with nonsyndromic RP ($N = 185$) or COD/CORD ($N = 29$) was collected for mutation analysis. In a previous study, we included 55 simplex males in our screening.[15] Individuals from this initial screening were also included in this current study if their pedigrees still met the study parameters (as defined below). Two hundred nine subjects were diagnosed by clinical examination, visual fields, and electroretinography. Subjects presented with a range of clinical presentations from early onset to later ages of onset of RP/COD/CORD. They were enrolled from ophthalmology clinics primarily in the United States, and a few were collected in Europe (Italy and Sweden). Five patients were referred to the study from a registry with self-reported inherited retinal disease. Informed consent was obtained from all subjects. Institutional Review Board of the University of Michigan approved the study protocol, and the research adhered to the tenets of the Declaration of Helsinki.

Pedigree Analysis

Pedigree information was gathered and analyzed for all patients. Subjects with a history of consanguinity or those whose family history was unknown (i.e., adopted) were excluded from the analysis. Simplex males were defined as subjects who had no family members affected with RP, COD, or CORD. Pedigrees were grouped into two different categories. In Category A, there were no additional reports of any female family members with any symptoms consistent with RP, COD, or CORD. In Category B, one or more females in the family reported having some symptoms, such as night blindness or color vision difficulties, which could be consistent with a carrier state for XLRP. However, none of these females was diagnosed as having RP, COD, or CORD.

Mutational Analysis

DNA was extracted from lymphocytes with Qiagen whole blood kits (Qiagen, Valencia, CA). Methods for *RP2* mutational analysis were reported previously.[10] For *RPGR* mutation screening, the primer sequences were reported previously for the analysis of exons 1 through 19[25] and ORF15.[32] Accuprime high fidelity Taq polymerase (Invitrogen, Grand Island, NY) was used to amplify various *RPGR* exons. PCR setup conditions were 100 ng of DNA per reaction, 2.5 μL of 10× Accuprime HF buffer, 0.5 μL of 10 μmol/L of each the forward and reverse primers, 0.1 μL of Accuprime HF Taq polymerase (5 U/μL) and water to 25-μL reaction volume. PCR conditions for all *RPGR* exons except *RPGR*-ORF15 were 94°C for 2 minutes followed by 10 cycles at 92°C for 20 seconds, 56°C for 30 seconds, and 68°C for 30 seconds; followed by 25 cycles at 92°C for 20 seconds, 60°C for 30 seconds, and 68°C for 30 seconds; followed by 10 minutes of extension at 68°C and hold at 4°C. *RPGR*-ORF15 exon was amplified by two sets of primers. Exon 15_1F primers and 4R amplified ~ 2 kb fragments, while the purine rich region was amplified with 3F/3R primers (see https://sph.uth.tmc.edu/retnet/disease.htm for the primers location and sequences) to amplify ~ 1 kb fragments. The PCR conditions were similar to other exons except that following annealing at 56°C and 60°C, extensions at 68°C were for 1 and 2.5 minutes for 3F/3R and 1F/4R fragments, respectively. Aliquots of the amplified PCR products were analyzed using 1% agarose gels, viewed using UVP bioimaging system (UVP, Upland, CA), and submitted to the University of Michigan Medical School Sequencing Core.

Mutational Data Analysis

Sequences were analyzed by two independent investigators with Sequencher (Gene Code Corporation, Ann Arbor, MI). All identified mutations were validated by another independent PCR analysis of each sample. Once a mutation was identified, additional screening was not performed. For *RP2* and *RPGR* exons 1 through 19, missense, nonsense, and splice site mutations as well as deletions were considered causative if they had been reported previously as mutations, if they were not published previously as polymorphisms, or if they were not detected in 96 male controls. Missense mutations were also analyzed for pathogenicity by the Polyphen and SIFT Programs. In ORF15, only nonsense and frame-shift mutations were considered disease causing.

Results

Among the 185 subjects with RP, mutations were identified in 28 patients; of these, analyses of samples from three patients revealed mutations in *RP2*, 13 in *RPGR* exons 1 through 14, and 12 in the *RPGR* ORF15 region (Table 1). Of the 29 subjects with COD/CORD, four mutations were identified in ORF15. As

Table 1. Simplex Males with *RPGR* or *RP2* Mutations

	Total Screened	*RP2*	*RPGR* 1–15	*RPGR* ORF15	Total with Mutations	*P* Value
COD/CORD						
Cat A	26	0	0	3	3 (11.5%)	4 (13.8%)
Cat B	3	0	0	1	1 (33%)	
RP						
Cat A	151	1	8	7	16 (10.6%)	28 (15.1%)
Cat B	34	2	5	5	12 (35.2%)	
	214	3	13	16	32 (15%)	

reported in other studies of patients with COD/CORD,[26,31–33] these mutations were located towards the 3′ end of ORF15. No *RP2* mutations were detected in subjects with COD/CORD. Mutations were identified in 10.7% (19/177) of Category A patients and 35.1% (13/37) of Category B patients. We found that 15.1% of simplex RP males and 13.8% of simplex COD/CORD males had mutations in the two XLRP genes (25/28 being in *RPGR*).

Of the three *RP2* mutations, two were small deletions (one being novel) and the third was a missense change that was predicted to be damaging by both Polyphen and SIFT (Table 2). An additional change (p.Thr87Ile) was considered originally to be a disease-causing mutation[10] because it was not detected in 96 male controls and has not been identified as a variant on the Exome Variant Server. However, analysis with pathogenicity programs Polyphen and SIFT consider the change to be a tolerated or benign variant, and we are now considering this change to be a variant of unknown significance. *RPGR* mutations included three missense, four splice site, three deletions, and two nonsense mutations; six of the identified mutations were novel (Fig.). In ORF15, eight frame-shift mutations and five nonsense mutations were identified; nine of these have not been reported previously. Each mutation was detected in one patient each, with the exception of c. 2236_2237delGA which was identified in four patients and c.154G>T which was present in two patients.

Discussion

In our initial XLRP screening cohort,[15] we analyzed 234 families including 55 subjects who were the only affected males in their family with RP yet believed to be X-linked because of the clinical presentation of an early onset of severe disease. Of these, 16 subjects had mutations in *RPGR* or *RP2*, with an overall mutation rate of 29%; 5% of the subjects carried mutations in *RP2*, 9% in *RPGR* exons 1 through 14, and 15% in

Table 2. Nature of Mutations Identified in the *RP2* (AJ007590) and *RPGR* (NM_001034853.1) Genes

Diagnosis	Exon	Nucleotide	Protein	Reference
RP2 mutations				
RP	1	c. 8 G>C	p. Cys3Ser	Jayasundera et al.[10]
RP	2	c. 409_411 del ATT	p. Ile37del	Breuer et al.[15]
RP	2	c. 803 del A	p. Lys268fs	Novel
RPGR mutations				
RP	1	c. 28+2 T>G	Splice	Novel
RP	2	c. 154 G>T	p. Gly52X	Meindl et al.[25]
RP	2	c. 155-2 A>G	Splice	Miano et al.[48]
RP	3	c. 173delT; 177delT	p. Met58fs	Novel
RP	5	c. 460G>C	p. Ala154Pro	Novel
RP	5	c. 469+2 T>A	Splice	Novel
RP	8	c. 869 del A	p. Glu290fs	Buraczynska et al.[49]
RP	8	c. 934 +1 G>A	splice	Miano et al.[48]
RP	10	c. 1216_1217 del CT	p. Leu406fs	Bader et al.[23]
RP	11	c. 1345 C>T	p. Arg449X	Breuer et al.[15]
RP	11	c. 1348 T>C	p. Cys450Arg	Novel
RP	14	c. 1699 A>T	p. Ile567Phe	Novel
RP	ORF15	c. 2088 del A	p. Leu696fs	Novel
RP	ORF15	c. 2227 G>T	p. Gly743X	Novel
RP	ORF15	c. 2234_2237 del GAGA	p. Arg745fs	Breuer et al.[15]
RP	ORF15	c. 2236_2237 del GA	p. Glu746fs	Vervoort et al.[29]
RP	ORF15	c. 2296_2299 del GGAG	p. Gly766fs	Novel
RP	ORF15	c. 2384del A	p. Glu795fs	Breuer et al.[15]
CORD	ORF15	c. 2442_2445 del AGAG	p. Val814fs	Vervoort et al.[29]
RP	ORF15	c. 2509 G>T	p. Glu837X	Novel
RP	ORF15	c. 2614 G>T	p. Glu872X	Novel
RP	ORF15	c. 2703delA	p. Glu903fs	Novel
COD	ORF15	c. 2893 G>T	p. Glu965X	Novel
CORD	ORF15	c. 2965 G>T	p. Glu989X	Novel
COD	ORF15	c. 3308_3309 del AT	p. Tyr1103fs	Novel

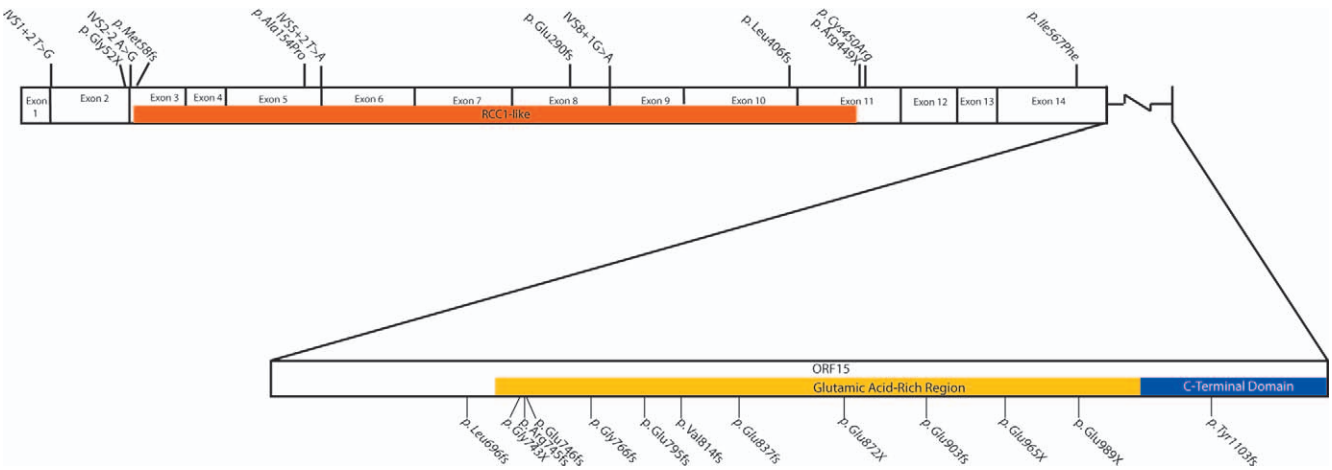

Figure. *RPGR* mutations identified in this report. Novel mutations are *italicized*.

ORF15. A subsequent study screened 187 male patients for *RP2* and *RPGR*; of these, 30 simplex patients suspected of having XLRP (based on visual acuity and myopia) were screened and 7% showed mutations in *RP2*, 3% in *RPGR* exons 1 through 14, and 3% in ORF15.[28] A more recent study of 127 French families included 25 isolated males suspected of XLRP based on early onset, rapid progression, and subnormal visual acuity.[26] In this group, 4% of the patients had mutations in *RP2*, 4% in *RPGR* exons 1 through 14, and 24% in *RPGR* ORF15. Another study of 37 families with RP, including five patients with no family history, found no X-linked mutations among the simplex subjects.[40] Finally, 141 families with possible X-linked inheritance and 39 simplex males were screened for mutations in *RP2* and *RPGR*, but no mutations were found in the simplex males.[41] In summary, these previous studies vary in their detection rates from 0% to 32%. The studies with the higher detection rates specifically selected for simplex males with suspected X-linked inheritance based on severity or clinical features of disease.[15,26,28] The two studies with no reported mutations in X-linked genes did not select the patients based on the phenotype.[40,41] These studies were also relatively small and varied in the countries of origin for their patient ascertainment. All these factors could contribute to observed variations in detection rates.

The current study in 214 simplex RD males reports the largest screening to date of X-linked mutations. The phenotype of our population varied and included patients with both early and late onset disease. In our cohort, we identified mutations in 15% of simplex RP and COD/CORD patients. In the subcategory of families with one or more female family members reporting history of decreased vision/night vision problems, our mutation detection rate was as high as 35%. We would therefore like to emphasize the importance of taking a targeted family history when making decisions about genetic testing and care.

An isolated case of retinal degeneration may be caused by an autosomal recessive, autosomal dominant (de novo and/or a family with reduced penetrance), or X-linked gene mutation. When clinicians are presented with an isolated male, clinical information may in some cases provide clues to the inheritance pattern. In others, examination of at-risk female(s) may identify a distinctive carrier phenotype in an X-linked disorder. However, in many instances, clinical data are not useful to determine the genetic basis of the disease. Therefore, the identification of the genetic defect should be an integral part of clinical management for retinal degeneration patients, in order to provide genetic counseling for recurrence risk to parents, offspring, and siblings. Moreover, with the success of gene-based treatment(s) for human *RPE65* disease[42,43] and with new possibilities for many retinal and macular diseases, including those caused by *RPGR* mutations,[7] the knowledge of the underlying gene defect will be valuable to determine which trials a patient might be eligible for in the future. The relatively high X-linked gene mutation frequency identified in this study strongly argues for characterizing the underlying cause of RP in simplex male patients.

Our study has important implications for the likely prevalence of X-linked mutations among simplex RD males. To determine the genetic cause in an isolated case, a retina clinician or geneticist is often presented with difficulty in formulating the appropriate plan of action for ordering genetic tests. Likely candidate genes for mutation screening of isolated nonsyndromic RP patients are 34 genes that have been identified for autosomal recessive RP (http://www.sph.uth.tmc.edu/Retnet/). Notably, *USH2A* gene mutations have been identified as being responsible for 7% to 23% of nonsyndromic ARRP[44–46] and, therefore, comprise one of the major causes of isolated RP. If we take into account that 50% to 60% of all RP are simplex cases, *USH2A* would become probably the most common cause of RP in the United States.[44] We should also emphasize the importance of ethnicity in guiding the genetic testing of simplex RP patients, as demonstrated by the presence of founder mutations in Ashkenazi Jewish patients.[47] The identification of mutations in 15% of the simplex male RD patients, reported here, would make *RPGR* a major cause of RP and CORD. Therefore, we suggest that *RPGR* should be included as a first tier gene in the screening strategy for simplex males with retinal degenerative disease.

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