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Expanding the Clinical and Molecular Heterogeneity of Nonsyndromic Inherited Retinal Dystrophies

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A cohort of 172 patients diagnosed clinically with nonsyndromic retinal dystrophies, from 110 families underwent full ophthalmologic examination, including retinal imaging, electrophysiology, and optical coherence tomography, when feasible. Molecular analysis was performed using targeted next-generation sequencing (NGS). Variants were filtered and prioritized according to the minimum allele frequency, and finally classified according to the American College of Medical Genetics and Genomics guidelines. Multiplex ligation-dependent probe amplification and array comparative genomic hybridization were performed to validate copy number variations identified by NGS. The diagnostic yield of this study was 62% of studied families. Thirty novel mutations were identified. The study found phenotypic intra- and interfamilial variability in families with mutations in *C1QTNF5*, *CERKL*, and *PROM1*; biallelic mutations in *PDE6B* in a unilateral retinitis pigmentosa patient; interocular asymmetry RP in 50% of the symptomatic *RPGR*-mutated females; the first case with possible digenism between *CNGA1* and *CNGB1*; and a *ROM1* duplication in two unrelated retinitis pigmentosa families. Ten unrelated cases were reclassified. This study highlights the clinical utility of targeted NGS for nonsyndromic inherited retinal dystrophy cases and the importance of full ophthalmologic examination, which allows new genotype—phenotype associations and expands the knowledge of this group of disorders. Identifying the cause of disease is essential to improve patient management, provide accurate genetic counseling, and take advantage of gene therapy—based treatments. *(J Mol Diagn 2020, 22: 532—543; https://doi.org/10.1016/j.jmoldx.2020.01.003)*

Inherited retinal dystrophies (IRDs) are a group of disorders characterized by the progressive death of retinal pigment epithelium (RPE) cells and photoreceptors leading to loss of visual function and legal blindness. Although the prevalence of each disorder is low individually, they affect approximately 1 per 4000 individuals globally.[1]

Retinitis pigmentosa (RP) is the most common form of IRD, characterized by primary rod dysfunction followed by loss of cone photoreceptors, which initially results in nyctalopia and visual field constriction. In later stages, cone degeneration leads to decreased visual acuity and loss of central visual field.[2] Macular dystrophies (MD) result from primary defects in RPE, rods, or cones restricted to the macular zone, which are distinct from cone dystrophy (CD)/cone-rod dystrophy (CRD). However, early loss or

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distortion of central vision is frequent in MD and CD/CRD.[3,4] From the clinical point of view, one disease may overlap with others depending on the type of the photoreceptor that is primarily affected and the degree of progression at the time of diagnosis. In addition, in 20% to 30% of RP patients, extraocular abnormalities are also present; such as in case of patients with Usher syndrome, Bardet-Biedl syndrome, Senior-Loken syndrome, or Alström syndrome, among others. Moreover, there is a wide phenotypic variability intra- and interfamily, incomplete penetrance, and uniparental isodisomy,[5] so the differential diagnosis between them can be complicated.

IRDs can display all the possible Mendelian inheritance patterns including forms of digenic inheritance and *de novo* mutations.[6–8] IRDs are characterized by high genetic and allelic heterogeneity. To date, 307 genes and loci have been identified in patients with nonsyndromic (NS) and syndromic IRDs (RetNet, *https://sph.uth.edu/retnet*, last accessed November 4, 2019). In addition, common mutations and hot spots are rare, and mutations in the same gene can present different types of inheritance patterns and clinical manifestations.

Despite this complexity, next-generation sequencing (NGS) allows us to make a definitive diagnosis, to offer an accurate genetic counseling, to improve the patient management, and to enable the inclusion in clinical trials, over a reduced period and low cost.[9,10]

Our aim was to evaluate the clinical utility of the targeted NGS in the diagnosis of 172 patients of IRDs and to establish new phenotype–genotype associations that allow us to broaden our knowledge about the physiopathology of this group of diseases.

Materials and Methods

Subjects and Clinical Classification

Our cohort included 172 patients diagnosed clinically with NS-IRD and 217 unaffected individuals for segregation analysis from 110 unrelated pedigrees. All of them were of Spanish origin, except fRPN-216 and fRPN-217, who were from Venezuela. Genetic testing was performed between April 2016 and December 2018. To validate the new panel designed, eight control patients were also analyzed, consisting of patients harboring a previously identified mutation (Supplemental Table S1).

Clinical diagnosis was established at the Hospital La Fe, Hospital de Manises, and Hospital General de Valencia (Spain). Phenotyping included medical history, pedigree mapping, and wide-ranging ophthalmic examination that are detailed in the supplemental material (Supplemental Table S2). Blood or salivary samples were obtained from all probands and available family members to extract genomic DNA using the manufacturer's protocol (MagNA Pure; Roche, Basel, Switzerland). Written informed consent was obtained from all participants or their legal guardians. This study was approved by the Hospital La Fe Ethics Committee in agreement with the Declaration of Helsinki.

NGS Panel Design

Our capture panel was designed using SureDesign version 3.5 software (Agilent Technologies, Santa Clara, CA) including all coding exons and their adjacent 25 bp of 117 genes associated with NS-IRDs (according to RetNet at the time of panel design, November 2015). The design was enriched, increasing the number of probes in the ORF15 region from *RPGR*. The design also included some deep-intronic regions of *USH2A*, *ABCA4*, *CEP290*, *OFD1*, and *PRPF31*, in which pathogenic mutations have been previously described (Table 1). The total size of the captured region was 490 Kb.

Library Preparation and Sequencing

The DNA library was prepared according to the SureSelectQXT protocol (Agilent Technologies). Generated libraries were sequenced with the MiSeq platform (Illumina, San Diego, CA) using a MiSeq version 2 (300 cycles) reagent kit (Illumina, San Diego, CA).

Data Analysis and Variant Interpretation

Base calling and quality scoring were performed by the Illumina RTA software application version 1.18.54. Sequences were aligned against the reference genome (GRCh37/hg19) and subsequently variants were identified using SureCall version 3.0 software (Agilent Technologies). Finally, detected variants were annotated employing wANNOVAR (wANNOVAR, *http://wannovar.wglab.org*).

The variants with a minor allele frequency $\leq$0.01 were evaluated in the Exome Aggregation Consortium and Genome Aggregation Database. The pathogenicity of the variants was assessed according to standards of the American College of Medical Genetics and Genomics (ACMG).[11] VarSome database (VarSome, *https://varsome.com*), Human Gene Mutation Database (HGMD Professional 2018.3; HGMD, *http://www.hgmd.cf.ac.uk*, last accessed August 7, 2019) and Locus Specific Mutation Databases (Locus Specific Mutation Databases, *http://grenada.lumc.nl/LSDB_list*, last accessed August 7, 2019) were used. The potential effect on the splicing of intronic and synonymous variants was evaluated using Human Splicing Finder version 3.1 algorithms (HSF and MaxEntScan; Human Splicing Finder, *http://www.umd.be/HSF*).

Sanger sequencing (BigDye Terminator kit version 3.1; Applied Biosystems, Foster City, CA) was performed to validate the putative pathogenic mutations and to perform segregation analysis. Moreover, deep intronic mutations not included in the panel were screened by Sanger sequencing in patients who remained with one or no mutated alleles.

Table 1 Regions Included in the Panel Design

Gene	Inheritance	Gene	Inheritance	Gene	Inheritance
ABCA4	AR	*GUCA1A*	AD	*RAX2*	AR/AD
ADAM9	AR	*GUCA1B*	AD	*RBP3*	AR
ADAMTS18	AR	*GUCY2D*	AR/AD	*RBP4*	AR
AIPL1	AR/AD	*HK1*	AD	*RD3*	AR
ARL2BP	AR	*IDH3B*	AR	*RDH12*	AR
ARL6	AR	*IMPDH1*	AD	*RDH5*	AR
BBS1	AR	*IMPG1*	AR/AD	*RGR*	AR/AD
BBS2	AR	*IMPG2*	AR	*RGS9*	AR
BEST1	AR/AD	*IQCB1*	AR	*RGS9BP*	AR
C1QTNF5	AD	*KCNJ13*	AR/AD	*RHO*	AD
C21orf2	AR	*KCNV2*	AR	*RIMS1*	AD
C2ORF71	AR	*KIZ*	AR	*RLBP1*	AR
C8orf37	AR	*KLHL7*	AD	*ROM1*	AD
CA4	AD	*LCA5*	AR	*RP1*	AR/AD
CABP4	AR	*LRAT*	AR	*RP1L1*	AR/AD
CACNA1F	XL	*MAK*	AR	*RP2*	XL
CACNA2D4	AR	*MERTK*	AR	*RP9*	AD
CDH3	AR	*MVK*	AR	*RPE65*	AR/AD
CDHR1	AR	*NEK2*	AR	*RPGR*	XL
CEP290	AR	*NEUROD1*	AR	*RPGRIP1*	AR
CERKL	AR	*NMNAT1*	AR	*SAG*	AR/AD
CHM	XL	*NR2E3*	AR/AD	*SEMA4A*	AD
CLRN1	AR	*NRL*	AR/AD	*SLC7A14*	AR
CNGA1	AR	*OFD1*	XL	*SNRNP200*	AD
CNGA3	AR	*OTX2*	AR/AD	*SPATA7*	AR
CNGB1	AR	*PDE6A*	AR	*TIMP3*	AD
CNGB3	AR	*PDE6B*	AR/AD	*TOPORS*	AD
CNNM4	AR	*PDE6C*	AR	*TTC8*	AR
CRB1	AR	*PDE6G*	AR	*TTLL5*	AR
CRX	AR/AD	*PDE6H*	AR	*TULP1*	AR
DHDDS	AR	*PITPNM3*	AD	*UNC119*	AD
DRAM2	AR	*POC1B*	AR	*USH1C*	AR
DTHD1	AR	*PRCD*	AR	*USH2A*	AR
EFEMP1	AD	*PROM1*	AR/AD	*ZNF408*	AR/AD
ELOVL4	AD	*PRPF3*	AD	*ZNF513*	AR
EYS	AR	*PRPF31*	AD	chr1:216064520-216064560	
FAM161A	AR	*PRPF4*	AD	chr1:94492980-94493020	
FLVCR1	AR	*PRPF6*	AD	chr12:88494940-88494980	
FSCN2	AD	*PRPF8*	AD	chr19:54633379-54633419	
GDF6	AR/AD	*PRPH2*	AD	chrX:13770172-13770212	
GNAT2	AR	*RAB28*	AR		

The design included all coding exons and their adjacent 25 bp of 117 genes and 5 deep-intronic regions of *USH2A*, *ABCA4*, *CEP290*, *OFD1*, and *PRPF31*. The total panel size was 490 kbp.

AD, autosomal dominant; AR, autosomal recessive; XL, X-linked.

CNV Analysis

Large rearrangements were screened in all probandi using the DECoN tool version 1.0.2.[12] Potential copy number variations (CNVs) were validated using multiplex ligation-dependent probe amplification: *EYS* (SALSA MLPA P328), *ABCA4* (SALSA MLPA P151 and P152), and *PRPF31* (SALSA MLPA P235) genes (all MRC-Holland, Amsterdam, the Netherlands); or CytoScan 750 K array (array Comparative Genomic Hybridization) in patients and family members. The multiplex ligation-dependent probe amplification, and array Comparative Genomic Hybridization results were analyzed with the Coffalyser.Net software version 140721.1958 (MRC-Holland) and Chromosome Analysis Suite software version 2.1 (Affymetrix, Santa Clara, CA), respectively.

Results

Validation of the NGS Panel

This strategy allowed the detection of all the variants previously found in the eight controls, which had different

mutations in several genes, including three missense variants, four frameshift, one nonsense, one homozygous splice-site mutation, and one deletion of five exons. Moreover, one of the frameshift mutations was identified in the ORF15 region of *RPGR*. So, the sensitivity of the study's NGS approach for those variants was 100%. The mean depth of the target regions was 158×, with 99.18% of captured bases covered by more than 20×, 96.99% more than 50×.

Clinical Assessment

According with the ophthalmologic data and the pedigree information, initially, 60 families were classified as RP, 20 as Stargardt disease (STGD), nine as CRD, eight as Best MD, four as autosomal dominant MD, five as Leber congenital amaurosis, two as achromatopsia, one as fundus albipunctatus, and one as adult-onset vitelliform MD. Furthermore, 57% were sporadic cases, 23% presented with autosomal recessive (AR) inheritance, 18% autosomal dominant (AD), and 2% X-linked (XL) (Supplemental Table S3).

Molecular Findings

A total of 68 of 110 unrelated NS-IRD studied families were genetically diagnosed by the sequencing of this panel, obtaining a detection rate of 62%. In that group, mutations were found in 27 different genes of 117 genes analyzed; and 83 different mutations were identified, 30 of which were novel. Among these variants, 36 (43%) were missense variants, 15 (18%) nonsense, 14 (17%) frameshift, 11 (13%) splice-site mutations, and only one (1%) inframe deletion. Moreover, six CNVs (7%) were found (Figure 1).

In addition, the variants with a minor allele frequency <0.01, except synonymous variants, identified in each patient studied by NGS and classified as "uncertain significance," "likely pathogenic," or "pathogenic" according to the ACMG, were reported in Supplemental Table S4.[13–94] Of these, 183 had never been described.

Thirty-one (52%) of the RP families included in the study were solved, where *USH2A* and *RPGR* were the most frequently mutated genes, accounting for seven and six of the solved cases, respectively. *ABCA4* was responsible for the disease in 15 of 20 STGD included families and one case of CRD, solving 75% of STGD cases (Figure 1).

Thirty-eight sporadic cases (60%) were resolved, of which 34 presented mutations in AR inheritance genes, three in AD inheritance genes, and one in XL inheritance. According to the genetic results, five initially AD retinitis pigmentosa (adRP) cases were reclassified to AR retinitis pigmentosa (arRP; fRPN-110) and to XL RP (fRPN-GB, fRPN-45, fRPN-97, fRPN-174), two arRP families to adRP (fRPN-AP, fRPN-168), one autosomal dominant MD family to late-onset retinal degeneration (LORD; fRPN-100), one Best MD case to CRD (fRPN-125), and one STGD case to Best MD (fRPN-39) (Supplemental Table S5).

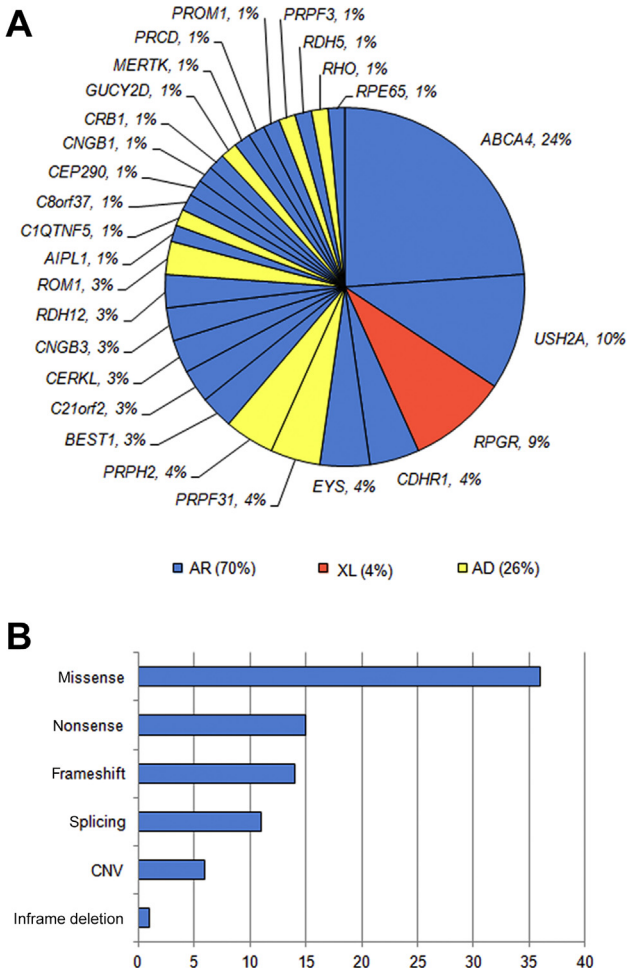

Figure 1 Percentage of patients with mutations in each represented gene and types of mutations. **A:** The genes implicated in cases with an identified disease-causing genotype are accompanied by the percentage of resolved patients in each gene. The meaning of the different colors is indicated in the visual key. **B:** Distribution of disease-causing variant types within the resolved cohort. AD, autosomal dominant; AR, autosomal recessive; CNV, copy number variation; XL, X-linked.

Coverage analysis using DECoN software suggested large deletions in RP-632 (*EYS*, chr6:64776188-64791961), RPN-443 (*EYS*, chr6:66005724-66006043), RPN-602 (*EYS*, chr6:65531457-65622655), RPN-317 (*ABCA4*, chr1:94520629-94520908), RPN-442 (*MERTK*, chr2:112656243-112786484), RPN-335 (*PRPF31*, chr19:54631372-54631835), and a gross duplication in the affected patients of fRPN-AP and fRPN-140 families (*ROM1*, chr11:62380729-62382341) (Supplemental Table S5).

The multiplex ligation-dependent probe amplification technique allowed confirmation in the patients, and segregation in the family members, of the heterozygous deletions in the *EYS*, *ABCA4*, and *PRPF31* genes. The heterozygous deletion of the whole *MERTK* gene in RPN-442 was confirmed by CytoScan 750 K array, showing a deletion of 1.8 Mbp including *BUB1*, *BCL2L11*, *ANAPC1*, *MERTK*, *FBLN7*, *RGPD8*, and *RGPD5* genes (chr2: 111371701-113132395).

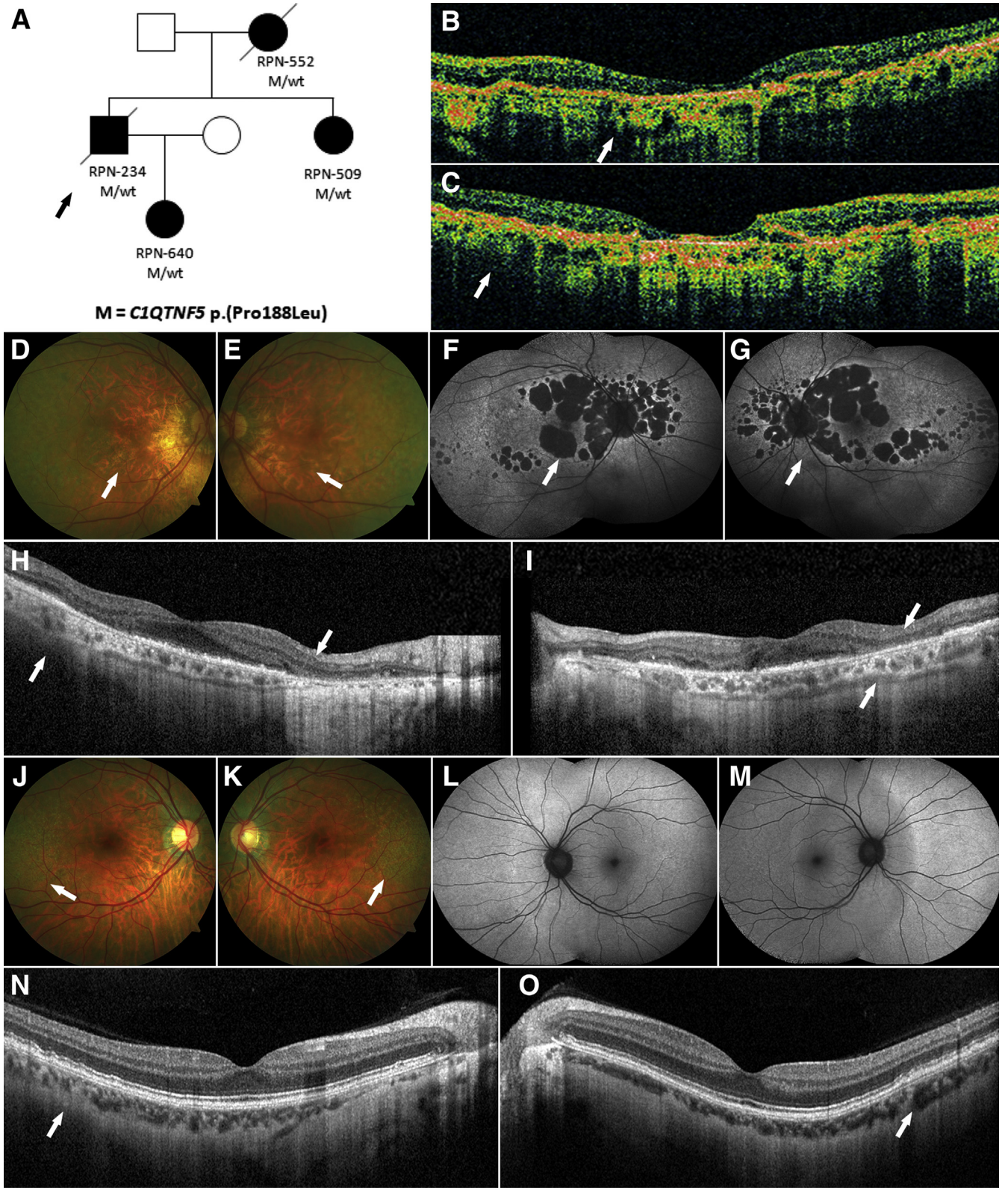

Figure 2 Clinical phenotyping of the late-onset retinal degeneration (LORD) family. **A:** Family pedigree reveals the mutation segregation in the fRPN-100 family showing autosomal dominant inheritance (The **arrow** indicates the proband). **B** and **C:** Patient RPN-234 at 65 years of age: optical coherence tomography (OCT) shows bilateral atrophy of retinal layers and markedly atrophied choroid (**arrows**). **D**—**I:** Patient RPN-509 at 64 years of age: color fundus photography shows areas of central retinal and peripapillar atrophy (**D** and **E**; **arrows**); fundus autofluorescence images show areas of hypoautofluorescence, serpinginosa-like, affecting the fovea and matching the areas of atrophy (**F** and **G**; **arrows**); OCT shows bilateral and asymmetric atrophy of outer retinal layer [inner segment/outer segment, external limiting membrane, and retinal pigment epithelium (RPE)] and choroid (**H** and **I**; **arrows**). **J**—**O:** Patient RPN-640 at 39 years of age: color fundus shows multiple drusen in the temporal area of the retina in both eyes as unique finding (**J** and **K**; **arrows**); fundus autofluorescence images are normal (**L** and **M**); OCT: RPE abnormalities corresponding to retina areas with drusen and choroidal atrophy in both eyes (**N** and **O**; **arrows**). wt, wild type.

Genotype—Phenotype Correlation

Phenotypic Intra- and Interfamilial Variability

In the fRPN-100 family, RPN-234 and his mother (RPN-552) were diagnosed with MD at 56 and 55 years of age, respectively (clinical examination of RPN-552 not available). He started with decreased visual acuity and nyctalopia in his 52nd year. At 65 years of age his best corrected visual acuity was hand movements bilaterally, and optical coherence tomography revealed severe RPE and choroid atrophy with thickening of the sub-RPE layer (Figure 2). Panel sequencing revealed a novel missense mutation in *C1QTNF5* (p.Pro188Leu) in RPN-234, which was classified as likely pathogenic according to the ACMG criteria. In the segregation analysis, this mutation was also identified in RPN-552 and in their clinically undiagnosed sister (RPN-509) and daughter (RPN-640). Subsequent ophthalmic examination in RPN-509 at 63 years of age revealed bilateral and asymmetric atrophy of outer retinal layer and choroid. She had experienced 4 years of nyctalopia with worsening of central vision in both eyes (Figure 2). Ophthalmic examination in RPN-640 at 39 years of age revealed multiple drusen in the temporal area of the retina at color fundus, corresponding with RPE abnormalities, drusen and choroidal atrophy peripherally showed in the optical coherence tomograph (Figure 2). She had no visual disturbance.

Phenotypic intrafamilial variability in the fRPN-43 family with mutations in *CERKL* and in the fRPN-BT family with mutations in *PROM1* were also observed. In addition, in the patient RPN-113, the homozygous p.(Arg283*) mutation in *CERKL* caused RP, whereas in RPN-475, the same genotype displayed CRD (Supplemental Table S2).

RPGR

The six resolved families having mutations in *RPGR*, included 20 mutated individuals: eight males and 12 females. All males except RPN-343 presented with early onset RP. Eight (67%) of the carrier women displayed RP, four of them presented interocular asymmetry, and the others were asymptomatic. In the fRPN-158 family, the probandus and his asymptomatic mother harbored p.(Asn305Lysfs*41) in *RPGR* and also p.(Cys140Ser) in *RHO*, classified by the authors as likely pathogenic.[56] The healthy carrier was fully examined at 43 years of age, and only myopia magna was found (Supplemental Figure S1–S4 and Supplemental Table S2).

ABCA4

The cases with mutations in *ABCA4* were classified according to the age of disease onset: early (≤15 years of age), middle (16 to 30 years of age), and late (>30 years of age). One third of the *ABCA4* mutated patients had early onset, at a mean of 10.6 (3 to 15) years of age. Forty-four percent presented with middle onset at a mean of 23.8 (16 to 30) years of age, and the rest of the group (22%) showed late onset at a mean of 43 (39 to 48) years of age. The most severe case in this group was RPN-478 with CRD. The allele p.(Arg1129Leu) was the most prevalent mutant allele in our *ABCA4*-mutated cohort with a prevalence of 28%, followed by p.(Gly1961Glu) with a prevalence of 9% (only index cases were considered).

Partially Diagnosed

Thirteen families with only one disease-associated IRD allele were identified. Nine cases had one IRD allele causing mutation in genes with AR inheritance, and the other four cases had one IRD allele causing mutation in genes that can follow AR as well as AD patterns of inheritance. In a sporadic RP case (RPN-536), p.(Thr631Met) in *CNGA1* and p.(Phe1051Leufs*12) in *CNGB1* in a compound heterozygous state were identified. Interestingly, proteins codified by these genes are members of the same channel (Supplemental Table S6).

Moreover, in two siblings initially diagnosed as adRP, one with unilateral RP, p.(Asn803Ser) and (p.Glu129Lys) in *PDE6B* in a compound heterozygous state were found. However, an asymptomatic sibling was also carrier of both variants in a heterozygous state. The latter underwent ophthalmic examination, but RP was ruled out, and the family remained without a complete genetic diagnosis (Supplemental Table S6).

Discussion

In the present study, the disease-causing specific mutation was found in 68 (62%) of the 110 analyzed families, a similar diagnostic yield compared with previous studies using a targeted NGS genes panel, whole-exome sequencing, or whole-genome sequencing in NS-IRD patients.[57,88,95,96] In the majority of sporadic IRD cases, the retinopathy is inherited in an AR mode with a lower recurrence risk for the offspring. However, the number of AD and XL cases is noteworthy. In this study, one male sporadic case with XL RP (RPN-323) and two sporadic cases with adRP (RPN-347, RPN-603) were identified. RPN-347, RPN-603, and the three affected siblings from the fRPN-168, previously classified as arRP, showed mutations in *PRPF31*. Segregation analysis showed incomplete penetrance, as is well known for this gene.[97,98] This study highlights the possibility that pathologic large deletions or duplications might be more frequent than estimated in IRD cases.[99] Therefore, in recessive or sporadic cases, when a homozygous change is identified, it must be kept in mind that compound heterozygosity with a CNV might be possible.

ROM1

Previous studies in murine models showed that overexpressed ROM1 is toxic for both photoreceptors.[100–103] In this study, the whole *ROM1* duplication was found in two independent families diagnosed with RP, supporting the toxic effect of ROM1 overexpression.

Phenotypic Intrafamilial Variability

C1QTNF5 has been related to LORD, an AD disorder characterized by onset in the fifth to sixth decade with nyctalopia, drusenoid deposits, RPE atrophy, and choroidal neovascularization.[104] Only a few pathogenic variants in *C1QTNF5* have been described.[104–106] The current study reports a novel heterozygous mutation in *C1QTNF5*, p.(Pro188Leu), identified in four affected relatives with the expected phenotypes for LORD. This family showed variability in the presentation of visual symptoms as previously described for LORD (Figure 2).[105,106] The patient RPN-640 is one of the youngest patients showing clinical symptoms with mutations in this gene.

Moreover, these familial cases were other examples of the characteristically phenotypic intrafamilial variability of the IRD.[107]

RPGR

To date, approximately 80% of the XL RP were caused by mutations in the *RPGR* gene, in particular in the hot spot region ORF15.[108]

RPGR-related RP is one of the most severe forms of IRD in males.[109] RPN-343 was the only case in our cohort with nyctalopia onset in his twenties, harboring a frameshift (p.Glu916Lysfs*173). As previously suggested by Tee et al,[108] the moderate phenotype of RPN-343 could be due to a longer wild-type ORF15 amino acid sequence able to perform some functions compared with the other *RPGR*-mutated males with upstream variants.

Moderate and, less frequently, severe phenotypes can also be seen in female carriers.[110,111] Thus, cases including affected females in several generations might be erroneously classified as AD.[112] Among the six XL RP families reported here, four were previously classified as AD. Although the interocular asymmetry within the *RPGR* heterozygous females have been described in isolated cases,[113,114] we highlight this because of the high prevalence in this cohort. Four of the six symptomatic women showed demonstrable RP asymmetry.

The several patterns described in this *RPGR* female carriers' cohort, including the interocular asymmetry, show the different clinical phenotypes that are deemed to happen as a result of random X-inactivation in early embryological development. Mosaic patterns have been seen in mice retinas due to the random X-inactivation.[115,116]

In the family fRPN-158, maybe the RP caused by p.(Cys140Ser) in *RHO* has not yet begun, or this variant might have a neutral effect. Following the criteria of the ACMG, this variant was reclassified as unknown significance.

ABCA4

The *ABCA4*: p.(Arg1129Leu) allele was the most prevalent allele in the Spanish STGD population (22.4%).[19] In this study's cohort, the allele p.(Arg1129Leu) was found in 10 of the 32 alleles studied in patients with the STGD phenotype. The single homozygous case for that allele, RPN-294, showed a disease onset at 24 years of age, which would indicate that this mutation would have a moderate effect. In the remaining compound heterozygous patients, the severity of the phenotype was heterogeneous, although, in general, patients with a truncating mutation in *trans* presented with an earlier disease onset than those with missense mutations. These findings are in accordance with the previous studies.[19]

It is estimated that 9.5% of the pathogenic *ABCA4* alleles are complex alleles.[117] In the current study, this was found in two cases: RPN-510, carrier of p.(Leu541Pro); p.(Ala1038Val) in *trans* with p.(Gly1961Glu), and RPN-317 carrier of an exon 16 deletion in *cis* with p.(Arg1129Leu) and p.(Arg602Trp) in the second allele. Both cases had an early onset. The RPN-510 genotype had been previously identified in several independent cases,[117] but RPN-317 was the first case with an exon deletion in a complex allele. Thus, a careful analysis of the variants is important to avoid missing information.

Partially Diagnosed

Not identifying pathological variants in the genes analyzed in a group of patients can be due to causative mutations in noncoding regions of these genes or in genes discovered after the panel design or in genes not yet related with retinal dystrophy. It may also be due to large rearrangements or other mechanisms of the pathology undetectable with this diagnostic strategy. One interesting case among the 13 partially diagnosed families is a sporadic RP patient that carried the mutation *CNGA1*: p.(Thr631Met) in a paternal allele and *CNGB1*: p.(Phe1051Leufs*12) in a maternal allele. It is tempting to speculate that mutant CNGB1 and CNGA1 protein lead to damaged heterotetramer CNG channel complex. However, digenism cannot be demonstrated, so this case is included among the partially diagnosed cases.

Another interesting case from this group is the fRPN-SF family diagnosed initially with adRP, in which we identified in two affected siblings the mutation p.(Glu129Lys) in *PDE6B*, previously described as likely pathogenic,[94] in *trans* with the novel missense p.(Asn803Ser) that was classified as likely pathogenic. Their also-affected father and paternal aunt carried p.(Glu129Lys) in homozygosity; consequently, we believed that the genetic diagnoses in that family were completed. Subsequent analysis in the remaining family members identified the two missense mutations in a sibling in which RP were clinically ruled out. So, the variant p.(Glu129Lys) was reclassified as uncertain significance, and the diagnosis of the fRPN-SF family remained incomplete.

Since 2015, when the panel was designed, over 40 novel IRD genes have been identified to date. Currently, 271

genes are known to be involved in IRD (RetNet, *https://sph.uth.edu/retnet/sum*, accessed November 5, 2019). However, the percentage of solved cases varies depending on the population studied, the pattern of inheritance, and the different types of IRD in the cohort. Besides this, the novel genes usually account only for one or two families. This percentage ranges from 30% to 70% independent of the number of genes included in the panel or even when whole-exome sequencing is performed.[94,96,118]

Conclusions

In summary, this study demonstrates that clinical patient characterization and targeted exome sequencing is a reliable tool for definitive diagnosis of NS-IRD. The phenotype−genotype correlation is reported in a large cohort of patients, expanding the knowledge of this group of disorders. The next challenge is to diagnose 100% of the patients, so they can benefit from upcoming gene-based therapeutic strategies.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2020.01.003*.

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