Abstract

Retinitis pigmentosa GTPase regulator (RPGR) gene sequence variants account for most of X-linked retinitis pigmentosa (XLRP). Symptoms such as nyctalopia typically begin in childhood, with increasing loss of peripheral visual field during teenage years, and progressive loss of central vision later in the disease process. *RPGR* is involved in ciliary function, with ciliary dysfunction now recognised as the mechanism underlying a large proportion of inherited retinal disease. There has been significant progress in identifying animal models to define the underlying disease mechanisms and to test gene replacement therapy. This progress, combined with advances in human retinal imaging, have culminated in multiple on-going gene therapy clinical trials. This review summarizes the molecular genetics, protein function, clinical phenotypes, animal models and the clinical trials for *RPGR*-associated RP.

Molecular Genetics of *RPGR*

The *RPGR* gene consists of 19 exons and is located on the short arm of the X chromosome (Xp11.4). Twenty-one *RPGR* splice-site variants have been identified in humans, as a result of complex alternative splicing, with expression in a variety of tissues including the testis, kidney, brain, lung, pancreas and retina. However, two main RPGR isoforms have been characterised as either tissue or cell specific.[1, 2]

Exons 1-19 encode an 815 amino acid, 90kDa protein, and account for one of the two main RPGR isoforms ($RPGR^{ex1-19}$, constitutive variant). RCC1 (Regulator of Chromosome and Condensation)-like domain (RLD) is located at the N terminal and is encoded by the first ten exons. This area consists of six tandem repeats with structure similar to the RCC1 protein, which is highly conserved across vertebrate species.[1] The *RPGR* gene derives its name from the properties conferred by RCC1, and it acts as a guanine nucleotide exchange factor, regulating a ubiquitous GTPase called Ran (Ras-related nuclear protein), which has a role in membrane transport and trafficking. All proteins known to directly interact with RPGR do so through the RLD, and all known splice-site variants of *RPGR* contain the RLD domain.

The other major human isoform is $RPGR^{ORF15}$, which is composed of 1152 amino acids (127kDa protein). $RPGR^{ORF15}$ and $RPGR^{Ex1-19}$ have the same N terminus, which is encoded by exons 1 to 14. However, beyond exon 14, the $RPGR^{ORF15}$ transcript is encoded by Open Reading Frame 15 (ORF15), which consists of exon 15 and part of intron 15, created by the skipping of exon 15's splice donor-site.[3] ORF contains numerous exonic splicing enhancers.[4] Up to 80% of *RPGR* damaging variants are located in this 1.6kb stretch of ORF15, making it a 'mutation hot spot'.[3] Its highly purine-rich sequence is thought to be responsible for its mutagenicity. $RPGR^{ORF15}$ is expressed in several tissues including the cochlea and the epithelium of the respiratory tract, but the most marked expression is found in the retina.[5] Almost all known *RPGR* variants causing retinal disease so far have been identified within the $RPGR^{ORF15}$ transcript. *RPGR* disease-causing variants

are estimated to account for 5% of all pedigrees with inherited retinal diseases.[6]

RPGR Function and Localization

RPGR interacts and forms complexes with a variety of proteins, including centrosomal, basal body, axonemal and microtubule transport proteins.[7] Previous studies have identified a protein now known as RPGRIP that interacts specifically with RPGR,[8, 9] and has been localised to the connecting cilia and rod outer segments.[9, 10] RPGR binds to RPGRIP via its RLD and is dependent upon the presence of RPGRIP at the connecting cilia for its correct localisation and function[11] RPGR anchored at the connecting cilia, regulates intracellular protein transport between the inner and outer segments,[9] and maintains correct localization and concentration of opsin. In a murine *RPGR* knock out model, mislocalization of opsin with ensuing degeneration was reported.[12] The multiple roles of RPGR are yet to be fully elucidated, including intracellular protein transportation and localization,[13] [14] and transport regulation within primary cilia and microtubule organisation.[15]

Clinical Phenotypes associated with *RPGR* Variants

i) *RPGR*-associated RP

Variants in *RPGR* can result in a range of phenotypes. The most common presentation is XLRP, having one of the most severe disease courses, with earlier onset of symptoms, often in the first decade, and relatively rapid deterioration, culminating in legal blindness by the third or fourth decade.[16] Fundoscopy can show areas of atrophic retinal pigment epithelium (RPE)/photoreceptor cell loss, and pigmentation to a greater extent in the mid-periphery (**Figure 1**). Static perimetry has been used to follow disease progression in *RPGR* patients and an exponential decline in mean sensitivity was reported.[17] Structural markers of disease severity and progression are of

interest prognostically, as well as for their relevance in establishing trial endpoints.[18] Optical coherence tomography (OCT) imaging shows disruption of the photoreceptor layer (ellipsoid zone, EZ) starting from the periphery (rod rich region) and gradually constricting towards the fovea (**Figure 1**). The rate of decline based upon reduction in EZ area (EZA), derived from *en face* images of macular OCT volume scans, has been reported in 38 patients with *RPGR*-XLRP.[19] A mean rate of 0.67mm^2 per year decline in EZA was calculated, with even faster rates in younger individuals. Similarly, younger subjects with *RPGR* were also demonstrated to have faster rates of progression with respect to constriction of the parafoveal hyperautofluorescent ring that is present in many patients with RP (46/96 patients, 48%).[18, 20] Adaptive optics imaging allows for *in vivo* cellular imaging,[21] and a good repeatability has been reported for *RPGR*-XLRP.[22]

ii) *RPGR*-associated cone dystrophy/cone-rod dystrophy (COD/CORD)

Variants in *RPGR* contribute to a minority (1-2%) of all COD/CORD cases.[6, 23] To date, all such patients have variants in the ORF15 portion of *RPGR*, with a propensity to be located at the 3' end of this region.[24-26] Typical features of *RPGR*-associated COD/CORD are photophobia, myopia, progressive reduction in central visual acuity and central visual field, and impaired colour vision; with later rod loss resulting in increasing nyctalopia and constricted visual fields. A correlation between higher myopic refractive error and faster rates of visual acuity loss has been reported.[27] COD/CORD affected males may exhibit parafoveal hyperautofluorescent rings (**Figure 2**), which behave in an opposite manner to those seen in RP; with disease progression, there is an increase in the size of such rings, with associated worsening in the pattern electroretinogram (PERG) P50 amplitude.[28, 29] OCT imaging usually shows disruption of the EZ at the fovea (cone rich region), which gradually extends towards the periphery (**Figure 2**).

iii) Female carriers of *RPGR* variants

A wide range of retinal phenotypes have been reported in female *RPGR*-carriers. One third of female carriers have neither symptoms nor evidence of retinal pigmentary changes; whilst about a quarter manifest complete RP or CORD disease expression, albeit most often a milder form compared to male sufferers; rarely are as severely affected as males. Forty percent of female carriers have been noted to have a tapetal-like reflex (TLR) at the macula on clinical examination.[30, 31] TLR has long been associated with XLRP carriers and is described as a golden, radial spoke-like pattern of hyperreflectivity at the posterior pole, which is most apparent on autofluorescence imaging.[30, 31] Mosaicism is believed, at least in part, to underlie the heterogeneity seen in carriers.[31, 32] This clinical heterogeneity may pose a challenge in establishing the mode of inheritance in an undiagnosed family, as the presence of affected females may suggest autosomal dominant inheritance. Moreover, this variability makes providing prognostic information to carriers difficult.

iv) Extraocular associations of *RPGR*-associated disease

RPGR is widely expressed in ciliated tissues and is implicated in a host of extraocular phenotypes/ciliopathies.[33] Electron microscopy of the sperm of patients with XLRP has revealed abnormalities in the axoneme. RPGR's crucial role in spermatogenesis was also identified from studies of a transgenic mouse, that was generated to overexpress *RPGR* and was found to have varying extents of abnormalities ranging from numerous structural defects of the flagellum to lack of mature spermatozoa, leading to infertility.[34] Associations with hearing impairment, as well as recurrent sinorespiratory infections from early childhood, are well-documented.[5, 35-37] Primary ciliary dyskinesia (PCD) is a multi-systemic disorder arising from functional and structural defects in the cilia. There is impaired mucociliary clearance leading to recurrent respiratory infections, bronchiectasis and sinusitis. In addition, it is associated with subfertility, due to ciliary abnormalities of the fallopian tube and defects in the sperm flagella affecting their motility. *RPGR* damaging variants have been identified as a rare cause of PCD.[38]

Phenotype-Genotype associations

More than 350 pathogenic *RPGR* variants have been described to date.[39] In most of these cases (up to 95%), the resulting phenotype will be XLRP.[40] *RPGR*-related COD/CORD variants more frequently localize at the 3' end of the gene,[25, 26, 41, 42] similar to variants associated with *RPGR*-associated atrophic macular degeneration.[43] Syndromic RP usually arises from variants at the 5' end of the gene.

In terms of variant localization and disease severity, the literature is controversial. There may be a trend for variants affecting the RLD of the N-terminus to result in more severe disease compared to variants in ORF15.[44] In one study, ORF15 variants were reported to result in greater ERG responses and more intact visual field compared to variants in exons 1-14.[45] However, other studies show no significant difference between ORF15 and non-ORF15 disease severity based on structural and functional measures.[17, 19] Variants located towards the 3' end of ORF15 have been associated with a milder phenotype compared to those found towards the 5' end; but again there is considerable variability both within and between failies.[46] It also remains uncertain whether the degree of associated myopia has a relationship with disease severity / progression.

Animal Models

A number of mouse and canine models exist.[7] A major disadvantage of those models is the relatively slow onset of photoreceptor degeneration compared to human disease. This observation has been reported for several murine models, and likely reflects species difference, rather than incomplete ablation of gene expression.

Two naturally occurring, distinct ORF15 variants in the canine Siberian husky breed result in different phenotypes. A 5-nucleotide deletion in *RPGR*

ORF15 (delGAGAA at position 1028-1032) gives rise to a premature stop codon with truncation of 230 residues, resulting in X-linked Progressive Retinal Atrophy 1 (XLPRA1) [47]. This phenotype is characterised by post-developmental onset, gradual photoreceptor degeneration that affects rods more than cones, in keeping with human RP.[47]. The second more severe phenotype, X-linked Progressive Retinal Atrophy 2 (XLPRA2), is caused by a 2-nucleotide deletion in ORF15 (del GA at position 1084_1085) resulting in frameshift and insertion of 34 amino acids prior to termination. Onset of XLPRA2 is during early retinal development and progression is rapid, with both rods and cones affected, more in keeping with human cone-rod dystrophy.[47]

One mouse model was generated by the deletion of *RPGR* exons 4-6 [12]. When examined 20 days post-natally, cone opsin mislocalisation to inner segments, nuclear and synaptic regions was seen (but not rod opsin), and rhodopsin levels were reduced in rods. Retinal structure was however comparable to wild type and ERG function was within normal limits, despite a lack of RPGR. Late degeneration was observed by 6 months, photoreceptor cell loss was apparent in this model, but connecting cilia structure was still maintained.[12] One naturally occurring murine model deficient in RPGR is the retinal degeneration 9 (Rd9) strain of mice. A 32-base pair duplication in ORF15 results in truncated protein product that is unstable. $RPGR^{ORF15}$ levels were undetectable in the retina of affected male mice [48]. A third RPGR deficient model has been created by the deletion of exon 1 [49]. These models carry a similar phenotype to the first murine model described above. In contrast, transgenic mice have been created with the introduction of mutant *RPGR* ORF15 (bearing a truncated purine rich repetitive region) into both wild type and *RPGR* knockout backgrounds,[50] with a greater deleterious effect when compared to *RPGR*-null mice,[50] and can be considered similar to the XLPRA2 canine model.[51]

Preservation of photoreceptor nuclei and inner/outer segments limited to treated areas has been observed with adeno-associated virus (AAV) gene augmentation both in murine and canine models.[52, 53] The arrest of disease

progression was also achieved in late stages of retinal degeneration in a canine model, suggesting a wide therapeutic window.[54] The ORF15 sequence contained within this AAV vector in the canine studies had ORF15 DNA sequence variations, that are likely due to the repetitive purine nucleotides.[55] This inherent mutability has been overcome with codon optimized sequence,[56] and abbreviation of the repetitive sequence.[52] Retinal rescue in *RPGR* knockout mice has been achieved by a transgene encoding a shortened form of the $RPGR^{ORF15}$.[57]

Toxic Effects of *RPGR* Overexpression

Transgenic mice engineered with multiple copies of *RPGR* in their genome have been found to have various structural and functional defects, the severity of which was proportional to the number of *RPGR* copies.[34] Mice with 4 or 5 copies had a lowered sperm count, while mice carrying 8 to 10 *RPGR* copies had a complete absence of sperm flagella. These findings are of note in designing therapies, which need to be efficacious but avoid potential issues related to overexpression.

Clinical Trials

Successful AAV gene augmentation has been performed in murine and canine models, with preservation of photoreceptor nuclei and inner/outer segments limited to treated areas.[52, 53] The arrest of disease progression was also achieved in late stages of retinal degeneration in a canine model, suggesting a wide therapeutic window.[54] Clinical trials in humans are summarized in **Table 1** and discussed below.

Three Phase 1/2 clinical trials (NCT03316560, NCT03252847 and NCT03116113) are delivering a submacular injection of AAV-gene therapy. Early results of NCT03116113 have been peer-reviewed. Eighteen patients

were treated during a dose escalation phase and followed for 6 months after subretinal delivery of an AAV8 encoding codon-optimized human *RPGR* (AAV8-coRPGR), with steroid-responsive subretinal inflammation in patients at the higher doses; thereby meeting the pre-specified primary safety endpoint. In six patients, improvement in retinal sensitivity on mesopic microperimetry was noted at 1 month and variably maintained up to last follow-up.[58] NCT03252847 explores the safety and efficacy of AAV5-RPGR with the primary end-point being the absence of safety events, and secondary outcome measures being improvement in visual function, retinal sensitivity, functional vision (mobility maze), and quality of life (QoL) improvement as measured by QoL questionnaires. The 12-month data presented from the dose escalation phase of the clinical trial (n=10, AAO 2020) demonstrated that the AAV5-RPGR was generally well tolerated and produced significant improvements in both retinal sensitivity on static perimetry and microperimetry, and also vision-guided mobility. NCT03316560 investigates rAAV2tYF-GRK1-*RPGR* with the primary outcome being the number of participants experiencing adverse events and clinically relevant hematology/clinical chemistry parameters. Secondary outcomes are changes from baseline in visual function by perimetry, visual acuity by ETDRS, retinal structure by imaging and a QOL questionnaire. Data from the dose escalation phase have been reported which show improvements in retinal sensitivity on microperimetry. NCT04517149, in contrast to all aforementioned trials, is designed to investigate the safety and efficacy of a single *intravitreal* administration of AAV-RPGR at two dose levels.[59]

Conclusions

Inherited retinal diseases contribute to significant disease burden.[60, 61] XLRP-*RPGR* is currently an incurable condition with a particularly aggressive course of progression, compared to other inheritance patterns of RP. Promising results from extensive preclinical studies have helped to pave the way to establish multiple on-going gene therapy trials for *RPGR*-RP which also report

early promise, with Phase 3 trials planned and due to commence in 2021/2022.

Legends

Figure 1: Retinal Imaging of *RPGR*-associated Retinitis Pigmentosa

Color fundus photograph (CFP, top) imaging with corresponding horizontal optical coherence tomography (OCT, bottom), of a patient with *RPGR*-associated retinitis pigmentosa. CFP with pigmentation in the mid-periphery, and OCT imaging with preservation of the foveal photoreceptor layer.

Figure 2: *RPGR*-associated Cone-Rod Dystrophies (CORD)

Fundus autofluorescence (FAF, top) imaging with corresponding horizontal optical coherence tomography (OCT, bottom), of a patient with X-Linked *RPGR*-associated CORD. Parafoveal ring of increased signal is visible on FAF, and ellipsoid zone atrophy is present on OCT.

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