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Authors

Gorin, MB

Zito, I

Plant, C

et al.

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MUTATION IN BRIEF

Novel Mutations of the *RPGR* Gene in RP3 Families

Ilaria Zito[1], Michael B. Gorin[2], Catherine Plant[1], Alan C. Bird[1], Shomi S. Bhattacharya[1], and Alison J. Hardcastle[1]

[1]Departments of Molecular Genetics and Clinical Ophthalmology, Institute of Ophthalmology, University College London, London, UK; [2]Department of Ophthalmology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

*Correspondence to Ilaria Zito, Department of Molecular Genetics, Institute of Ophthalmology, University College London, 11-43 Bath Street, London, EC1V 9EL; Tel: 44 171 608 6987; Fax; 44 171 608 6863; E-mail; ilaria.zito@ucl.ac.uk

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X-linked retinitis pigmentosa is a severe retinal degeneration characterized by night blindness and visual field constriction, leading to complete blindness within the third decade of life. Mutations in the *RPGR* gene (retinitis pigmentosa GTPase regulator), located on Xp21.1 in the RP3 region, have been associated with an RP phenotype. Further to our previous mutation screening of *RPGR* in families segregating with the RP3 locus, we have expanded this study to include other 8 RP3 pedigrees. Here we report the results of this expanded study and the identification of five mutations in *RPGR*, four of which are novel (IVS6+5 G>A, 950-951delAA, 963 T>C, EX8del) and one of which occurs in the donor splice site of intron 1 (IVS1+1 G>A). These findings bring the proportion of "RP3 genotypes" with a mutation in this gene to 27% (10/37). © 2000 Wiley-Liss, Inc.

KEY WORDS: retinitis pigmentosa GTPase regulator, *RPGR*, RP3, X-linked retinitis pigmentosa, XLRP, mutation analysis

INTRODUCTION

   X-linked retinitis pigmentosa (XLRP) is a severe form of retinal degeneration manifesting in the first years of life of affected males due to haploinsufficiency. The first clinical symptoms of the disease include night blindness and reduced visual acuity as a consequence of the degeneration of rods in the periphery of the retina. As disease progresses cones start to degenerate producing a gradual impairment of the central vision which culminates in total blindness within the third or forth decade of life.

   Two genes causing retinitis pigmentosa have been cloned on the short arm of the X chromosome: the *RPGR* gene (retinitis pigmentosa GTPase regulator; MIM# 312610) on Xp21.1 in the RP3 region (Meindl et al. 1996) and the *RP2* gene on Xp11.3 in the RP2 region (Shwann et al. 1998). By a combination of haplotype and linkage analysis these appear to be the two major XLRP loci accounting respectively for over 70% and for 20-25% of familial disease (Ott et al. 1980, Teague et al. 1994).

   Worldwide effort in trying to unravel the complete genetic picture underlying this devastating disease led to the identification of another two XLRP loci in two single families. These two loci (RP23 on Xp22.3, Harcastle et al. submitted and RP24 on Xq24, Gieser et al. 1998) appear to account for only a small proportion of disease in XLRP

families.

The RP3 gene (*RPGR*) was cloned in 1996 (Meindl et al.) and is composed of 19 exons encoding an ubiquitously expressed protein product of 815 amino-acids. More recently a new retina-specific exon (exon 15a) has been identified (Kirschner et al 1999). This exon is contained within intron 15 and it introduces a new stop codon producing a predicted gene product of 646 instead of 815 amino acids. The N-terminal portion of the gene shares sequence homology with RCC1, a regulator of chromosome condensation that interacts with small nuclear GTPases (Meindl et al. 1996, Roepman et al. 1996). It has recently been demonstrated that the RCC1-like domain is able to interact with the δ subunit of phosphodiesterase (δPDE, Linari et al. 1999). However the role of *RPGR* in the retina and its involvement in the etiology of retinitis pigmentosa have not yet been resolved.

Mutations in *RPGR* seem to account for approximately 20-25% of XLRP families (Meindl et al. 1996, Roepman et al. 1996, Buraczynska et al. 1997, Zito et al. 1999, Miano et al. 1999) which does not correlate with the large proportion of families segregating with the RP3 locus (70-75%). Additional coding sequences, the regulatory region of the gene or even a different gene close to *RPGR* may be involved in the proportion of familial disease still unaccounted for.

Further to our previous study (Zito et al. 1999) we here report the results of a screening carried out on eight new families segregating with the RP3 locus and the identification of five *RPGR* mutations, bringing the proportion of familial "RP3 genotypes" with a mutation in this gene to 27%.

MATERIALS AND METHODS

XLRP families and controls

By pedigree and clinical analysis an X linked form of RP was established. The diagnosis was based on ophthalmological tests including fundus examination, visual field assessment, flourescein angiography and electroretinogram (ERG) recordings. Blood samples were collected for each family and DNA was extracted using the Nucleon II kit (Scotlab Limited, Strathclyde, Scotland) according to manufacturer instructions. The ethnic background of the families used in this study is as follows: family A is from the USA, family B is from New Zealand, families C, D and E are from the UK, the other three families that were screened in this study and in which mutations were not identified are from Belgium (two families) and from the UK (one family).

Mutation detection by direct automated sequencing of PCR products

All 19 exon fragments plus exon 15a of the *RPGR* gene were amplified with intronic primers described in Meindl et al. (1996) and in Kirschner et al. (1999). PCRs were carried out as previously described and special conditions were used for the amplification of exon 1 (Zito et al. 1999). PCR products were examined by agarose gel electrophoresis prior to sequencing. An aliquot of the amplification product (8 µl) was then purified by the addition of 1 U shrimp alkaline phosphatase (SAP; Amersham Life Science, Buckinghamshire, UK) and 1 U Exonuclease I (United States Biochemical) in SAP buffer, and incubation at 37°C for 30 min followed by 80°C for 15 min. Five µl of the purified DNA sample was then used for cycle sequencing using Big Dye Terminator cycle sequencing kit following manufacturers instructions. Reactions were then electrophoresed on an ABI 373A automated sequencer.

Restriction enzyme digestion analysis

Exon 8 PCR fragments were digested with *Bss*S I restriction enzyme (New England BioLabs, Hertfordshire, UK). PCR reactions were carried out as described and 5µl of the PCR product were checked on an agarose gel prior to digesting. The remaining 20µl were brought to a final volume of 30µl by the addition of 10X NE Buffer 3 (New England BioLabs, Hertfordshire, UK), 6U *Bss*S I enzyme and H_2O. Digestion was performed at 37°C for 16-18 h.

RESULTS AND DISCUSSION

Direct sequencing of PCR products of all the 19 exons plus exon 15a of *RPGR* in eight families segregating with the RP3 locus, led to the identification of five mutations in five families (summarized in table 1).

Table 1: Mutations identified in the *RPGR* gene in four XLRP families with an RP3 genotype

Family	Intron/Exon	Nucleotide alteration*	Amino acid no	Consequence
A	Intron 1	IVS1+1 G>A	-	Splicing
B	Intron 6	IVS6+5 G>A	-	Splicing
C	Exon 8	950-951delAA	297	Frameshift
D	Exon 8	963 T>C	302	C302R
E	Exon 8	Deletion (uncharacterized)	-	Deletion

*Position according to GenBank No. U57629

In family A, the mutation identified was a G>A transition 1 nucleotide downstream of the exon1/ intron1 boundary (IVS1+1 G>A). Fresh blood of patients from this family is not available for RNA extraction hence we cannot demonstrate that this mutation gives rise to an aberrant splicing product. However exon 1 was sequenced in 80 control chromosomes and this change was not detected. In addition sequence analysis of exon 1 in the family demonstrated segregation of the mutation with disease and sequence analysis of the entire gene in the family did not reveal any other sequence alterations, therefore it is likely that the G>A transition observed affects normal splicing and is the causative mutation in this family. This mutation has also been already identified in another family (Zito et al 1999), therefore strengthening its role as a pathogenic mutation.

It is interesting to observe that in previous reports describing *RPGR* mutations, exon 1 was often excluded from the analysis, due to amplification difficulties. However this is the third potential mutation described for this exon out of a total of 44 mutations reported for the gene to date (Meindl et al. 1996, Roepman et al. 1996, Buraczynska et al. 1997, Zito et al. 1999, Miano et al. 1999, this study). The three exon 1 mutations are all within the 5' consensus donor splice site and two of them are identical. Considering also that exon 1 comprises less than 2% of the entire coding region of the gene, this donor splice site may be considered a potential mutational hot spot of the gene.

Family B carries a novel G>A change five nucleotide downstream of the exon6/inron6 boundary (IVS6+5 G>A). This nucleotide is part of the 5' consensus donor splice site and is 80% conserved in mammals (Horowitz and Krainer 1994), it is therefore likely that a sequence variation at this position affects normal RNA splicing. This change cosegregates with disease in the family and over 200 control chromosomes were sequenced to exclude the possibility of a polymorphism.

In family C a novel 2-bp deletion was observed at nucleotide position 950-951 (950-951delAA). This deletion causes a frameshift that is predicted to create 47 novel amino acids and a new stop codon that produces a protein product of 344 amino acids with 471 amino acids missing. The mutation segregates with the disease in the family.

In family D we observed a novel 963T>C transition, producing a Cys>Arg change at codon 302. This is a non-conservative amino acid change at a conserved codon. The T>C change creates a restriction site for *Bss*S I enzyme which was used to demonstrate segregation of the mutation with disease in the family and lack of the same mutation in 100 control chromosomes. Sequencing of the entire coding region of the gene in this family showed no other variations.

In family E exon 8 failed to amplify in the affected members of the family. To exclude the possibility of sequence alterations within the primer sequences new primers were designed still spanning the exon and intron/exon boundaries (8b-F: atccttgtcttgtatttgccc and 8b-R: ccagttctataaataatataacag). The new primers again failed to amplify in the affected members of the family. The deletion needs further characterization, as unfortunately the full genomic sequence for *RPGR* is not available and PCR across exons 7-9 has been carried out with no resulting amplification product (probably due to the size of the introns). Southern blot analysis is currently

unachievable due to a shortage of DNA. However, the mutation segregates with the disease in the family and the remaining coding sequence of the gene presents no alterations.

   With these three mutations in exon 8 the number of mutations described for this exon to date is 9 (Meindl et al 1996, Roepman et al. 1996 Buraczynska et al. 1997, Miano et al. 1999) out of a total of 44 mutations (20.5%). This suggests that exon 8 has an important role in the etiology of RP, especially considering that three of these mutations are missense mutations pinpointing at least three crucial amino acids contained within this exon.

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