Inherited retinal dystrophies (RD) are a group of diseases characterized by a progressive degeneration of photoreceptors and retinal pigmented epithelium cells (RPE) leading to visual impairment. Of all types of inherited RD, retinitis pigmentosa (RP; MIM 268000) is the most prevalent subset, with a prevalence of 1:4000 people.1 It is characterized by primary degeneration of rod photoreceptors. Typically, night blindness is the first symptom of the disease, followed by a loss of peripheral vision and, in most of the cases, cone degeneration in the late stage. Leber congenital amaurosis (LCA; MIM 204000), with a prevalence of approximately 1:30000,2 is the earliest and most severe form of inherited RD and is responsible for congenital impaired vision or blindness. Congenital nystagmus and a nonrecordable electroretinogram (ERG), before 1 year of life, are frequent signs of the disease, which may be accompanied by sluggish or absent pupillary reaction and eye poking. Cone–rod dystrophy (CRD; MIM 120970), with a prevalence of approximately 1:35000,3 is characterized by primary cone dysfunction in the early stage and subsequent rod degeneration. Clinical manifestations include photoversion, reduced visual acuity, color vision defects, and paracentral scotomas. Nystagmus may be present in some cases. Within macular dystrophies (MD), Stargardt disease type I (STGD1; MIM 248200) is the most common
TABLE 1. Contribution of the Mutations in the Most Frequent RD Genes to the Different Phenotypes in the Spanish Population

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotypes (%)</th>
</tr>
</thead>
</table>
| ABCA4    | STGD (70.5)\(^9\)  
|          | arCRD (36.6)   |
| USH2A    | Usher syndrome (62.9)\(^10\)  
|          | arRP (7)       |
| RHO      | adRP (21)\(^11\)  
| CRB1     | LCA (14)\(^12\)  
|          | EORP(9)        |
| CERKL    | arRP (4.8)\(^13\) 
| RP1      | EORP (4.5)\(^14\)  
|          | adRP (3.3)\(^13\) |

juvenile MD, with a prevalence of 1:10000.\(^4\) It is characterized commonly by early-onset visual acuity loss, although it also can appear later in life. Approximately 20% to 30% of RD cases have been associated with extraocular symptoms leading to a diverse range of syndromes. Among them, Usher syndrome, an association of RP with hearing impairment, is the most frequent syndromic RD. It accounts for approximately 15% to 20% of all RP cases. Another representative syndromic form, accounting for 5% to 6% of cases of RP, is Bardet-Biedl syndrome in which RP is associated with obesity, polydactyly, cognitive impairment, hypogonadism, and renal dysfunction.\(^5\)

All RD show a marked clinical and genetic heterogeneity. For RP, the most frequent RD, all Mendelian inheritance patterns have been described: autosomal dominant (adRP), accounting for 15% to 25% of cases, autosomal recessive (arRP), representing 35% to 50%, and X-linked (xlRP), accounting for 7% to 15%.\(^5\) Approximately 40% of RP cases are sporadic. Other inheritance patterns also exist, such as maternal, digenic, triallelic, and isodisomy.\(^6\)–\(^8\) Mutations in more than 200 genes have been identified for the different forms of RD (RetNet; available in the public domain at https://sph.uth.tmc.edu/RetNet/). Some of them have been associated with more than one phenotype or inheritance pattern as displayed in Table 1,\(^9\)–\(^14\) which shows the most prevalent RD genes in the Spanish population.

The tremendous heterogeneity of this group of diseases makes the genetics of RD really complicated. Multiple findings underscore this heterogeneity: Different mutations in the same gene may cause different phenotypes (the PRPH2 gene involved in adRP and adMD, ABCA4 in arCRD and STGD, USH2A in arRP or Usher type II, RPGR in xlRP and xlMD), RD due to mutations in the same gene may display different inheritance patterns (autosomal dominant or recessive due to mutations in the RP1, CRX, and NR2E3 genes), and the same mutation may exhibit intra- or interfamilial phenotypic variability (mutations in the BEST1 and PRPH31 genes). Moreover, to our knowledge genotype-phenotype correlations have not been fully established yet.

This scenario shows the real challenge linked to the molecular characterization of RD. The high frequency of RD variants random carriers has been widely described.\(^15\)–\(^16\) The aim of this article is to know the basis of RD intrafamilial locus and allelic heterogeneity to further discriminate the contribution of the monoallelic mutations to the pathology.

Patients and Methods

Patients

Patients clinically diagnosed with RD were recruited from the Fundacion Jimenez Diaz Hospital (Madrid, Spain). The study was reviewed and approved by the Ethics Committee of the hospital, and adhered to the tenets of the Declaration of Helsinki and further reviews. Informed consent was obtained from all subjects before their participation in this study.

We included 2468 nonsyndromic and syndromic unrelated Spanish families with RD who had been studied according to the molecular methods described below: 873 of them (55.4%) were fully characterized. They were distributed in 354 (40%) of MD, 308 (35%) of RP, 107 (12%) of syndromic RD, 52 (6%) of LCA, and 52 (6%) of CRD cases.

Clinical Evaluation

Diagnosis of RD was focused mainly on measurements of visual acuity (VA) and visual field (VF) tests, fundus examination, and ERG responses. Differential diagnosis of RP, LCA, CRD, MD, STGD, and syndromic RP forms (Usher type II and Bardet-Biedl syndromes) was determined conforming to their respective mode of inheritance (genetic classification was performed according to the study of Ayuso et al.\(^17\)) and the clinical criteria described in multiple studies.\(^2\)\(^,\)

Molecular Analysis

Molecular characterization of the RD families was performed by combining the following genotyping tools: APEX technology-based commercial genotyping chip (Asper Biotech, Tartu, Estonia)\(^9\)\(^,\)\(^12\)\(^,\)\(^13\)\(^,\)\(^15\)\(^,\)\(^24\), direct mutational screening by denaturing high pressure liquid chromatography (dHPLC), single-strand conformational polymorphism (SSCP), high resolution melt (HRM), multiplex ligation-dependent probe amplification (MLPA), or Sanger sequencing\(^2\)\(^,\)\(^12\), indirect analysis by microsatellites, whole-genome homozygosity mapping using high-resolution commercial single nucleotide polymorphism (SNP) arrays from Affymetrix (Santa Clara, CA, USA) or Illumina (San Diego, CA, USA)\(^12\)\(^,\)\(^14\)\(^,\)\(^25\)\(^,\)\(^26\), or next-generation sequencing (NGS) technologies using 2 targeted RD gene panels, including more than 70 genes, or by whole exome sequencing (WES).\(^14\)\(^,\)\(^27\)\(^,\)\(^28\)

All identified variants were annotated according to the nomenclature recommendations of the Human Genome Variation Society. To predict the potential impact of the variants on protein function, missense mutations were analyzed by bioinformatics programs, including Sorting Intolerant from Tolerant (SIFT; available in the public domain at http://sift.jcvi.org) and Polymorphism Phenotyping v2 (Polyphen-2; available in the public domain at http://genetics.bwh.harvard.edu/pph2). The effect on splicing of the variants identified was analyzed by different softwares: Analyzer Splice Tool (AST; available in the public domain at http://ibis.tau.ac.il/ssa/SpliceSiteFrame.htm) and Berkeley Drosophila Genome Splice Site Prediction (BDGP; available in the public domain at http://www.fruitfly.org/seq_tools/splice.html). All changes were checked by Sanger sequencing, and segregation of the potentially pathogenic mutations was confirmed in all cases within the family and with the pathology.

Selection of Cases

Among the 873 fully characterized families, we searched for diverse mechanisms of intrafamilial genetic heterogeneity, including disease-causing mutations in more than one gene within the same family (locus heterogeneity) and/or different disease-causing mutations in the same gene within the same family (allelic heterogeneity).
RESULTS

Disease-Causing Mutations in More Than One RD Gene in the Same Family

We specifically described 5 of the 873 fully characterized families (0.6%) from our cohort, in which more than one RD gene were segregating within the same family. The initial clinical examination demonstrated distinct phenotypes (macular versus peripheral forms, early-onset versus congenital versus late-onset forms) that, in all cases except one, were clearly differentiated between patients within the family.

Family RP-0184 is a very large pedigree with 7 different branches and inbreeding events, in which 5 of the subfamilies were studied (Fig. 1). All members of this family were from a particular area of central Spain. The affected members of 2 of the subfamilies (VI:6, subfamily 1 and VI:10, subfamily 7) presented a clearly distinct phenotype with bilateral sensorineural hearing impairment along with typical symptoms of RP, characteristics of Usher II form (Table 2). Direct sequencing of the USH2A gene revealed that these individuals were compound heterozygotes for different mutations in the USH2A gene: p.Glu767Serfs*21/p.Cys425Phefs*4 (individual VI:6, subfamily 1) and p.Glu767Serfs*21/p.Arg303His (individual VI:10, subfamily 7). These findings were consistent with the phenotype (Table 2). In all other branches, the affected individuals presented an early-onset RP phenotype. In the subfamily 3, the genotyping chip revealed the p.Tre49Met mutation in homozygosis in the RDH12 gene in the proband (VII:1). In the other branch (subfamily 6), still uncharacterized by conventional methods, we tested mutations in the proband, who had a very similar early-onset RP phenotype, with a NGS RD resequencing gene panel. Thus, we found a mutation in a third additional gene to be segregating in the family. The novel variant p.Arg149Trp was found in homozygosis in the TULP1 gene. This variant was predicted as highly pathogenic after in silico analysis, it is located in a very highly conserved region and it was discarded in 150 control alleles. The change was carried in the proband and in his affected sibling, and segregated with the pathology and exclusively within this subfamily (Table 2). It is not ruled out that other genes also would be involved in this family, as there still is one branch in study in which any contribution from these 3 genes was excluded, remaining yet molecularly uncharacterized.

FIGURE 1. Pedigrees of the RD families with disease-causing mutations in more than one RD gene within the family. The proband is marked by an arrow in each case. The genotype of each affected member is represented below the individual symbol, being "m, m1, m2, m3, and m4" the different mutated alleles and "?" individuals uncharacterized yet. All the variants were confirmed to be exclusive of each particular subfamily, being excluded in the rest. (A) Disease-causing mutations in three different genes within this family: USH2A, segregating with Usher II syndrome, and RDH12 and TULP1 with an EORP. NS, nonstudy. (B) Mutations in the PDE6B and in a new candidate gene were found in the 4 affected siblings with an EORP phenotype. (C) One mutation in the CERKL gene and a combination of distinct CRB1 alleles was found in this pedigree, causing different phenotypes within the affected members in the family. (D) Mutations in the C2orf71 and BBS1 genes were found within this family, segregating with their RP and Bardet-Biedl syndrome phenotypes, respectively. (E) Mutations in the CRB1 and ABCA4 genes segregating with LCA and STGD phenotypes, respectively, in the same family.
Table 2. Clinical Findings in Patients With Disease-Causative Mutations in More Than One RD Gene in the Same Family

<table>
<thead>
<tr>
<th>Family</th>
<th>Subfamily</th>
<th>ID</th>
<th>Gene</th>
<th>Mutations</th>
<th>References</th>
<th>First Symptoms and Course</th>
<th>Age of Ophthalmic Evaluation, y</th>
<th>BCVA RE/LE</th>
<th>Visual Field RE/LE</th>
<th>ERG</th>
<th>Fundus Aspect</th>
<th>Additional Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subfamily-3</td>
<td>VII:1</td>
<td>RDH12</td>
<td>p.T49M/p.T49M</td>
<td>32</td>
<td>NB (3 y), diminished VA (5 y), diminished VF (3 y)</td>
<td>15</td>
<td>0.7/0.2</td>
<td>Inferior nasal scotoma with reduced sensibility in the remaining field</td>
<td>Diminished Pale optic disc, retina vessels attenuation and bone spicule pigmentation Macular alteration</td>
<td>Normal hearing acuity (15 y)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Subfamily-6</td>
<td>VI:16</td>
<td>TULP1</td>
<td>p.R419W/p.R419W</td>
<td>Novel</td>
<td>NB (4 y), diminished VA (4 y), diminished VF (4 y)</td>
<td>36</td>
<td>LP/LP</td>
<td>ND</td>
<td>ND</td>
<td>Macular unstructured and atrophy in left macula</td>
<td>Cataract (50 y), nystagmus</td>
</tr>
<tr>
<td></td>
<td>Subfamily-7</td>
<td>VII:10</td>
<td>USH2A</td>
<td>p.E767S*21/p.R306H</td>
<td>30, 33</td>
<td>NB (25 y), diminished VA (25 y), diminished VF (36 y)</td>
<td>54</td>
<td>0.6/0.5</td>
<td>10/10</td>
<td>NR</td>
<td>Slightly pale optic disc, retina vessels attenuation and bone spicule pigmentation, normal macula</td>
<td>Cataract 30 y, bilateral sensorineural hearing impairment (7 y)</td>
</tr>
<tr>
<td></td>
<td>Subfamily-2</td>
<td>II:2</td>
<td>PDE6B</td>
<td>p.Q298R/p.Q298R</td>
<td>54</td>
<td>NB (7 y), diminished VA and VF</td>
<td>65</td>
<td>0.4/CF 1m</td>
<td>ND</td>
<td>ND</td>
<td>Pale optic disc, retina vessels attenuation and bone spicule pigmentation covering the entire retina</td>
<td>Cataract, ocular hypertension</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II:4</td>
<td>PDE6B</td>
<td>p.Q298R/p.Q298R</td>
<td>54</td>
<td>NB (7 y), diminished VA and VF</td>
<td>67</td>
<td>0.6/CF</td>
<td>ND</td>
<td>ND</td>
<td>Pale optic disc</td>
<td>Cataract (21 y), glaucoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II:5</td>
<td>New candidate gene</td>
<td>p.Q298R/p.Q298R</td>
<td>51</td>
<td>NB (8 y), diminished VA and VF</td>
<td>51</td>
<td>0.1/0.4</td>
<td>Severe scotoma</td>
<td>ND</td>
<td>Pale optic disc</td>
<td>Cataract</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II:6</td>
<td>PDE6B</td>
<td>p.Q298R/p.Q298R</td>
<td>54</td>
<td>NB (9 y), diminished VA and VF</td>
<td>54</td>
<td>0.5/0.25</td>
<td>10/10</td>
<td>ND</td>
<td>Pale optic disc, dispersed bone spicule pigmentation</td>
<td>Cataract (25 y)</td>
</tr>
<tr>
<td>MD-0092</td>
<td>Subfamily-1</td>
<td>IV:1</td>
<td>CERKL</td>
<td>p.R257H/p.R257</td>
<td>35</td>
<td>NB (30 y), diminished VA (16 y) and VF (28 y)</td>
<td>36</td>
<td>0.2/0.2</td>
<td>Central scotoma Pathologic flash both eyes</td>
<td>Pale optic disc, slightly retina vessels attenuation and extensive RPE macular atrophy well delimited</td>
<td>Photosensitivity (16 y)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Subfamily-2</td>
<td>III:4</td>
<td>CRB1</td>
<td>p.1167_G169del/p.C948Y</td>
<td>36, 37</td>
<td>NB (40 y), diminished VA (11 y) and VF (11 y)</td>
<td>70</td>
<td>CF 3 cm/CF 3 cm</td>
<td>Absolute scotoma</td>
<td>ND</td>
<td>General RPE and macular atrophy</td>
<td>Cataract, photophobia, hypermetropia, astigmatism</td>
</tr>
<tr>
<td></td>
<td>Subfamily-3</td>
<td>III:6</td>
<td>CRB1</td>
<td>p.C948Y/p.C948Y</td>
<td>37</td>
<td>NB (6 y), diminished VA (5 y) and VF (6 y)</td>
<td>59</td>
<td>LP/amaurotic</td>
<td>ND</td>
<td>ND</td>
<td>Cataract (LE), ocular hypertension RE, nystagmus (from born)</td>
<td>Cataract (LE), ocular hypertension RE, nystagmus (from born)</td>
</tr>
<tr>
<td>Family</td>
<td>Subfamily</td>
<td>ID</td>
<td>Gene</td>
<td>Mutations</td>
<td>References</td>
<td>First Symptoms and Course</td>
<td>Age of Ophthalmic Evaluation, y</td>
<td>BCVA RE/LE</td>
<td>Visual Field RE/LE</td>
<td>ERG</td>
<td>Fundus Aspect</td>
<td>Additional Findings</td>
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<tr>
<td>RP-0622</td>
<td>III:1</td>
<td>C2ORF71</td>
<td>p.I210F/p.I210F</td>
<td>25 NB (18 y), diminished VA (25 y) and VF (26 y)</td>
<td>27</td>
<td>0.4/0.1</td>
<td>Absolute scotoma RE</td>
<td>Abolished</td>
<td>Pale optic disc, retina vessels attenuation and bone spicule pigmentation, macular unstructured and atrophy in left macula</td>
<td>Color alteration, cataract (27 y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>II:7</td>
<td>BBS1</td>
<td>p.M390R/p.M390R</td>
<td>38 NB (3 y), diminished VA (3 y) and VF (3 y)</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Maculopathy with RPE atrophy, hyperpigmentation, few central yellowish flecks, slight temporal papillary pallor, no constriction of retinal vessels</td>
<td>Polydactyly, intellectual disability Photophobia, myopia, and astigmatism (14 y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP-0280</td>
<td>II:1</td>
<td>ABCA4</td>
<td>p.N1805D/p.N1805D</td>
<td>39 No NB or restriction of VF, loss of VA</td>
<td>26</td>
<td>0.1/0.1</td>
<td>No restriction</td>
<td>Slightly reduced amplitude for rod, mixed cone-rod, cone single flash, and cone flicker</td>
<td>Roundish pigments distributed across the entire retina, including peripheral retina, posterior pole, and macular region</td>
<td>Hyperopia, astigmatism, and nystagmus</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>II:4</td>
<td>CRB1</td>
<td>p.C948Y/p.W822*</td>
<td>57.56 NB (14 y), diminished VF (2 y), and reduction central VA (14 y)</td>
<td>14</td>
<td>0.1/0.2</td>
<td>Concentrically constricted with small remaining central and nasal islands (&lt;10°)</td>
<td>Not discernable from noise anymore</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ID, identification; BCVA, best corrected visual acuity; OD, right eye; OS, left eye; NB, night blindness; ND, no data; NR, nonrecordable; CF, counting fingers; LP, light perception.
The consanguineous RP-1712 family (Fig. 1) has 4 affected siblings with very similar phenotypes in terms of age of onset and progression, suggestive of an early-onset RP (Table 2). The genotyping chip revealed the previously described p.Gln298* mutation in homozygosis in the \textit{PDE6B} gene, in only 3 of the siblings (II:2, II:4, and II:6). This change was confirmed by Sanger sequencing. The other affected sibling (II:5) was heterozygous for the mutation and no second pathogenic allele was found. Other pathological variants in associated genes in this patient were excluded by further NGS targeted RD gene resequencing. Thus, homozygosity mapping and WES was performed allowing the identification of a pathological variant in homozygosis in a new candidate gene (manuscript in preparation).

Pedigree MD-0092 has 3 different branches with suspected inbreeding in one of them. This family presented various affected individuals of different generations with different phenotypes and age of onset (Fig. 1, Table 2). Possible pseudodominance was discarded. The affected sibling (II:5) was heterozygous for the mutation and no second pathogenic allele was found. Other pathological variants in associated genes in this patient were excluded by further NGS targeted RD gene resequencing. Thus, homozygosity mapping and WES was performed allowing the identification of a pathological variant in homozygosis in a new candidate gene (manuscript in preparation).

We included 2 additional families reported previously. One of these, described by Nishimura et al.\textsuperscript{25} was the RP-0622 family, with 2 affected individuals of different generations and a consanguinity event (Fig. 1). Initial clinical findings led us to suspect the existence of intrafamilial heterogeneity due to the different segregation of nonsyndromic RD and extraocular symptoms in the affected members. The proband presented typical clinical features of RP as summarized in Table 2, while his maternal aunt (II:7) showed additional intellectual disability and polydactyly, dealing with characteristic symptoms of BBS. Homozygosity mapping and sequencing revealed causative mutations in two ciliary genes: \textit{C2orf71} in the proband and \textit{BBS1} in his aunt, consistently segregating with their respective phenotypes.

The RP-0280 family, with two affected siblings, was described previously by Rivero-Alvarez et al.\textsuperscript{29} The proband...
### TABLE 3. Clinical Findings in Patients Showing Intrafamilial Variability due to Different Mutations in the Same RD Gene

<table>
<thead>
<tr>
<th>Family</th>
<th>Subfamily</th>
<th>ID</th>
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<th>BCVA RE/LE</th>
<th>Visual Field RE/LE</th>
<th>ERG</th>
<th>Fundus Aspect</th>
<th>Additional Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP-0714</td>
<td>II:5</td>
<td>5</td>
<td>ABCA4</td>
<td>c.4253+4C&gt;T</td>
<td>43</td>
<td>NB (50 y), diminished VA (10 y), diminished VF (30 y)</td>
<td>40</td>
<td>≤ 0.1/≤ 0.1</td>
<td>Central scotoma</td>
<td>ND</td>
<td>ND</td>
<td>Photophobia</td>
</tr>
<tr>
<td></td>
<td>III:3</td>
<td>4</td>
<td>ABCA4</td>
<td>c.4253+4C&gt;T/p R1129L</td>
<td>45, 44</td>
<td>Diminished VA (22 y)</td>
<td>22</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Photophobia</td>
</tr>
<tr>
<td></td>
<td>Subfamily-1</td>
<td>IV:3</td>
<td>CRB1</td>
<td>p.C948Y/p.C948Y</td>
<td>37</td>
<td>NB birth, diminished VA (30 y), diminished VF (20 y)</td>
<td>48</td>
<td>Amaurosis</td>
<td>Absolute scotoma</td>
<td>NR</td>
<td>Difficult to evaluate due to leukoma</td>
<td>Nystagmus, dense cataracts, corneal leukoma secondary to keratoconus, microphthalmus</td>
</tr>
<tr>
<td></td>
<td>Subfamily-2</td>
<td>V:2</td>
<td>CRB1</td>
<td>p.C948Y/p.I1100T</td>
<td>37, 42</td>
<td>ND</td>
<td>21</td>
<td>0.1/0.2</td>
<td>&lt;5°</td>
<td>NR</td>
<td>Bone spicule pigmentations, pale papilla, constricted arterioles</td>
<td>Nystagmus (7 m)</td>
</tr>
<tr>
<td>LCA0038</td>
<td>Subfamily-1</td>
<td>V:1</td>
<td>CRB1</td>
<td>p.C984*/p.I1001N</td>
<td>45, 23</td>
<td>Diminished VA</td>
<td>1.5</td>
<td>ND</td>
<td>Partially preserved central vision with reduced sensitivity in inferior VF</td>
<td>NR</td>
<td>Slightly pale optic disc, attenuation of retinal vessels, granular and grayish aspect of RPE, dense yellowish area in all macular region</td>
<td>Nystagmus, photophobia</td>
</tr>
<tr>
<td></td>
<td>Subfamily-2</td>
<td>III:4</td>
<td>CRB1</td>
<td>p.D564Y/p.I11001N</td>
<td>23</td>
<td>NB (5 y), diminished VA (3 y), diminished VF (3 y)</td>
<td>51</td>
<td>LP/IP</td>
<td>Almost absolute scotoma</td>
<td>ND</td>
<td>ND</td>
<td>Cataracts (40 y)</td>
</tr>
<tr>
<td></td>
<td>III:5</td>
<td>5</td>
<td>CRB1</td>
<td>p.D564Y/p.I11001N</td>
<td>23</td>
<td>NB (12 y), diminished VA (12 y), diminished VF (20 y)</td>
<td>51</td>
<td>LP/IP</td>
<td>Almost absolute scotoma</td>
<td>ND</td>
<td>ND</td>
<td>Cataracts (40 y)</td>
</tr>
<tr>
<td></td>
<td>Subfamily-3</td>
<td>III:12</td>
<td>CRB1</td>
<td>p.I11001N/p.Y1161C</td>
<td>23, 46</td>
<td>NB (45 y), diminished VA (40 y), diminished VF (52 y)</td>
<td>55</td>
<td>0.5/0.4</td>
<td>Annular scotoma</td>
<td>NR</td>
<td>Pale optic disc, retina vessels attenuation and bone spicule pigmentation, peripapilar atrophy, normal macula</td>
<td>Photophobia, hearing loss (55%) 36 y, diplopia (27 y) corrected by vita mins, hypermetropia, astigmatism, subcapsular cataract both eyes</td>
</tr>
</tbody>
</table>
(II-4) presented a severe early-onset RP, while her affected sibling (II-1) had a typical STGD phenotype (Fig. 1). The genotyping chip revealed a missense mutation in homozygosis in the \(ABCA4\) gene in the individual II-1, segregating with the disease and within the family. The proband was heterozygous for the \(ABCA4\) mutation, but no additional variants were found in the second mutated allele in this gene. Further investigation, testing mutations by screening on the genotyping chip in combination with DHPLC, revealed that the proband was compound heterozygous for mutations in the \(CRB1\) gene (Table 2).

**Intrafamilial Phenotypic Variability due to Different Mutations in the Same RD Gene**

It has been described previously that mutations in the same gene are implicated in several RD cases, associated with a wide range of phenotypic manifestations.\(^{30,41}\) In our cohort of patients we found 3 of 873 families (0.3%) harboring different mutations associated with distinct phenotypes, specifically in two of the most common RD genes: \(CRB1\) and \(ABCA4\).

The RP-0714 family has two affected members: a woman with a CRD phenotype (II-3), born to consanguineous parents, and her affected daughter (III-3) who points to a STGD phenotype and a slower progression at the time of writing (Fig. 2, Table 3). In the genotyping chip, \(c.4253+1C>T\) mutation in the \(ABCA4\) gene, putatively affecting splicing, was found in the mother (in homozygosis) and in her daughter (in compound heterozygosity with a second mutation not present in her mother, p.Arg1129Leu). Bioinformatic evaluation of the \(c.4253+1C>T\) mutation predicts that it decreases the strength of the 5′ splice site (from 0.99 to 0.77) of exon 28, putatively leading to exon skipping, correlating with a severe phenotype when in homozygosis.

The families RP-0069 and LCA-0058 were described previously.\(^{12,42}\) Both are extended families with distinct branches and various affected individuals from different generations (Fig. 2). The LCA and EORP phenotypes can be distinguished in each of the branches. By a combination of methods, different mutations in the \(CRB1\) gene were found, segregating within the family and with the associated phenotypes (Table 3).

**Discussion**

The clinical and genetic heterogeneity of RD has been widely described.\(^{37,48}\) The extended mechanism of RD heterogeneity can complicate the work of clinicians and geneticists when seeking an accurate diagnosis. This report aims to assess the mutational load in known arRP genes in the general population. Not to mislead false inheritance patterns, such as pseudo-dominance (in pedigrees RP-0622 and MD-0092) or ambiguous inheritance patterns, could be observed.

As reported here, there is great variability in intrafamilial genetic heterogeneity in RD. One common heterogeneity mechanism is to find two different RD genes segregating in the same family. This could be explained by the high rate of coincidental carriers in the general population, as we described. This mechanism frequently occurs in the \(ABCA4\) and \(CRB1\) genes, which are highly involved in either locus and allelic heterogeneity in the Spanish population, as reflected in the present research, as well as in other populations.\(^{50,51}\) Moreover, dealing with extended families with multiple branches, like RP-0184 and LCA-0058, and/or with consanguinity events, such as RP-0622, RP-1712, RP-0184, and RP-0714 families, increases the probability of finding intrafamilial genetic heterogeneity events, as previously described.\(^{52}\)

In the MD-0092 pedigree with genetic and phenotypic heterogeneity, we found a remarkable finding in \(CRB1\), one of the genes involved mostly in genetic heterogeneity. In one of the individuals (III-4), we found the “\(\text{a priori}\)” uncertain significant clinical p.Ile167_Gly169del variant. This change was reported in other five families from our cohort,\(^{13}\) always in combination with other \(CRB1\) pathogenic allele, which may suggest to be an hypomorphic allele.

From a large cohort of 873 fully genetically characterized Spanish families, we identified a total of 8 pedigrees in which mutational load contributes to intrafamilial heterogeneity, which represents a frequency of almost 1%. Other complementary studies will be necessary, including NGS techniques, which help us to estimate the real rate of mutational load promoting RD intrafamilial heterogeneity.

To our knowledge, this is the first time that a systematic research of RD intrafamilial heterogeneity has been done. This study is an essential step toward identifying the genetic mechanisms underlying RD to discern the real contribution of the individual pathological variants in the disease, especially in the NGS era, when mutant alleles not underlying the pathology may be found.\(^{53}\) The collection of these sets of genetic mechanisms and their frequency is important in establishing a better genotype-phenotype correlation and to provide accurate genetic counseling, since events, such as pseudodominance (in pedigrees RP-0622 and MD-0092) or ambiguous inheritance patterns, could be observed.

Although this kind of research must be performed for each particular population, the present study evidences the estimated frequency of overall mutation load, which contributes to RD intrafamilial heterogeneity in a large cohort of Spanish population. Furthermore, this is essential in patient management and especially in disease treatment, as locus and allelic heterogeneity represent a barrier to the improvement of therapies focused on correcting the primary genetic defect.

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