Updating the Genetic Landscape of Inherited Retinal Dystrophies

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Inherited retinal dystrophies (IRD) are a group of diseases characterized by the loss or dysfunction of photoreceptors and a high genetic and clinical heterogeneity. Currently, over 270 genes have been associated with IRD which makes genetic diagnosis very difficult. The recent advent of next generation sequencing has greatly facilitated the diagnostic process, enabling to provide the patients with accurate genetic counseling in some cases. We studied 92 patients who were clinically diagnosed with IRD with two different custom panels. In total, we resolved 53 patients (57.6%); in 12 patients (13%), we found only one mutation in a gene with a known autosomal recessive pattern of inheritance; and 27 patients (29.3%) remained unsolved. We identified 120 pathogenic or likely pathogenic variants; 30 of them were novel. Among the cone-rod dystrophy patients, ABCA4 was the most common mutated gene, meanwhile, USH2A was the most prevalent among the retinitis pigmentosa patients. Interestingly, 10 families carried pathogenic variants in more than one IRD gene, and we identified two deep-intronic variants previously described as pathogenic in ABCA4 and CEP290. In conclusion, the IRD study through custom panel sequencing demonstrates its efficacy for genetic diagnosis, as well as the importance of including deep-intronic regions in their design. This genetic diagnosis will allow patients to make accurate reproductive decisions, enroll in gene-based clinical trials, and benefit from future gene-based treatments.

Keywords: inherited retinal dystrophies, diagnosis, custom-panels, gene, pathogenic, deep-intronic

INTRODUCTION

Inherited retinal dystrophies (IRD) are a group of diseases characterized by the progressive death or dysfunction of photoreceptors that leads to vision loss and, in some cases, legal blindness. IRDs have a prevalence of one case in 3,000 individuals (Sahel et al., 2015). Depending on the photoreceptor initially affected, IRD can be classified into cone, rod-cone, or cone-rod dystrophies in those cases in which both are affected at one time. Moreover, they can manifest as either isolated (70–80% of the total) or part of one of the 80 syndromes that have been estimated to be associated with IRD (Ayuso and Millan, 2010; Tatour and Ben-Yosef, 2020).

This group of diseases has a wide clinical spectrum and number of involved genes, currently reaching 271 for syndromic and non-syndromic forms (Retnet1, December 2020); explaining why IRDs have such a high clinical and genetic heterogeneity. Furthermore, there is a high inter and intrafamily variability, variable expression, and incomplete penetrance (Farrar et al., 2017). IRDs can follow different patterns of inheritance, including autosomal recessive, autosomal dominant,
X-linked, mitochondrial mode, and some other less common, such as uniparental isodisomy or digenic inheritance (Kajiwara et al., 1994; Dryja et al., 1997; Rivolta et al., 2002; Ayuso and Millan, 2010; Parmeggiani et al., 2011; Neveling et al., 2012). These issues, together with the fact that 50% are sporadic cases, make it even more complicated to determine the mode of inheritance, genetic diagnosis, and genetic counseling (Perea-Romero et al., 2021).

With the advent of next generation sequencing (NGS), the ratio of diagnosis has risen to 50–70%, which was difficult to achieve a few years ago (Carss et al., 2017; Sanchis-Juan et al., 2018; Rodriguez-Muñoz et al., 2020). Some different approaches, such as custom panel designs, whole exome sequencing (WES), and whole genome sequencing (WGS), have been implemented to study the molecular mechanisms of IRD. Currently, these new sequencing techniques are essential to obtain an early and accurate genetic diagnosis, which is necessary to offer a correct genetic counseling to patients and their families (Salmaninejad et al., 2019).

In this study, we analyzed 92 patients, who were previously clinically diagnosed with IRD, with two custom panels for the main aim of achieving genetic diagnoses.

MATERIALS AND METHODS

Cohort Selection

We selected 92 patients, belonging to 90 different Spanish families, with a clinical diagnosis of non-syndromic IRD, except for one who had a clinical suspicion of a syndromic IRD. Patient DNA was isolated from peripheral blood with the automatic extractor QIAsymphony (QIAGEN).

Patients underwent complete ophthalmologic examinations, including OCT (Heidelberg Spectralis OCT Bluepeak, Heidelberg, Germany; Topcon 3D OCT 2000, Tokyo, Japan; CIRRUS OCT Zeiss, Oberkochen, Germany), ERG (Roland RETI-port/scan21, Brandenburg, Germany), eye fundus (Visucam NM/FA Zeiss, Oberkochen, Germany), visual acuity measure (BCVA), and evoked potentials and visual fields (Carl Zeiss Humphrey Field Analyzer, Oberkochen, Germany). A clinical questionnaire, which collected the main IRD characteristics, and an informed consent were completed by each patient. This study was approved by the Hospital La Fe Ethics Committee, in agreement with the Declaration of Helsinki.

Panel Design and Sequencing

Sixty-three patients were sequenced with a gene panel design (PV1) that included 117 genes involved in a non-syndromic IRD, and their flanking intronic regions (±25 base pairs) (Rodríguez-Muñoz et al., 2020). Moreover, the panel of genes contained five intronic regions of ABCA4, OFD1, USH2A, CEP290, and PRPF31, in which pathogenic variants had been previously identified (Den Hollander et al., 2006; Littink et al., 2010; Vaché et al., 2012; Webb et al., 2012; Braun et al., 2013; Supplementary Table 1).

The remaining 29 cases were analyzed with an updated version of the custom panel (PV2) that had 114 genes and all the deep-intronic variants described in the last few years in ABCA4 and USH2A (Vaché et al., 2012; Braun et al., 2013; Zernant et al., 2014; Bawens et al., 2015, 2019; Liquori et al., 2016; Baux et al., 2017; Fadaie et al., 2019; Khan et al., 2019; Sangermano et al., 2019; Supplementary Table 2).

The patients’ libraries were prepared in accordance with the SureSelect QXT protocol (Agilent Technologies) and sequenced on a MiSeq platform (Illumina, San Diego, CA) in 300 cycles with 2 × 150 base pairs reads.

Data Analysis

The reads alignment against the reference hg19 genome, variant calling, and annotation of all the identified variants were carried out with the Alissa resource (Agilent Technologies). The obtained variants were filtered based on a MAF (minor allele frequency) ≤ 0.01 according to the ExAC2 and gnomAD5 databases. In order to evaluate the pathogenicity of the detected variants, we also evaluated specific databases such as ClinVar8, Locus Specific Data Base9, and HGMD professional10. To evaluate the potential effect of novel missense variants, we used the in silico predictors included in Varsome2. The putative effect on the splicing process was performed with HSF (Human SplicingFinder6), NNSplice8, and SpliceAI9. Finally, the IGV view finder11 allowed the examination of all detected variants in every read.

The novel variants identified in this study were classified according to the standards of the American College of Medical Genetics and Genomics (ACMG) (Richards et al., 2015).

Copy Number Variation Analysis

A copy number variations (CNV) analysis was performed using the DECoN bioinformatic tool version 1.0.2 (Fowler et al., 2016).

We also studied large rearrangements by Multiplex Ligation-dependent Probe Amplification (MLPA; MRC Holland) in patients harboring one pathogenic variant in USH2A (probemixes P361 and P362), EYS (probemix P328-A3), and ABCA4 (probemixes P151 and P152). The multiplex ligation-dependent probe amplification results were analyzed by the Coffalyser. Net software version 140721.1958 (MRC-Holland).

Sanger Sequencing and Segregation Analysis

The candidate variants identified in each patient were validated by Sanger sequencing (Big Dye Terminator v1.1 or v3.1, Applied

1http://exac.broadinstitute.org/
2https://gnomad.broadinstitute.org/
3http://www.ncbi.nlm.nih.gov/clinvar/
4https://grenada.humc.nl/LSDB_list/Lsdb
5https://portal.biobase-international.com/cgi-bin/portal/login.cgi
6https://varsome.com/
7http://www.umdb.de/HSF
8https://www.fruitfly.org/seq_tools/splice.html
9https://mobidetails.iurc.montp.inserm.fr/MD/
10http://software.broadinstitute.org/software/igv/
Pathogenic variants in more than one IRD gene (autosomal recessive gene, and we also described six patients who carry with more than two pathogenic variants in the same autosomal recessive gene (RPN-670). We reported five cases autosomal dominant genes and two additional variants in an autosomal recessive gene, two patients (of two families) who carried a pathogenic variant in two different autosomal recessive genes, among which, we could not carry out the segregation analysis to harbored another heterozygous pathogenic variant in the same gene; however, we could not carry out the segregation analysis to confirm it as likely pathogenic.

Copy number variants analysis with the bioinformatic tool allowed us to detect two putative deletions: a deletion in PRPF31, which was properly validated with MLPA (patient RPN-717), and a homozygous deletion of exon 11 of PROM1 in patient RPN-709. In this last patient, the exon 11 did not have reads in the alignment and was not amplified by PCR; however, there was PCR amplification for the flanking exons, thus, reinforcing the hypothesis of a homozygous deletion of the exon.

The individual RPN-728 presented nephropathy and alterations in the brain MRI (mild hypoplasia of the cerebellar vermis and slightly elongated superior cerebellar peduncles) in addition to retinal degeneration, suggesting a Joubert syndrome.

The panel sequencing allowed us to identify two pathogenic variants in CEP290, c.4966_4967delGA (p.Glu1656Asnfs*3) and c.2817G > T (p.Lys939Asn). On the other hand, the panel analysis in the RPN-708 revealed a pathogenic variant in the OTX2 gene (Table 1). Although this gene has been implicated in other diseases such as microphthalmia and retinal degeneration with pituitary dysfunction (OMIM: 610125; 610125), our patient only referred a macular dystrophy (MD) phenotype.

Because of the inclusion of the deep-intronic regions, we solved two cases who carried deep-intronic pathogenic variants in ABCA4 and CEP290 (RPN-750, RPN-734) (Table 1).

RESULTS

We obtained a mean depth of 190 × per patient. In 98.6% of the patients, at least 88% of the bases were covered with a sequencing depth of coverage ≥ 50 × and 95% of the bases were covered with at least 20 ×. Moreover, we obtained a 70% of on-target reads.

We solved 53 patients belonging to 52 families (53/92, ratio of 57.6%): 17 (of 17 families) which had disease-causing variants in autosomal dominant genes, 33 (of 32 families) with autosomal recessive genes, two patients (of two families) who carried a pathogenic variant in an X-linked gene and one patient who carried two different pathogenic variants in two different autosomal dominant genes and two additional variants in an autosomal recessive gene (RPN-670). We reported five cases with more than two pathogenic variants in the same autosomal recessive gene, and we also described six patients who carry pathogenic variants in more than one IRD gene (Table 1). Among the unresolved patients, 13% carried one pathogenic variant in an autosomal recessive IRD gene, with a higher prevalence of cases with a heterozygous variant in ABCA4, followed by USH2A and RPGRIP1 (Table 2). In the remaining 29.3% cases, no pathogenic variant was identified.

We identified a total of 120 pathogenic or likely pathogenic variants (of which, 85 were unique), including: 65 missense, 15 nonsense, 17 frameshift, 16 splice-site variants, two pathogenic deep-intronic variants, one in-frame deletion, one synonymous, and three CNV (Figure 1). Twenty-nine variants were first described in this study (Tables 1–3). In line with this, we identified a high number of mutated-genes, nine autosomal dominant genes (highest prevalence of PRPH2); 19 autosomal recessive genes, among which, ABCA4 and USH2A stand out; and finally, one X-linked gene, RPGR (Figure 2). All identified novel variants were classified as pathogenic or likely pathogenic according to the ACMG criteria except for variant c.2470_2478del (p.Lys824_Glu826del) in PDE6B, which remained as a variant of uncertain significance. This patient harbored another heterozygous pathogenic variant in the same gene; however, we could not carry out the segregation analysis to confirm it as likely pathogenic.

Copy number variants analysis with the bioinformatic tool allowed us to detect two putative deletions: a deletion in PRPF31, which was properly validated with MLPA (patient RPN-717), and a homozygous deletion of exon 11 of PROM1 in patient RPN-709. In this last patient, the exon 11 did not have reads in the...

DISCUSSION

A total of 92 patients, previously clinically diagnosed with IRD, were analyzed by two IRD-custom panels. These gene panels allowed us to find a genetic diagnosis in 53 patients of 52 different families (Table 1), with a diagnostic ratio of 57.6%, which is within the average of other studies (50–70%) (Ellingford et al., 2016; Di Resta et al., 2018; Wang et al., 2018; Jespersgaard et al., 2019; Zenteno et al., 2020).

In recent years, several deep-intronic mutations have been described as pathogenic due to their effect in the splicing process by leading to the introduction of a pseudoexon (PE) in the coding sequence (Vaché et al., 2012; Braun et al., 2013; Zernant et al., 2014; Bauwens et al., 2015, 2019; Liquori et al., 2016; Baux et al., 2017; Fadaie et al., 2019; Khan et al., 2019; Sangermano et al., 2019). The inclusion of these regions in the present study allowed us to detect two deep intronic variants and, therefore, solve the molecular diagnosis in two more families. The variant CEP290: c.2991 + 1655A > G, detected in patient RPN-750, is one of the most prevalent in Leber congenital amaurosis (LCA) associated with CEP290 (Den Hollander et al., 2006; Coppieters et al., 2010). Coppieters et al. reported this variant with a frequency of 49% in the CEP290-associated LCA cases. Also, we identified the deep-intronic variant c.4539 + 2064C > T in ABCA4. Deep-intronic variants in ABCA4 were reported to be involved in 2.1–17.9% of the STGD cohorts (Braun et al., 2013; Zernant et al., 2014; Bauwens et al., 2015; Bax et al., 2015; Zaneveld et al., 2015; Schulz et al., 2017); our finding of 7.1% of cases within the STGD patients fits into the observed frequencies. Furthermore, the development of new therapeutical approaches, such as antisense oligonucleotides (AONs), entailed a new advantage for deep-intronic variants correction, which highlights the importance of detecting these changes for future treatments. In this sense, several studies have proven the therapeutic potential of AONs strategy and a phase I/II clinical trial for patients harboring the CEP290 c.2991 + 1655A > G mutation using this type of therapy was conducted (Burke et al., 2012; Slijkers et al., 2016;
| Family | Patient | Clinical diagnosis | Gene | Nucleotide change | Protein change | Zygosity | References |
|--------|---------|--------------------|------|-------------------|---------------|----------|------------|
| $FRPN$-51 | RP-129 | STG | ABCA4 (NM_000350.3) | c.1804C > T, c.982G > T | p.(Arg602Trp), p.(Glu328*) | Heterozygous | Lewis et al., 1999 |
| $FRPN$-267 | RP-666 | Reverse BCAMD/STG/RP | ABCA4 (NM_000350.3) | c.5714 + 1G > A | p.? | Heterozygous | This study |
| $FRPN$-268 | RP-668 | CD | CRB1 (NM_201253.3) | c.5882G > A | p.(Gly1961Glu) | Heterozygous | Allikmets et al., 1997 |
| $FRPN$-269 | RP-670 | NA | RP1 (NM_0006269.2) | c.3157del | p.(Val1973*) | Heterozygous | Le Quesne Stabej et al., 2012 |
| $FRPN$-270 | RP-671 | RP | USH2A (NM_206933.4) | c.4732C > T | p.(Arg1578Cys) | Heterozygous | Le Quesne Stabej et al., 2012 |
| $FRPN$-271 | RP-672 | RP | CNGB1 (NM_0012975.7) | c.2267G > T | p.(Glu806Glu) | Heterozygous | Fishman, 2003 |
| $FRPN$-272 | RP-673 | RP | USH2A (NM_206933.4) | c.12575G > A, c.4880del | p.(Arg4192His), p.(Leu1627Argfs*35) | Heterozygous | Ávila-Fernández et al., 2010 |
| $FRPN$-273 | RP-674 | MD | BEST1 (NM_0071193.3) | c.5714 + 5G > A | p.? | Heterozygous | Cremer, 1998 |
| $FRPN$-274 | RP-675 | RP | RPGR (NM_00134853.2) | c.309G > A | p.(Gly1033Arg) | Heterozygous | Collin et al., 2008 |
| $FRPN$-275 | RP-676 | RP | EYS (NM_001142800.2) | c.9468T > A | p.(Arg220Trp) | Heterozygous | Payne et al., 1998 |
| $FRPN$-276 | RP-677 | RP | USH2A (NM_206933.4) | c.6718A > G | p.(Thr2240Ala) | Heterozygous | Günzler et al., 2010 |
| $FRPN$-277 | RP-678 | RP | CRB1 (NM_201253.3) | c.481G > A | p.(Tyr164His) | Homozygous | Corton et al., 2013 |
| $FRPN$-278 | RP-679 | STG | ABCA4 (NM_000350.3) | c.3386G > T | p.(Ser1130Arg) | Heterozygous | Allikmets et al., 1997 |
| $FRPN$-279 | RP-680 | RP | USH2A (NM_206933.4) | c.2953G > A | p.(Arg984Cys) | Homozygous | Lewis et al., 1999 |
| $FRPN$-280 | RP-681 | RP | ABCA4 (NM_000350.3) | c.3386G > T | p.(Ser1130Arg) | Heterozygous | Allikmets et al., 1997 |
| $FRPN$-281 | RP-682 | MD | BEST1 (NM_0071193.3) | c.247G > T | p.(Val83Phe) | Heterozygous | Fishman, 2003 |
| $FRPN$-282 | RP-683 | RP | RPGR (NM_00134853.2) | c.1366del | p.(Glu456Alafs*20) | Hemizygous | Fishman, 2003 |
| $FRPN$-283 | RP-684 | RP | EYS (NM_001142800.2) | c.658C > T | p.(Arg216His) | Homozygous | Hu et al., 2020 |
| $FRPN$-284 | RP-685 | RP | USH2A (NM_206933.4) | c.12573G > A | p.(Arg4192His) | Heterozygous | Ávila-Fernández et al., 2010 |
| $FRPN$-285 | RP-686 | RP | ABCA4 (NM_000350.3) | c.3386G > T | p.(Ser1130Arg) | Heterozygous | Allikmets et al., 1997 |
| $FRPN$-286 | RP-687 | RP | ABCA4 (NM_000350.3) | c.6148G > C, c.440dup | p.(Val2050Leu), p.(Gly1481Trpfs*29) | Heterozygous | Lewis et al., 1999 |
| $FRPN$-287 | RP-688 | RP | ABCA4 (NM_000350.3) | c.3386G > T, c.634C > T | p.(Arg1129Leu), p.(Arg216His) | Hemizygous | Hu et al., 2020 |
| $FRPN$-288 | RP-689 | RP | ABCA4 (NM_000350.3) | c.647C > A | p.(Asp216His) | Homozygous | Lotery, 2001 |

(Continued)
TABLE 1 | Continued

| Family | Patient | Clinical diagnosis | Gene | Gene deletion | Nucleotide change | Protein change | Zygosity | References |
|--------|---------|--------------------|------|---------------|------------------|----------------|----------|------------|
| FRPN-299<sup>1</sup> | RPN-707 | RP | USH2A (NM_206933.4) | | c.13811 + 2T > G | p.? | Heterozygous | Besnard et al., 2014 |
| FRPN-300<sup>1</sup> | RPN-708 | NA | OTX2 (NM_001270525.2) | | c.6387T > A | p.(Leu213*) | Heterozygous | This study |
| FRPN-301<sup>1</sup> | RPN-709 | RP | PROM1 (NM_006017.2) | | deletion exon 11 | p.? | Homozygous | This study |
| FRPN-302<sup>1</sup> | RPN-710 | MD/BVMD | PRPH2 (NM_000322.5) | | c.641G > A | p.(Cys214Tyr) | Heterozygous | Trujillo et al., 2001 |
| FRPN-303<sup>1</sup> | RPN-711 | RP | USH2A (NM_206933.4) | | c.14803C > T | p.(Arg4935)* | Heterozygous | Baux et al., 2007 |
| FRPN-307<sup>1</sup> | RPN-715 | RP | RHO (NM_005539.3) | | c.512C > A | p.(Pro171Gln) | Heterozygous | Antiñolo et al., 1994 |
| $FRPN-308$<sup>2</sup> | RPN-717 | RP | ABCA4 (NM_000350.3) | | Gene deletion | p.? | Heterozygous | Allikmets et al., 1997 |
| FRPN-309<sup>2</sup> | RPN-718 | RP | USH2A (NM_206933.4) | | c.2276G > T | p.(Cys759Phex) | Homozygous | Rivolta et al., 2000 |
| FRPN-312<sup>2</sup> | RPN-721 | RP | RHO (NM_000350.3) | | c.512C > A | p.(Pro171Leu) | Heterozygous | Stone et al., 2017 |
| FRPN-315<sup>2</sup> | RPN-725 | MD/STG | ABCA4 (NM_000350.3) | | c.6310C > T | p.(Gln2104)* | Heterozygous | This study |
| FRPN-316<sup>2</sup> | RPN-726 | RP | NRL (NM_001354788.3) | | c.149C > T | p.(Ser50Leu) | Heterozygous | Koyanagi et al., 2019 |
| FRPN-318<sup>2</sup> | RPN-728 | Joubert syndrome | CEP290 (NM_025114.4) | | c.4966-4967del | p.(Glu1656Asnf*3) | Heterozygous | Sheck et al., 2018 |
| FRPN-320<sup>2</sup> | RPN-730 | MD/STG | PRPH2 (NM_000322.5) | | c.2817G > T | p.(Lys939Asnx) | Heterozygous | Srivastava et al., 2017 |
| FRPN-322<sup>2</sup> | RPN-732 | RP | PDE6B (NM_000283.4) | | c.1920 + 1G > A | p.? | Heterozygous | Díñez et al., 2020 |
| FRPN-323<sup>2</sup> | RPN-733 | MD | PRPH2 (NM_000322.5) | | c.2470_2478del | p.(Lys824_Glu826del) | Heterozygous | This study |
| FRPN-324<sup>2</sup> | RPN-734 | MD/CD | ABCA4 (NM_000350.3) | | c.421T > G | p.(Tyr151His) | Heterozygous | Díñez et al., 2020 |
| FRPN-325<sup>2</sup> | RPN-735 | NA | EYS (NM_001142800.2) | | c.854del | p.(Thr2973Leufs*23) | Heterozygous | This study |
| FRPN-326<sup>2</sup> | RPN-736 | MD/STG | RDHS5 (NM_002905.3) | | c.537G > A | p.(Trp179?) | Heterozygous | This study |
| FRPN-327<sup>2</sup> | RPN-737 | MD/STG | PRPH2 (NM_000322.5) | | c.1.1920 + 1G > A | p.? | Heterozygous | This study |
| FRPN-332<sup>2</sup> | RPN-738 | MD/STG | BBS1 (NM_024649.4) | | c.4539 + 2064C > T | [p.: p.(?, Arg1514Leufs*36)] | Heterozygous | Salles et al., 2019 |
| FRPN-335<sup>2</sup> | RPN-745 | RP | PRGR (NM_001034853.2) | | c.3113C > T | p.(Ala1038Val) | Heterozygous | Zernant et al., 2014; Bauwens et al., 2019 |
| FRPN-337<sup>2</sup> | RPN-747 | RP | ABCA4 (NM_000350.3) | | c.3386G > T | p.(Glu1129Leu) | Heterozygous | Allikmets et al., 1997 |
| FRPN-339<sup>2</sup> | RPN-749 | STG | ABCA4 (NM_000350.3) | | c.2670G > A | p.(Arg1129Leu) | Heterozygous | Allikmets et al., 1997 |
| $FRPN-340$<sup>2</sup> | RPN-750 | LCA | CEP290 (NM_025114.4) | | 2T | c.2991 + 1655A > G | p.? | Homozygous | Den Hollander et al., 2006 |

Novel pathogenic variants are highlighted in bold font.

FRPN, family number; FRPN-“<sup>1</sup>” families studied with PV1; FRPN-“<sup>2</sup>” families studied with PV2; $FRPN$, families in which segregation analysis was performed; RPN, patient number; RP, retinitis pigmentosa; MD, macular dystrophy; p.?, unknown protein effect; STG, stargardt; LCA, leber congenital amaurosis; BCAMD, benign concentric annular macular dystrophy; CD, cone dystrophy; CRD, cone-rod dystrophy; BVMD, best vitelliform macular dystrophy; NA, not available. The “*” symbol corresponds to: stop-codon according to the format of mutations nomenclature from the Human Genome Variation Society (HGVS).

In our study, ABCA4 and USH2A are the most frequent mutated-genes, similar to other studies (Bernardis et al., 2016; Ezquerra-Inchausti et al., 2018; Jespersgaard et al., 2019). In ABCA4, 10% of alleles are reported to be complex (Shroyer et al., 2001; Zhang et al., 2015), a value that increased to 25% in our results. Moreover, we reported the variant c.3386G > T (p.Arg1129Leu) in ABCA4 in 37.5% of the resolved patients with mutations in this gene (Table 1), which was not surprising because this variant appears to be very frequent in the Spanish population as we can observe in a study performed by Del Pozo-Valero et al. (2020). On the other hand, variants with an allelic frequency higher than 1% (MAF < 0.01) are usually ruled out as
**TABLE 2** | Patients in which only one pathogenic variant in a recessive gene has been identified.

| Family | Patient | Clinic diagnosis | Gene | Nucleotide change | Protein change | Zygosity | References |
|--------|---------|------------------|------|-------------------|----------------|----------|------------|
| FRPN-243 | RPN-645 | RP | USH2A (NM_206933.4) | c.2276G > T | p.(Cys759Phe) | heterozygous | Rivolta et al., 2000 |
| FRPN-272 | RPN-678 | STG | ABCA4 (NM_000350.3) | c.754G > T | p.( Gly252Cys) | heterozygous | Bravo-Gil et al., 2016 |
| FRPN-283 | RPN-691 | RP | CDHR1 (NM_033100.3) | c.783G > A | (p.Pro261 = ) | heterozygous | Glockle et al., 2014 |

Novel pathogenic variants identified are highlighted in bold font.

FRPN, family number; FRPN-1, families studied with PV1; FRPN-2, families studied with PV2; RPN, patient number; RP, retinitis pigmentosa; STG, stargardt; NA, not available; p.?, unknown protein effect; SNHL, sensorineural hearing loss; XL, X-linked. The * symbol corresponds to: stop-codon according to the format of mutations nomenclature from the Human Genome Variation Society (HGVS).

**FIGURE 1** | Representation of the total alleles identified in this study classified according to the alteration type. The X-axis refers to the different types of variants identified in this study, and the Y-axis concerns the total allele count for each type of variant. Pathogenic variants identified in this study are represented in green, while those previously described are in orange.

the cause of disease in prioritization analyses; however, in some cases, the frequency of some variants, although > 1%, is higher in patients than in healthy individuals, suggesting that they are causal for the disease. Examples of this have been reported for ABCA4. For instance, the variant c.5603A > T (p.Asn1868Ile) was presented in STGD patients four times more frequently than expected (Zernant et al., 2017), and it is a disease-causing variant in 5% of patients when it is in trans with a severe allele in ABCA4. This variant was initially considered benign, however, recent studies consider it pathogenic with reduced
| Gene         | Mutation         | Classification | Frequency (gnomAD Ex) | Pathogenicity scores<sup>a</sup> | Conservation score (GERP)<sup>b</sup> | ClinVar | HGMD<sup>c</sup> |
|-------------|-----------------|----------------|-----------------------|--------------------------------|---------------------------------------|---------|-----------------|
| ABCA4       | c.2953G > A     | Likely pathogenic | NF                    | 12 of 13                        | 5.1979                                | NA      | NA              |
|             | c.4880del       | Pathogenic      | 0.0000159             | NA                              | 5.6399                                | NA      | NA              |
|             | c.5714 + 1G > A | Pathogenic      | 0.00000398            | NA                              | 4.7399                                | NA      | NA              |
| CEP290      | c.6310C > T     | Pathogenic      | NF                    | NA                              | 5.6799                                | NA      | NA              |
|             | c.7394_7395del  | Pathogenic      | 0.0000598             | NA                              | 5.42                                  | NA      | NA              |
| CNGA3       | c.673 + 5G > T  | Likely pathogenic | 0.0000199             | NA                              | 5.09                                  | NA      | NA              |
|             | c.3339 + 5G > C | Likely pathogenic | 2 of 2                | NA                              | NA                                    | NA      | NA              |
| CRG1        | c.481G > A      | Likely pathogenic | 0.0000579             | NA                              | 1.8514                                | NA      | NA              |
|             | c.1194del       | Pathogenic      | NF                    | NA                              | 6.07                                  | NA      | NA              |
| GUCA1A      | c.66C > A       | Likely pathogenic | 0.00000795            | NA                              | 5.75                                  | NA      | NA              |
|             | c.673 + 5G > T  | Likely pathogenic | 0.00000398            | NA                              | 5.4499                                | NA      | NA              |
|             | c.7394_7395del  | Pathogenic      | NF                    | NA                              | 5.09                                  | NA      | NA              |
| OTX2        | c.638T > A      | Pathogenic      | NF                    | NA                              | 5.21                                  | NA      | NA              |
| PROM1       | exon 11 del     | Pathogenic      | NF                    | NA                              | 5.32                                  | NA      | NA              |
| RHO         | c.647C > A      | Likely pathogenic | 0.0000128             | NA                              | 4.19                                  | NA      | NA              |
|             | c.1079_1080del  | Uncertain significance | 0.0000128            | NA                              | 4.57                                  | NA      | NA              |
| RPRH2       | c.440dup        | Pathogenic      | NF                    | NA                              | 5.69                                  | NA      | NA              |
| USH2A       | c.695C > T      | Likely pathogenic | 0.0000159             | 12 of 13                        | 5.0999                                | NA      | NA              |
|             | c.13894C > T    | Likely pathogenic | 0.00000797            | 8 of 13                         | 5.21                                  | NA      | NA              |

Column “Classification” refers to classification according to AMCG. <sup>a</sup>Pathogenicity Scores from https://varsome.com/for missense variants (accessed November 2020) (BayesDel_addAF, DANN, DEOGEN2, EIGEN, FATHMM-MKL, LIFT-S2, M-CAP, MVP, MutationAssessor, MutationTaster, PrimateAI, REVEL, and SIFT). Represent predictors supporting the pathogenic effect against the total of available predictors. <sup>b</sup>GERP conservation score based on the reduction in the number of substitutions in the multi-species sequence alignment compared to the neutral expectation using the genomes of 35 mammals. Range: -12.3 to 6.17 (most conserved). <sup>c</sup>HGMD public version (accessed November 2020). p.?, unknown protein effect; NF, no found; NA, not applicable. The "*" symbol corresponds to: stop-codon according to the format of mutations nomenclature from the Human Genome Variation Society (HGVS).
penetrance (Zernant et al., 2017; Cremers et al., 2018; Runhart et al., 2018). For autosomal dominant cases, PRPH2 was found to be responsible for 14.8% of the resolved cases (Table 1), whilst a prevalence of 10.3% in PRPH2 was the highest obtained thus far (Manes et al., 2015).

Concerning the patients who carry mutations in more than one IRD gene (Table 1), it is estimated that 2.7 billion individuals worldwide (36%) are carriers of an IRD disease-causing mutation, whereas 5.5 million are expected to be affected (Hanany et al., 2020). In this study, we diagnosed patient RPN-670, who was a carrier of disease-causing variants in RP1, PRPH2, and USH2A (Table 1). Despite the fact that any relative displays any symptoms, we cannot confirm that PRPH2 and RP1 variants are de novo, as DNA from family members was not available for segregation analysis. Incomplete penetrance can also be suggested, since it has been described for patients carrying pathogenic variants in PRPH2 and RP1 (Dietrich, 2002; Boon et al., 2008; Thiadens et al., 2012; Coco-Martín et al., 2020).

It is important to remark that the fact of being a carrier of pathogenic variants in more than one gene can have a major impact on the reproductive choices for IRD patients, as well as impact their eligibility for gene-specific genetic therapies. This finding outstands the significance of achieving an exhaustive diagnosis.

The OTX2 gene is associated with syndromic diseases, such as microphthalmia and retinal degeneration with or without pituitary dysfunction (OMIM: 610125; 610125). In this study, we also identified a nonsense variant in the OTX2 gene in a family (FRPN-300) (Table 1) with an autosomal dominant mode of inheritance but only retinal symptoms. Similarly, two autosomal dominant families with heterozygous pathogenic variants in OTX2 with only retinal degeneration patterns were previously reported (Vincent et al., 2014). Eleven truncating and two missense pathogenic variants have been described in this gene (LOVD accessed on April 7, 2021) and both type of variants have been associated with both syndromic and non-syndromic cases (Vincent et al., 2014; Slavotinek et al., 2015; Ellingford et al., 2016; Wang et al., 2016; Bryant et al., 2017; Patel et al., 2018; Sanchez-Navarro et al., 2018). Thus, a correlation between the type of mutation and the clinical diagnosis cannot be assessed.

A clear difference of the use of PV1 or PV2 concerning the solved cases (57.1% with the PV1 and 58.6% with PV2) did not exist. Strikingly, the rate of partially solved cases is almost half for the PV1 (11.1%) when compared to the PV2 (19.35%). It would be expected that the number of partially solved cases would be lower with a panel that includes all the deep-intronic mutations reported to date for ABCA4 and USH2A. In our opinion, this could be due to the lower sample size analyzed with the PV2.

In general, custom panel designs remain an excellent option for the genetic diagnosis of IRD. However, due to the rapid increase of the number of genes involved, some groups prefer to use alternative approaches, such as WES (Lee et al., 2015; Riera et al., 2017; Zhang et al., 2018). Both sequencing strategies have their pros and cons. A specific panel of genes will allow the study of known IRD at a high time/effectiveness ratio regardless of the clinical diagnosis, which is an advantage in those cases in which the clinical diagnosis is not well defined or overlaps between different clinical entities. Furthermore, it allows not only the possibility of including a greater number of probes in repetitive regions such as ORF15 of the RPGR gene, which is highly involved in X-linked IRD cases (Vervoort et al., 2000; Megaw et al., 2015; Chiang et al., 2018; Charng et al., 2019),
but also reported deep-intronic pathogenic variants (González-del Pozo et al., 2018; Di Scipio et al., 2020). On the other hand, WES made it possible to find mutations in novel IRD-related genes without updating the panel in low prevalent genes not included for the sake of increasing the depth of coverage or to identify novel IRD genes. The price reduction for high throughput sequencing has made the costs between the panels and WES quite even, so, it does not make the difference between the panels and WES. Taking this into account, the choice between both depends on the preferences of the clinician/researcher in terms of comprehensiveness, accuracy, and time consumption.

Currently, there is no significant difference between the panels and WES concerning the diagnostic rates, as the WES rate did not reach > 70% (Lee et al., 2015; Riera et al., 2017; Wang et al., 2018; Zhang et al., 2018; Liu et al., 2020). Therefore, further studies, including introns, other non-coding regions and epigenetics, are needed to achieve a comprehensive molecular diagnosis of IRD.

DATA AVAILABILITY STATEMENT

The data presented in this study are deposited in the European Genome-phenome Archive (EGA), Study: EGAS00001005369 and Dataset: EGAD00001007755.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Hospital La Fe Ethics Committee. Written informed consent to participate in this study was provided by the participants’ legal guardian/next of kin.

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AUTHOR CONTRIBUTIONS

EA, JM, and GG: conceptualization. BG and AR: methodology. BG, AR, EA, and TJ: resources. BG: writing—original draft preparation. BG, AR, EA, TJ, GG, and JM: writing—review and editing. GG and JM: supervision. JM: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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