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Genomic screening of *ABCA4* and array CGH analysis underline the genetic variability of Greek patients with inherited retinal diseases

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ABSTRACT

Background: Retinal dystrophies are a clinically and genetically heterogeneous group of disorders which affect more than two million people worldwide. The present study focused on the role of the *ABCA4* gene in the pathogenesis of hereditary retinal dystrophies (autosomal recessive Stargardt disease, autosomal recessive cone-rod dystrophy, and autosomal recessive retinitis pigmentosa) in patients of Greek origin.
Materials and methods: Our cohort included 26 unrelated patients and their first degree healthy relatives. The *ABCA4* mutation screening involved Sanger sequencing of all exons and flanking regions. Evaluation of novel variants included sequencing of control samples, family segregation analysis and characterization by *in silico* prediction tools. Twenty five patients were also screened for copy number variations by array-comparative genomic hybridization.
Results: Excluding known disease-causing mutations and polymorphisms, two novel variants were identified in coding and non-coding regions of *ABCA4*. Array-CGH analysis revealed two partial deletions of *USH2A* and *MYO3A* in two patients with nonsyndromic autosomal recessive retinitis pigmentosa.
Conclusions: The *ABCA4* mutation spectrum in Greek patients differs from other populations. Bioinformatic tools, segregation analysis along with clinical data from the patients seemed to be crucial for the evaluation of genetic variants and particularly for the discrimination between causative and non-causative variants.

1. Introduction

Inherited retinal diseases affect millions of individuals worldwide. They include many different types with more than 238 causative genes identified up to date (RetNet: https://sph.uth.edu/retnet/ July 2015). Significant achievements have been made in the elucidation of the genetic causes of retinal diseases and mutation screening strategies have improved dramatically during the past two decades (Xu et al., 2014). However it is still difficult to clarify the pathophysiologic mechanisms associated with specific genotypes due to the nature of the tissue that needs to be studied. Types of retinal diseases include retinitis pigmentosa, Stargardt's Disease and cone dystrophies (Berger et al., 2010).

Retinitis pigmentosa (RP) accounts for one half of inherited retinal diseases. RP is a progressive, degenerative disease of the retina with clinical hallmarks which include night blindness, loss of peripheral vision and subsequent loss of central vision. Clinical examination reveals 'bone spicule' pigmentary deposits, retinal vessel attenuation and characteristic electroretinogram patterns. More than 3100 mutations, in more than 50 genes, cause nonsyndromic RP. In addition to genetic and mutational heterogeneity, there is extreme variation in clinical phenotypes as well as overlapping clinical symptoms (Daiger et al., 2013).

Stargardt's disease 1 (STGD1, OMIM# 248200) is one of the most frequent types of inherited macular dystrophy in childhood. Clinical features include degeneration of cones in the macula which begins within the first two decades of life. The disease can be inherited in an autosomal recessive fashion and it is caused by mutations in the ATP-binding cassette subfamily A group 4 (*ABCA4*) gene (OMIM# 601691) which contains 50 exons and spans over 100 kb (Allikmets et al., 1998; Allikmets et al., 1997a). ABCA4 is a large transmembrane protein, expressed in retinal photoreceptors and localized to the outer segments disk membrane of rods, acting as a retinoid flipase involved in all-trans-

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retinal transport (Sun and Nathans, 1997; Molday et al., 2009). Mutations in *ABCA4* gene lead to abnormal protein and to accumulation of high levels of lipofuscin in the retinal pigment epithelium cells.

Mutations in *ABCA4* have also been associated with other forms of retinal dystrophies, such as autosomal recessive RP (arRP) and autosomal recessive cone-rod dystrophy (CORD), as well as age-related macular degeneration. These forms are characterized as *ABCA4*- associated retinopathies (Klevering et al., 2005; Cremers et al., 1998; Maugeri et al., 2000; Allikmets et al., 1997b; De La Paz et al., 1999; Martinez-Mir et al., 1998; Humphries et al., 1997; Shroyer et al., 2001). With the use of improved tools in routine laboratory diagnostics, such as *ABCA4* microarray mutation screening and next-generation sequencing, more than 800 mutations in *ABCA4* have been reported, highlighting extreme allelic heterogeneity (HGMD Professional; http://www.biobase-international.com/product/hgmd) (Zernant et al., 2011; Jin et al., 2014; Ernest et al., 2009; Zhang et al., 2014). Carrier frequency of *ABCA4* varies significantly between different populations. Many variants are common in specific geographic and ethnic groups (Maugeri et al., 2000; Weleber, 1994; Rivera et al., 2000; Valverde et al., 2006; Consugar et al., 2015; Chacon-Camacho et al., 2013; Westerfeld and Mukai, 2008; Riveiro-Alvarez et al., 2013). According to a previous study, six large deletions have been identified in 40 cases with syndromic and nonsyndromic RP (Garcia-Garcia et al., 2014). No large copy number variants were identified in another study using custom aCGH with high-density probes for genetic loci of *ABCA4*-associated disease (Zernant et al., 2014) (Seeliger et al., 2001). Custom aCGH containing probes tiling *PCDH15* gene revealed large duplications in Usher patients (Liu et al., 2015). Up to date, a very restricted number of studies attempted to describe the genetic basis of Greek patients with these types of retinopathies (Sung and Chuang, 2010).

In this study we sequenced the coding and adjoining intronic regions of the *ABCA4* gene and attempted to describe the phenotypic- genotypic correlation in unrelated Greek families, with autosomal recessive RP, autosomal recessive CORD and STGD1. Our results included known and novel variants. The pathogenicity of novel variants was investigated with segregation analysis in cases where family members were available and 200 control samples were screened for each novel variant. Further evaluation of each variant was attempted with the use of bioinformatic tools and in relation to the clinical phenotype.

2. Materials and methods

2.1 Patients

Our cohort included a total of 26 unrelated patients (n = 6 arRP, n = 10 STGD1 and n = 6 CORD) and 44 healthy first degree relatives (parents), all of Greek origin, who requested genetic testing by the Department of Medical Genetics, Medical School, University of Athens. Ophthalmologic evaluation of all individuals involved full ophthalmologic examination, fundus examination, fluorescein angiography, color vision testing, full-field electroretinograms (ff-ERG) and in some cases optical coherence tomography. All patients underwent electroretinogram tests following the ISCEV (International Society for Clinical Electrophysiology of Vision) standards. The diagnosis of STGD1 patients was based on the reduced central vision bilaterally and on the presence of “dark choroid” sign of fluorescein angiography. Most patients presented yellow flecks and atrophic lesions of choriocapillaris and RPE around the macula. The clinical features of autosomal recessive CORD patients included reduced visual acuity and color vision at the onset of the disease, followed by night blindness and loss of peripheral vision at later stages. The differential diagnosis between STGD1 and CORD patients was based on clinical examination and ff-ERG. Of note, ff-ERG of CORD patients showed loss of both cone and rod-mediated responses. Patients diagnosed with arRP initially presented difficulties at night vision while progressive loss of peripheral vision and visual acuity followed. The fundoscopic examination revealed bone spicule-like pigmentation extended from the peripheral retina to the macula. Patients' scotopic ff-ERG was affected severely and before the reduction of photopic ff-ERG signs.

The genetic study was conducted in accordance with the principles of the Declaration of Helsinki and with the institutional guidelines defined by the ethics committee of the Faculty of Medicine of the National and Kapodistrian University of Athens. After the first session of genetic counseling, informed consent was obtained from all participants and a peripheral blood sample was collected.

2.2 DNA extraction

DNA was extracted from peripheral blood lymphocytes, automatically, with the use of MagAttract DNA Blood Mini M48 Kit (Qiagen GmBh, Hilden, Germany).

2.3 Mutation analysis

Mutation screening of *ABCA4* involved direct sequencing of amplified exons. The 50 exons of *ABCA4* were amplified with M13-tagged specific primer sets. Flanking intronic sequences of approximately 100 bp of all exons, were also included. Public genome sequence databases were used for the design of PCR primers (NCBI: http://www.ncbi.nlm.nih.gov, ENSEMBL: http://www.ensembl.org). The primers were designed using Primer 3 (Rozen and Skaletsky, 2000). Primer sequences and PCR conditions are shown in Supplementary Table 1. The PCR protocol used for the amplification of the specific fragments was based on the manufacturer's recommendations (HotStarTaq MasterMiKit, Qiagen, Hilden, Germany). A single-step enzymatic clean-up of PCR products was performed (ExoSAP-IT kit, Affymetrix, High Wycombe, United Kingdom), followed by sequencing reaction of all PCR products in a ABI3500 sequencer (Applied Biosystems, Inc., NY, USA) using the BigDye® Terminator v3.1/Sequencing Standard Kit (Applied Biosystems, Inc., NY, USA). The sequencing reaction was performed in both forward and reverse directions. Mutation nomenclature followed Human Genome Variation Society recommendations (www.hgvs.org/mutnomen). DNA mutation numbering was based on the *ABCA4* Ref Seq mRNA sequence (GenBank accession number NM_000350.1), with nucleotide +1 being the A of the ATG translation initiation codon, while the corresponding amino acid residues were similarly numbered, with +1 identifying the initial methionine of ABCA4 (GenBank ABCA4 Ref Seq NP_000341.2).

2.4 Mutation evaluation

We have attempted to evaluate the consequence of known and novel variants with family segregation analysis and *in silico* bioinformatic tools in combination with the clinical phenotype. The *in silico* tools used in this study are the following: SIFT (Kumar et al., 2009; Sim et al., 2012), PolyPhen-2 (Adzhubei et al., 2010), PMut (Ferrer-Costa et al., 2004), PROVEAN (Choi et al., 2012), Human Splicing Finder (HSF3.0) (Desmet et al., 2009) and Mutation Assessor (Reva et al., 2011). Frequency of each novel variant was investigated using 200 control samples, both males and females of Greek origin.

2.5 Array-comparative genome hybridization

In total, 25 patients diagnosed with RP, STGD1 and CORD and 2 unaffected family members, were analyzed for copy number variations (CNVs) using comparative genomic hybridization arrays (aCGH) as previously described (Birnbach et al., 1994). In brief, aCGH was performed using standard protocols with SurePrint G3 Human CGH 4x180K microarray platform (Agilent Technologies, Santa Clara, CA, USA). Each array contains approximately 180,000 60-mer oligonucleotide probes. Labeling was performed using the SureTag DNA labeling kit (Agilent technologies). The microarrays were hybridized in a rotisserie oven at 67 °C for

24 h according to manufacturer's recommendations. The arrays were scanned using an Agilent DNA microarray scanner (G2565CA, software version 4.8.4.1) and the spot intensities were measured using the Agilent Feature Extraction Software (version 10.7.3.1). Raw data were analyzed by Agilent Cytogenomics software (version 3.0.2.11). The cut-off for recording an alteration was ≥4 consecutive probes. For the interpretation of the results, the following databases were used: database of genomic variants (http://projects.tcag.ca/variation/), DECIPHER database (http://www.sanger.ac.uk/PostGenomics/decipher/), and the International Standards for Cytogenomic Arrays (https://www.iscaconsortium.org/).

3. Results

3.1 Mutation and CNV detection

In total, mutations were detected in 86% (22/26) of the patients studied with STGD1, autosomal recessive RP and autosomal recessive CORD. The findings include 16 known mutations (12 missense, 1 nonsense and 3 splicing) and 2 novel genetic variants with unknown pathogenic significance. The patients' clinical diagnosis, the identified mutations and bioinformatic results are shown in Tables 1-4. Possible candidate genes associated with retinal dystrophies from aCGH results along with the clinical diagnosis are shown in Table 5.

3.1.1 arRP cases

ABCA4 mutations were identified in 6 arRP patients out of 10 that were studied. Only one mutation was found in 4 patients; 3 of them carried known pathogenic mutations and one of them carried the mutation p.His423Arg, which has previously been reported (Shroyer et al., 2001; Webster et al., 2001) and has been registered as non pathogenic in the Retina International database (http://www.retina-international.org/). The p.Arg18Trp mutation was found in a female adult patient with arRP. This mutation has previously been described in patients with STGD1 (Gerber et al., 1998). In addition, a p.Arg212Cys mutation was found in a male adult patient with arRP, along with c.6282 + 7G > A, an intronic variant which has been reported as non-pathogenic (Webster et al., 2001). Known variations including p. Ser2255Ile and c.6282 + 7G > A were found in two siblings; a 14-year-old boy and his 16-year-old sister, both affected with arRP (Webster et al., 2001; Ducroq et al., 2002).

Results from aCGH analysis from a female patient with arRP indicated the existence of a 247 kb deletion (chromosome 1 position 216,090,502 to 216,337,490, hg19). The deletion, which was not found in her healthy daughter and grandson, included the *USH2A* gene, from intron 21 to intron 38. This patient also carried the mutation c.6282 + 7G > A at intron 45 of *ABCA4.* A 79 kb deletion (chromosome 10, position 26,278,622 to 26,357,780, hg19), which involved the *MYO3A* gene was detected in a patient with arRP. This patient also carried p.Arg212Cys and c.6282 + 7G > A mutations (exon 6 and intron 45 of *ABCA4*, respectively).

3.1.2 CORD cases

Six CORD patients, with autosomal recessive inheritance pattern, were screened for *ABCA4* mutations. Mutation at only one allele of the *ABCA4* gene was identified in those patients. Three patients carried the mutation p.His423Arg, which is considered non pathogenic as mentioned above. Another patient carried the p.Arg943Gln mutation which has already been reported in CORD patients (Klevering et al., 2004). The p.Thr897Ile mutation was identified in another patient with autosomal recessive CORD. This mutation has been reported in previous studies in STGD1 patients (Webster et al., 2001; Simonelli et al., 2000; Fumagalli et al., 2001). A novel intronic variation, c.161-5T > C, at intron 2 was also found at a CORD patient in heterozygous state.

3.1.3 STGD1 cases

The p.Ala1598Asp and p.Gly1961Glu mutations were found in compound heterozygosity in the *ABCA4* gene in a male STGD1 patient. Thep.Leu541Pro and p.Ala1038Val mutations found in cis, according to family segregation analysis were identified in three STGD1 patients, along with other variations (p.Gly1961Glu and c.4352 + 1G > A). A

Table 1
Summary of mutation details and *in silico* analysis

Variation ID	Amino acid change	Nucleotide change	Exon/ Intron	PolyPhen-2	SIFT	PROVEAN	Mutation taster	Pmut	Mutation Assessor	HSF
CM980003	p.Arg18Trp	c.52C > T	1	Prob. Dam. (1)	Dam. (0)	Del. (−6314)	Dis. Caus. (101)	Path. (7)	Medium (2.92)	N/A
CM015072	p.His423Arg	c.1268 A > G	10	Benign (0)	T (0.39)	Neut. (2.141)	Polym. (29)	Neut. (5)	Neutral (−2.255)	N/A
CM003575	p.Thr897Ile	c.2690C > T	18	Benign (0.054)	T (0.18)	Neut. (−1.659)	Dis. Caus. (89)	Path. (5)	Low (1.1)	N/A
CM024629	p.Arg943Gln	c.2828G > A	19	Benign (0.010)	T (0.16)	Neut. (−0.999)	Polym. (43)	Path. (4)	Low (0.855)	N/A
Novel	–	c.161-5T > C	int2	N/A	N/A	N/A	N/A	N/A	N/A	Disruption of motifs for SRp40 protein
CM980004	p.Arg212Cys	c.634C > T	6	Prob. Dam. (1)	Dam. (0)	Del. (−3.906)	Dis. Caus. (180)	Path. (2)	Medium (2.395)	N/A
CS003628	–	c.6282 + 7G > A	int45	N/A	N/A	N/A	N/A	N/A	N/A	Disruption of ESE motifs
CM023000	p.Ser2255Ile	c.6764G > T	49	Benign (0)	T (0.12)	Neut. (−0.989)	Polym. (142)	Path. (1)	Neutral (0.255)	N/A
CM003386	p.Ala1598Asp	c.4793C > A	34	Poss. Dam. (0.870)	Dam. (0.01)	Neut. (−2.285)	Dis. Caus. (126)	Path. (8)	Medium (2,33)	N/A
CM970016	p.Gly1961Glu	c.5882G > A	42	Prob. Dam. (1)	Dam. (0)	Del. (−6.589)	Dis. Caus. (98)	Path. (8)	Neutral (−0.545)	N/A
CM990022	p.Leu541Pro	c.1622 T > C	12	Prob. Dam. (0.994)	Dam. (0,01)	Del. (−5.853)	Dis. Caus. (98)	Neut. (6)	Medium (3,145)	N/A
CM970006	p.Ala1038Val	c.3113C > T	21	Benign (0.009)	T (1)	Neut. (−0.235)	Dis. Caus. (64)	Path. (2)	Neutral (0.625)	N/A
CS982057	–	c.5714 + 5G > A	int40	N/A	N/A	N/A	N/A	N/A	N/A	Donor splice site affected
CD003502	p.Val1973X	c.5917delT	43	N/A	N/A	Del. (−4.527)	Dis. Caus. (6)	N/A	N/A	N/A
Novel	p.Leu1525Val	c.4573C > G	31	Poss. Dam. (0.5)	T (0.09)	Neut. (−2.287)	Dis. Caus. (32)	Neut. (2)	Low (1.9)	N/A
CS099669	–	c.4352 + 1G > A	int29	N/A	N/A	N/A	N/A	N/A	N/A	Donor splice site affected, disruption of ESE motifs
CM015083	p.Arg1108His	c.3323G > A	22	Prob. dam. (0.984)	Dam. (0)	Del. (−4.341)	Dis. Caus. (29)	Path. (7)	Low (1.915)	N/A
CM015077	p.Ala854Thr	c.2560G > A	16	Benign (0.119)	T (1)	Neut. (0.694)	Dis. Caus. (58)	Neut. (3)	Neutral (0.545)	N/A

Abbreviations: N/A: non-applicable, PolyPhen-2: Polymorphism Phenotyping v2 (http://genetics.bwh.harvard.edu/pph2/), SIFT: Sorting Intolerant From Tolerant (http://sift.jcvi.org/), PROVEAN: Protein Variation Effect Analyzer (http://provean.jcvi.org/index.php), Mutation taster: http://www.mutationtaster.org/, Pmut: http://mmb2.pcb.ub.es:8080/PMut/, Mutation Assessor: http://mutationassessor.org/, HSF: Human Splicing Finder (http://www.umd.be/HSF3/HSF.html), Prob. Dam: probably damaging, Poss. Dam: possibly damaging, Dam: damaging, T: tolerated, Del: deleterious, Dis Caus: disease causing, Polym: polymorphism, Path: pathological, Neut: neutral, ESE: exonic splicing enhancer.

Table 2
Summary of clinical phenotype and genotype for each patient

Patient	Sex	Clinical diagnosis	Inheritance manner	Variation ID	Amino acid change	Nucleotide change
1	F	RP	AR	CM980003	p.Arg18Trp	c.52C > T
2	F	CORD	AR	CM015072	p.His423Arg	c.1268 A > G
3	F	STGD1	AR	CM015072	p.His423Arg	c.1268 A > G
4	F	CORD	AR	CM015072	p.His423Arg	c.1268 A > G
5	F	CORD	AR	CM003575	p.Thr897Ile	c.2690C > T
6	F	CORD	AR	CM024629	p.Arg943Gln	c.2828G > A
7	M	STGD1	AR	CD003502	p.Val1973X	c.5917delG
8	M	CORD	AR	novel	–	c.161-5T > C
9	M	RP	AR	CM980004	p.Arg212Cys	c.634C > T
				CS003628	–	c.6282 + 7G > A
10	M	RP	AR	CM015072	p.His423Arg	c.1268 A > G
11	F	RP	AR	CS003628	–	c.6282 + 7G > A
12	M	CORD	AR	CM015072	p.His423Arg	c.1268 A > G
13	F	RP	AR	CS003628	–	c.6282 + 7G > A
				CM023000	p.Ser2255Ile	c.6764G > T
14	M	RP	AR	CS003628	–	c.6282 + 7G > A
15	M	STGD1	AR	CM003386	p.Ala1598Asp	c.4793C > A
				CM970016	p.Gly1961Glu	c.5882G > A
16	F	STGD1	AR	CM990022	p.Leu541Pro	c.1622 T > C
				CM970006	p.Ala1038Val	c.3113C > T
				CM970016	p.Gly1961Glu	c.5882G > A
17	F	STGD1	AR	CS982057	–	c.5714 + 5G > A
				CD003502	p.Val1973X	c.5917delT
18	M	STGD1	AR	novel	p.Leu1525Val	c.4573C > G
19	F	STGD1	AR	CM990022	p.Leu541Pro	c.1622 T > C
				CM970006	p.Ala1038Val	c.3113C > T
				CS099669	–	c.4352 + 1G > A
20	M	STGD1	AR	CM990022	p.Leu541Pro	c.1622 T > C
				CM970006	p.Ala1038Val	c.3113C > T
				CS099669	–	c.4352 + 1G > A
21	M	STGD1	AR	CM990022	p.Leu541Pro	c.1622 T > C
				CM970006	p.Ala1038Val	c.3113C > T
				CS099669	–	c.4352 + 1G > A
22	F	STGD1	AR	CM015083	p.Arg1108His	c.3323G > A
				CM015072	p.His423Arg	c.1268 A > G
				CM015077	p.Ala854Thr	c.2560G > A
				CS982057	–	c.5714 + 5 > A

Abbreviations: M: male, F: female, RP: Retinitis Pigmentosa, CORD: Cone-Rod dystrophy, STGD1: Stargardt's disease, AR: autosomal recessive.

novel mutation, p. Leu1525Val, was found in exon 31 in a STGD1 patient. Three known genetic variations were identified in a female patient with STGD1 and in her brother, who was also affected; two missense mutations (p.Arg1108His and p.Ala854Thr in exons 22 and 16 respectively) and a mutation which affects the splicing process, c.5714 + 5G > A in intron 40. Due to known variants that have been previously described in a small number of patients, both known and unknown variants were analyzed with bioinformatic tools. Specifically, Polyphen-2, SIFT, PROVEAN, Mutation Taster, Pmut and Mutation Assessor were employed for variations in coding sequences, whereas Human Splicing Finder was used for intronic variations. Detailed results from *in silico* analysis tools along with allele frequencies from 1000 Genomes Project (www.1000genomes.org) are shown in Supplementary Table 2.

Table 3
Clinical information for patients which were biallelic for *ABCA4* following segregation analyses

Patient No	Allele 1	Allele 2	Clinical diagnosis	Age	Age of onset	BCVA OD	BCVA OS	ff-ERG/ cone	ff-ERG/ rod	mERG	Additional clinical characteristics
9	c.643C > T	c.6282 + 7G > A	arRP	40	18	0.02	0.04	↓	↓↓	n/a	night blindness, narrowed retinal vessels, bone spicule-like pigmentation at periphery
13	c.6282 + 7G > A	c.6764G > T	arRP	35	14	0.04	0.06	↓	↓	↓ at macula bilaterally	Night blindness, bone spicule-like pigmentation at periphery
15	c.4793C > A	c.5882G > A	STGD1	30	12	0.14	0.16	↓	N	n/a	Small central scotomas within 30–40°
16	c.[1622 T > C;3113C > T]	c.5882G > A	STGD1	31	20	0.16	0.18	N	N	↓ at macula bilaterally	Small central scotoma
17	c.5714 + 5G > A	c.5917delT	STGD1	38	18	0.08	0.1	↓	N	n/a	Small central scotoma
19	c.[1622 T > C;3113C > T]	c.4352 + 1G > A	STGD1	12	10	0.04	0.04	↓	N	n/a	Dark choroid sign on IVFA
20	c.[1622 T > C;3113C > T]	c.4352 + 1G > A	STGD1	18	4	0.02	0.02	↓	N	↓ at macula bilaterally	Dark choroid sign on IVFA
21	c.[1622 T > C;3113C > T]	c.4352 + 1G > A	STGD1	11	7	0.04	0.04	↓	N	n/a	Dark choroid sign on IVFA
22	c.3323G > A	c.5714 + 5G > A	STGD1	35	18	0.14	0.12	↓	N	↓ at macula bilaterally	Dark choroid sign on IVFA

Abbreviations: BCVA: best-corrected visual acuity, OD: right eye, OS: left eye, ff-ERG: full-field electroretinogram, mERG: multifocal electroretinogram, ↓ reduced, ↓↓ severely reduced, IVFA: intravenous fluorescein angiography, n/a not available.

Table 4
Summary of details of novel mutations and *in silico* analysis

Novel mutations and *in silico* analysis									
Amino acid change	Nucleotide change	Exon/ intron	PolyPhen-2	SIFT	PROVEAN	Mutation taster	Pmut	Mutation assessor	HSF3
p.Leu1525Val	c.4573C > G	31	Pos. Dam. (0.5)	T (0.09)	Neutral (−2.287)	Dis. Caus. (32)	Neutral (2)	Low (1.9)	Activation of an exonic cryptic donor site. Alteration of an exonic ESE site. Potential alteration of splicing.
–	c.161-5T > C	int2	N/A	N/A	N/A	N/A	N/A	N/A	No significant splicing motif alteration detected.

Abbreviations: N/A: non-applicable, PolyPhen-2: Polymorphism Phenotyping v2 (http://genetics.bwh.harvard.edu/pph2/), SIFT: Sorting Intolerant From Tolerant (http://sift.jcvi.org/), PROVEAN: Protein Variation Effect Analyzer (http://provean.jcvi.org/index.php), Mutation taster: http://www.mutationtaster.org/, Pmut: http://mmb2.pcb.ub.es:8080/PMut/, Mutation Assessor: http://mutationassessor.org/, HSF: Human Splicing Finder (http://www.umd.be/HSF3/HSF.html), Poss. Dam: possibly damaging, Dam: damaging, T: tolerated, Dis Caus: disease causing, ESE: exonic splicing enhancer.

4. Discussion

In the current study, we attempted to develop a potential sensitive and robust protocol for screening mutations in Greek patients diagnosed with STGD1, CORD and arRP. This protocol included the amplification and sequencing of all coding and flanking intronic regions of the *ABCA4* gene. A total of 18 genetic variations were detected, two of which, to the best of our knowledge have not been described elsewhere. In cases where no more than one mutation was detected, an alternative approach could be performed including screening of the entire *ABCA4* genomic locus for the identification of deep intronic variants. Previous studies have suggested that analysis of deep intronic *ABCA4* variants could facilitate the elucidation of the genetic causality underlying retinal dystrophies (Zernant et al., 2014; Lee et al., 2016; Bax et al., 2015).

Despite the small number of cases screened, we found several mutations that frequently occur in other populations (Rivera et al., 2000; Burke et al., 2012), including p.Gly1961Glu and p.Ala1038Val. According to previous studies (Sciezynska et al., 2015; Lambertus et al., 2015) a combination of p.[Leu541Pro; Ala1038Val] and/or a truncating ABCA4 mutation resulted in an early disease onset. The [p.Leu541Pro; p.Ala1038Val] complex allele was identified in 4 STGD1 patients. Three of them carried the c.4352 + 1G > A mutation at the second allele. They presented reduced visual acuity and rapid impairment of central vision with age of onset 4 to 10 years old. Therefore, our results are in line with the aforementioned studies.

A female STGD1 patient carried the [p.Leu541Pro; p.Ala1038Val] complex allele, which has been previously related to severe phenotype including rapid reduction in visual acuity in parallel with chorioretinal atrophy (Sciezynska et al., 2015). An additional mutation, the p.Gly1961Gln, was detected on the other allele, which has been correlated with a milder end of the disease spectrum and with more severe phenotypes when patients carry additional *ABCA4* variants (Burke et al., 2012). Herein, the female patient presented a severe phenotype with reduced visual acuity and age of onset 20 years old. Multifocal ERG revealed significant reduction in macular function against the peripheral field of 30° and optic coherence tomography revealed diffused thinning of the neuroepithelium, particularly in the area of central fovea. Fluorescein angiography revealed punctate hyperfluorescence in the macula region bilaterally.

In addition, the p.Arg18Trp and p.Thr897Ile mutations were identified in patients with RP and CORD respectively, both with positive family history for autosomal recessive inheritance. Nevertheless, those mutations have been previously reported in STGD1 patients and may have a diverse impact on different patients, depending on the second allele (Heathfield et al., 2013).

The p. Leu1525Val affords a novel mutation detected in a STGD1 patient with age of onset seven years of age and remarkable worsening at the age of 14. More specifically, for this patient clinical examination revealed yellow-white flecks at the posterior pole as well as the presence of a mild destruction of foveal outer retinal structure. Notably, p.Leu1525Val was absent from all 200 control samples screened, while based on several *in silico* approaches, it was assessed as non pathogenic (SIFT, PROVEAN and PMut). Polyphen-2 assessed it as possibly damaging, whilst Mutation Taster characterized this mutation as disease causing, but with low score (32) (Table 1). Furthermore, according to Mutation Assessor, this is a mutation with low impact on protein function but with score 1.9, close to the score of disease-associated variants. HSF3.0 revealed that this nucleotide substitution may affect the splicing process by activating an exonic cryptic donor site and by altering an exonic splicing enhancer site. This mutation probably contributed to the disease phenotype in association with other gene mutations, or at positions of *ABCA4* that were not analyzed, such as deep intronic sequences. No pathogenic CNVs were identified with aCGH analysis for this patient. It is generally accepted that the use of *in silico* tools could contribute to the characterization of novel variants. In the current study results from bioinformatic tools were contradicting, making their contribution inadequate. Subsequently a potential alternative could lead to functional studies, with all the related issues arising from the nature of the affected tissue.

The novel variant c.161-5T > C, identified in intron 2 of a CORD patient, was also absent in 200 control samples from Greek individuals. No significant splicing motif alteration was predicted by HSF3.0. Further functional studies need to be done in order to clarify the role of these alterations in the splicing process. All mutations were found in heterozygosity, indicating the huge allelic heterogeneity of *ABCA4* and the complex haplotypes of patients with these retinal diseases. This fact makes genetic counseling and prognosis even more difficult.

We also performed array-comparative genomic hybridization (aCGH), for the detection of large deletions or duplications, on patients with one known *ABCA4* mutation identified with Sanger sequencing and on patients with no *ABCA4* mutations. Array-CGH revealed large deletions in two out of the 25 cases studied. The first patient was diagnosed with nonsyndromic, autosomal recessive RP and was found to carry a heterozygous 247 kb deletion at 1q41, resulting in partial

Table 5
Summary of clinically significant CNV findings

Submicroscopic rearrangements (CNVs) in our patients with retinal dystrophy				
Patient no	Phenotype	CNVs: chromosomal region and size (Mb)	Possible candidate genes related to RD (OMIM)	Other findings at *ABCA4* (exon/intron)
9	arRP	Del 10p12.1 (0,079)	*MYO3A*(606808)	p.Arg212Cys (exon 6), c.6282 + 7G > A (intron 45)
11	arRP	Del 1q41 (0,247)	*USH2A*(608400)	c.6282 + 7G > A (intron 45)

Abbreviations: RD: retinal dystrophies, arRP: autosomal recessive retinitis pigmentosa, CNVs: copy number variations.

deletion (intron 21 to intron 38) of the *USH2A* gene. This deletion was not identified in the patient's healthy daughter and grandson. Partial deletion of *USH2A* has been previously described in only one patient with nonsyndromic RP (Garcia-Garcia et al., 2014). Specific point mutations in *USH2A* have also been related to nonsyndromic autosomal recessive RP (Lenassi et al., 2015; Hartong et al., 2006; McGee et al., 2010; Xu et al., 2011). Partial *USH2A* deletions have been reported only in Usher syndrome patients in several studies so far (Krawitz et al., 2014; Steele-Stallard et al., 2013; Shzeena et al., 2015). Therefore, taking into consideration the family segregation analysis and previous studies, we conclude that the 247 kb deletion at 1q41 is the possible cause of the disease.

In the second case, a male patient with nonsyndromic autosomal recessive RP, a 79 kb deletion at 10p12.1, which involved the *MYO3A* gene, was identified. The *MYO3A* gene codes a highly conserved protein, myosin VIIA, which is expressed mainly in retina and cochlea (Les Erickson et al., 2003; Walsh et al., 2002). Sequencing variants of *MYO3A* have been associated with autosomal dominant and recessive hearing impairment (Liu et al., 1997a; Liu et al., 1997b) and with Usher syndrome in patients from diverse origins (Sodi et al., 2014; Ouyang et al., 2005; Jaijo et al., 2007; Le Quesne Stabej et al., 2012). No family segregation analysis was possible for the patient of our cohort, who also carried mutations p.Arg212Cys and c.6282 + 7G > A (exon 6 and intron 45 of *ABCA4* respectively). The contribution of the partial heterozygous deletion of *MYO3A* to the pathogenesis of the disease cannot be definitely supported. Expansion of genetic testing to larger cohorts will allow the evaluation of novel findings.

The identification of the genetic causes that lead to the clinical phenotype of hereditary retinal dystrophies, such as STGD1, arRP or CORD is very important for the accurate clinical diagnosis, prognosis and genetic counseling and eventually could also contribute to the therapeutic approach. In the current study, the suggested mutation screening protocol in combination with segregation analyses, patients' clinical data and *in silico* approaches, seemed to be valuable, in Greek patients carrying more than one mutation and diagnosed with STGD1, arRP or autosomal recessive CORD. In Greek patients with no identified *ABCA4* mutation(s) entire screening of the specific genomic locus for the identification of deep intronic variants or next generation sequencing is necessary.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mgene.2016.02.002.

Conflict of interest

None of the authors disclosed any conflict of interest.

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