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**ORIGINAL ARTICLE**

# Panel-based next-generation sequencing identifies novel mutations in Bulgarian patients with inherited retinal dystrophies

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## Abstract

**Background:** Next-generation sequencing (NGS)-based method is being used broadly for genetic testing especially for clinically and genetically heterogeneous disorders, such as inherited retinal degenerations (IRDs) but still not routinely used for molecular diagnostics in Bulgaria. Consequently, the purpose of this study was to evaluate the effectiveness of a molecular diagnostic approach, based on targeted NGS for the identification of the disease-causing mutations in 16 Bulgarian patients with different IRDs.

**Methods:** We applied a customized NGS panel, including 125 genes associated with retinal and other eye diseases to the patients with hereditary retinopathies.

**Results:** Systematic filtering approach coupled with copy number variation analysis and segregation study lead to the identification of 16 pathogenic and likely pathogenic variants in 12/16 (75%) of IRD patients, 2 of which novel (12.5%): *ABCA4*-c.668delA (p.K223Rfs18) and *RP1*-c.2015dupA (p.K673Efs*25). Mutations in the *ABCA4, PRPH2, USH2A, BEST1, RP1, CDHR1*, and *RHO* genes were detected reaching a diagnostic yield between 42.9% for Retinitis pigmentosa cases and 100% for macular degeneration, Usher syndrome, and cone-rod dystrophy patients.

**Conclusion:** Our results confirm the usefulness of targeted NGS approach based on frequently mutated genes as a comprehensive and successful genetic diagnostic tool for IRDs with significant impact on patients counseling.

**KEYWORDS**

inherited retinal degeneration, molecular diagnostics, novel mutations, targeted next generation sequencing

# 1 | INTRODUCTION

Inherited retinal degenerations (IRDs) are a group of clinically and genetically heterogeneous diseases characterized by progressive degeneration of photoreceptors and/or the retinal pigment epithelial cells. Altogether, IRDs have a prevalence ranging from 1 in 2000 to 1 in 3000, affecting almost two million people worldwide (Berger et al., 2010) and thus represent the most frequent cause of visual dysfunction in people of working age. Such conditions therefore have a highly significant impact on quality of life and health economics.

To date, over 300 genetic loci have been shown to be associated with non-syndromic and syndromic forms of IRD (http://www.sph.uth.tmc.edu/Retnet), with a wide range of clinical presentations and rates of progression. This high genetic heterogeneity complicates the process of prioritization in examining the genes known to be associated with IRDs. Next-generation sequencing (NGS) is a powerful technique that enables rapid and cost-effective parallel sequencing of large number of genes. Although whole-exome sequencing offers the potential to locate disease-causing mutations in novel genes, in practice diagnosis rates in whole-exome and targeted-sequencing studies are similar (Farrar et al., 2017), suggesting that the coding regions responsible for the majority of IRDs have already been located. Targeted analysis of disease-specific candidate genes, which provides deep coverage of the targeted sequences, has been widely used in the molecular diagnosis of IRD because it allows a cost-effective test, better functional interpretation of sequence variations, and overcomes the limitations of computational analysis related to the large amount of data generated by high-throughput sequencing platforms (Bravo-Gil et al., 2016; Consugar et al., 2015).

IRDs represent a diverse group of progressive, visually debilitating diseases that can lead to blindness in which mutations in genes that are critical to retinal function are responsible for progressive photoreceptor cell death and vision loss. The development of treatments to modify the rate of disease progression has been limited to date, with the best example of treatment success is gene augmentation therapy for IRD caused by mutations in the *RPE65* gene (MIM*180069), which received US Food and Drug Administration (FDA) approval, which in fact represented the first FDA-approved gene therapy for any genetically inherited disease (Bennett et al., 2016). With the implementation of the new sequencing technologies, an increasing number of deep-intronic variants resulting in pseudoexon insertion (and therefore disruption of the reading frame) are being described. By delivering antisense oligonucleotides (chemically modified small RNA molecules that bind complementarily to the pre-mRNA) in several cellular and animal models, the pre-mRNA splicing of *CEP290* (MIM*610142) (Gerard et al., 2012), *USH2A* (MIM*608400) (Slijkerman et al., 2016), *OPA1* (MIM*605290) (Bonifert et al., 2016), *CHM* (MIM*300390) (Garanto et al., 2018), or *ABCA4* (MIM*601691) (Albert et al., 2018) has been corrected. One of the most frequently occurring mutations causing up to 15% of the Leber congenital amaurosis (LCA) in some populations is a deep-intronic mutation in *CEP290* (MIM*610142), c.2991+1655>G, that creates a pathogenic new splice site and pseudoexon with a premature transcription stop codon. Currently, a phase 1/2 clinical trial using antisense oligonucleotides to restore splicing in LCA patients carrying this mutation is ongoing with very promising results (Cideciyan et al., 2019). The same principle of using antisense oligonucleotides targeting deep-intronic splice site IRD-associated mutations suppressed pseudoexon expression of *OPA1* (MIM*605290) (Bonifert et al., 2016), *USH2A* (MIM*608400) (Slijkerman et al., 2016), and *ABCA4* (MIM*601691) (Albert et al., 2018) in patient-derived fibroblasts.

To date, the application of NGS in the clinic testing in Bulgaria has been limited to targeted techniques such as clinical exome sequencing which gives a diagnostic yield of nearly 80% for IRD cases but is not effective for the detection of deep-intronic mutations. We developed a targeted NGS panel (SureSelect, Agilent) for genetic diagnostic testing of patients with IRD, optic atrophy, Leber hereditary optic neuropathy, and glaucoma, which allowed us to identify not only coding point mutations and in/dels, but also copy number variations (CNVs).

# 2 | METHODS

## 2.1 | Ethics statement

The institutional board of the Ethics Committee of Medical University of Sofia (Bulgaria) approved all experiments including patient DNA and that of their relatives. An informed consent form was signed by all participants. The study adhered to the tenets of the Declaration of Helsinki for research involving human subjects.

Please refer to the Supporting Information for additional sections of the Material and Methods.

# 3 | RESULTS

## 3.1 | Clinical data

The 16 index patients showed various clinical manifestations (see Table S1, Figure 2). Among identified patients,

5 were females, 11 were male, and ages at the time of examination ranged from 7 to 46. A total of 14 cases out of 16 presented night blindness as the first symptom, whereas the remaining families showed other initial symptoms such as decreased visual acuity or visual field constriction. Best-corrected visual acuity (BCVA) of affected individuals ranged from 0.2 to 0.8. Age of onset and subsequent fundus appearance were different among families (Table S1, Figure 2).

## 3.2 | Targeted sequencing

A capture panel covering 125 retinal disease genes (Table S2) and deep-intronic mutations (Table S3) was used to identify the genetic cause of 16 IRD patients. Common mitochondrial mutations leading to Leber hereditary optic neuropathy were added to the panel (Table S4). Next-generation sequencing experiment was run on the Illumina MiSeq platform using 150 bp paired-end reads. The coding regions of the genes present in the panel were covered by 161 times on average (range between 109x and 222x) and 98% of the base pairs were covered by at least 20 reads (Table S5). On average, 500 single nucleotide variants and 70 insertions/deletions were identified per sample. Application of variant calling, filtering, and annotation of the sequencing data was performed as described above. This process concluded with an average of two candidate variants per sample to be validated and co-segregated by Sanger sequencing.

As expected, coverage was low (<20 times per base) or null for GA-rich repetitive region ORF15 of *RPGR* (NM_001034853.2). This particular region was analyzed by Sanger sequencing for patients with no pathogenic variants after NGS. However, the rest of the gene was well covered and on average 81% of the whole gene was read by at least 20X. There were no other uncovered targets.

The identification of causative mutations paragraph is available as Supporting Information.

## 3.3 | Mutation spectrum

Using targeted NGS 16 pathogenic variants, located in the *ABCA4* (NM_000350.3), *PRPH2* (NM_000322.5), *USH2A* (NM_206933.4), *BEST1* (NM_004183.4), *RP1* (NM_006269.2), *CDHR1* (NM_033100.4), and *RHO* (NM_000539.3) genes, were identified in 12 (9 autosomal-recessive, ar, and 3 autosomal-dominant, ad) out of 16 index patients. Causative mutations are listed in Table 1.

Homozygous or compound heterozygous mutations in *ABCA4* were the most prevalent for ar cases (5/9 patients, 55.6%). Eight disease-associated variants in this gene were found in 5 Stargardt disease cases making it the most frequently mutated gene in our cohort (5/16 patients, 31.3%). The *ABCA4* mutation spectrum included 1 novel variant, c.668delA (p.K223Rfs18) (Figure 3a), and 7 previously reported mutations among which 6 missense and 1 frameshift changes (Table 1). All of the probands except patient RD52-II:1 were identified as compound heterozygotes. In families RD30 (Figure 3a) and RD70, the detected variants of *ABCA4* co-segregated with the subject phenotype—both parents of the probands were found to carry one mutant allele without clinical manifestations (Figure 1). For families RD59, RD52, and RD62, one or two parents were not available for segregation analysis but according to the collected patient data they were asymptomatic.

Mutations within the *USH2A* gene were found in two cases with arRD from our patient group. Two *USH2A* mutations were detected in family RD50, p.R2509Gfs* and p.R303H, and both co-segregated with Usher syndrome in compound heterozygosity. A homozygous deletion of exons 22–24 was detected in patient RD57-II:1 displaying symptoms of non-syndromic arRP (Table 1). Confirmation of the deletion region was performed by MLPA (Figure 3c).

One index case with parental consanguinity, RD64-II:1 (Figure 1, Figure 2m), was found to carry a novel homozygous mutation, *RP1*-c.2015dupA (p.K673Efs*25), which is predicted to cause a loss-of-function allele. This variant was absent from the public databases gnomAD, dbSNP, EVS, ClinVar, and HGMD. Segregation study showed that both parents were heterozygous for the mutation (Figure 3b).

The rest of the mutated genes were *CDHR1, PRPH2, BEST1*, and *RHO*, all involved only in one family (Figure 1, Table 1). Based on our genetic screening, one case could be clinically reclassified. Patient RD66-II:1 was initially diagnosed as Stargardt′s disease but after the genetic screening clinical phenotype of this case turned out to be cone-rod dystrophies (with mutation in *CDHR1*). Detailed clinical examination and the genetic data for this case are described by Mermeklieva et al. (Mermeklieva et al., 2021).

Unsolved cases section is available as Supporting Information.

## 4 | DISCUSSION

Parallel testing of many genes significantly increased the diagnostic yield and represents the most cost-effective diagnostic tool for genetically and clinically heterogeneous diseases such as IRDs (Bravo-Gil et al., 2016). For example, in the case of Retinitis pigmentosa and the technology used it is possible to detect mutations in 30–80% of patients (Daiger et al., 2013). The overall diagnostic yield

**TABLE 1** Summary of 12 patients carrying pathogenic and likely pathogenic variants in known IRD genes

| Patient | Inheritance | Gene | Allele state | Ex | cDNA | Protein | ACMG classification | References |
|---|---|---|---|---|---|---|---|---|
| RD30-II:1 | ar | ABCA4 | Het | 43 | c.5917delG (M1) | p.V1973*fs | Pathogenic | Gerth et al. (2002) |
|  |  | ABCA4 | Het | 6 | c.668delA (M2) | p.K223Rfs18 | Likely pathogenic | This study |
| RD36-II:1 | ad | PRPH2 | Het | 1 | c.136C>T (M3) | p.R46* | Pathogenic | Coco et al. (2010) |
| RD50-II:1 | ar | USH2A | Het | 40 | c.7524delT (M4) | p.R2509Gfs* | Pathogenic | Bonnet et al. (2016) |
|  |  | USH2A | Het | 6 | c.908G>A (M5) | p.R303H | Likely pathogenic | Yan et al. (2009) |
| RD52-II:1 | ar | ABCA4 | Hom | 43 | c.5917delG (M1) | p.V1973*fs | Pathogenic | Rivera et al. (2000) |
| RD56-III:1 | ad | BEST1 | Het | 6 | c.652C>T (M6) | p.R218C | Pathogenic | Milenkovic et al. (2011) |
| RD57-II:1 | ar | USH2A | Hom | 22–24 | c.4628-?_4987+?del (M7) | p.G1543_E1663delinsE | Pathogenic | Van Cauwenbergh et al. (2017) |
| RD59-II:1 | ar | ABCA4 | Het | 12; 21 | c.[3113C>T (M8); c.1622T>C (M9)] | p.[A1038V; L541P] | Pathogenic | Tracewska et al. (2019) |
|  |  | ABCA4 | Het | 13 | c.1798G>T (M10) | p.D600Y | Pathogenic | Fishman et al. (2003) |
| RD62-II:1 | ar | ABCA4 | Het | 12; 21 | c.[3113C>T (M8); c.1622T>C (M9)] | p.[A1038V; L541P] | Pathogenic | Tracewska et al. (2019) |
|  |  | ABCA4 | Het | 40 | c.5603A>T (M11) | p.N1868I | Pathogenic | Runhart et al. (2018) |
| RD64-II:1 | ar | RP1 | Hom | 4 | c.2015dupA (M12) | p.K673Efs*25 | Likely pathogenic | This study |
| RD66-II:1 | ar | CDHR1 | Hom | 17 | c.2522_2528delTCTCTGA (M13) | p.I841Sfs*119 | Pathogenic | Arno et al. (2016) |
| RD70-II:1 | ar | ABCA4 | Het | 47 | c.6419T>A (M14) | p.L2140Q | Likely pathogenic | Passerini et al. (2010) |
|  |  | ABCA4 | Het | 22 | c.3322C>T (M15) | p.R1108C | Pathogenic | Ezquerra-Inchausti et al. (2018) |
| RD71-II:1 | ad | RHO | Het | 5 | c.1040C>T (M16) | p.P347L | Pathogenic | Dryja et al. (1990) |

*Note:* M1–M16: Mutations found in patients; RefSeq: *ABCA4* (NM_000350.3), *PRPH2* (NM_000322.5), *USH2A* (NM_206933.4), *BEST1* (NM_004183.4), *RP1* (NM_006269.2), *CDHR1* (NM_033100.4), *RHO* (NM_000539.3).

Abbreviations: ar, autosomal recessive; ad, autosomal dominant; Ex, exon; Het, heterozygous; Hom, homozygous.

**FIGURE 1** Pedigrees of IRD families and segregation analysis of identified variants. Individuals are identified by pedigree number. Squares indicate males, circles indicate females, slashed symbols indicate deceased, solid symbols indicate affected individuals, open symbols indicate unaffected individuals, black arrow indicates the proband. Consanguinity is marked by a double horizontal line. Sequencing chromatograms showing mutation segregation in each pedigree except RD57 where three-exon deletion in *USH2A* (NM_206933.4) was found.

of NGS testing of IRD patients reported in this type of study using gene panel-based testing varies from 55 to 60% suggesting that around 50% of these patients do not receive a molecular diagnosis after genetic testing (Khan et al., 2017). In this study, we apply a customized panel covering 125 genes to identify pathogenic variants in 16 Bulgarian families. All cases showed consistent results between clinical and genetic data.

The analyzed cohort comprised 5 Stargardt's disease (STGD), 2 Macular dystrophies (MD), 7 Retinitis pigmentosa (RP), 1 cone-rod dystrophies (CRD), and 1 Usher syndrome (USH) cases. According to the molecular results, 12 of them were solved (detection rate of 75%) revealing 16 different mutations located in seven genes: *ABCA4, PRPH2, USH2A, BEST1, RP1, CDHR1,* and *RHO* (Table 1). Among the identified variants 6 (6/16, 37.5%) were loss-of-function mutations including 1 nonsense and 5 frameshift mutations, and 9 (56.3%, 9/16) were missense changes. All variants considered as causative in the complete cohort were confirmed by Sanger sequencing therefore no false positives were found giving a specificity of 100%. Additionally, one large deletion of three exons was
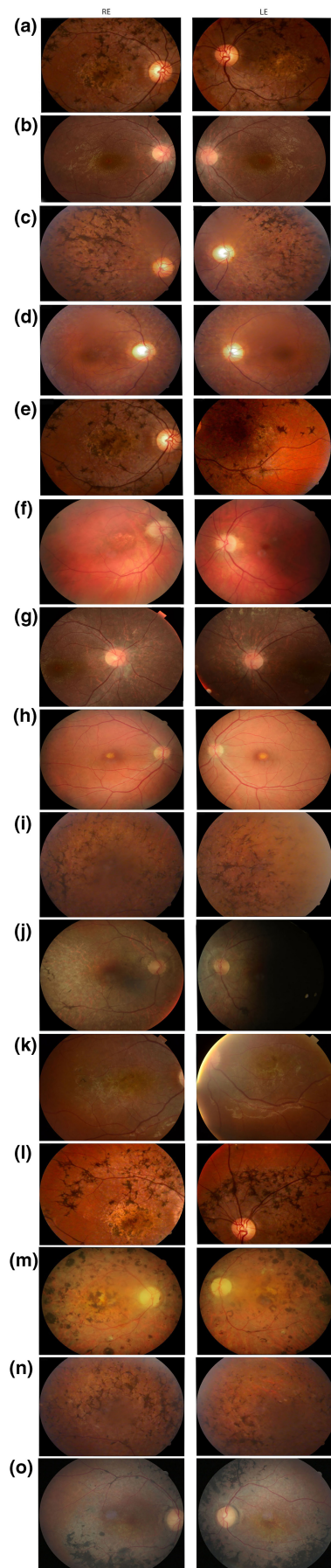
**FIGURE 2** Fundus photographs of both eyes of patients (a) RD30-II:1 (31 years), (e) RD30-II:1 (31 years), (k) RD59-II:1 (13 years), (l) RD62-II:1 (36 years), and (n) RD70-II:1 (25 years) showing bilateral optic disc pallor, narrowed retinal vessels, and macular pigmentary deposits characterized by different *ABCA4*-mutations; (b) RD36-II:1 (46 years) carrying *PRPH2*-mutation resulted in fleck-shaped subretinal yellowish deposits and a RPE defect in the macula; patients with various syndromic and non-syndromic RP phenotypes classical changes including attenuation of the retinal vessels, waxy pallor of the optic disc, retinal atrophy, and pigmentary deposits resembling bone spicules in peripheral retina seen in (c) RD50-II:1 (31 years) carrying *USH2A*-mutations; (d) RD51-II:1 (28 years); (f) RD53-II:1 (38 years); (g) RD54-II:1 (26 years); (j) RD58-II:1 (40 years); (i) RD57-II:1 (36 years) carrying *USH2A* homozygous deletion; (m) severe rod-cone dystrophy signs with macular involvement in patient RD64-II:1 (29 years) carrying RP1 homozygous mutation; (o) 11-year-old male patient RD71-II:1 with *RHO*-associated RP showing bone spicule pigmentation in the mid-periphery, vessel attenuation; and (h) fundus photograph of a 33-year-old female patient RD56-III:1 with *BEST1*-associated MD showing vitelliform lesions in a typical "egg yolk" shape in the center of the macula. RE: Right eye, LE: Left eye.

found in the gene *USH2A* in pedigree RD57. MLPA was performed to confirm the presence of this CNV.

Defects in the *ABCA4* (MIM*601691) gene have been described in *ABCA4*-associated retinopathies, including STGD, MD, CRD, RP, and other recessive retinal dystrophies (Schulz et al., 2017). It is the only gene known to be associated with autosomal recessive STGD with over 800 mutations (Lee et al., 2016). Genetic analysis of our 5 patients with presumed STGD (Figure 2a,e,k,l,n) revealed 8 different mutations in the *ABCA4* gene and a detection rate of 100% meaning that all cases were completely characterized (with two mutant alleles detected).

Most of the patients (4; n = 5) (80%) were compound heterozygotes. Only one patient (subject RD52-II:1, Figure 2e) was found to be homozygous (Table 1). A prevalence of one mutation, c.5917delG, representing 30% (3, n = 10) of all *ABCA4* alleles, was observed. This 1-bp deletion has previously been suggested to be quite severe in the homozygous state but has also been associated with a milder phenotype and late-onset STGD in compound heterozygous patients (Gerth et al., 2002). In our study, the homozygous frameshift mutation c.5917delG found in patient RD52-II:1 was associated with an early disease manifestation (onset 5–6 years) and a general photoreceptor dysfunction, compatible with early-stage cone and rod dysfunction accompanied by a clearly reduced macular function (Figure 2e). The combination of this mutation and a newly found *ABCA4* 1-bp deletion, c.668delA (p.K223Rfs18) (Figure 3a), resulted in a similar phenotype in patient RD30-II:1 – a relatively early age at onset (9–10 years) with widespread RPE atrophy and yellow flecks around
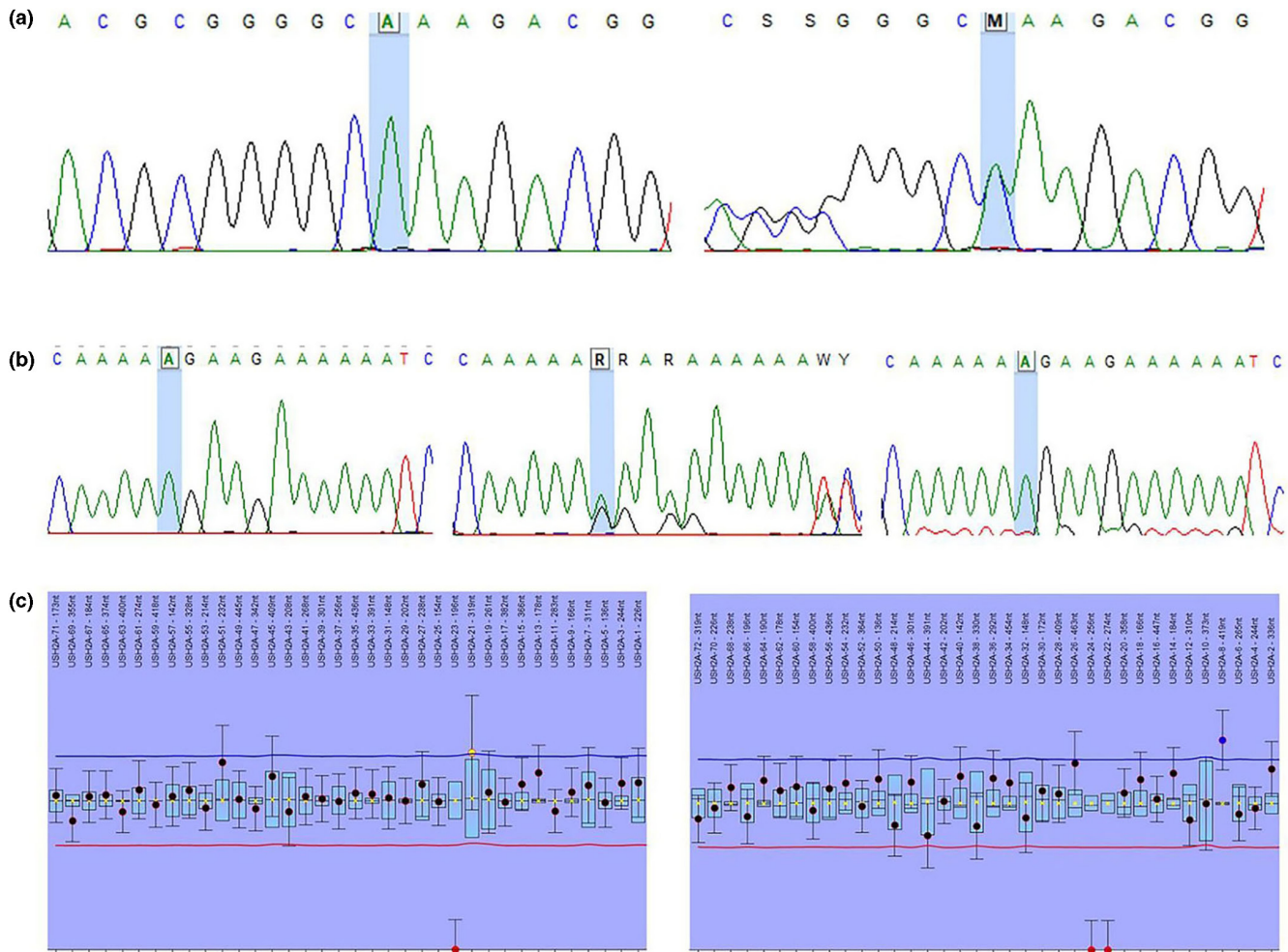
**FIGURE 3** Sequencing chromatograms showing (a) DNA sequences of normal control (left) and the heterozygous deletion of one nucleotide A, c.668delA, in exon 6 of *ABCA4* (NM_000350.3) resulting in a loss-of-function allele p.K223Rfs18 in patient RD30-II:1 (right); (b) DNA sequences of normal control (left), heterozygous (middle), and homozygous (right) insertion of one nucleotide A, c.2015dupA, in exon 4 of *RP1* (NM_006269.2) resulting in a homozygous loss-of-function allele p.K673EfsX25 in patient RD64-II:1; (c) CNVs analysis and MLPA profile for the exon 22–24 deletion of *USH2A* (NM_206933.4) found in patient RD57-II:1. Ratios below 0.7 were considered deletions; those above 1.2 were considered to be duplications. Deletion of exon 23 is shown on the first pane (MLPA probemix P361) and deletion of exons 22 and 24 is shown on the second pane (MLPA probemix P362).

macula (Figure 2a). The functional role of the mutation c.668delA found in patient RD30-II:1 is not yet known. This mutation is predicted to create a premature truncation after just 223 amino acids (human ABCA4 is a large single polypeptide of 2273 amino acids), which is likely to render the protein to be nonfunctional or lead to nonsense-mediated mRNA decay. Looking at the protein structure, the p.K223Rfs18 mutation should disrupt the first exocytoplasmic domain (ECD1) abolishing the C-terminal part of the protein by removing important domains such as transmembrane α-helices (TMs) TM2-12, the second exocytoplasmic domain (ECD2) and the nucleotide-binding domains (NBD1 and NBD2) (Trezza et al., 2017). In combination with the other loss-of-function allele, c.5917delG, both mutations may result in a nearly nonfunctional *ABCA4* transporter. This would be in agreement with the young age at onset and the progressive photoreceptor dysfunction observed in subject RD30-II:1.

Two mutations were detected more than once, which are p.L541P and p.A1038V both found on the same chromosome as a complex allele in 2 alleles in 2 STGD patients. The two subjects, RD59-II:1 (Figure 2k and 4a) and RD62-II:1 with advanced stage STGD and diffuse atrophy (Figure 2l), displayed similar phenotypes at age 13 years including a delayed ffERG implicit time, severely reduced mERG amplitudes, prolonged mERG implicit times, and bilateral outer retinal disruption on OCT images. They both carried the p.L541P-p.A1038V (Tracewska et al., 2019) complex allele and p.D600Y (Fishman et al., 2003) and p.N1868I (Runhart et al., 2018) heterozygous mutations, respectively.
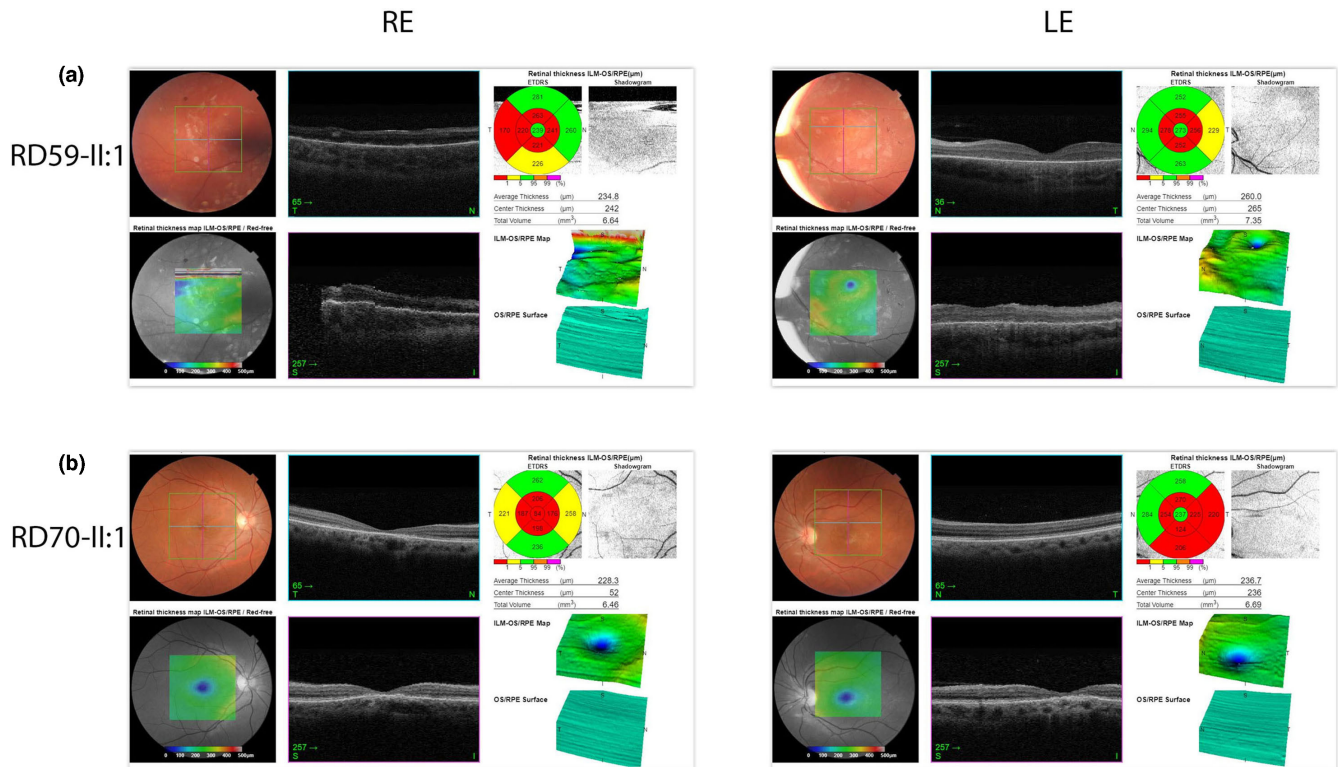
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**FIGURE 4** Representative photographs of probands (a) RD59-II:1 (13 years) and (b) RD70-II:1 (25 years) both affected by STGD. For each scan, left pane top: Fundus photographs showing a central atrophic lesion with flecks around the macula; left pane bottom: FA images showing fluorescence blocking caused by the pigment mottling in the macular region, hyperfluorescent flecks extended to the midp018peripheral retina; *middle pane top and bottom*: Macular OCT scans showing a reduced thickness of the attenuated retina and an altered reflectivity in the choroid, RPE, and the outer segments of the photoreceptors in both eyes; *right pane top*: Macular thickness significance map, the central innermost 1-mm-diameter circle represents the central subfield; inner superior, inner nasal, inner inferior, and inner temporal areas bounded by the 3-mm-diameter circle form the inner macula; outer superior, outer nasal, outer inferior, and outer temporal areas bounded by the 6-mm-diameter circle form the outer macula; *right pane bottom*: 3-D surface maps: The ILM-RPE, displaying the retinal thickness in three dimensions. ILM: Inner limiting membrane; RPE: Retinal pigment epithelium; RE: Right eye, LE: Left eye.

Progression of STGD over 23 years can be seen in patient RD62-II:1 (Figure 2l).

Two biallelic *ABCA4* mutations, p.L2140Q and p.R1108C, were found in patient RD70-II:1 with STGD (Figure 2n and 4b). Genetic variant c.6419T>A (p.L2140Q) has been previously reported as pathogenic in patients with STGD and central vision impairment (Passerini et al., 2010). The second mutation, c.3322C>T (p.R1108C), is common genetic cause in patients with STGD (Ezquerra-Inchausti et al., 2018) and is associated with an age of onset between 10 years for homozygous individuals being categorized as moderate to severe based on clinical studies (Curtis et al., 2020), which is in line with our data.

All affected members carrying *ABCA4* mutations complained of an early-onset markedly decreased vision acuity in both eyes along with an increasing difficulty in dark adaptation and a variable loss in color vision. On fundus examination, typical presentations were observed, including some pigment mottling, beaten-bronze

macular appearance, and yellow-white flecks around maculae (Figure 2a,e,k,l,n). Atrophic changes in the photoreceptors and a disruption of the foveal RPE are demonstrated on the OCT image of the probands RD59-II:1 and RD70-II:1. Lipofuscin deposits can be detected within the parafoveal RPE in both patients. The fluorescein angiogram displayed fluorescence blocking due to the pigment mottling in the macular, hyperfluorescent flecks extended to the midperipheral retina (Figure 4). Determining the status of the photoreceptor layer on OCT may provide an assessment of the central visual function.

The high coverage obtained with our NGS approach allowed us to perform a CNV analysis in our cohort providing the genetic diagnosis for one family. In the index patient with late-onset nonsyndromic RP (RD57-II:1, Figure 2i) a homozygous deletion of exons 22 to 24, c.4628-?_4987+?del (p.G1543_E1663delinsE), of the *USH2A* gene was found (Figure 3c). This mutation has previously been reported in compound heterozygosity with other pathogenic variants in a patient with simplex

RP (Van Cauwenbergh et al., 2017). The *USH2A* gene is known to cause Usher syndrome type 2A, an autosomal recessive disorder characterized by mild to severe hearing impairment and RP (MIM*276901) in 85% of cases (Yan & Liu, 2010). The gene has been also associated with autosomal recessive isolated RP in 23% of cases (MIM*613809) (McGee et al., 2010).

The patient RD57-II:1 had late-onset RP with initial night blindness since age 18 years and slow disease progression. A specific retinal phenotype included characteristic macular hyperautofluorescent ring, midperipheral outer retinal atrophy, and attenuated retinal vasculature were seen (Figure 2i). No systemic disease manifestations were recorded. Although the parents were not available for segregation analysis, there was no family history of retinal or auditory abnormalities. Based on this and the nature of the *USH2A* CNV found in this patient autosomal recessive trait was confirmed. There are multiple evidences that genomic rearrangements resulting in CNVs are responsible for IRDs in several genes including not only *USH2A*, but also *PRPF31, EYS*, and X-linked *RPGR* and *CHM* genes (Ezquerra-Inchausti et al., 2018). Van Cauwenbergh et al. identified CNV in 3 patients out of 57 analyzed, with mutations in *USH2A, HGSNAT*, and *RCBTB1* genes (Van Cauwenbergh et al., 2017). A recent study has established a prioritization of IRD genes according to genomic features and CNV events and these authors even recommend performing routinely a targeted CNV screening in the most common top-ranked IRD genes (Van Schil et al., 2018).

In addition, we found two pathogenic changes in *USH2A* in compound heterozygote, p.R2509Gfs* (Bonnet et al., 2016) and p.R303H (Yan et al., 2009), and thus confirmed the molecular diagnosis of USH in patient RD50-II:1 (Figure 2c).

The consanguineous RD64 family segregating a homozygous *RP1* mutation (Figure 1) was classified as an autosomal recessive RP (Figures 2m and 3b). Mutations in the retinitis pigmentosa 1 gene (*RP1*, MIM*180100) have been described in both, autosomal dominant and recessive RP forms, and account for approximately 5.5% of adRP cases and only 1% of arRP cases, depending on the type and the position of the mutations (Hartong et al., 2006). Although the novel frameshift mutation p.K673Efs*25 is located in the protein domain where mutations were usually found to cause adRP (Chen et al., 2010), the severe phenotype of the proband argues in favor of this mutation causing premature termination of RP1 and resulting in recessive early-onset RP. Moreover, there were no antecedents to retinal degeneration in the family. We could confirm the frameshift mutation in a heterozygous state in the nonaffected parents suggesting that incomplete penetrance,

previously reported in *RP1* forms (Gamundi et al., 2006), could also be present in this family.

The family RD66 harboring the known frameshift mutation in *CDHR1* (MIM*609502) (Arno et al., 2016) was reclassified as autosomal recessive CRD. Mutations in *CDHR1* encoding the cadherin-related family member 1 protein are implicated in recessively inherited retinopathies ranging from retinitis pigmentosa to macular and cone-rod dystrophy (Arno et al., 2016). The proband RD66-II:1 was initially diagnosed as STGD and fundus flavimaculatus but after molecular testing and further reexamination the clinical diagnosis was revised to CRD. Electrophysiological tests of this patient demonstrated severely affected generalized cone function accompanied by the reduced functional activity of rods during scotopic stimulation—both atypical for hereditary macular dystrophies, such as STGD, but typical for CRDs (Mermeklieva et al., 2021). Similar clinical and electrophysiological findings were previously described in a family with *CDHR1* mutation (Arno et al., 2016).

Lastly, for the three remaining cases, RD36-II:1 (Figure 2b), RD56-III:1 (Figure 2h), and RD71-II:1 (Figure 2o), mutations were identified in genes typically responsible for certain autosomal dominant retinopathies. These were: a heterozygous mutation c.136C>T (p.R46X) in the *PRPH2* gene (MIM*179605) (Coco et al., 2010), c.652C>T (p.R218C) mutation in the *BEST1* gene (MIM*607854) (Milenkovic et al., 2011), and c.1040C>T (p.P347L) mutation in the *RHO* gene (MIM*180380) (Dryja et al., 1990). This allowed us to confirm the clinical diagnoses of adMD for families RD36 and RD56 segregating heterozygous mutations in *PRPH2* (MIM*179605) and *BEST1* (MIM*607854) genes, respectively, as well as adRP for pedigree RP71 carrying heterozygous mutation in *RHO* (MIM*180380) gene.

Mutations were not detected in four cases (RD51, RD53, RD54, and RD58) of our cohort (25%, 4/16), all affected by RP (Figure 2d,f,g,j, respectively). Enrichment-based NGS methods typically do not capture the hot spot exon ORF15 of *RPGR* (MIM*312610), implicated in X-linked retinopathies. Overall, *RPGR* mutations are reported to cause 25% to 70% of xlRP cases and, thus, are the most common RP gene (Berger et al., 2010). In our study, no mutations in ORF15 were found using NGS and Sanger sequencing approaches.

The patients with negative results following targeted sequencing of the 125 IRD genes were analyzed for several genes known to be prone to CNV formations, such as *USH2A* and *EYS* responsible for autosomal dominant form of RP, and the autosomal dominant genes *RHO, IMPDH1, RP1*, and *PRPF31*. Despite some studies that have described these genes as the main containing CNVs (Bujakowska et al., 2017), MLPA failed to detect

rearrangements in those patients. Possible genetic cause of the disease in these patients could be the presence of mutations in deep intronic regions, promoter regions or 5′ and 3′ untranslated regions, not included in our gene panel. Furthermore, it is possible that the gene responsible for the disease had not yet been identified, and therefore it could not be included in our panel.

In summary, in this study, we applied NGS to a cohort of 16 Bulgarian patients using a comprehensive panel of 125 IRD genes. The achieved detection rate of 75% strongly supports the application of the targeted NGS strategy as an effective tool for the diagnosis of patients affected by inherited retinal degenerations, handling the heterogeneity of these diseases. Further improvements in NGS technologies, such as the second more extensive genome analysis—whole-exome sequencing, and the discovery of novel genes involved in the disease will likely improve the molecular diagnosis of IRD. Using this approach, gene defects underlying ad and ar retinal diseases in a patient cohort were determined for the first time in Bulgaria. Novel mutations were found, expanding the mutation spectrum of IRDs.

## AUTHOR CONTRIBUTIONS

Kunka Kamenarova and Radka Kaneva conceived and designed the analysis. Kunka Kamenarova and Kalina Mihova collected the data. Nevyana Veleva, Elena Mermeklieva, Bilyana Mihaylova, Galina Dimitrova, Alexander Oscar, Iliyan Shandurkov, and Sylvia Cherninkova made the clinical examination and recruited the patients. Kunka Kamenarova and Kalina Mihova performed NGS. Kalina Mihova performed the bioinformatics and confirmation analyses. Kunka Kamenarova wrote the manuscript. Nevyana Veleva, Elena Mermeklieva, Bilyana Mihaylova, Galina Dimitrova, Alexander Oscar, Iliyan Shandurkov, and Sylvia Cherninkova critically appraised and revised the manuscript. Radka Kaneva reviewed and complemented the manuscript and gave the final approval for the work to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work.

## CONFLICT OF INTEREST
The authors declare no conflict of interest.

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