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ORIGINAL INVESTIGATION

Next-generation sequencing-based molecular diagnosis of 82 retinitis pigmentosa probands from Northern Ireland

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Abstract Retinitis pigmentosa (RP) is a group of inherited retinal disorders characterized by progressive photoreceptor degeneration. An accurate molecular diagnosis is essential for disease characterization and clinical prognoses. A retinal capture panel that enriches 186 known retinal disease genes, including 55 known RP genes, was developed. Targeted next-generation sequencing was performed for a cohort of 82 unrelated RP cases from Northern Ireland, including 46 simplex cases and 36 familial cases. Disease-causing mutations were identified in 49 probands, including 28 simplex cases and 21 familial cases, achieving a solving rate of 60 %. In total, 65 pathogenic mutations were found, and 29 of these were novel. Interestingly, the molecular information of 12 probands was neither consistent with their initial inheritance pattern nor clinical diagnosis. Further clinical reassessment resulted in a refinement of the clinical diagnosis in 11 patients. This is the first study to apply next-generation sequencing-based, comprehensive molecular diagnoses to a large number of RP probands from Northern Ireland. Our study shows that molecular information can aid clinical diagnosis, potentially changing treatment options, current family counseling and management.

L. Zhao and F. Wang contributed equally to this work.

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Introduction

Retinitis pigmentosa (RP; MIM#268000) refers to a group of inherited retinal diseases characterized by progressive photoreceptor apoptosis and retinal degeneration. RP is the most common form of hereditary retinal degeneration with a prevalence of approximately 1:3,500 to 1:4,000 (Wang et al. 2005; Haim 2002) affecting more than one million individuals worldwide (Chang et al. 2011). The typical clinical manifestations of RP include night blindness and tunnel vision. Some patients may eventually develop complete blindness. The phenotype of RP usually occurs alone, as nonsyndromic RP affecting only the eye. In some rare cases, RP can also be accompanied with other

clinical symptoms affecting additional organs. For example, patients with Usher syndrome suffer both RP and hearing loss. RP is a highly genetically heterogeneous disease. First, more than 50 genes are known to be associated with nonsyndromic RP (RetNet; http://www.sph.uth.tmc.edu/Retnet/) and nearly 3,100 pathogenic mutations have been reported (Chang et al. 2011). Second, the inheritance pattern of RP involves all modes: autosomal-dominant (adRP), autosomal-recessive (arRP), X-linked (xlRP), and digenic forms (Anasagasti et al. 2013; Neveling et al. 2012; Kajiwara et al. 1994). Third, the molecular basis of RP overlaps with other retinal diseases. Different mutations in the same genes, or sometimes even the exact same mutations, can cause different retinal diseases (Wang et al. 2014).

Because of the heterogeneity of RP, accurate molecular diagnosis is essential for meaningful patient counseling as it can provide specific disease characterization and prognostic information. Hitherto, standard methods of genetic testing for RP include Sanger sequencing, arrayed primer extension (APEX) and next-generation sequencing (NGS). Sanger sequencing is the gold standard of sequencing, however, it is costly for large-scale sequencing. APEX only analyzes previously reported mutation loci and thus misses novel mutations, leading to a low diagnosis rate (Avila-Fernandez et al. 2010; Zeitz et al. 2009). NGS is currently considered the most efficient method for mutation screening. One approach of NGS is target sequencing, which limits testing to known disease-causing genes. For instance, our laboratory has developed a retinal capture panel to systematically test over 150 known retinal disease genes for pathogenic mutations in RP and Leber congenital amaurosis patients (Wang et al. 2013, 2014). The NGS-based targeted sequencing is superior in both time and cost compared to other methods, which makes it an optimal approach for the molecular diagnosis of RP.

It is known that the prevalence of causative genes and the mutation spectrum can vary significantly among different ethnicity groups. This is especially notable in relatively isolated populations or those with a higher consanguineous rate. For example, in Israeli and Palestinian patient populations, *FAM161A* mutations account for about 12 % of arRP cases (Bandah-Rozenfeld et al. 2010), whereas in North America *FAM161A* is responsible for only 1 % of arRP cases (Venturini et al. 2014). Furthermore, within a certain ethnic background, the frequency of a specific mutant allele may vary geographically. As an example, the well-known c.2299delG, p.(Glu767Serfs) mutation in *USH2A* is frequently found in European patients. This mutation accounts for 47.5 % of *USH2A* alleles in Denmark (Dreyer et al. 2008), while the allelic frequency is 31 % in the Netherlands (Pennings et al. 2004) and 10 % in France (Aller et al. 2010). The mutation frequency may become common as a result of the founder effect and may change due to genetic drift. Therefore, characterizing the mutation spectrum of a certain RP cohort can provide more comprehensive knowledge of the disease.

In this study, we performed NGS-based targeted sequencing in 82 unrelated RP cases from Northern Ireland; 46 were simplex cases and 36 were familial cases. The capture panel covered 55 RP genes and 131 other retinal disease genes. To our knowledge, this is the first study that performed NGS-based comprehensive molecular diagnosis on a large number of RP probands from Northern Ireland. Our study demonstrated that an NGS-based molecular diagnosis can facilitate a clinical diagnosis that better defines the disease and helps with family planning and patient management.

Materials and methods

Clinical diagnosis and sample collection

A cohort of 82 RP patients and other family members were ascertained at the Department of Ophthalmology (BHSCT) and Centre for Experimental Medicine (Belfast, UK). All patients had a detailed clinical history and underwent full ophthalmic evaluation including visual acuity testing, visual fields testing, fundal examination, and electroretinography. Retinitis pigmentosa was diagnosed on the basis of the typical fundal features (bone spicule retinal pigmentation, arteriolar attenuation, and optic disc pallor), visual field constriction, and an attenuated or abolished electroretinogram. Pedigrees are constructed based on interview. Available additional family members both affected and unaffected were also recruited. Genomic DNA of patients was extracted from peripheral blood. The research was conducted in accordance with the Tenets of the declaration of Helsinki. Ethical permission was granted through ORECNI and all patients gave written consent to participate in the study.

Retinal capture panel design

A capture panel of retinal disease genes was designed by our group which has been successfully applied for the molecular diagnosis of RP and Leber congenital amaurosis patients (Wang et al. 2013, 2014; Fu et al. 2013a). In this study, we updated the capture panel to include 23 newly reported retinal disease genes. The panel consisted of 994,088 bp covering 3,720 exons in 186 known retinal disease genes (RetNet; http://www.sph.uth.tmc.edu/Retnet/), including 55 known RP genes that had been reported at the time of panel design (Table S1).

Library preparation and capture sequencing

Pre-capture Illumina paired-end libraries were generated according to the manufacturer's protocol. Briefly, ~1 µg of patient's genomic DNA was sheared into 300–500 bp fragments. The DNA fragments were end-repaired and a single adenine base was added to the 3′ ends using Klenow exonuclease. Illumina Y-shape index adapters were ligated to the repaired ends, and DNA fragments were PCR amplified for eight cycles after ligation. The DNA libraries were quantified by the PicoGreen fluorescence assay kit (Invitrogen, Carlsbad, CA, USA). In each capture reaction, 50 pre-capture DNA libraries were pooled together. The targeted DNA was captured, washed and recovered using Agilent Hybridization and Wash Kits (Agilent Technologies, Santa Clara, CA, USA). Captured libraries were sequenced on Illumina HiSeq 2000 (Illumina, San Diego, CA, USA) as 100 bp paired-end reads, following the manufacturer's protocol.

Bioinformatics analysis

Paired-end sequencing reads were obtained for each sample. Reads were mapped to human reference genome hg19 using Burrows–Wheeler Aligner (BWA version 0.6.1) (Li and Durbin 2009). Base quality recalibration and local realignment were performed using the Genome Analysis Tool Kit (GATK version 1.0.5974) (McKenna et al. 2010). AtlasSNP and AtlasIndel2 (Challis et al. 2012) were used to call single-nucleotide polymorphisms (SNPs) and small insertions and deletions (INDELs).

Since RP is a rare Mendelian disease, polymorphisms that appear at a higher than 0.5 % frequency (for recessive variants) or 0.1 % frequency (for dominant variants) in at least one of the following databases were considered too frequent to be pathogenic and therefore excluded from further analysis: the 1000 Genome (build 20110521 and 20101123) (Genomes Project C et al. 2010, 2012), dbSNP135 (Sherry et al. 2001), NHLBI exome sequencing database (Fu et al. 2013b), NIEHS exome sequencing database (Genomes Project C et al. 2010), and our internal control databases. After frequency-based filtering, ANNOVAR (Wang et al. 2010) was used to predict protein-coding changes and filter out synonymous variants. Furthermore, mutations known to cause retinal diseases were identified by searching against HGMD professional database (Stenson et al. 2013). Finally, dbNSFP (version 2.3) (Liu et al. 2013), a program that compiles prediction scores from six prediction algorithms [SIFT (Ng and Henikoff 2003), Polyphen2 (Adzhubei et al. 2010), LRT (Chun and Fay 2009), MutationTaster (Schwarz et al. 2010), MutationAssessor (Reva et al. 2011) and FATHMM (Shihab et al. 2013)] and three conservation scores [Phylop (Siepel et al. RECOMB 2006), GERP++ (Davydov et al. 2010) and Siphy (Garber et al. 2009; Lindblad-Toh et al. 2011)], was used to predict the pathogenicity of novel missense variants. The details of the method are described in supplementary material. The prediction of novel missense variants is listed in Table S2.

Causative mutation prioritization

For each patient, we looked for causative variants using the following prioritization strategy:

1. Reported pathogenic variants in RP genes.
2. Novel severe loss-of-function (LOF) variants (stop-gain, splicing, frameshift, fail-to-start) in RP genes.
3. Novel missense variants in RP genes. The missense variants must be predicted to be deleterious by dbNSFP as described in the Sect. "Materials and methods".
4. Pathogenic variants in other retinal disease genes.

All the variants should be consistent with the known pattern of inheritance of the respective gene (i.e., homozygous/compound heterozygous for recessive genes and heterozygous for dominant genes). For the familial cases, we specifically looked for variants in genes that matched the inheritance patterns predicted from the pedigrees.

Sanger sequencing validation and family segregation test

All putative mutations identified by NGS were validated using Sanger sequencing and tested for co-segregation if additional affected family members are available. Primers were designed using Primer3 (Rozen and Skaletsky 2000). To ensure the quality of Sanger sequencing, the amplicons were designed to have a boundary around 100 bp away from the mutation. Then the amplicons (~400 bp) were Sanger sequenced on Applied BioSystems (ABI) 3,730 × 1 capillary sequencer (Applied Biosystems Inc., Foster City, CA, USA). The Sanger sequencing results were analyzed using Sequencher (version 5.0).

Results

82 Unrelated Northern Ireland families with RP patients were recruited

A total of 82 well-characterized RP families from Northern Ireland were recruited for this study. Among these families, 36 had two or more affected members, while the remaining 46 with only one affected member are considered as simplex cases. Based on the pedigree information, 26.8 % (22/82) of the families were arRP, 13.4 % (11/82) of the

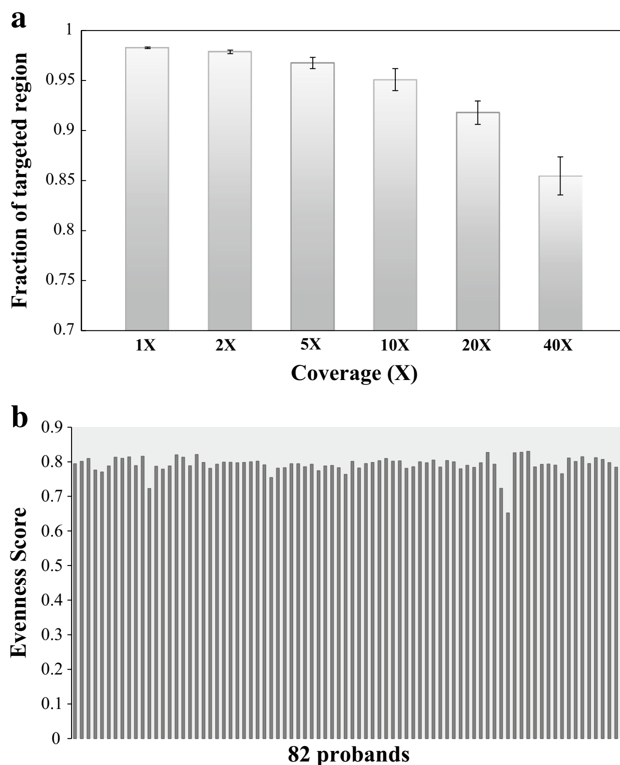

Fig. 1 High-quality next-gen sequencing results were obtained. **a** Coverage distribution plot shows the fraction of targeted region (*y* axis) covered by at least certain coverage (*x* axis). **b** The evenness scores of capture sequencing results from 82 RP probands

families were adRP, 3.7 % (3/82) were xlRP, and 56.1 % (46/82) were simplex.

High-quality NGS results were obtained

Capture NGS was performed on all 82 RP families. DNA from one affected member of each family was selected, captured and sequenced. Within the design region, an average of 141× coverage was achieved for all samples. 95.1 % of bases had coverage of >10×, 91.8 % of bases had coverage of >20× and 85.5 % of bases had coverage of >40×, indicating that sufficient coverage was achieved to enable high variant detection sensitivity (Fig. 1a). To test if the coverage of target region was evenly distributed, an evenness score was calculated for each sample as described previously (Fig. 1b) (Mokry et al. 2010). On average, the evenness score for all the 82 probands was 0.8, suggesting a nearly uniform distribution was achieved.

Pathogenic mutations were identified in 49 probands

An average of 732 variants, including 672 SNPs and 60 small INDELs, were initially identified for each sample in the targeted region. After all filtering and annotation steps (see Sect. "Materials and methods"), an average of 8.2 SNPs and 1.8 INDELs per patient remained and were therefore considered as candidate pathogenic variants. Through the mutation prioritization procedure (see Sect. "Materials and methods"), we identified pathogenic mutations in 49 probands, including 28 simplex cases and 21 familial cases, and achieved a solving rate of 60 % (49/82) (Tables 1 and 2).

Simplex cases

Out of the 46 simplex RP cases, 28 (61 %) were identified as carrying pathogenic or putative pathogenic mutations in known retinal disease genes. Overall, 41 mutations were identified in the simplex RP cases and 20 of them were novel. Among these novel mutations, six were LOF mutations, including four frameshift and two nonsense mutations. The remaining fourteen were novel missense variants that passed multiple frequency-based filters and were predicted to be pathogenic by dbNSFP (Table S2). Genotypes of the patients are detailed in Table 1.

According to the identified mutations, the inheritance pattern of two of the simplex probands was autosomal dominant (proband Rp25, proband Rp29), two probands were X-linked cases (proband Rp349B, proband Rp232A) rather than simplex, and the remaining 24 probands carry mutations in autosomal-recessive genes. In most cases, the diagnosis of simplex RP is strongly biased towards a recessive model; however, it is possible that the simplex cases are due to mutations in dominant RP genes. For proband Rp25 further assessment of family members was carried out. Both Rp25′s parents were deceased but reported as unaffected. However, a history of blindness was reported in the paternal grandfather and two great-uncles, making the inheritance pattern likely to be autosomal dominant.

A total of 16 causative genes were observed in our simplex cohort. The most prevalent mutated gene was *USH2A*, which explained disease in eight probands. Among the 16 causative genes, eight of them are known RP genes, which accounted for 18 (64 %) simplex cases. Interestingly, pathogenic mutations in eight other retinal disease genes (*CDH23*, *VPS13B*, *MYO7A*, *CLRN1*, *RS1*, *CACNA1F*, *PHYH*, and *NPHP4*) were found in 10 (36 %) probands, including five previously reported alleles, two novel LOF alleles, and eight novel missense alleles. The minor allele frequency (MAF) and pathogenicity predictions for the novel missense alleles are listed in Table S2. For these 10 simplex probands, the molecular information is inconsistent with the original clinical diagnosis. This could be due to the difficulty of assigning a more precise clinical diagnosis at the time of the initial visit, or a novel genotype–phenotype correlation as proposed in Wang et al. (2014).

Table 1 Summary of 28 simplex cases carrying pathogenic mutations

ID	Type	Gene	NM ID	Genotype	cDNA change	Protein change	References
Probands carrying pathogenic mutations in known RP genes							
Rp44	Simplex	*ABCA4*	NM_000350	Homozygous	c.2617T > C	p.(Phe873Leu)	(Webster et al. 2001)
Rp14	Simplex	*ABCA4*	NM_000350	Homozygous	c.161G > A	p.(Cys54Tyr)	(Lewis et al. 1999)
Rp171	Simplex	*ABCA4*	NM_000350	Heterozygous	c.1805G > A	p.(Arg602Gln)	(Briggs et al. 2001)
				Heterozygous	c.4469G > A	p.(Cys1490Tyr)	(Wiszniewski et al. 2005; Sun et al. 2000)
Rp141	Simplex	*ABCA4*	NM_000350	Heterozygous	c.3352C > G	p.(His1118Asp)	Novel
				Heterozygous	c.1317G > A	p.(Trp439*)	(Rivera et al. 2000; Fujinami et al. 2013a)
Rp167	Simplex	*CNGB1*	NM_001297	Homozygous	c.2957A > T	p.(Asn986Ile)	(Simpson et al. 2011)
Rp170	Simplex	*USH2A*	NM_206933	Heterozygous	c.4645C > T	p.(Arg1549*)	(Baux et al. 2007)
				Heterozygous	c.9371 + 1G > C	p.(?)	(Le Quesne Stabej et al. 2012)
RD1200002	Simplex	*USH2A*	NM_206933	Heterozygous	c.4714C > T	p.(Leu1572Phe)	(Song et al. 2011)
				Heterozygous	c.8740C > T	p.(Arg2914*)	(McGee et al. 2010)
				Heterozygous	c.2299delG	p.(Glu767Serfs)	(Eudy et al. 1998; Aller et al. 2010)
Rp311B	Simplex	*USH2A*	NM_206933	Heterozygous	c.4714C > T	p.(Leu1572Phe)	(Song et al. 2011)
				Heterozygous	c.3309C > A	p.(Tyr1103*)	Novel
				Heterozygous	c.2299delG	p.(Glu767Serfs)	(Eudy et al. 1998; Aller et al. 2010)
Rp400B	Simplex	*USH2A*	NM_206933	Heterozygous	c.2276G > T	p.(Cys759Phe)	(Rivolta et al. 2000)
				Heterozygous	c.9371 + 1G > C	p.(?)	(Le Quesne Stabej et al. 2012)
				Heterozygous	c.4618G > A	p.(Asp1540Asn)	Novel
Rp159	Simplex	*USH2A*	NM_206933	Heterozygous	c.4714C > T	p.(Leu1572Phe)	(Song et al. 2011)
				Heterozygous	c.2299delG	p.(Glu767Serfs)	(Eudy et al. 1998; Aller et al. 2010)
				Heterozygous	c.4106C > T	p.(Ser1369Leu)	(Cremers et al. 2007)
Rp87	Simplex	*USH2A*	NM_206933	Heterozygous	c.13094G > A	p.(Trp4365*)	Novel
				Heterozygous	c.4106C > T	p.(Ser1369Leu)	(Cremers et al. 2007)
Rp4	Simplex	*USH2A*	NM_206933	Heterozygous	c.1813T > C	p.(Cys605Arg)	Novel
				Heterozygous	c.10073G > A	p.(Cys3358Tyr)	(McGee et al. 2010)
Rp86	Simplex	*USH2A*	NM_206933	Heterozygous	c.4714C > T	p.(Leu1572Phe)	(Song et al. 2011)
				Heterozygous	c.10073G > A	p.(Cys3358Tyr)	(McGee et al. 2010)
				Heterozygous	c.2299delG	p.(Glu767Serfs)	(Eudy et al. 1998; Aller et al. 2010)
Rp182	Simplex	*PDE6B*	NM_000283	Heterozygous	c.2116A > T	p.(Lys706*)	(McLaughlin et al. 1995)
				Heterozygous	c.299G > A	p.(Arg100His)	(Neveling et al. 2012)
Rp244	Simplex	*RP1*	NM_006269	Heterozygous	c.5673G > T	p.(Leu1891Phe)	Novel
				Heterozygous	c.2826_2827insA	p.(Ser943Lysfs)	Novel
Rp1	Simplex	*LRAT*	NM_004744	Heterozygous	c.569G > A	p.(Arg190His)	Novel
				Heterozygous	c.298G > T	p.(Gly100Cys)	Novel

Table 1 continued

ID	Type	Gene	NM ID	Genotype	cDNA change	Protein change	References
Rp29	Simplex	*IMPDH1*	NM_000883	Heterozygous	c.968A > G	p.(Lys323Arg)	(Wada et al. 2005)
Rp25	Simplex	*PRPF31*	NM_015629	Heterozygous	c.772_773del2insCAACATGCAACATCAT	p.(Thr258Glnfs)	Novel
Probands carrying pathogenic mutations in other retinal disease genes							
Rp78	Simplex	*CDH23*	NM_022124	Homozygous	c.5237G > A	p.(Arg1746Gln)	(Bolz et al. 2001)
Rp112	Simplex	*CDH23*	NM_022124	Heterozygous	c.8878G > A	p.(Val2960Ile)	Novel
				Heterozygous	c.419G > A	p.(Arg140His)	Novel
Rp399A	Simplex	*CDH23*	NM_022124	Heterozygous	c.7466G > A	p.(Arg2489His)	Novel
				Heterozygous	c.5237G > A	p.(Arg1746Gln)	(Bolz et al. 2001)
Rp83	Simplex	*VPS13B*	NM_017890	Heterozygous	c.6732 + 1G > A	p.(?)	(Kolehmainen et al. 2004)
				Heterozygous	c.11746_11747del	p.(Ala3917Thrfs)	Novel
Rp41	Simplex	*MYO7A*	NM_000260	Heterozygous	c.631A > G	p.(Ser211Gly)	Novel
				Heterozygous	c.2904G > T	p.(Glu968Asp)	(Bharadwaj et al. 2000)
Rp76	Simplex	*CLRN1*	NM_174878	Homozygous	c.190G > A	p.(Gly64Arg)	Novel
Rp349B	Simplex	*RS1*	NM_000330	Hemizygous	c.520C > T	p.(Arg174Trp)	Novel
Rp232A	Simplex	*CACNA1F*	NM_005183	Hemizygous	c.2237A > C	p.(Asn746Thr)	Novel
Rp58	Simplex	*PHYH*	NM_001037537	Homozygous	c.403G > A	p.(Gly135Arg)	Novel
Rp131	Simplex	*NPHP4*	NM_015102	Heterozygous	c.3859C > G	p.(Gln1287Glu)	(Hoefele et al. 2005)
					c.3506delC	p.(Pro1169Glnfs)	Novel

Table 2 Summary of 21 familial cases carrying pathogenic mutations

ID	Type	Gene	NM ID	Genotype	cDNA change	Protein change	References
Probands carry pathogenic mutations in known RP genes							
Rp113	arRP	*ABCA4*	NM_000350	Homozygous	c.3211_3212insGT	p.(Ser1071Cysfs)	(Allikmets et al. 1997)
Rp105	arRP	*ABCA4*	NM_000350	Homozygous	c.2041C > T	p.(Arg681*)	(Maugeri et al. 1999)
Rp125	arRP	*ABCA4*	NM_000350	Heterozygous	c.6416G > C	p.(Arg2139Pro)	Novel
				Heterozygous	c.1519G > T	p.(Asp507Tyr)	(Fujinami et al. 2013b)
Rp375B	arRP	*ABCA4*	NM_000350	Heterozygous	c.161G > A	p.(Cys54Tyr)	(Green et al. 1999)
					c.43_48del6insC	p.(Trp15Alafs)	Novel
Rp124	arRP	*BBS1*	NM_024649	Homozygous	c.1169T > G	p.(Met390Arg)	(Estrada-Cuzcano et al. 2012)
Rp79	arRP	*CRB1*	NM_201253	Heterozygous	c.2129A > T	p.(Glu710Val)	(Clark et al. 2010)
				Heterozygous	c.2234C > T	p.(Thr745Met)	(den Hollander et al. 1999)
Rp73	arRP	*CERKL*	NM_201548	Homozygous	c.847C > T	p.(Arg283*)	(Tuson et al. 2004)
Rp128	arRP	*CERKL*	NM_201548	Homozygous	c.847C > T	p.(Arg283*)	(Tuson et al. 2004)
Rp69	arRP	*IMPG2*	NM_016247	Homozygous	c.829-1G > T	p.(?)	Novel
Rp116	arRP	*PROM1*	NM_006017	Heterozygous	c.1355_1356insT	p.(Tyr453Leufs)	Novel
				Heterozygous	c.622delA	p.(Thr208Leufs)	Novel
Rp107	arRP	*USH2A*	NM_206933	Heterozygous	c.14453C > T	p.(Pro4818Leu)	(Aller et al. 2006)
				Heterozygous	c.3187_3188del	p.(Gln1063Serfs)	(Seyedahmadi et al. 2004)
Rp229	arRP	*USH2A*	NM_206933	Heterozygous	c.10073G > A	p.(Cys3358Tyr)	(McGee et al. 2010)
				Heterozygous	c.14458_14505del	p.(Ala4820_Pro4835del)	Novel
Rp55	arRP	*USH2A*	NM_206933	Heterozygous	c.769G > A	p.(Gly257Arg)	(Le Quesne Stabej et al. 2012)
				Heterozygous	c.2276G > T	p.(Cys759Phe)	(Rivolta et al. 2000)
Rp114	arRP	*PDE6B*	NM_000283	Homozygous	c.1547T > C	p.(Leu516Pro)	(Clark et al. 2010)
Rp289	arRP	*PDE6B*	NM_000283	Heterozygous	c.1895T > C	p.(Phe632Ser)	Novel
				Heterozygous	c.2116A > T	p.(Lys706*)	(McLaughlin et al. 1995)
Rp142	adRP	*SNRNP200*	NM_014014	Heterozygous	c.2042G > A	p.(Arg681His)	(Benaglio et al. 2011)
RD1200008	adRP	*PRPH2*	NM_000322	Heterozygous	c.1A > T	p.(Met1Leu)	Novel
Rp181	xlRP	*RP2*	NM_006915	Hemizygous	c.352C > T	p.(Arg118Cys)	(Bader et al. 2003)
Rp296	xlRP	*RPGR*	NM_000328	Hemizygous	c.778 + 1G > C	p.(?)	(Shu et al. 2007)
Probands carry pathogenic mutations in other retinal disease genes							
Rp278B	adRP	*PITPNM3*	NM_031220	Heterozygous	c.1878G > C	p.(Gln626His)	(Kohn et al. 2007)
Rp150	xlRP	*CHM*	NM_000390	Heterozygous	c.498_499del	p.(Leu167Argfs)	Novel

To further investigate the two possibilities for these probands, we either reviewed the available clinical data and imaging or performed further clinical assessment. One proband (Rp131) was confirmed to be affected by RP, while the rest of nine probands (Rp78, Rp112, Rp399A, Rp83, Rp41, Rp76, Rp349B, Rp232A, and Rp58) were re-diagnosed to other retinal diseases (Table S4).

Proband Rp131 who carries compound heterozygous mutations in *NPHP4* remained a diagnosis of RP after clinical reassessment. Mutations in *NPHP4* are associated with nephronophthisis type 4, a renal disease, and with Senior–Loken syndrome type 4, a combination of nephronophthisis and retinitis pigmentosa (Hoefele et al. 2005; Otto et al. 2002). However, Rp131 did not show any clinical signs of nephrolithiasis; therefore, the mutation in *NPHP4* must not be expressing clinically in the kidneys in this patient, and proband Rp131 was confirmed as RP.

Proband Rp58 is an interesting case of clinical re-diagnosis. The patient carries a putative pathogenic homozygous mutation c.403G > A, p.(Gly135Arg) in *PHYH*. *PHYH* was previously reported to cause Refsum disease (Jansen et al. 2004) which is characterized by early-onset RP with variable symptoms including, but not limited to, ataxia, neuropathy, hearing loss, and anosmia. Patients with Refsum disease usually have night blindness and retinal degeneration in their late childhood or early adulthood, and as the disease progresses, other symptoms may appear. Some patients will not develop other symptoms until their 40 or 50 s (Wanders et al. 1993). Therefore, it is very difficult to distinguish Refsum disease and RP if the disease is at the early stage. We revisited proband Rp58 and other available family members. Rp58 had developed mild cerebellar ataxia and hearing loss in later years. Interestingly, two sons of Rp58 showed learning disability and dyslexia

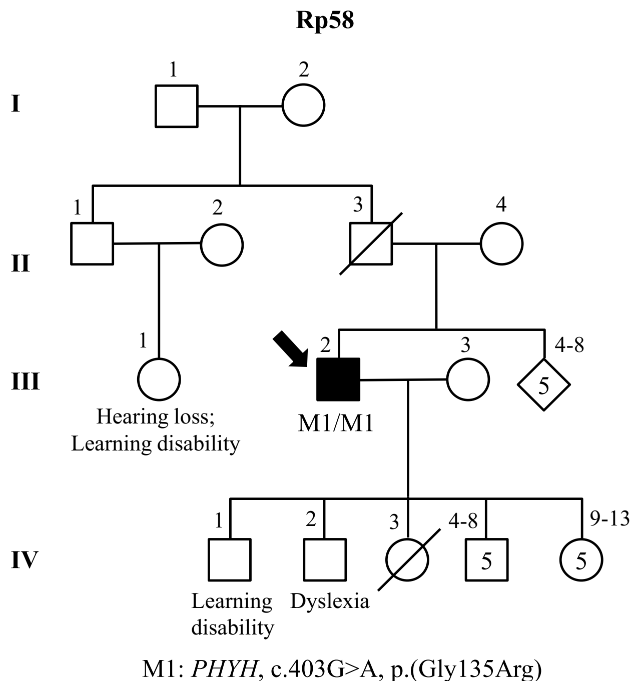

Fig. 2 Pedigrees and mutations of proband Rp58. The patient carried a putative pathogenic homozygous mutation c.403G > A, p.(Gly135Arg) in *PHYH*, and was refined to Refsum disease

(Fig. 2). Considering both the clinical reassessment and the molecular information, Rp58 was re-diagnosed to Refsum disease and dietary treatment was started.

Proband Rp83 carries compound heterozygous LOF mutations in *VPS13B*, which was reported to cause Cohen syndrome (Kolehmainen et al. 2004). The features of Cohen syndrome vary widely among affected individuals, and one of the features is retinal degeneration (Chandler et al. 2002), which is phenotypically similar to RP. We revisited patient Rp83 and other syndromic features were revealed, including learning difficulties, clumsiness, characteristic facial features, progressive retinochoroidal dystrophy, and myopia. Therefore, proband Rp83 was re-diagnosed to Cohen syndrome.

The remaining five re-diagnosed patients carry pathogenic mutations in genes that are known to cause Usher syndrome (Rp78 with *CDH23* mutations, Rp112 with *CDH23* mutations, Rp399A with *CDH23* mutations, Rp41 with *MYO7A* mutations, and Rp76 with *CLRN1* mutations). After clinical reassessment, all five probands were found to have a mild hearing loss in addition to RP, and were reclassified as Usher syndrome patients. Interestingly, patient Rp399A had posed a diagnostic difficulty. Although the patient had typical features of a pigmentary retinopathy, there was no history of nyctalopia. The patient's mother had contracted rubella while pregnant with patient Rp399A and the family was keen to establish definitively whether the patient had nonprogressive retinopathy due to rubella or whether this was an inherited progressive disorder for the purposes of genetic counseling.

Familial cases

Out of 36 familial cases, 21 probands (58 %) were identified as carrying putative pathogenic mutations in known retinal disease genes, as shown in Table 2. These 21 solved familial cases are from 15 arRP, three adRP and three xlRP families. For the 15 solved arRP cases, there were in total 22 variants identified, including 15 previously reported variants and seven novel variants. The seven novel variants included four LOF mutations, one nonframeshift deletion and two missense mutations. The novel missense variants were filtered with 0.5 % frequency in multiple control databases, and predicted to be pathogenic by dbNSFP (Table S2). Among the three solved adRP cases, two probands carry previously reported mutations and one proband (RD120008) carries a fail-to-start mutation in dominant RP gene *PRPH2*. For the three xlRP cases, two probands carry reported mutations known to cause RP and one proband carries a LOF mutation in *CHM*.

For some familial cases, the inheritance modes obtained from the pedigree did not match with the mutations identified in the patients. For example, proband Rp73 was initially classified as xlRP according to the pedigree (Fig. 3a), as all the 5 patients were male and none of the female family members were affected. Since this family was at risk of xlRP, the male offspring of a carrier mother has a 50 % chance of having the disease. To prevent the transmission of RP, the daughters of affected members were undergoing embryonic testing. However, with the molecular diagnosis, proband Rp73 was found to carry a reported homozygous stop-gain mutation in *CERKL* on chromosome 2, which suggested that proband Rp73 in fact had arRP. To confirm this finding, we performed segregation on this family. The segregation test was consistent with the molecular diagnosis, saving the family from performing taxing offspring selection.

In our familial cases, we identified pathogenic mutations in two genes that have not been previously linked to RP (*PIPTNM3*, *CHM*) but are known to cause other retinal dystrophies. To resolve these ambiguous cases, we reviewed the patients and performed a clinical reassessment. After revisiting the patients, they were re-diagnosed to other retinal diseases (Table S4).

In the case of proband Rp278B (Fig. 3b), the pedigree appeared to show adRP. We identified a known heterozygous mutation c.1878G > C, p.(Gln626His) in *PITPNM3*. The mutation was reported to cause autosomal-dominant cone dystrophy (Kohn et al. 2007) and patient Rp278B was re-diagnosed to dominant cone dystrophy. However, when we

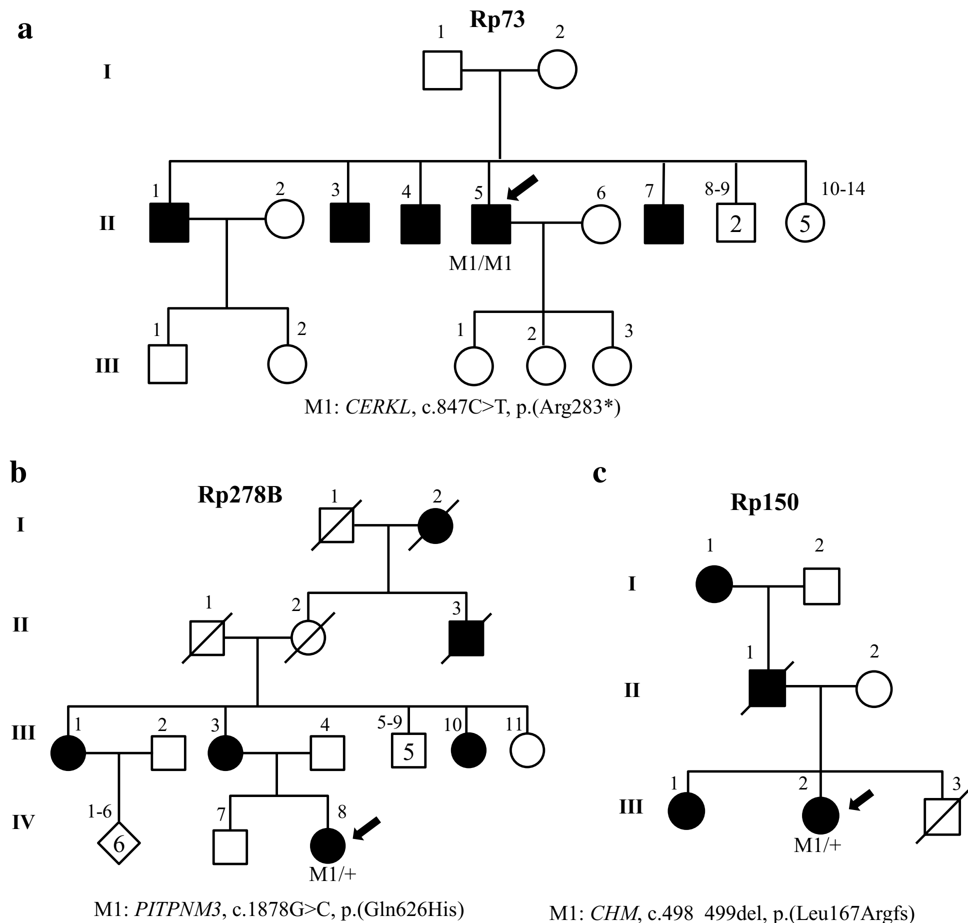

Fig. 3 Pedigrees and mutations of proband Rp73, Rp278B, and Rp150. **a** Proband Rp73 carried a homozygous mutation c.847C > T, p.(Arg283*) in *CERKL*, and was refined to arRP from xlRP. **b** Proband Rp278 carried a heterozygous mutation c.1878G > C, p.(Gln626His) in *PITPNM3*, and was refined to cone dystrophy. **c** Proband Rp150 carried a heterozygous mutation c.498_499del, p.(Leu167Argfs) in *CHM*, and was refined to choroideremia

performed a segregation test on other affected family members, this mutation was not shared by the patient's affected mother and aunts. One possible explanation is that the affected members of this family have different types of retinal diseases that are caused by different genetic mutations.

Another case is proband Rp150 (female) (Fig. 3c), which was identified as carrying a heterozygous frameshift mutation in *CHM*. Mutations in this gene are known to cause choroideremia, an X-linked eye disorder characterized by progressive degeneration of the choroid, retinal pigment epithelium, and retina. A hemizygous mutated male is fully affected while the female heterozygous carriers usually show mild fundus abnormalities (irregular pigmentation of the retinal periphery) which are typically subclinical. Yet, some female carriers may also develop the full choroideremia phenotype (van den Hurk et al. 1997; Francois 1971). Choroideremia can be confused with RP since both have symptoms of night blindness and tunnel vision. The difference is that the loss of vision in choroideremia often starts as an irregular ring that gradually expands both centrally and out toward the extreme periphery (Coussa and Traboulsi 2012). In our case, proband Rp150 might be a female choroideremia carrier.

Collectively, as shown in Tables 1 and 2, 65 pathogenic mutations were identified in 49 probands, including 28 simplex cases and 21 familial cases. Twenty-nine (44.6 %) of 65 pathogenic mutations identified were novel (Table 3). Most of these mutations were nonsynonymous (61.5 %) while a significant proportion is frameshift (16.9 %) and stop-gain (12.3 %). As shown in Table S4, among all simplex and familial RP cases, there are in total 12 probands showing inconsistency between the molecular information and the original clinical diagnosis. After clinical reassessment, 11 of 12 subjects were reclassified in terms of their retinal disease on the basis of the mutation analysis.

Discussion

In this study, we performed an NGS-based molecular diagnosis on 82 well-characterized RP probands from Northern Ireland, including 46 simplex cases and 36 familiar cases. Our method successfully solved 49 out of 82 probands, achieving a solving rate of 60 %.

Our results demonstrate that NGS-based molecular information can contribute to precise clinical diagnoses

Table 3 Classifications of all identified putative pathogenic mutations

	Novel	Previously reported
Missense	16	24
Frameshift	8	3
Stop-gain	2	6
Splicing	1	3
Fail-to-start	1	0
Nonframeshift	1	0
Total	29 (44.6 %)	36 (55.4 %)

enabling better disease management and accurate family counseling. Clinical manifestations of a number of retinal diseases are similar, especially for syndromic RP where some syndromes are late-onset and it can be difficult to distinguish these retinal diseases from nonsyndromic RP by clinical examination alone, even with a high index of clinical suspicion. Our approach can provide accurate molecular information to better define the disease manifestation. Patients with a precise diagnosis can then take advantage of any treatment available in a timely fashion. For example, Rp58 was re-diagnosed as Refsum disease. Unlike nonsyndromic RP, Refsum disease can be modified by diet, and preventative treatment can slow the neurological degeneration (Baldwin et al. 2010; Wanders et al. 1993); however, the clinical manifestations of Refsum disease are very subtle at an early stage. Therefore, a molecular diagnosis increases our understanding of how the patient's disease will progress and allows the possibility of an earlier diagnosis and treatment in other family members. Further, as shown by proband Rp73, the characterization of genetic defects can help with family birth planning to minimize the risk of transmitting the disease to offspring. Moreover, the molecular testing of patient Rp399A helps resolve the diagnostic dilemma which was due to a history of maternal rubella, and confirmed a diagnosis of Usher Syndrome with mutations in the *CDH23* gene. Finally, an accurate molecular diagnosis is the first step concerning eligibility for gene therapy (den Hollander et al. 2010).

It is also worth noting that simplex cases are often thought to be recessive since the parents of patients are assumed to be unaffected, however, 2/28 of our simplex cases were identified to carry heterozygous mutations in autosomal-dominant genes. One explanation could be a de novo mutation in the patient which results in only one affected member in the pedigree. It is also possible that patients carry dominant mutations inherited from their parents, but the mutation displays incomplete penetrance in the parents causing them not to manifest the disease phenotype. Here for example, proband Rp25 was identified to carry a heterozygous frameshift mutation in *PRPF31* which is known to cause dominant RP. Both parents of the patient were deceased but reported as unaffected, however, a history of blindness was reported in the paternal grandfather and great-uncles. This suggests patient Rp25 is very likely to be adRP, and the unaffected parents could be due to incomplete penetrance.

Our patient cohort has a different mutation spectrum from patient cohorts of other ethnicities. For instance, mutations in *EYS* were frequently found in Chinese RP cases (Wang et al. Unpublished data), while we observed no pathogenic mutations in *EYS*. Furthermore, recurrent mutations were identified in our cohort. The most frequent mutations were c.4714C > T, p.(Leu1572Phe) and c.2299delG, p.(Glu767Serfs) in *USH2A*, shared by 4 probands (RD1200002, Rp311B, Rp159, Rp86). The genotypes of these 4 probands around this region are listed in Table S5. The shared SNPs may suggest specific haplotypes and indicate the founder effect. Recent studies on Irish population history suggested that a large proportion of Irish population was originated from northern Spain. Interestingly, the *USH2A* haplotype identified in our cohort is also found to be widespread in Spanish RP and Usher patients (Najera et al. 2002; Aller et al. 2010), which supports the close link between Irish and Spanish population.

In our cohort, we were able to solve a significantly lower fraction of adRP than xlRP or arRP patients. One reason is for this is that it is difficult to confidently verify that lone novel missense mutations cause disease. In the cases where DNA of other affected members was not available, a segregation test could not be performed. As a result, we could not confidently report the candidate mutations. Among our adRP cases, we did identify novel putative pathogenic missense mutations in three adRP families (Table S3). We also identified some novel missense mutations with lower confidence levels in unsolved simplex cases that failed to pass our rigorous criteria.

About 35 % of our cases do not have even low confidence candidates. For these unsolved patients, we have made every effort to ensure accurate clinical diagnoses and although it is possible that some cases are phenocopies, this is unlikely given that all cases have been followed clinically for many years and all show progression of their disease with the expected electrophysiological findings. Another explanation for this is that the disease-causing genes were not included in our designed panel. Therefore, we are performing whole-exome sequencing on all negative cases, the results of which will be presented in a future manuscript. A further possibility is that the patients' phenotype is caused by novel disease genes. An additional explanation could be pathogenic intronic mutations that were not captured in our panel and copy number variations that were difficult to detect cause disease in these patients.

In summary, our approach identified the genetic cause of 60 % of disease in our patient cohort from Northern

Ireland. A total of 31 novel mutations were found. Our study indicated that molecular information can aid clinical diagnosis and help with patient treatment and management, particularly highlighted by three patients and their families (Rp58, Rp73 and Rp399A). Further improvements in NGS technology together with the discovery of novel RP genes will undoubtedly boost the success rate of NGS-based diagnostic approaches in RP in the future.

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Conflict of interest The authors declare no conflict of interest.

Ethical standards This research was conducted in accordance with the Tenets of the declaration of Helsinki. Ethical permission was granted through ORECNI and all patients gave written consent to participate in the study.

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