Further Associations between Mutations and Polymorphisms in the *ABCA4* Gene: Clinical Implication of Allelic Variants and Their Role as Protector/Risk Factors

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Purpose. Mutations in *ABCA4* have been associated with autosomal recessive Stargardt disease, autosomal recessive cone-rod dystrophy, and autosomal recessive retinitis pigmentosa. The purpose of this study was to determine (1) associations among mutations and polymorphisms and (2) the role of the polymorphisms as protector/risk factors.

Methods. A case-control study was designed in which 128 Spanish patients and 84 control individuals were analyzed. Patient samples presented one or two mutated alleles previously identified using ABCR400 microarray and sequencing.

Results. A total of 18 previously described polymorphisms were studied in patients and control individuals. All except one presented a polymorphisms frequency higher than 5% in patients, and five mutations were found to have a frequency >5%. The use of statistical methods showed that the frequency of the majority of polymorphisms was similar in patients and controls, except for the IVS10+5delG, p.Asn1868Ile, IVS48+21C>T, and p.Arg943Gln polymorphisms. In addition, IVS48+21C>T and p.Arg943Gln were found to be in linkage disequilibrium with the p.Gly1961Glu and p.Arg602Trp mutations, respectively.

Conclusions. Although the high allelic heterogeneity in *ABCA4* and the wide spectrum of many common and rare polymorphisms complicate the interpretation of clinical relevance, polymorphisms were identified that may act as risk factors (p.Asn1868Ile) and others that may act as protection factors (p.His423Arg and IVS10+5 delG). (*Invest Ophthalmol Vis Sci.* 2011;52:6206–6212) DOI:10.1167/iovs.10-5743

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Supported by FIS (Health-Care Research Fund) 06/0027; Fundacion Mutua Madrileña (30,171/005); CIBERER (06/07/0036); EviGeno-Ret LSHG-CT-2005-512,036; and FIS through the Instituto de Salud Carlos III (PI06/0027) (JA-L).

Submitted for publication April 20, 2010; revised September 6, 2010; accepted September 23, 2010.

Disclosure: **J. Aguirre-Lamban**, None; **J.J. González-Aguilera**, None; **R. Riveiro-Alvarez**, None; **D. Cantalapiedra**, None; **A. Avila-Fernandez**, None; **C. Villaverde-Montero**, None; **M. Corton**, None; **F. Blanco-Kelly**, None; **B. Garcia-Sandoval**, None; **C. Ayuso**, None

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Stargardt disease (STGD, MIM 248200) is the most common hereditary macular dystrophy, with an estimated prevalence of at least 1:10,000.[1] It is characterized by a juvenile to young-adult onset, reduction in central vision, progressive bilateral atrophy of the retinal pigment epithelium (RPE), and the appearance of orange-yellow flecks around the macula and/or midperiphery of the retina.[2] The *ABCA4* gene, which causes recessive STGD, was mapped to the short arm of chromosome 1 (1p21–p13).[3] *ABCA4* is a large gene consisting of 50 exons[4] and encodes the *ABCA4* protein, a member of the ATP-binding cassette (ABC) transport superfamily. It is involved in the transport of vitamin A derivatives across the membrane of the photoreceptor outer segment discs.[5,6] Mutations in *ABCA4* have been described in a number of inherited retinal disorders, including autosomal recessive STGD (arSTGD),[4] autosomal recessive retinitis pigmentosa (arRP),[7] autosomal recessive cone-rod dystrophy (arCRD),[8] and age-related macular degeneration (AMD).[9] To date, some 500 variants have been identified in *ABCA4.* The mutation spectrum ranges from single base substitutions to deletions of several exons, although the majority of reported changes are missense mutations, followed by nonsense mutations, small insertions/deletions, and mutations affecting RNA splicing. Indeed, two or more alterations in *ABCA4* occurring in *cis* configuration (complex allele) have been identified and reported.[10,11] However, a high number of these changes were considered to be nonpathogenic polymorphisms and therefore did not contributed to pathologic lesions, though in some cases it has not been an easy task to establish whether they were associated with the disease. Consequently, genetic analyses of *ABCA4* variations were complicated by substantial allelic heterogeneity.

We selected two subsets of Spanish patients: with one or two mutant *ABCA4* alleles as well as a group of control individuals. Once the most frequent polymorphisms and mutations in both groups were noted, statistical analyses were performed to assess (1) whether the presence of a polymorphism correlated (or was associated) with any mutations and (2) the role of the polymorphisms as protector/risk factors.

Patients and Methods

A case-control study was designed. Methods are described as follows.

Recruitment of Patients

We studied arSTGD, arCRD, and arRP patient groups. The present molecular study was reviewed and approved by the Ethics Committee of the Hospital (Fundacion Jimenez Diaz) and adhered to the tenets of the Declaration of Helsinki (www.wma.org). Informed consent was

Investigative Ophthalmology & Visual Science, August 2011, Vol. 52, No. 9

obtained from all patients after they received a full explanation of the nature and purposes of the study procedures. Ophthalmologic and electrophysiological examinations were performed according to existing protocols. Examinations included patient and family history, best-corrected visual acuity testing, central and peripheral visual fields, fluorescein angiography, electro-oculography, and electroretinography, all according to the protocols recommended for vision testing by the International Society for Clinical Electrophysiology of Vision.[12,13] Diagnosis of STGD was determined according to a history of a recessive mode of inheritance, bilateral central vision loss with a beaten-bronze appearance and/or the presence of orange-yellow flecks in the retina from the posterior pole to the midperiphery, typical dark choroid on fluorescein angiography, and normal to subnormal electroretinograms (ERGs). Diagnosis of CRD was based on initial complaints of blurred central vision with no history of night blindness, poor visual acuity, color vision impairment, funduscopic evidence of atrophic macular degeneration, peripheral disturbances including pigment clumping and/or pigment epithelial thinning, and greater or earlier loss of cone than rod ERG amplitude. RP was diagnosed in patients who developed night blindness early in life or experienced peripheral vision loss, pigmentary retinal degeneration, and markedly reduced scotopic ERG.

Recruitment of Controls

Healthy control individuals were recruited from anonymous blood donors enrolled in the Blood Service of the hospital. Subjects were previously given an informed consent form indicating their nationality, age, and gender. Therefore, individuals who had arrived recently in the country were excluded from the study. The control group was free of any type of ocular disease.

Molecular and Statistical Methods

The analysis included a total of 128 samples from Spanish patients with one or both mutated alleles previously identified using both ABCR400 genotyping microarray and sequencing: The results for these processors were 106/128 (82.8%) arSTGD, 15/128 (11.7%) arCRD, and 7/128 (5.4%) arRP. Next, 84 samples from Spanish control individuals without any ocular disease were studied. From the mutational spectrum of *ABCA4*, we selected the disease-associated alleles, which had a frequency >5% in our patient subset, to determine their association with concrete polymorphisms acting in *cis*.

Genotyping

Peripheral blood samples were taken and genomic DNA was extracted using an automated DNA extractor (BioRobot EZ1, Qiagen, Hilden, Germany). Patients and control samples were analyzed for variants on the ABCR400 microarray (www.asperbio.com), as described elsewhere.[14] To confirm the obtained results, the 50 *ABCA4* exons, including intron-exon boundaries, were PCR-amplified[15] and directly sequenced.[16]

Confirmation of *cis*-Acting Polymorphisms by High-Resolution Melting Analysis and Direct Sequencing

Melting curve analysis with a high-resolution melting instrument (LightCycler480; Roche, Mannheim, Germany) provided a sensitive and specific tool for the detection of variations in DNA. Therefore, this technique was used to detect polymorphisms associated with the most frequent (>5%) *ABCA4* disease-associated alleles.

The PCR amplification mixture included 2.0 mM $MgCl_2$, 10 μL commercial reagent mixture (PCR Master Mix 2× from Roche Reaction Mix Kit), 5.4 μL H_2O, and 0.2 mM primers. We also added 25 ng of DNA template. PCR amplification began with the first denaturation step at 95°C for 10 minutes, followed by 30 denaturation cycles at 95°C for 20 seconds, annealing at an appropriate temperature for each exon for 20 seconds and extension at 72°C for 40 seconds. Following amplification, a program for generating melting curves was carried out at 95°C for 1 minute, 40°C for 1 minute, and 65°C for 1 second followed by a cooling program at 40°C for 30 seconds.

To confirm the polymorphisms in exons 10 and 41 of ABCA4, a sequencing reaction was performed.[16] To determine whether the polymorphisms were acting in cis or trans, DNA samples from the patients' parents were also analyzed for these variants.

Statistical Analyses

The frequency of the analyzed polymorphisms was evaluated by testing the control and patient groups with the Hardy-Weinberg equilibrium test. Hardy-Weinberg equilibrium of the genotype distribution of the controls was tested by a χ^2 goodness-of-fit test. The statistical significance of nonassociation between different variables was performed with a χ^2 or Fisher's exact test as appropriate. Odds ratios (ORs) and the 95% confidence intervals (CIs) for risk of Stargardt were calculated after univariate regression. For the statistic models, a commercially available software program (SPSS v. 16.0; SPSS, Inc., Chicago, IL) was used. The reported *P* values were two sided. Probabilities were considered significant whenever *P* was lower than 0.05.

Evaluation of the Functional Impact of Coding SNPs Using a Sequence Homology Tool (SIFT)

SIFT takes a query sequence and uses multiple alignment information to predict tolerated and deleterious substitutions for every position of the query sequence. It is a multistep procedure that, given a protein sequence, (1) searches for similar sequences, (2) chooses closely related sequences that may share similar function, (3) obtains the multiple alignment of these chosen sequences, and (4) calculates normalized probabilities for all possible substitutions at each position from the alignment. Substitutions at each position with normalized probabilities less than a tolerance index of 0.05 are predicted to be intolerant or deleterious; those greater than or equal to 0.05 are predicted to be tolerated. The analysis was performed by allowing the algorithm to search for homologous sequences using the default settings (UniProt-TrEMBL 39.6 database, median conservation of sequences of 3.00, and allowance to remove sequences >90% identical with query sequence).

Evaluation of the Functional Impact of Coding SNPs Using a Structural Homology-Based Method (PolyPhen)

PolyPhen prediction is based on straightforward empiric rules that are applied to the sequence, phylogenetic, and structural information characterizing the substitution. PolyPhen then searched for 3D protein structures, multiple alignments of homologous sequences, and amino acid contact information in several protein structure databases, calculated position-specific independent counts (PSIC) scores for each of the two amino acid residues entered (the original residue and the SNP), and then computed the PSIC scores difference of the two residues. The higher a PSIC score difference, the higher functional impact a particular amino acid substitution is likely to have. A PSIC score difference of 1.5 and above is considered to be damaging. The query options were left with default values.

Results

Genotype distributions of the mutations and polymorphisms were in Hardy-Weinberg equilibrium for control and patient groups.

Mutational Spectrum of *ABCA4* in the Two Cohorts Studied

We selected disease-associated alleles from the mutational spectrum of *ABCA4* with a major frequency (>5%) in our patients' subset to determine their association with specific polymorphisms (Table 1). A total of five disease-associated

TABLE 1. Most Frequent *ABCA4* Disease-Associated Alleles Identified in the Patient Cohort

Exon	Nucleotide Change	Aminoacid Change	Patients *n* (%)	Allele Frequency *n* (%)	Controls *n* (%)	Allele Frequency *n* (%)	*P*
23	c.3386G>T	p.Arg1129Leu	34 (26.6)	38 (14.8)	0 (0.0)	0 (0.0)	0.000
42	c.5882G>A	p.Gly1961Glu	18 (14.1)	18 (7.0)	1 (1.2)	1 (0.6)	0.001
13	c.1804C>T	p.Arg602Trp	8 (6.3)	9 (3.5)	0 (0.0)	0 (0.0)	0.020
22	c.3211insGT	Frameshift	7 (5.5)	7 (2.7)	0 (0.0)	0 (0.0)	0.029
45	c.6179T>G	p.Leu2060Arg	7 (5.5)	7 (2.7)	0 (0.0)	0 (0.0)	0.029

Note that only the p.Gly1961Glu substitution was identified in both patients and control groups.

alleles were detected: four missense variants and one in-frame deletion. Interestingly, only the p.Gly1961Glu substitution was identified in both patient and control groups.

ABCA4 Polymorphisms in the Two Cohorts Studied

A total of 18 polymorphisms were detected in patients and controls: 15 missense changes (nine of which were synonymous variants) and three intronic changes that were not predicted to alter the splicing of the corresponding exon. Interestingly, the frequency of *ABCA4* polymorphisms was higher than 5% in both patient and control groups, except for p.Pro327Pro (patients, 1.6%; controls, 0%) and IVS48+21T>C (patients, 10.2%; controls, 0%) (Table 2, Fig. 1). All polymorphisms are in Hardy-Weinberg equilibrium in patients and controls.

Mutations and Polymorphisms in the Patient Cohort

ABCA4 mutation screening demonstrated that the most frequent (>5%) disease-associated alleles were the following: p.Arg1129Leu, p.Gly1961Glu, p.Arg602Trp, c.3211insGT, and p.Leu2060Arg (Table 1; Fig. 1). Following this, the mutations were compared to the 18 polymorphisms located in *cis* using the Pearson's χ^2 test, thus making it possible to find any association existing between them.

p.Arg1129Leu

The most frequent mutation in the group of patients was p.Arg1129Leu, detected in 34 patients and with a frequency of 26.6% (Table 1). Once this variant was compared to *cis*-acting polymorphisms, statistical analyses demonstrated significant differences among p.Arg1129Leu and the p.His423Arg and IVS33+48C>T variants: 94.1% of the p.Arg1129Leu patients also had the p.His423Arg change ($P = <0.001$), and 100% of them also carried the IVS33+48C>T polymorphism ($P = 0.003$) (Table 3). In contrast, the p.Pro1401Pro, p.Asn1868Ile, p.Leu1894Leu, and p.Leu1938Leu variants were less frequently detected among the p.Arg1129Leu patients (Table 3). Despite this, p.His423Arg, IVS33+48C>T, p.Pro1401Pro, p.Leu1894Leu, and p.Leu1938Leu polymorphisms were detected in similar frequencies between p.Arg1129Leu patients and the control population, since no significant differences existed ($P = 0.108$, 0.542, 0.605, 0.130, and 0.394 respectively).

p.Gly1961Glu

The p.Gly1961Glu mutation was the second most frequent missense variant, with a frequency of 14.1% (Table 1). p.Gly1961Glu was found in association with the following polymorphisms: p.Leu1894Leu in 100% of the patients ($P < 0.001$), p.Pro1948Pro in 94.4% ($P < 0.001$), p.Leu1938Leu in 89.9% ($P < 0.001$), p.Asp2095Asp in 83.3% ($P < 0.001$, and IVS10+5delG in 55.6% of the cases ($P = 0.005$). Similarly, IVS48+21C>T was found to exist in association with p.Gly1961Glu in 72.2% of 18 individuals ($P < 0.001$) and was not found in the remaining patients (Table 3). Moreover, this polymorphism was not present in control samples (Table 2; Fig. 1). However, two polymorphic variants were found to be less frequently associated with the p.Gly1961Glu mutation in

TABLE 2. Most Frequent *ABCA4* Polymorphisms Found in Patients and Controls

Exon	Nucleotide Change	Amino Acid Change	Patients *n* (%)	Allele Frequency *n* (%)	Controls *n* (%)	Allele Frequency *n* (%)	*P*
—	**IVS48+21C>T**	**SPLICE**	**13 (10.2)**	**13 (5.1)**	**0 (0.0)**	**0 (0.0)**	**0.003**
—	**IVS10+5 delG**	**SPLICE**	**36 (28.1)**	**40 (15.6)**	**39 (46.4)**	**43 (25.6)**	**0.006**
40	**c.5603A>T**	**p.Asn1868Ile**	**27 (21.1)**	**30 (11.7)**	**9 (10,7)**	**9 (5.3)**	**0.049**
19	c.2828G>A	p.Arg943Gln	13 (10.2)	15 (5.8)	3 (3.6)	3 (1.8)	0.076
45	c.6249C>T	p.Ile2083Ile	14 (10.9)	15 (5.8)	16 (19.0)	18 (10.7)	0.098
49	c.6764G>T	p.Ser2255Ile	13 (10.2)	13 (5.1)	15 (17.9)	16 (9.5)	0.105
10	c.1268A>G	p.His423Arg	68 (53.1)	84 (32.8)	54 (64.3)	60 (35.7)	0.108
40	c.5682G>C	p.Leu1894Leu	70 (54.7)	90 (35.1)	37 (44.0)	41 (24.4)	0.130
42	c.5843CA>TG	p.Pro1948Leu	13 (10.2)	13 (5.1)	14 (16.7)	15 (8.9)	0.164
8	c.981C>T	p.Pro327Pro	2 (1.6)	2 (0.8)	0 (0.0)	0 (0.0)	0.250
6	c.635G>A	p.Arg212His	8 (6.3)	11 (4.3)	8 (9.5)	8 (4.7)	0.377
41	c.5814A>G	p.Leu1938Leu	40 (31.3)	48 (18.7)	31 (36.9)	35 (20.8)	0.394
44	c.6069C>T	p.Ile2023Ile	17 (13.3)	17 (6.6)	14 (16.7)	15 (8.9)	0.495
	IVS33+48C>T	SPLICE	109 (85.2)	170 (66.4)	74 (88.1)	93 (55.3)	0.542
28	c.4203C>A/T	p.Pro1401Pro	10 (7.8)	10 (3.9)	5 (6.0)	5 (2.9)	0.605
10	c.1269C>T	p.His423His	8 (6.3)	8 (3.1)	4 (4.8)	4 (2.4)	0.647
42	c.5844A>G	p.Pro1948Pro	36 (28.1)	42 (16.4)	23 (27.4)	25 (14.9)	0.906
46	c.6285T>C	p.Asp2095Asp	39 (30.5)	43 (16.8)	25 (29.8)	27 (16.1)	0.913

Variants revealing significant differences between both groups are shown in bold.

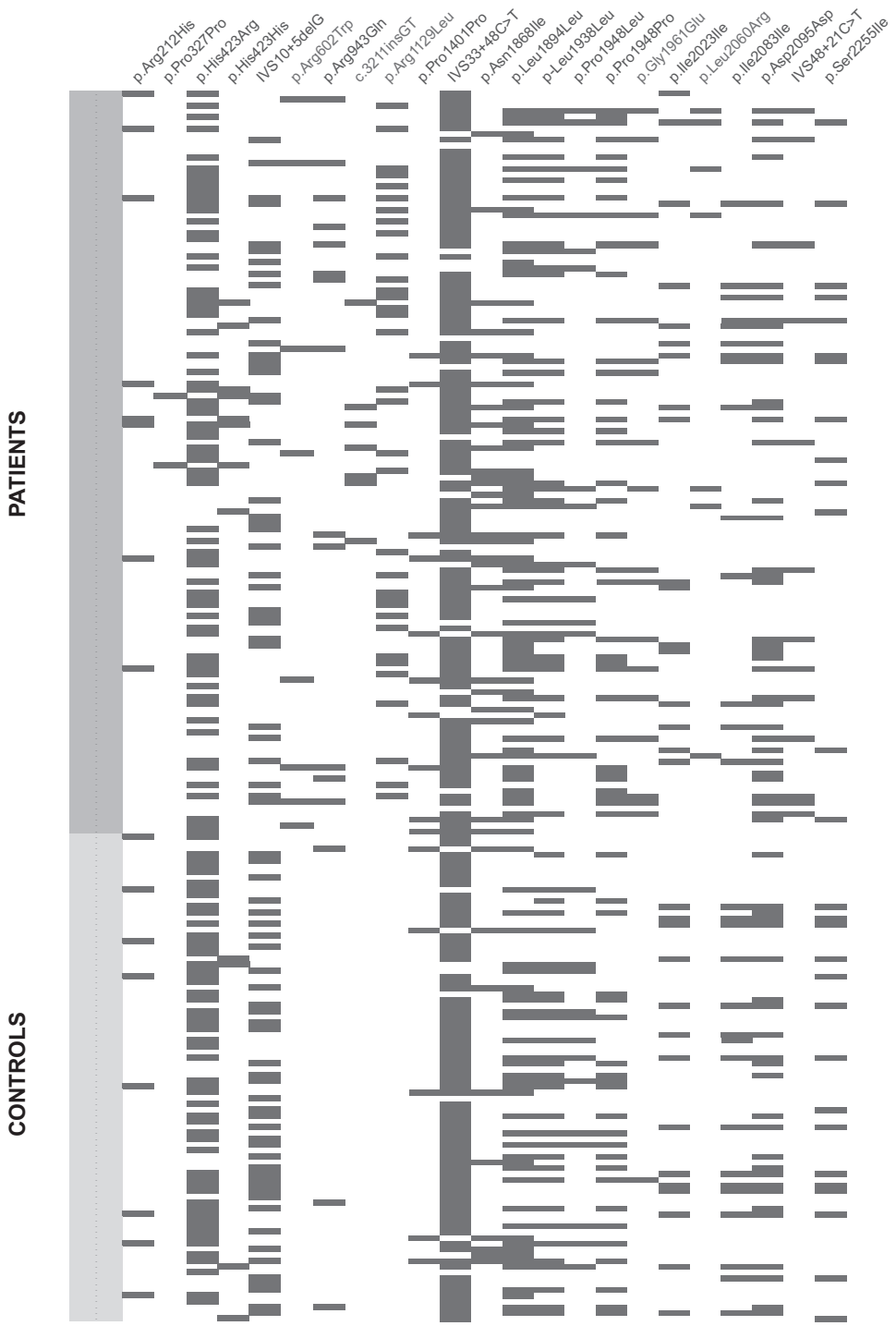

FIGURE 1. Representation of most frequent mutations and SNPs.

the patient group: p.Asn1868Ile ($P = 0.013$) and p.His423Arg ($P = 0\ 0.023$) (Table 3). The p.Asn1868Ile variant was found in higher proportion in patients than in control individuals ($P = 0.049$) (Table 2) and was not present in the 82% of the carrier patients of the p.Gly1961Glu mutation ($P = 0.013$) (Table 3).

p.Arg602Trp

The p.Arg602Trp mutation was found to be the third most prevalent missense variant, with a frequency of 6.3% (Table 1). Using the Pearson's χ^2 test, a significant association was detected between this alteration and the p.Arg943Gln polymorphism ($P < 0.001$), since 62.5% of the patients showed both sequence variants. Moreover, p.Arg943Gln was found in higher proportion in patients than in control individuals ($P = 0.076$) (Table 2; Fig. 1). In contrast with this, the p.Leu1938Leu variant was less frequently detected among patients with p.Arg602Trp and had a frequency of 0% ($P = 0.05$) (Table 3).

c.3211insGT

The fourth most frequent mutation was c.3211insGT (5.5%) (Table 1). This variant was significantly associated with p.His423Arg ($P = 0.014$), p.Asn1868Ile ($P < 0.001$), and p.Leu1894Leu ($P = 0.016$), as 100% of the patients carried both the mutation and these polymorphisms (Table 3).

TABLE 3. Association between the Most Frequent *ABCA4* Polymorphisms and Mutations

	Patients				
	Variants	**Frequency**	***P***	**Status**	**Predicted Effect**
Mutation, *n* (%)	**p.Arg1129Leu**	**34 (26.6)**			
Present polymorphisms	**p.His423Arg**	**94.1%**	**0.000**	**Associated**	Risk
	IVS33+48C>T	**100%**	**0.011**	**Associated**	Risk
	IVS10+5delG	20.6%	0.049	Associated	Protector
	p.Leu1938Leu	17.6%	0.033	Associated	Protector
	p.Ser2255Ile	2.9%	0.054	Associated	Protector
Mutation, *n* (%)	**p.Gly1961Glu**	**18 (14.1)**			
Present polymorphisms	**p.Pro1948Pro**	**94.7%**	**0.000**	**Associated**	Risk
	p.Leu1938Leu	**89.5%**	**0.000**	**Associated**	Risk
	p.Asp2095Asp	**78.9%**	**0.000**	**Associated**	Risk
	IVS48+21C>T	**70.0%**	**0.000**	**Associated**	Risk
	IVS10+5delG	**57.9%**	**0.008**	**Associated**	Risk
	p.His423Arg	31.6%	0.016	Associated	Protector
	p.Asn1868Ile	18.7%	0.039	Associated	Protector
Mutation, *n* (%)	**p.Arg602Trp**	**8 (6.3%)**			
Present polymorphisms	**p.Arg943Gln**	**62.5%**	**0.000**	**Associated**	Risk
	p.Pro1401Pro	25%	0.044	Associated	Protector
	p.Leu1938Leu	0%	0.041	Associated	Protector
Mutation, *n* (%)	**c.3211insGT**	**7 (5.5%)**			
Present polymorphisms	**p.His423Arg**	**100%**	**0.021**	**Associated**	Risk
	p.Asn1868Ile	**100%**	**0.000**	**Associated**	Risk
	IVS10+5delG	0%	0.047	Associated	Protector
Mutation, *n* (%)	**p.Leu2060Arg**	**7 (5.5%)**			
Present polymorphisms	**p.Leu1938Leu**	**100%**	**0.000**	**Associated**	Risk
	p.Pro1948Leu	**100%**	**0.000**	**Associated**	Risk
	p.His423Arg	14.3%	0.019	Associated	Protector

p.Leu2060Arg

The fifth most frequently detected mutation was p.Leu2060Arg (5.5%) (Table 1). This change was found to be associated with p.Leu1894Leu ($P = 0.016$), p.Leu1938Leu, and p.Pro1948Leu polymorphic variants in 100% of the cases ($P < 0.001$). However, p.His423Arg was less frequently detected among patients harboring p.Leu2060Arg, since the frequency of this occurrence was 14.3% ($P = 0.05$) (Table 3).

Rare Sequence Variants

The p.Pro327Pro polymorphism was found in a very low frequency in patients (1.56%) and was not found at all in controls, and no significant differences were observed ($P = 0.250$).

Except for the IVS10+5delG, p.Asn1868Ile, and IVS48+21C>T polymorphisms, the remaining polymorphic variants whose frequency was >5% failed to show any significant differences between patients and control individuals (Table 2; Fig. 1).

Polymorphisms and Risk

Carriers of the p.His423Arg and IVS10+5 delG variants have a diminished risk of disease compared to normal homozygous variant (OR: AG+GG = 0.46; 95% CI, 0.25–0.86; $P = 0.015$; and OR: N/D + DD = 0.45; 95% CI, 0.25–0.80; $P = 0.007$, respectively; Table 4). Carriers of the T variant (homozygous and heterozygous) of p.Asn1868Ile are at more than double the risk of developing the disease than normal homozygous variant (OR: AT+TT = 2.23; 95% CI, 1.01–5.01; $P = 0.05$; Table 4).

DISCUSSION

One hundred twenty-eight Spanish patients affected with different retinopathies and carrying either one or both disease-causing *ABCA4* mutated alleles were selected to find associations between polymorphisms and mutations. In this work, 84 Spanish control samples were also studied and analyzed. Using statistical analysis, we determined the most frequent mutations (>5%) and polymorphisms detected by using genotyping microarray. Five mutations and 18 polymorphisms were found to be the most prevalent in both patients and control individuals.

We have used the term of risk or protector factor according to our results and in the context of our population.

The p.His423Arg and IVS10+5 delG polymorphisms show a protective effect of the disease (OR: AG+GG = 0.46; 95% CI, 0.25–0.86; $P = 0.015$, and OR: N/D + DD = 0.45; 95% CI,

TABLE 4. Polymorphisms and Risk of Stargardt Disease

Polymorphism	**Genotype**	**Cases ($n = 128$) *N***	**Controls ($n = 84$) *N***	**OR**	**95% CI**	***P***
p.His423Arg	AA	60	30	1 (reference)		
	AG+GG	68	54	0.464	0.250–0.863	0.015
IVS10+5 delG	Normal	92	45	1 (reference)		
	N/D+D/D	36	39	0.452	0.254–0.804	0.007
p.Asn1868Ile	AA	101	75	1 (reference)		
	AT+TT	27	9	2.23	0.990–5.015	0.05

0.25–0.80; $P = 0.007$, respectively). However, carriers of the variant sequences of the p.Asn1868Ile polymorphism exhibit a more than twofold risk of developing the disease than normal homozygous variant (OR: AT+TT = 2.23; 95% CI, 1.01–5.01; $P = 0.05$; Table 4). Moreover, using a SIFT and a Polyphen prediction, the p.Asn1868Ile variant affected protein function.

We observed a negative association between some polymorphisms and particular mutations. This is the case of the p.His423Arg polymorphism and the p.Gly1961Glu and p.Leu2060Arg mutations ($P = 0.023$; Table 3); in other cases, however, the same polymorphism was found in association with the pArg1129Leu and c.3211insGT mutations. Similarly, the p.Asn1868Ile polymorphism is negatively associated with both p.Gly1961Glu and pArg1129Leu mutations and in positive association with the c.3211insGT mutation (Table 3).

As expected, no mutations were present in control samples, with the exception of p.Gly1961Glu (1.19%). It also was found in other control populations, although less frequently.[15,17] The p.Gly1961Glu variant is the second most frequent mutation in our group of patients, although it has been described as the most frequent variant in European populations.[14,15] We observe that the p.Gly1961Glu and IVS48+21C>T variants are in linkage disequilibrium, and the high frequency of the p.Gly1961Glu mutation in Europe may be the result of a founder effect. The p.Gly1961Glu variant was found in 18 (out of 128) STGD patients and was considered a moderate allele.[9] However, there is no linkage disequilibrium, since five out of 18 STGD patients did not carry the IVS48+21C>T polymorphism. One control individual was seen to carry the p.Gly1961Glu mutation but not the IVS48+21C>T polymorphism. In a Danish population, both *ABCA4* gene variants were found, though no possible association was analyzed.[18] However, in a German population, 18 individuals were found to have the IVS48+21C>T polymorphism, of whom 17 had the p.Gly1961Glu mutation.[15] As a contrast, p.Asn1868Ile and p.His423Arg are negatively associated with the p.Gly1961Glu mutation. p.Asn1868Ile appears at a higher frequency in patients than in controls ($P = 0.049$), although previous studies have found the variant to occur in higher, albeit insignificant, frequency among the control population.[19]

The IVS10+5delG polymorphism was first described in 2001 by Webster et al.[18] in a study on a Danish population. We found this polymorphism, IVS10+5delG, to be associated with the p.Gly1961Glu mutation ($P = 0.005$). In addition, this polymorphism was found in higher proportion in control individuals than in patients ($P = 0.006$), thus suggesting a protective effect.

The most frequent missense disease-associated allele was p.Arg1129Leu (26.6%), a mutation that does not appear in control individuals. This mutation is hardly present at all in other populations.[20] Appearing in 34 of 128 patients, the mutation is associated with both p.His423Arg and IVS33+48C>T polymorphisms ($P = 0.001$ and 0.003, respectively). The p.Pro1401Pro, p.Asn1868Ile, p.Leu1894Leu, and p.Leu1938Leu polymorphisms, on the other hand, appeared less frequently among patients with the p.Arg1129Leu mutation. Except for p.Asn1868Ile, the remaining polymorphisms were found in a similar number of patients and normal control subjects (Table 2). These facts suggest that the variants do not contribute to the disease and that the p.Arg1129Leu mutation is more recent than these polymorphisms.

The p.Arg943Gln polymorphism is in linkage disequilibrium with the p.Arg602Trp mutation in Spanish STGD ($P < 0.001$, Table 3). However, in other European studies the p.Arg943Gln variant was detected in linkage disequilibrium with the p.Gly863Ala mutation,[18,19,20] and [21] but this mutation has a low frequency in our series of patients, and no association analysis was performed. This change has been described showing diminished activity of the ABCA4 protein.[11,22] However, using a SIFT and a Polyphen prediction, the p.Arg943Gln variant would not affect the protein function, so it is not clear how it affects the phenotype.

The p.Pro327Pro variant is a synonymous (silent) variant. It was previously described by Webster et al.[20] in 2001. We have found this variant in 1.6% (2 out of 128) of patients but in none of the controls, although these differences were not significant. This change could be categorized as a rare variant with unclassifiable pathogenicity.

We found an association between p.Arg602Trp and p.Arg943Gln that has not been observed in other populations. On the other hand, we found the p.Arg1129Leu mutation in 26.6% (34 out of 128) of patients, and these results are in accordance with those of previous studies describing this mutation as the most frequent among Spanish people.[23] These findings are in contrast with those obtained from other populations in which the frequency is low. In this sense, it is worth noting that some major polymorphisms found in the United States, Canada,[20,24] the United Kingdom,[21] and Japan[25] also appear with similar frequency in the Spanish population.

Except for the IVS10+5delG, p.Asn1868Ile and IVS48+21C>T polymorphisms, the remaining polymorphic variants showed no significant differences between patients and control individuals. Therefore, these changes were categorized as nonpathogenic polymorphisms and did not contribute to pathologic conditions. Several polymorphisms have been described with a limited pathogenicity, so it is important to perform functional studies that clarify the role of these changes.

In addition, we hypothesized that CG doublets could represent mutation hot spots. Eleven of the 18 (61.1%) most frequent polymorphisms, including the IVS48+21C>T variant, were located in hot spots of the *ABCA4* gene. However, IVS10+5delG and p.Asn1868Ile did not represent mutational hot spots. The change of nucleotide was produced in either a cytosine or in a guanine. The mutation hot spots of the polymorphisms were located throughout protein. This could explain the presence of similar polymorphisms in Spanish populations as well as in other Europeans, Americans, and Japanese.

In conclusion, the p.His423Arg and IVS10+5delG polymorphisms have a protective effect, whereas the p.Asn1868Ile polymorphism is a risk factor for disease. We observed negative and positive associations between different polymorphisms and mutations, suggesting an interaction (when positive) with development of the disease phenotype.

Acknowledgments

The authors thank all patients and controls for their participation in this study.

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