Mutations in the X-Linked Retinitis Pigmentosa Genes *RPGR* and *RP2* Found in 8.5% of Families with a Provisional Diagnosis of Autosomal Dominant Retinitis Pigmentosa

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Purpose. We determined the fraction of families in a well-characterized cohort with a provisional diagnosis of autosomal dominant retinitis pigmentosa (adRP) that have disease-causing mutations in the X-linked retinitis pigmentosa GTPase regulator (*RPGR*) gene or the retinitis pigmentosa 2 (*RP2*) gene.

Methods. Families with a provisional clinical diagnosis of adRP, and a pedigree consistent with adRP but no male-to-male transmission were selected from a cohort of 258 families, and tested for mutations in the *RPGR* and *RP2* genes with di-deoxy sequencing. To facilitate testing of *RPGR* in "adRP" families that had no male members available for testing, the repetitive and purine-rich ORF15 of *RPGR* was subcloned and sequenced in heterozygous female subjects from 16 unrelated families.

Results. Direct sequencing of *RPGR* and *RP2* allowed for identification of a disease-causing mutation in 21 families. Of these "adRP" families 19 had *RPGR* mutations, and two had *RP2* mutations. Subcloning and sequencing of ORF15 of *RPGR* in female subjects identified one additional *RPGR* mutation. Of the 22 mutations identified, 15 have been reported previously.

Conclusions. These data show that 8.5% (22 in 258) of families thought to have adRP truly have X-linked retinitis pigmentosa (XLRP). These results have substantive implications for calculation of recurrence risk, genetic counseling, and potential treatment options, and illustrate the importance of screening families with a provisional diagnosis of autosomal inheritance and no male-to-male transmission for mutations in X-linked genes. Mutations in *RPGR* are one of the most common causes of all forms of retinitis pigmentosa. (*Invest Ophthalmol Vis Sci.* 2013;54:1411–1416) DOI:10.1167/iovs.12-11541

Retinitis pigmentosa (RP) is an inherited retinal degeneration that affects approximately one in 3,500 individuals, with an estimated total of 1.5 million patients worldwide.[1] RP is caused by progressive loss of rod and cone photoreceptors. Patients experience night blindness followed by loss of visual fields, usually culminating in legal and often complete blindness. Clinical hallmarks of RP include bone spicule deposits, attenuated retinal blood vessels, optic disc pallor, visual field loss, and abnormal, diminished or nonrecordable electroretinographic responses (ERG).[2,3]

RP can be inherited in an autosomal dominant (adRP), autosomal recessive (arRP), or X-linked (XLRP) manner, with rare digenic and mitochondrial forms.[4,5] RP also is associated with several syndromic disorders, such as Bardet-Biedl and Usher syndrome.[3,5] To date, mutations in 23 genes are known to cause adRP, mutations in 36 genes cause arRP, and mutations in 3 genes cause XLRP.[6] These genes encode proteins involved in various retinal functions, including phototransduction, photoreceptor outer-segment structure, and pre-mRNA splicing.[7,8] Despite the large number of RP genes, mutations cannot be identified in 30 to 35% of patients with adRP.[9]

One possible cause of disease in these remaining adRP patients is that some "adRP" families without mutations in known adRP genes may, in fact, have XLRP. Although XLRP is thought to affect male subjects only, many documented cases of *RPGR* and *RP2* mutations cause disease in carrier female subjects, thus giving an impression of occurrence in sequential generations simulating Mendelian dominant transmission.[10–15] Historically, *RPGR* carrier female subjects were thought to have much milder (if any) symptoms compared to male subjects with comparable *RPGR* mutations.[12,15] More recently, it has been reported that carrier female subjects exhibit a range of phenotypes that can vary from asymptomatic to severe retinal disease similar to male subjects.[10,12,13] The presence of "affected" or, at least, partially manifesting female subjects with an absence of male-to-male transmission in a pedigree may lead to misinterpretation. Also, some adRP genes, such as *PRPF31* and *TOPORS*, may exhibit incomplete penetrance, which can complicate further the distinction between adRP and XLRP pedigrees.[6,8]

X-linked forms of RP account for 10 to 20% of all RP families.[16] Mutations in the *RPGR* gene are the most common causes of XLRP, accounting for over 70% of XLRP.[10,14,16] *RPGR*

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mutations also may account for more than 15% of simplex (isolated) male subjects with RP.[10,17] *RPGR* localizes to the connecting cilium of photoreceptors and is believed to have a role in protein transport.[18,19] Approximately 60% of disease-causing mutations in *RPGR* are found in ORF15.[10,14] ORF15's sequence is highly repetitive and purine-rich.[14] This type of sequence often promotes polymerase arrest and slipped strand mispairing, thus making ORF15 a hotspot for mutations.[14,16] ORF15 is a component of alternate splice variants of *RPGR* that are expressed predominantly in the retina, as is exon 9a.[14,20]

Mutations in the *RP2* gene account for approximately 15% of XLRP cases.[10,16] The *RP2* gene is believed to have a role in trafficking proteins to the plasma membrane and maintaining Golgi cohesion.[21] Unlike *RPGR*, disease-causing mutations in *RP2* are found spread more uniformly throughout the gene.

The goal of our study was to identify the fraction of families with a provisional diagnosis of adRP that have disease-causing mutations in the X-linked genes *RPGR* and *RP2*. Families without male-to-male transmission were selected from a well-characterized adRP cohort, and screened for mutations in *RPGR* and *RP2* with di-deoxy sequencing. The highly repetitive and purine-rich nature of the ORF15 exon of *RPGR* complicates examination of the ORF15 sequence, which is compounded further by the presence of many benign polymorphic insertions or deletions (indels).[14,16] The indels make dideoxy sequencing difficult due to overlapping frame shifted reads. To facilitate the testing of *RPGR*, ORF15 was subcloned and sequenced in families that had no male members available for testing and whose female members were heterozygous for polymorphic indels in ORF15.

Methods

Samples

To identify the fraction of families with a provisional diagnosis of adRP that have disease-causing mutations in X-linked genes, a well-characterized adRP cohort was screened for mutations in *RPGR* and *RP2*. This cohort has been described in detail previously.[9,22–28] Families in the cohort, currently 258 total, have a high likelihood of having adRP based on pedigree analysis. Pedigrees show either the presence of three or more generations, with male and female subjects among all affected family members, or two affected generations with male-to-male transmission. These requirements reduce but do not fully eliminate the likelihood of including X-linked families. Families without previously identified mutations were selected for testing in this study. DNA was extracted from whole blood or saliva samples with commercial kits as described previously.[22,29,30]

The study was performed in accordance with the tenets of the Declaration of Helsinki, and informed consent was obtained from each individual tested or from parents or guardians for individuals under age 18. This study was approved by the Committee for the Protection of Human Subjects of the University of Texas Health Science Center at Houston and by the respective human subjects' review boards at each of the participating institutions.

Sequence Analysis

A minimum of one affected individual from each family without male-to-male transmission was selected and screened for mutations in *RPGR* and *RP2* (Fig. 1). Male subjects were tested preferentially whenever available. Genomic DNA was amplified in a 12.5 μL reaction volume for 35 cycles with AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA) and M13-tailed primers designed to flank exons 1 to 19, and 9a of *RPGR*, and exons 1 to 5 of *RP2*. (Primer sequences are available upon request.) *RPGR*-ORF15 was amplified for 40 cycles with HotStar HiFidelity DNA Polymerase (Qiagen, Valencia, CA) and the following primers: 5′-GACTAAACCCATAATATCCAAATCCA-3′ and 5′-GCCAAAATTTACCAGTGCCTCCTAT-3′. PCR product was treated with ExoSAP-IT (Affymetrix, Santa Clara, CA) and sequenced using BigDye v1.1 (Applied Biosystems). Exons 1 to 19 and 9a of *RPGR*, and exons 1 to 5 of *RP2* were sequenced bidirectionally with M13 primers, and ORF15 was sequenced unidirectionally with a set of seven nested primers (sequences available upon request). Sequence reactions were purified with BigDye Xterminator purification kit, run on a 3500 Genetic Analyzer, and analyzed by SeqScape v2.7 or Sequencing Analysis (Applied Biosystems).

Subcloning

Three hundred nanograms of genomic DNA were amplified in each of two 50 μL reactions for 38 cycles with HotStar HiFidelity DNA Polymerase (Qiagen) and the following primers: 5′-GCGATGCGGCCGC GACTAAACCCATAATATCCAAATCCA-3′ 5′-GCTGTCGACGCCAAAATT TACCAGTGCCTCCTAT-3′. PCR products were purified with the QIAquick PCR purification kit (Qiagen) and the manufacturer's protocol. The purified PCR products and an exon trap cloning vector pET01 (Boca Scientific, Boca Raton, FL) were digested with SalI and NotI restriction enzymes (New England BioLabs, Ipswich, MA) and recommended protocols. Restriction digests were purified with the QIAquick Gel Extraction Kit (Qiagen) and the manufacturer's protocol.

Digested PCR products and vectors were ligated with Quick T4 DNA Ligase (New England BioLabs) and transformed into DH5α competent cells (Applied Biosystems) by the manufacturers' protocols. Ten colonies from each transformation reaction were inoculated into 4 mL of LB broth and grown overnight at 37°C. After inoculation, the colonies were pelleted, and plasmid DNA was isolated by the QIAprep Spin Miniprep Kit (Qiagen) and the manufacturer's protocol. *RPGR*-ORF15 was sequenced from each clone as described above. A minimum of eight clones were sequenced per sample. After analysis of the sequence data, each set of clones was divided into two groups based on which allele of *RPGR*-ORF15 had been inserted into the clone. Additionally, a minimum of two clones per allele were identified to insure each allele was identified accurately.

Results

Individuals Tested

The samples selected for this project included a subset of family members from our previously described adRP cohort. Families in this cohort, based on pedigree analysis, had a high likelihood of having adRP. A minimum of one individual from most families was screened previously for mutations in the known adRP genes, including complete coding regions of CA4, CRX, FSCN2, IMPDH1, NRL, PRPF31, RDS, RHO, ROM1, RP9, and TOPORS, and mutation hot spots of RP1, PRPF3, PRPF8, NR2E3, *KLHL7*, and SNRNP200.[22–26] These analyses identified likely disease-causing adRP mutations in 163 of the 258 families (63.7%), which were excluded from this study.[22–26]

Of the remaining 95 families in the cohort, 56 were selected for analysis of *RPGR* and *RP2* because they had a provisional diagnosis of adRP and a multigenerational pedigree consistent with adRP but lacked male-to-male transmission. Sequencing of *RPGR* included exons 1 to 19, 9a, and ORF15. Sequencing of *RP2* included exons 1 to 5. Sequencing of both genes included all known coding exons and 15 base-pairs of intronic sequence at intron–exon junctions.

RPGR

A proband from each of the 56 families without male-to-male transmission was tested for mutations in *RPGR* by di-deoxy sequencing. Whenever possible, a male subject was selected for analysis to avoid issues with the polymorphic indels seen routinely in ORF15 of *RPGR*.[14,16] Sequence analysis revealed

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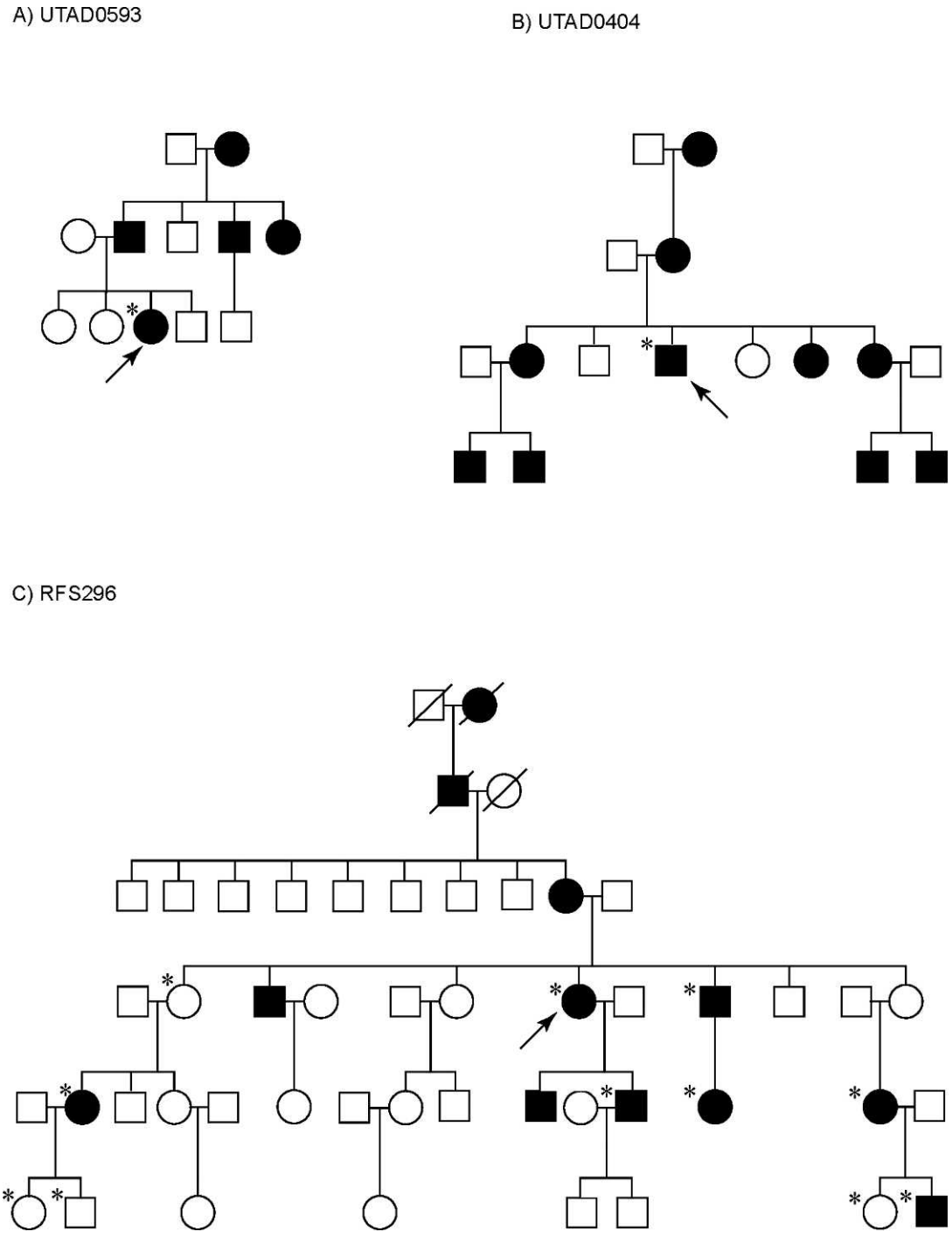

Figure 1. Three pedigrees used to illustrate the inclusion of families in our study that had a family history consistent with an autosomal dominant mode of inheritance and no male-to-male transmission. * Indicates patients who were investigated genetically. (**A**) UTAD0593: A p.Glu922Glyfs*156 mutation was identified in ORF15 of *RPGR* in this family. (**B**) UTAD0404: A p.Thr99Asnfs*13 mutation was identified in Exon 4 of *RPGR* in this family. (**C**) RFS296: A p.Gly65Asp mutation was identified in Exon 2 of *RPGR* in this family.

RPGR mutations in 19 of the 56 families analyzed, which included two mutations that appeared in more than one family (see Table).[14,17,31–35] Once a disease-causing mutation was identified in a family, additional members of that family were tested when available, and segregation analysis confirmed the mutation's likelihood of being disease-causing. Each mutation that was not excluded as a known polymorphism segregated appropriately with disease. Of these 19 mutations, 13 have been reported previously.[14,17,31–36]

The *RPGR* mutations identified included four nonsense, two splice-site, 10 deletion, and three missense mutations. Of these, 11 were found in the ORF15 exon, and the two splicing mutations were found in introns 8 and 13. The remaining six *RPGR* mutations were found throughout exons 1 to 19. Although two of the nonsense mutations and one of the deletions identified in the ORF15 exon (families UTAD0663, RFS191, and RFS354, respectively) have not been reported previously to our knowledge, many other nonsense mutations

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TABLE. *RPGR* (NM_001034853) and *RP2* (NM_006915) Mutations in "adRP" Cohort

Family ID	Gene	Exon	Mutation	Protein Change	Classification	Reported Previously
RFS296	*RPGR*	Exon 2	c.194G > A	p.Gly65Asp	Missense	Reported previously[32]
UTAD0036	*RPGR*	Exon 2	c.194G > A	p.Gly65Asp	Missense	Reported previously[32]
UTAD0404	*RPGR*	Exon 4	c.297_306del	p.Leu100Glnfs*30	Deletion	Novel
RFS211	*RPGR*	Exon 8	c.865A > G	p.Ile289Val	Missense	Reported previously[31]
UTAD0170	*RPGR*	Intron 8	c.934+1G > T	Splicing	Splicing	Novel
UTAD0488	*RPGR*	Exon 11	c.1377_1378del	p.Leu460Ilefs*2	Deletion	Reported previously*,[35]
UTAD0028	*RPGR*	Intron 13	c.1573-8A > G	Splicing	Splicing	Reported previously[34]
UTAD0514	*RPGR*	Exon 14	c.1636G > T	p.Glu546X	Nonsense	Novel
UTAD0663	*RPGR*	ORF15	c.2188G > T	p.Gly730X	Nonsense	Novel
RFS191	*RPGR*	ORF15	c.2212G > T	p.Gly738X	Nonsense	Novel
UTAD0242	*RPGR*	ORF15	c.2218G > T	p.Glu740X	Nonsense	Reported previously[17]
UTAD0442	*RPGR*	ORF15	c.2340del	p.Ala781Argfs*34	Deletion	Reported previously[35]
UTAD0008	*RPGR*	ORF15	c.2405_2406del	p.Glu802Glyfs*32	Deletion	Reported previously[14]
UTAD0201	*RPGR*	ORF15	c.2405_2406del	p.Glu802Glyfs*32	Deletion	Reported previously[14]
UTAD0537	*RPGR*	ORF15	c.2405_2406del	p.Glu802Glyfs*32	Deletion	Reported previously[14]
UTAD0498	*RPGR*	ORF15	c.2442_2445del	p.Gly817Lysfs*2	Deletion	Reported previously[14]
UTAD0005	*RPGR*	ORF15	c.2517_2518del	p.Glu841Glyfs*237	Deletion	Reported previously[33]
BCM-AD900	*RPGR*	ORF15	c.2625dupA	p.Gly876Argfs*203	Duplication	Reported previously[14]
UTAD0593	*RPGR*	ORF15	c.2763_2764del	p.Glu922Glyfs*156	Deletion	Reported previously[14]
RFS354	*RPGR*	ORF15	c.3106del	p.Glu1036Lysfs*53	Deletion	Novel
RFS119	*RP2*	Exon 2	c.688_692del	p.Lys230Glnfs*3	Deletion	Reported previously[36]
RFS021	*RP2*	Exon 4	del EX04-flanking†	del EX04-flanking†	Deletion	Novel

* Previously reported as c.1376_1377del; p.Val459fs*461, but changed in chart to fit current mutation nomenclature.[35]
† A 12.5 Kb deletion of exon 4 and flanking regions.

and frameshift mutations (e.g., caused by the deletion in the RFS354 family) in ORF15 have been shown to cause RP.[17] The c.1636G > T nonsense mutation identified in the UTAD0514 family also has not been reported previously to our knowledge, but other nonsense mutations in exon 14 of *RPGR* have been shown to be pathogenic in RP patients.[35] While the exact c.934+1G > T splicing mutation identified in the UTAD0170 family has not been reported previously to our knowledge, two different substitutions at this same position are known to cause disease.[34] The c.297_306del deletion identified in the UTAD0404 family caused a frameshift at amino acid 100, which is predicted to code for the incorporation of 30 incorrect amino acids before protein termination. While this novel mutation has not been reported previously, small deletions in *RPGR* have been reported in many families with XLRP, and those that result in a frameshift/early termination mutation are always pathogenic.[37]

Of the 56 families in the cohort who met criteria for inclusion in the study, 16 had no male members available for testing and had affected female members who, by conventional sequencing, were heterozygous for polymorphic indels in ORF15. (Homozygous female subjects were sequenced by conventional methods.) In-frame polymorphic indels make it difficult to analyze ORF15 sequence in a heterozygous state. To facilitate testing of *RPGR*, the repetitive and purine-rich exon ORF15 of *RPGR* was subcloned and sequenced in these 16 families. Analysis of the sequence data and exclusion of known polymorphisms revealed one additional *RPGR* mutation, a previously reported c.2625dupA duplication in ORF15, in the BCM-AD900 family (see Table).[14] Identification of this mutation brings the total number of *RPGR* mutations to 20.

RP2

Probands from the 56 families included in the study with a provisional clinical diagnosis of adRP but no male-to-male transmission also were screened for mutations in the *RP2* gene by di-deoxy sequencing. Sequence analysis revealed *RP2* mutations in two families (see Table). Each of these mutations was a deletion. The c.688_692del deletion found in exon 2 has been reported previously.[36] The second *RP2* mutation was a 12.5 kb deletion including all of exon 4, and some 5′ and 3′ flanking intronic regions. Due to a mononucleotide run around each breakpoint, the exact location of the exon 4 deletion breakpoints is ambiguous. The approximate location of these breakpoints can be defined as 46,725,365 to 46,737,820 base pairs (bp) on chromosome X (hg19). While this specific mutation has not been reported previously to our knowledge, other disease-associated mutations that eliminate all of exon 4 have been reported.[38–40] Once a disease-causing mutation was identified in a family, additional family members were tested, and segregation was confirmed in each case.

DISCUSSION

Families from a well-characterized adRP cohort that lacked male-to-male transmission were selected and screened for mutations in *RPGR* and *RP2* by di-deoxy sequencing. *RPGR* was tested further by subcloning and sequencing ORF15 in families that had no male members available for testing and whose female members were heterozygous for polymorphic indels in ORF15. This allowed for identification of a significant fraction of families with a provisional diagnosis of adRP that, in fact, have disease-causing mutations in the X-linked genes *RPGR* or *RP2*.

Combining data from these two methods showed that 8.5% of families (22 of 258) in our "adRP" cohort that were thought to have adRP actually have XLRP (Fig. 2). Of these mutations, 20 (7.8%) were found in *RPGR*, and two (0.8%) were found in *RP2*. The identified disease-causing mutations included a range of missense, nonsense, splicing, deletion, and duplication mutations. The mutations were located throughout the genes analyzed, most being located in ORF15 of *RPGR*. With the identification of these mutations in X-linked genes, disease-attributable mutations can be reported in 72% of our adRP cohort.

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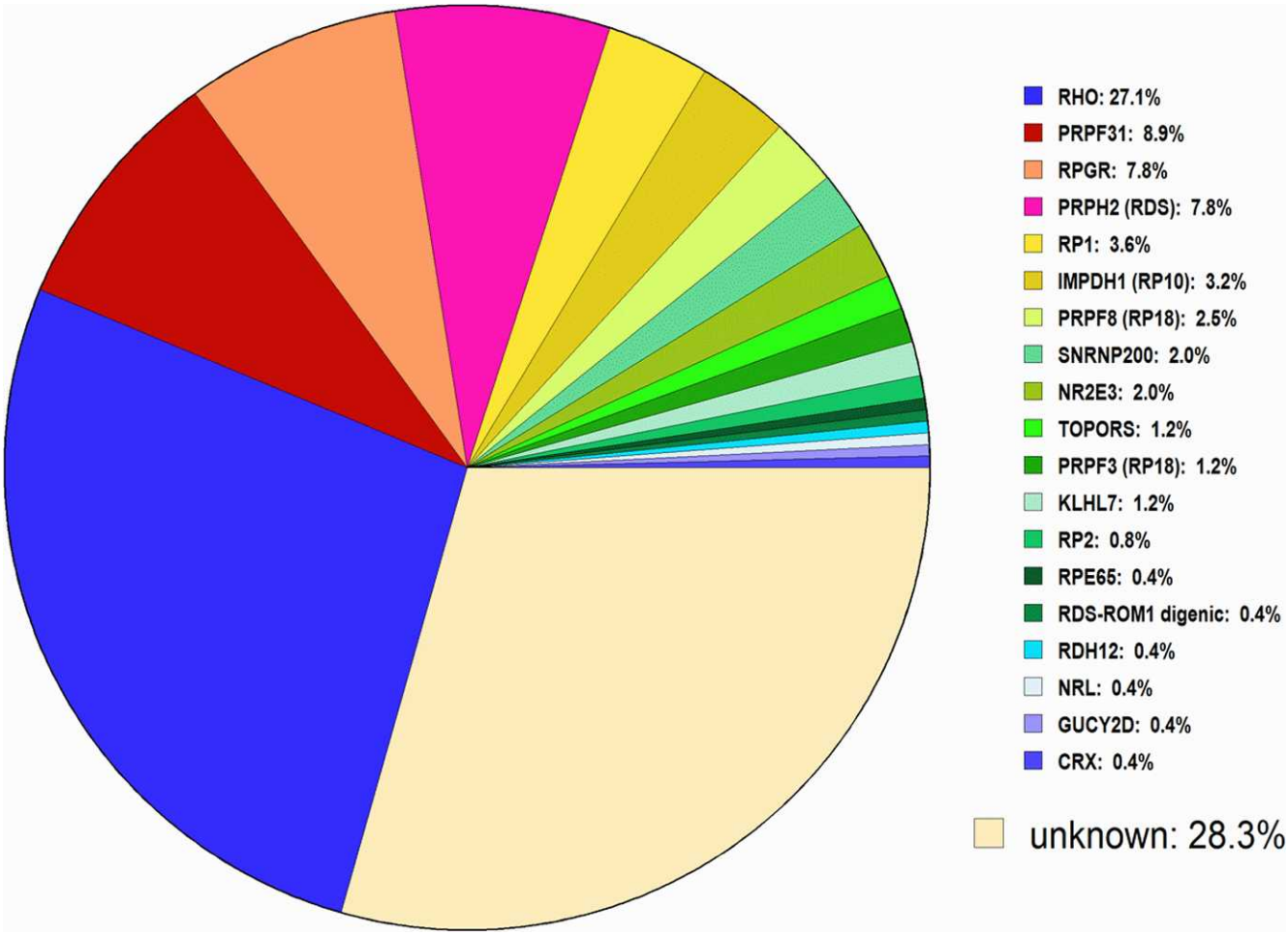

Figure 2. Prevalence of mutations in genes causing dominant RP. *RPGR* mutations have been identified in 20 of the 258 (7.8%) adRP cohort families. *RP2* mutations have been identified in 2 of the 258 (0.8%) adRP cohort families.

Identification of *RPGR* and *RP2* mutations in 8.5% of our cohort suggest that many families not previously considered candidates for XLRP should be screened for mutations in X-linked RP genes. While existence of mildly affected or asymptomatic females is still often a hallmark of XLRP families, it is not always the case. Females can be affected as severely as their male counterparts when multiple family members are examined.[10,12,13] Further, when examined in isolation, females with X-linked mutations often have symptoms comparable with the spectrum seen normally in dominant RP. Also, due to its highly repetitive nature, *RPGR*-ORF15 is not amendable to testing using next generation sequencing methods. Given its high mutation frequency, *RPGR*-ORF15 should be the subject of alternate approaches, such as Sanger sequencing tailored specifically for this exon.

Based on conservative estimates, RP accounts for half of all patients with inherited retinopathies. AdRP accounts for approximately 30% of non-syndromic RP, XLRP for approximately 15%, and simplex (isolated) cases for approximately 30%.[5] From this and other studies, *RPGR* mutations account for 70% of XLRP, 8% of adRP, and 15% of isolated male cases.[10,14] Without considering the number of affected individuals per family, this implies that *RPGR* mutations account for 15% of all RP cases or 7% of all inherited retinopathies. Based on an estimated 200,000 cases in the United States, this suggests 15,000 individuals are affected by *RPGR* mutations in this country, making it one of the most common causes and possibly the most common cause of RP.

Identification of frequent XLRP mutations illustrates the importance of screening families with a provisional diagnosis of autosomal inheritance and no male-to-male transmission for mutations in X-linked genes. Rather than "recessive" or "dominant," the mode of inheritance in these families is described best as X-linked retinitis pigmentosa with complete penetrance in hemizygous males and incomplete penetrance and variable expressivity in carrier females. Identifying XLRP mutations in RP patients originally thought to have a dominant pattern of disease transmission has significant implications for calculation of recurrence risk, genetic counseling, and treatment when evaluating RP patients and families.

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