(-195T, L-12X, 298-2G, T117 N, A159T, R229S, 997+2A, E274X, A331T, H364R, D389G, 1256delC, R433H, N461I, C472S) in the tissue-nonspecific alkaline phosphatase (TNSALP) gene in patients with hypophosphatasia. *Hum Mutat* 2000;**15**:293.
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RPGR mutation associated with retinitis pigmentosa, impaired hearing, and sinorespiratory infections

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Retinitis pigmentosa (RP) is a progressive retinal degeneration that affects about 1 in 4000 of the population.[1] Approximately 15-30% of patients with RP have X linked retinitis pigmentosa (XLRP), which is the most severe form of RP consistently manifesting early in life.[2 3] Night blindness is usually present in early childhood with loss of peripheral visual fields and ultimately central vision, resulting in registered blindness by the end of the third decade. Female carriers display a broad spectrum of fundus appearances ranging from normal to extensive retinal degeneration.[4–6]

XLRP is genetically heterogeneous with two major loci, RP2 (Xp11.23) and RP3 (Xp21.1). Both disease genes have now been identified (respectively *RP2*[7] and *RPGR*[8–10]) with *RP2* mutations causing disease in approximately 15% of XLRP families,[11 12] while *RPGR* mutations are reportedly more common, accounting for up to 75% of XLRP.[10] Two other rare loci for XLRP have also been described on Xp22 and Xq26-27.[13 14]

Hong *et al*[15] described the phenotype and pathology of an *RPGR* knockout mouse model. They showed the subcellular localisation of RPGR to the photoreceptor connecting cilia, and in the absence of RPGR partial mislocalisation of essential outer segment proteins. These data suggest a putative role for RPGR in the retina, controlling movement of essential proteins from the inner to the outer segment of photoreceptors via the connecting cilia. Several groups have recently identified a retina specific RPGR interacting protein (RPGRIP1).[16–18] This protein also localises to the photoreceptor connecting cilium and is thought to be a structural component of the ciliary axoneme.[18] Subsequent mutation screening in patients suffering from retinal diseases has identified mutations in *RPGRIP1* as a cause of Leber congenital amaurosis.[19 20]

In this report, we present the phenotype of a family suffering from XLRP associated with hearing loss, sinusitis, and chronic recurrent respiratory tract infections. To identify the causative gene on the X chromosome, we performed haplotype analysis with subsequent mutation screening of candidate genes. The new phenotype described is associated with a mutation in the *RPGR* gene, and highlights the significance of RPGR protein kinociliary function in non-ocular tissue.

Key points

- We report a novel systemic phenotype associated with XLRP, with patients suffering from hearing loss, sinusitis, and chronic chest infections, suggesting a mutation in a gene involved in ciliary function.
- The phenotype overlaps those described for primary ciliary dyskinesia and Usher syndrome.
- Genetic analysis of this family has identified a frameshift mutation in exon 8 of the *RPGR* gene.
- A gene in close proximity to *RPGR*, *TCTEL1*, was also examined for cSNPs as a potential phenotypic modifier locus; none was detected.
- Our findings show that mutations in the *RPGR* gene are associated with a complex phenotype broadening the clinical spectrum of disease, and provide supporting evidence for an essential ciliary function for RPGR in the retina and other tissues.
- RPGR and interacting partners involved in kinociliary function in a variety of tissues may therefore represent attractive candidate genes for other diseases, such as primary ciliary dyskinesia or hearing loss.

PATIENTS AND METHODS

Patients and controls

Appropriate informed consent was obtained from the family and control volunteers under investigation. An X linked form of retinitis pigmentosa was established by pedigree analysis, clinical examination, and ophthalmological tests. Blood samples were collected from each available member of the family and from controls and DNA extracted using the Nucleon II Kit (Scotlab Limited) according to the manufacturer's instructions. Clinical characterisation included ophthalmic and systemic history, visual field testing, and fundus examination. In addition fundus photography was performed. Three

The first two authors contributed equally to this work

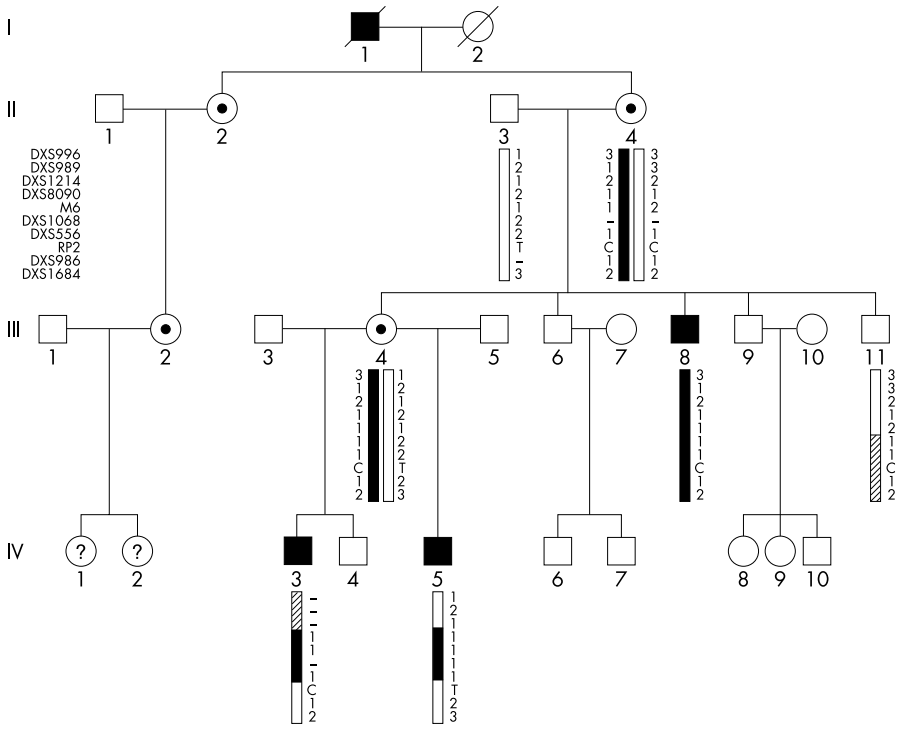

Figure 1 Pedigree of family 4462 showing haplotypes constructed around the RP2 and RP3 loci showing disease segregation with the RP3 locus. The polymorphic variant detected within the *RP2* gene is also shown and does not segregate with disease. The affected haplotype is shown as a shaded chromosome. Hatched chromosomes represent uninformative regions.

subjects (fig 1, II.4, III.4, and IV.5) underwent electrophysiological investigation; subject IV.3 was unable to attend because of renal dialysis. Electro-oculographic responses (EOG), full field electroretinography (ERG), and pattern electroretinograms (PERG) were recorded to incorporate the International Society for Clinical Electrophysiology of Vision (ISCEV) standards.[21–23] Hearing loss was assessed in one affected male (III.8) by pure tone audiometry and systemic disease history was provided by the patients' physicians.

Genotyping

Microsatellite markers on the X chromosome were used to generate haplotypes of affected, carrier, and unaffected members of the family (primers and conditions available at http://www.gdb.org/). Haplotypes were constructed assuming the minimal number of recombination events. PCRs were carried out in 10 µl reactions in the presence of 1 µCi α^{32}P-dCTP, 0.5 U *Taq* polymerase, 200 µmol/l each of dATP, dGTP, dTTP, and 20 µmol/l of dCTP, 50 pmol of each primer, 30 mmol/l Tris-HCl, pH 8.5, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, and 0.01% gelatine. Amplification conditions were 95°C for five minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at the primer specific temperature for 15 seconds, and extension at 72°C for 30 seconds. A final extension followed for five minutes at 72°C. Amplified products were mixed with 6 µl of formamide sample buffer and 3 µl aliquots were electrophoresed in 6% denaturing polyacrylamide gels. Electrophoresis was carried out at a constant power of 90 W for between two and five hours depending on fragment size. The gels were then fixed in 10% methanol/10% acetic acid solution for five minutes, dried onto Whatman paper, and analysed by autoradiography.

Mutation screening

The coding sequence and intron/exon boundaries for the *RP2*, *RPGR*, and *TCTE1L* genes were amplified as described previously.[11 24 25] PCR products were examined by agarose gel electrophoresis before sequencing. The *TCTE1L* gene was amplified as described by Roux *et al*,[25] except exon 1 primers were redesigned (TCTE1L1F - TGAAGTGACGCCTGGCGTTG and TCTE1L1R - AGAGGAAGGCGGGAGTGGG) and annealed at 60°C. An aliquot of each amplification product (8 µl) was then purified by the addition of 1 U shrimp alkaline phosphatase (SAP, Amersham Life Science) and 1 U Exonuclease I (United States Biochemical) in SAP buffer, and incubated at 37°C for 30 minutes followed by 80°C for 15 minutes. Five µl of the purified DNA sample was then used for cycle sequencing using Big Dye Terminator cycle sequencing kit following the manufacturer's instructions. Reactions were then electrophoresed on an ABI 373A automated sequencer.

RESULTS

During the genetic and clinical analyses of families diagnosed with XLRP, a family with additional systemic features was identified. The four generation pedigree structure of this family is shown in fig 1.

Clinical assessment

Ophthalmic phenotype

Affected males manifested night blindness in early childhood, had constricted visual fields by early teens, and were registered as legally blind by 20 years of age. Gross fields to confrontation in affected males IV.3 (aged 25) and IV.5 (aged 16) were less than 10°. Intraretinal bone spicule pigmentation was observed in IV.3 (fig 2). Female subjects II.4 and III.4 were asymptomatic, had normal fields to confrontation, and showed sparse peripheral intraretinal pigmentation (fig 2). Carrier females and affected males were myopic. Visual acuities were as follows for the right and left eyes respectively: II.4, 20/30 and 20/20; III.4, 20/40 and 20/30; IV.3, 20/30 and 20/30; and IV.5, 20/30 and 20/30. Levels of myopia were recorded for carrier females II.4 (5.00/−1.25 diopters and −7.50 diopters) and III.4 (−10 diopters). Electrophysiological phenotype was ascertained in one affected male patient (IV.5) and two carrier females (II.4 and III.4, fig 3). No recordable ERG could be

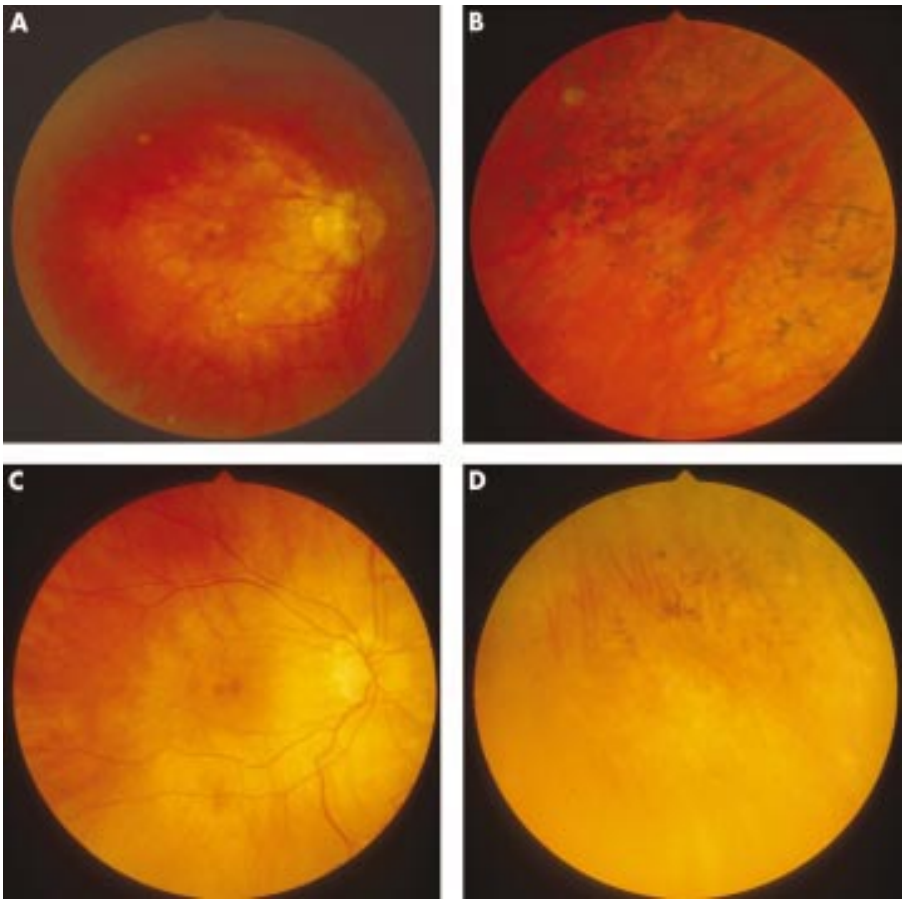

Figure 2 Fundus photos of (A) affected male IV.3, right macula showing retinal thinning and (B) typical intraretinal bone spicule pigmentation in peripheral retina, (C) right macula of carrier female III.4 showing mild retinal thinning, and (D) sparse intraretinal bone spicule pigmentation in the periphery.

detected in the affected male (IV.5) under any stimulus conditions; PERG was also undetectable confirming severe macular involvement. Both carrier females (II.4 and III.4) show delayed 30 Hz flicker ERG with mild delay also present in photopic single flash b wave. Rod specific ERGs do not show definite abnormality, but maximal response a wave is mildly subnormal in subject III.4. PERG is subnormal in both carrier females tested. EOG light rise was abolished in IV.5 and reduced in the carrier females. The ocular phenotype therefore fits the classical description of X linked retinitis pigmentosa, with very severe ERG abnormality in affected males and abnormal ERGs in female heterozygotes in keeping with carrier status.

Systemic phenotype

In this family, however, the classical ocular features of XLRP were associated with additional systemic symptoms in both hemizygous males and heterozygous females.

One of the most striking and obvious additional features was that affected subjects and carriers required hearing aids. Both males and females suffered severe recurrent ear infections from very early childhood continuing into adulthood. All affected males, and carrier female III.4 (aged 44), had progressive hearing loss and required hearing aids. Affected male I.1 (who had died) also had hearing loss and was described as deaf. The exception is carrier female II.4 (aged 68), who had recurrent ear infections, but did not require a hearing aid. The family had not been exposed to harmful environmental influences, such as excessive noise. Predominantly high frequency hearing impairment was recorded for affected male III.8 by pure tone audiometry (PTA, fig 4). Both left and right ear are similarly affected showing hearing loss between 4000 and 8000 Hz. Averaging the thresholds at these two frequencies is the convention to describe the degree of hearing loss predominant at high frequency. Thus, $(20 + 25 + 40 + 45) \div 4 = 32.5$, which indicates that hearing loss in this subject is mild, since the averaged threshold is less than 40 dB hearing loss. The audiogram could be consistent with a diagnosis of sensorineural hearing loss, in view of the hearing loss in the high frequencies. However, a conductive hearing component may also have contributed to this phenotype. Unfortunately, PTA data for other members of the family are not available.

In addition to retinitis pigmentosa and hearing loss, affected males and carrier females also suffered from severe recurrent sinus infections resulting in sinusitis. The three affected males experienced chronic recurrent chest infections starting in early childhood, with episodes of bronchitis, which continued into adulthood. Affected male IV.3 had nasal polyps and renal failure. The patient's affected brother (IV.5) and affected uncle (III.8) did not have renal failure, and carrier females do not seem to suffer from milder renal insufficiency. Although renal failure does not, therefore, segregate with the visual and hearing loss in this family, it remains possible that renal failure in patient IV.3 is part of the spectrum of symptoms for this syndrome.

In summary, the three affected males in this family had classical XLRP associated with hearing loss, sinusitis, and chronic recurrent chest infections, with renal failure in one person. Carrier females (II.4, III.2, and III.4) had a milder systemic phenotype, suffering recurrent ear infections and sinusitis without the chronic chest infections. Obligate carrier

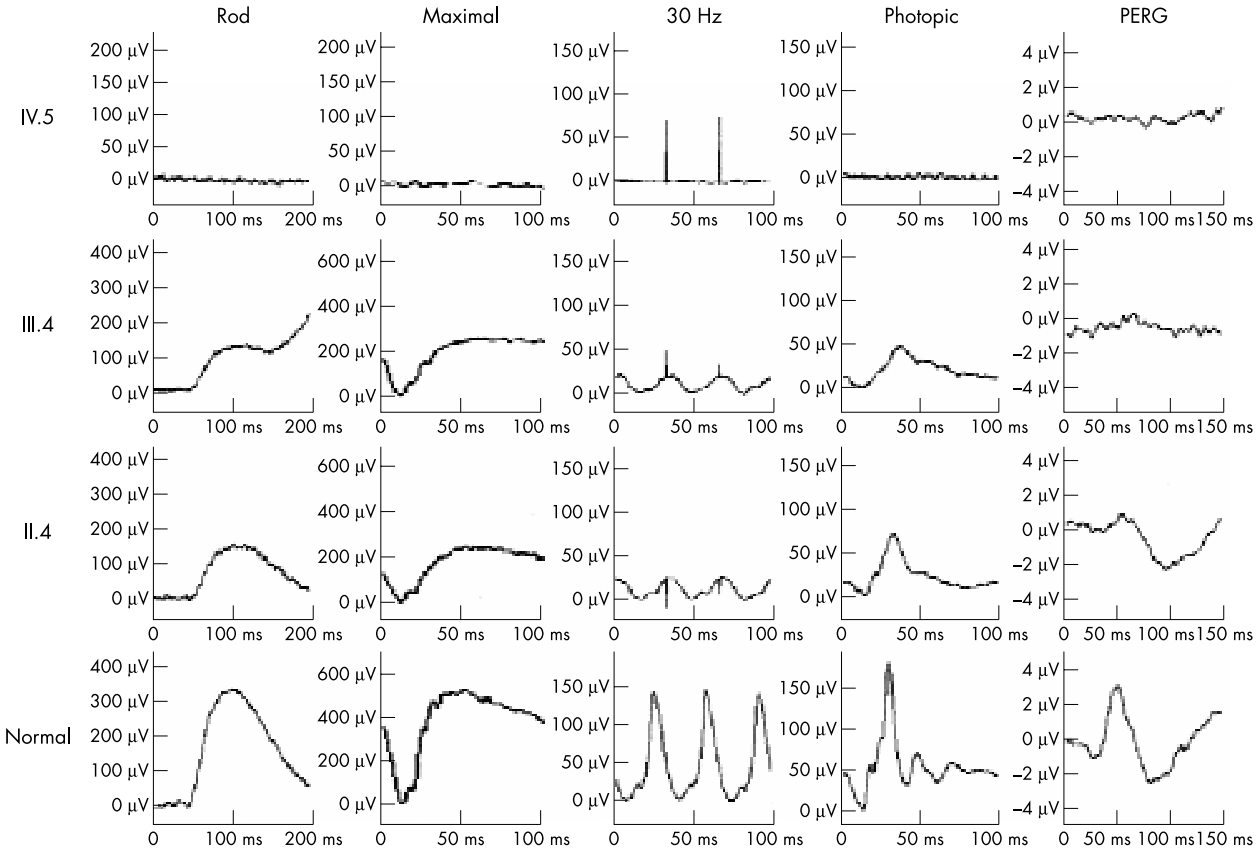

Figure 3 ERGs from an affected male (IV.5) and two carrier females (II.4 and III.4). The affected male shows no definite ERG under any stimulus conditions (note the changes in scale); PERG is also undetectable. The two carrier females show increased implicit time 30 Hz flicker ERGs, with mild change in photopic single flash b wave implicit time. Maximal response a wave is mildly subnormal in III.4. Both carriers show marked PERG reduction.

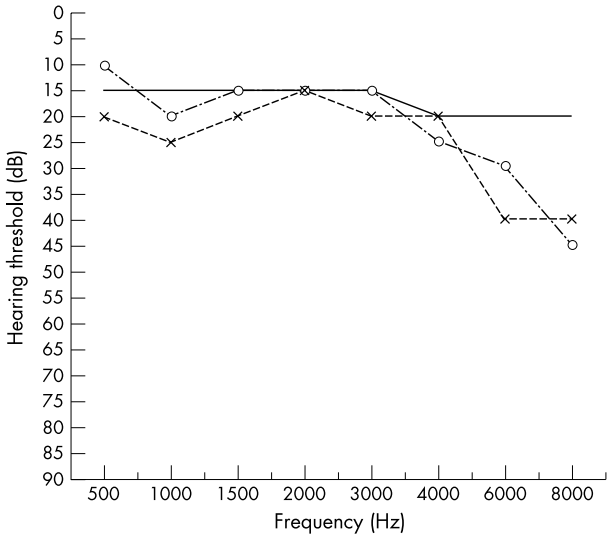

Figure 4 Pure tone audiometry for affected male III.8 showing bilateral hearing loss prominent at high frequencies. The continuous line represents the expected normal threshold for his age, patient right threshold = O, patient left threshold = X.

III.4 had progressive hearing loss and required a hearing aid. The ocular phenotype of II.4 and III.4 was consistent with carrier status for XLRP. Unaffected members of this family had no symptoms of either the ocular or systemic phenotype described.

The phenotype for this family is therefore XLRP associated with progressive hearing loss, sinusitis, and chronic recurrent chest infections.

Haplotype analysis and mutation screening

Since disease in this family appeared to be X linked through pedigree structure and phenotypic evaluation, we performed haplotype analysis using X chromosome markers to locate the disease gene interval. Haplotype analysis showed disease segregation between the markers DXS1214 and DXS986 spanning the *RP2* and *RPGR* genes. The *RP2* gene was screened for mutations by PCR exon amplification followed by direct sequencing.[11] *RP2* was considered a possible candidate since mutations were known to cause XLRP, and the encoded protein is ubiquitously expressed, with a potential role in tubulin biogenesis and hence ciliary function.[7 26] No mutations were identified in this gene. However, a polymorphism was identified in exon 3 (844C>T[18]) which did not segregate with the disease, thus refining the disease interval (fig 1).

Subsequently, *RPGR* was then analysed in our family.[8 9 10] We detected EST matches from olfactory and lung epithelial cells by BLAST and NIX analysis and UniGene database searches (http://www.ncbi.nlm.nih.gov/ and http://www.hgmp.mrc.ac.uk/) suggesting that this ubiquitously expressed gene is present in the tissues of interest. Sequence analysis of *RPGR* showed a 2 bp deletion in exon 8 (845-846delTG, fig 5). This frameshift mutation at residue 262, which segregates with disease, is predicted to introduce 19 new amino acids and a premature stop codon, resulting in a truncated protein of 280 residues. All exons upstream of exon 8 were also entirely sequenced to exclude the possibility of a second alteration, which may have a combinatory effect with the exon 8 mutation; no other alterations were detected.

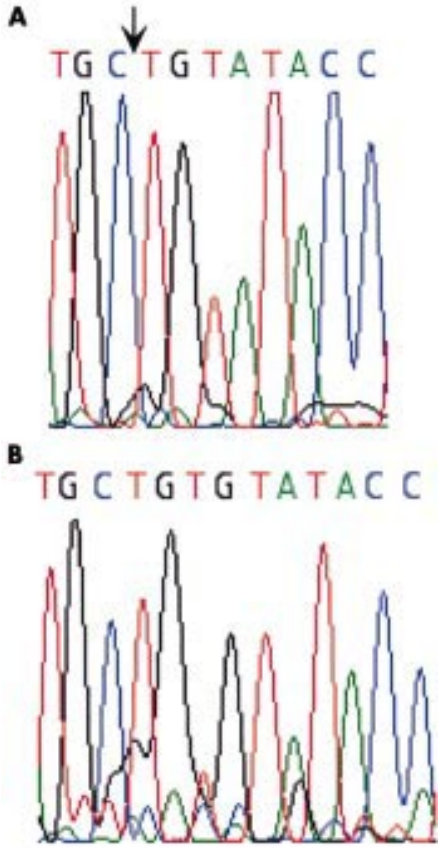

Figure 5 Electropherograms depicting patient mutation and wild type sequence in exon 8 of the *RPGR* gene. (A) TG deletion at nucleotide position 845-846 in an affected male from family 4462. (B) Wild type sequence from a population sample of the corresponding region of *RPGR*.

It is likely that the genetic background of subjects within this pedigree contributes to the additional systemic phenotypes observed. Since overlapping symptoms have been observed in numerous XLRP patients and at least one other XLRP pedigree (see Discussion), we hypothesised that a predisposing locus could be closely linked to *RPGR* on the X chromosome. The *TCTE1L* gene is approximately 500 kb distal to *RPGR* and has been shown to be expressed in lung, trachea, kidney, and brain, among other tissues and detects ESTs from olfactory and lung epithelial cells and the organ of Corti[25 28] (BLAST and UniGene searches at http://www.ncbi.nlm.nih.gov/). The TCTE1L protein forms part of the cytoplasmic dynein light chain of the microtubule motor complex, and may be involved in tissue specific cargo binding activities since other members of this protein family mediate specific interactions, for example, with rhodopsin.[29 30] The *TCTE1L* gene therefore presented an attractive positional and functional modifier locus for the phenotype described. To determine if a linked locus could predispose subjects to susceptibility to the systemic pathology observed, we screened the five exons of the *TCTE1L* gene for cSNPs and no polymorphic variants were detected in the affected males.

DISCUSSION

Phenotypic overlap with other syndromes

The systemic phenotype in this family has similarities with those observed in immotile cilia syndrome (ICS1) or primary ciliary dyskinesia (PCD, MIM 242650). PCD is a congenital respiratory disease characterised by impaired mucociliary clearance caused by cilia ultrastructural abnormalities.[31 32] PCD patients suffer from chronic bronchiectasis and sinusitis, usually associated with male infertility, but do not reportedly suffer from recurrent ear infections or deafness.[33] Approximately half of the patients with PCD also display situs inversus (Kartagener syndrome, MIM 244400). One causative gene for PCD has recently been identified, *DNAI1*, a dynein intermediate chain gene on chromosome 9p13-21, with mutations in this gene shown to cause axoneme ultrastructural abnormalities in two families.[34] Genetic and phenotypic heterogeneity are features of PCD, with a locus identified on chromosome 5p14-15,[34 35] and potential linkage to 11 other chromosomal intervals.[36] However, no linkage to the X chromosome or association with RP and deafness has previously been reported.

Usher syndrome is defined by an association of sensorineural deafness with RP, with three distinct clinical subtypes (I, II, and III) of variable severity and extensive genetic heterogeneity.[37] Usher syndrome is the most frequent cause of sensorineural deafness accompanied by blindness, although two of the causative genes have also been implicated in isolated deafness[38] or isolated retinitis pigmentosa.[39]

Although hearing loss in our family is associated with retinitis pigmentosa, the mode of inheritance, nature, and onset of hearing loss, and chronic infections leading to sinusitis and bronchitis distinguish the phenotype in this family from Usher syndrome types I, II, and III. Hearing loss in our family appears relatively mild and progressive with a sensorineural component, but the chronic infections suffered by the patients may also contribute to an acquired conductive hearing impairment. Unfortunately, we were not able to record bone conduction thresholds in this family, so we conclude that hearing loss is likely to be mixed, but not proven. It is difficult to say whether the patients have any loss of vestibular function without thorough neuro-otological evaluation, but family members did not report any dizziness/balance problems. The lack of reported symptoms may have been because of the progressive nature of the condition which allowed for vestibular compensation. The presence of an X linked form of Usher-like phenotype has been suggested, but no locus on the X chromosome has been described. In one report, however, retinitis pigmentosa with deafness (described as Usher syndrome) was associated with bronchiectasis and immotile cilia syndrome, and the possibility of an X linked mode of inheritance could not be excluded.[40]

The major sites of pathology in this new phenotype, causing hearing loss and other disabling systemic abnormalities in association with XLRP, suggest structural, degenerative, or developmental kinociliary defects.

RPGR mutations and ciliary abnormalities

Several lines of evidence support our findings that mutant *RPGR* causes XLRP with associated generalised cilia abnormalities. In 1992, Van Dorp *et al*[41] reported a family who suffered from XLRP with associated susceptibility to respiratory infections in the majority of affected males. The patients suffered from recurrent bronchitis and sinusitis, described as indistinguishable from immotile cilia syndrome, but did not suffer sterility or deafness. In a subsequent publication, a mutation was identified in this family in the *RPGR* gene, namely a G to T transversion at position +1 of the 5′ donor splice site of intron 5, predicted to result in aberrant splicing.[42] This additional phenotype, reported by Van Dorp *et al*,[41] overlaps the one described here with the exception of the associated hearing loss, present in our family. Independent studies describing the prevalence of deafness in association with RP[43] identified a group of patients that did not fit into previously described clinical categories (that is, not Usher syndrome). Hearing impairment in three families with XLRP was reported by Rosenberg *et al*,[43] and a mutation in *RPGR* had previously been identified in one of these families.[9 44] This mutation is described as a 6.4 kb deletion which disrupts the

3′ end of *RPGR* removing the last six exons. Both affected males and carrier females from this family had hearing difficulties.[43]

Other studies have centred around examination of cilia in patients with RP. Several reports examining nasal mucosa and sperm in heterogeneous groups of patients suggest increased incidence of abnormal cilia in XLRP patients.[45–47] However, the molecular basis for these observations remains undetermined.

Although the physiological role of RPGR in retina and other tissues is not fully understood, compelling evidence for the importance of RPGR in ciliary function comes from recent studies of animal models and the identification of RPGR interacting proteins.[15–20] It is possible, therefore, that other cell specific ciliary proteins exist in the lung, trachea, inner ear, and nasal passages which bind RPGR, and that this interaction may be compromised in the family described in this report.

Mutation type and modifiers of phenotype

The novel mutation we have identified results in partial loss of the RCC1 domain (exon 8, 845-846delTG) and downstream sequence. This protein truncation mutation occurs upstream of many other protein truncation and missense mutations previously reported to cause XLRP. It is unclear, however, why the phenotype of this protein truncation mutation is different from others reported to result in only an ocular phenotype.[48] Perhaps persistent but milder systemic infections in other families remain undetected or may not have been reported, and exposure to infections and subsequent disease manifestation varies widely.

Mutations in the *RPGR* gene have also been detected in families with X linked cone-rod dystrophy and X linked macular degeneration (as opposed to the rod-cone degeneration observed in classical XLRP), widening the clinical spectrum associated with mutant *RPGR* and highlighting the fact that other factors modulate the phenotype.[49–51] The factor(s) underlying the significant variability of the pathogenic expression of *RPGR* remain to be identified.

The genotype at a particular locus may account for an inter-individual susceptibility that can both increase or decrease the risk to develop the pathology by modulating mechanisms involved in the pathogenesis. We hypothesised that a closely linked gene which segregated with the primary *RPGR* mutation could be acting as a modifier gene in this family, since association with the symptoms described in this report are more common than previously suspected. *TCTE1L* lies approximately 500 kb distal to RPGR and presents an interesting functional candidate which is expressed in the tissues involved in the systemic disease associated with XLRP. No cSNPs were identified. Predisposing SNPs may lie outside those gene regions tested, and other loci on the autosomes can not be excluded as predisposing factors; however, if autosomal SNPs are involved in disease expression, they are predicted to be common owing to the occurrence of disease in more than one pedigree. It is now essential to collect a cohort of families with these overlapping phenotypes to determine the factors involved in disease expression.

Further evidence for this new syndrome being primarily an *RPGR* gene disorder comes from colleagues who have identified a family with an almost identical phenotype, XLRP, hearing loss, and recurrent respiratory tract infections. On the basis of our findings, they examined the *RPGR* gene and found a missense mutation in exon 6 (Iannaccone *et al*, in preparation). The data show that the families are unrelated and that different mutations in *RPGR* can result in overlapping phenotypes implicating ciliary dysfunction in a variety of tissues. In addition, Iannaccone *et al* describe expression of RPGR in human cochlea and bronchial and sinus epithelial lining.

Future studies towards unravelling the function of RPGR in the retina will need to be expanded to include analyses of multiple ciliated epithelial tissues. The identification of RPGR binding partners within these tissues may identify other specific proteins capable of interacting with RPGR. It would be of interest to evaluate the mouse and dog models of *RPGR* disease[15 52] with a view to examining structure/function and development of the ciliated epithelium of the respiratory tract, sinuses, and inner ear, for example, in addition to re-evaluating the patients already described as harbouring *RPGR* mutations as a cause of XLRP.

SUMMARY

In conclusion, we describe a new phenotype of typical X linked retinitis pigmentosa associated with hearing loss, chronic respiratory tract infections, and sinusitis caused by a mutation in *RPGR*. The systemic phenotypes are predicted to be variable, accentuated by repeated infections of the respiratory tract and consequent upon impaired mucociliary clearance (as described for PCD). Phenotypic variation between families may be caused by *RPGR* mutation type, genetic background, environmental effects, or a combination of these factors. Additional families will need to be investigated for SNPs on the X chromosome in proximity to *RPGR* to explore fully any phenotypic modification caused by adjacent loci. *RPGR* and interacting partners involved in kinociliary function in a variety of tissues may also represent attractive candidate genes for other phenotypes such as primary ciliary dyskinesia or isolated hearing loss.

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