WDR36 and P53 Gene Variants and Susceptibility to Primary Open-Angle Glaucoma: Analysis of Gene-Gene Interactions

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PURPOSE. To investigate the role of *WDR36* and *P53* sequence variations in POAG susceptibility.

METHODS. The authors performed a case-control genetic association study in 268 unrelated Spanish patients (POAG1) and 380 control subjects matched for sex, age, and ethnicity. *WDR36* sequence variations were screened by either direct DNA sequencing or denaturing high-performance liquid chromatography. *P53* polymorphisms p.R72P and c.97-147ins16bp were analyzed by single-nucleotide polymorphism (SNP) genotyping and PCR, respectively. Positive SNP and haplotype associations were reanalyzed in a second sample of 211 patients and in combined cases ($n = 479$).

RESULTS. The authors identified almost 50 *WDR36* sequence variations, of which approximately two-thirds were rare and one-third were polymorphisms. Approximately half the vari-

ants were novel. Eight patients (2.9%) carried rare mutations that were not identified in the control group ($P = 0.001$). Six Tag SNPs were expected to be structured in three common haplotypes. Haplotype H2 was consistently associated with the disease ($P = 0.0024$ in combined cases). According to a dominant model, genotypes containing allele P of the *P53* p.R72P SNP slightly increased glaucoma risk. Glaucoma susceptibility associated with different *WDR36* genotypes also increased significantly in combination with the *P53* RP risk genotype, indicating the existence of a genetic interaction. For instance, the OR of the H2 diplotype estimated for POAG1 and combined cases rose approximately 1.6 times in the two-locus genotype H2/RP.

CONCLUSIONS. Rare *WDR36* variants and the *P53* p.R72P polymorphism behaved as moderate glaucoma risk factors in Spanish patients. The authors provide evidence for a genetic interaction between *WDR36* and *P53* variants in POAG susceptibility, although this finding must be confirmed in other populations. (*Invest Ophthalmol Vis Sci.* 2011;52:8467-8478) DOI:10.1167/iov.11-7489

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Glaucoma is a genetically heterogeneous optic neuropathy originated by the apoptotic death of retinal ganglion cells and one of the most common causes of definitive blindness worldwide. The disease progresses slowly, leading to atrophy of the optic nerve, visual field loss, and eventually blindness.^{1,2} Primary open-angle glaucoma (POAG; Online Mendelian Inheritance in Man [OMIM] 137760) is the most common type of glaucoma, characterized by a complex inheritance, adult onset (older than 40 years), a gonioscopically open angle, and a reduced outflow facility that originates elevated intraocular pressure (IOP). The Human Genome Organization gene nomenclature database (<http://www.genenames.org/index.html>) contains 14 loci for POAG (GLC1A-N), but only three causative genes have been identified in Mendelian forms of glaucoma (*MYOCILIN* [*MYOC*; OMIM 601652], *OPTINEURIN* [*OPTN*; OMIM 602432], and *WD REPEAT-CONTAINING PROTEIN 36* [*WDR36*; OMIM 609669]). Sequence variations of these three genes are also involved in non-Mendelian POAG, accounting for only 5% to 10% of cases. Moreover, approximately 15 additional genes have been associated with POAG,³ although most of them are only reported in single studies. Among them, hypomorphic mutations of the *CYTOCHROME P4501B1* (*CYP1B1*, OMIM 601771) gene in the heterozygous state have been identified as a risk factor in POAG in approximately 7% of patients from different populations.⁴⁻⁸ Recently, heterozygous mutations in the *NTF4* gene have been associated with POAG in approximately 1.7% of patients of European origin.⁹

WDR36 was identified as the causative gene of adult-onset POAG, located on 5q22.1 (GLC1G), in approximately 6% of the studied families.¹⁰ The analysis of *WDR36* in nonfamilial POAG cases in different populations has provided diverse results,^{11–16} indicating that variants of this gene may act in certain populations as a causative or modifier gene for POAG. Alternatively, it cannot be ruled out that these sequence variations are genetic markers that are not directly involved in POAG development. *WDR36* is involved in 18S RNA processing and nucleolar homeostasis.^{17,18} Depletion of *WDR36* mRNA in human trabecular meshwork cells in culture causes apoptotic cell death and the upregulation of several mRNAs, including that of *P53*.¹⁸

The *P53* tumor suppressor gene (*TUMOR PROTEIN P53*; OMIM 191170) encodes a transcription factor that activates the expression of genes involved in growth arrest or apoptosis in response to multiple forms of cellular stress. It is upregulated in neurodegenerative diseases and toxic neuronal injury, promoting cell death by apoptosis.¹⁹ The individual role of *P53* polymorphisms in POAG has also been investigated with controversial results.^{20–26}

Loss-of-function of zebrafish *wdr36* activates the p53 stress-response pathway, suggesting that coinheritance of defects in genes of this route may modify the impact of *WDR36* variants on POAG.¹⁷ In addition, using a yeast model, it has been shown that POAG-associated *WDR36* sequence variants introduced into the structurally homologous protein Utp21, and expressed in a deficient STI1 genetic background, affect cell viability, thus supporting the theory that *WDR36* participates in polygenic forms of glaucoma²⁷ and provide evidence for a possible genetic interaction between the two genes.

Here we analyze the joint effect of *WDR36* and *P53* genetic variants in Spanish POAG patients. We found that rare *WDR36* variants, particularly those located in regulatory regions, and the common *P53* p.R72P polymorphism are associated with POAG. We report a large number of novel *WDR36* sequence variations and provide the first evidence for a genetic interaction between *WDR36* and *P53* genes in glaucoma susceptibility.

SUBJECTS AND METHODS

Subjects

A first sample of 268 unrelated POAG patients (POAG1 group) and 380 controls were investigated. In addition, we analyzed a group of 40 ocular hypertension (OHT) patients. The POAG patients were recruited in the Ophthalmology Departments of three Spanish hospitals: Complejo Hospitalario Universitario de Albacete, Hospital Arnau de Vilanova, Valencia, and Hospital Clínico San Carlos, Madrid. For replication of positive single-nucleotide polymorphism (SNP) and haplotype associations identified in the first group of patients, a second sample of 211 POAG patients (POAG2), with features similar to those of POAG1, was enrolled at the Ophthalmology Departments of Hospital Clínico San Carlos and Instituto Oftalmológico Fernández-Vega, Oviedo. The study protocol was approved by the ethics committees of the hospitals involved in the study and followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all the recruited subjects. POAG was diagnosed if all the following criteria were met: exclusion of secondary causes, open anterior chamber angle (Shaffer grade III or IV), IOP higher than 21 mm Hg, characteristic optic disc changes (e.g., vertical cup-to-disc ratio >0.3, thin or notched neuroretinal rim or disc hemorrhage), and characteristic visual field changes. Control subjects matched for sex, age, and ethnicity were recruited from among those who attended the clinic for conditions other than glaucoma, including cataracts, floaters, refractive errors, and itchy eyes. They also underwent full ocular exploration, including IOP measurement, determination of best visual acuity with optical correction, gonioscopy, and eye fundus examination. All patients were

checked for the absence of mutations in the coding regions of *MYOC*, *CYP11B1*, and *OPTN*.

Mutation Screening

Genomic DNA was extracted from peripheral leukocytes of all studied subjects with a purification kit (QIAamp DNA Blood Mini Kit; Qiagen, Valencia, CA) according to the manufacturer's protocol. The promoter (nucleotides -1 to -664) and 23 exons, including intronic flanking regions, of the *WDR36* gene were PCR amplified using primers indicated in Supplementary Table S1 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>) and conditions indicated in Supplementary Materials and Methods (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>). PCR products of the promoter and exons 1 and 4 to 19 were directly sequenced, as previously described.⁴ Exons 2, 3, 20, 21, 22, and 23 were screened for mutations by denaturing high-performance liquid chromatography (dHPLC) on a DNA analysis system (Helix; Varian, Palo Alto, CA), as indicated in Supplementary Materials and Methods (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>). Samples with altered dHPLC profiles were considered to potentially have a sequence variation and were sequenced bidirectionally.

The *P53* Arg72Pro (exon 4) polymorphism was genotyped by an SNP genotyping assay (TaqMan; Applied Biosystems), using conditions described in Supplementary Materials and Methods (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>). Genotypes for the *P53* c.97–147ins16bp (rs17878362, intron 3) polymorphism were determined as described previously.²⁸ PCR products were analyzed by capillary electrophoresis (3130 Genetic Analyzer; Applied Biosystems). The overall genotyping success rate for the two genes exceeded 90%.

Statistical Analysis

Each polymorphism was tested for deviation from Hardy-Weinberg equilibrium in cases and controls by the χ^2 test with one degree of freedom using SNPstats²⁹ and haploview.³⁰ POAG risk (odds ratios [ORs] and 95% confidence intervals [CIs]) was analyzed for association with polymorphisms by logistic regression using SNPstats. Generally, the most frequent allele among controls was used as the reference class. POAG risk was also analyzed for the combined effect of *WDR36* and *P53* polymorphisms using SNPstats. Bonferroni correction was applied by multiplying *P* values by the number of SNPs analyzed.³¹ The remaining statistical analyses are described in Supplementary Materials and Methods (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>).

Haplotype Construction

Haplotype reconstruction was performed with the expectation-maximization algorithm in marker data software (PowerMarker v. 3.22; <http://www.powermarker.net>).³²

RESULTS

In this case-control genetic association study, we initially analyzed 268 POAG cases (POAG1 sample) and 380 controls. We also included 40 patients previously diagnosed with OHT. The three groups were ethnically matched and had no significant differences regarding age and sex except that OHT patients were younger than the rest of the subjects (Table 1). Screening of the *WDR36* gene sequence revealed 48 single-nucleotide variants (46 substitutions, 1 deletion, 1 insertion), excluding variant c.-70A>G, which was identified in a normal tension glaucoma (NTG) patient (Fig. 1). All the variants were present in the heterozygous state and were classified according to their frequency as rare variants (minor allele frequency [MAF] <5% in at least one of the groups [Table 2]) and common polymorphisms [MAF >5% in the two groups; Supplementary Tables S1

TABLE 1. Demographic and Clinical Characteristics of the Participants in the Study

Variable	Control (n = 380)	OHT ³³ (n = 40)	POAG1 (n = 268)	POAG2 (n = 211)	POAG Combined (n = 479)
Age, mean ± SD	66.7 ± 13.0	56.5 ± 11.9†	66.1 ± 11.6	68.1 ± 10.1	67.2 ± 12.2
Female, %	49.7	47.5	54.1	46.9	52.4
Male, %	50.3	52.5	45.9	53.1	47.6
IOP, mean ± SD	15.0 ± 3.0	17.7 ± 4.2*†	17.2 ± 4.3*†	18.03 ± 5.6*†	17.5 ± 4.9*†
C/D OD, mean ± SD	0.1 ± 0.0	0.3 ± 0.2†	0.6 ± 0.3†	0.7 ± 0.2†	0.6 ± 0.2†

C/D, cup-disc ratio of optic nerve.
* IOP measured under medical treatment.
† *P* > 0.001 compared with the control group.

and S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>). Most of these variants²⁹ were located in noncoding regulatory regions (4 in the promoter, 6 in UTRs, and 19 in terminal intron regions; Fig. 1A) and 19 in coding regions (Fig. 1B). Of the coding variants, 15 were nonsynonymous and 4 were synonymous (Fig. 1B). As far as we know, 21 of the mutations have been identified in this study for the first time (Fig. 1, Table 2, and Supplementary Table S2 [<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>]).

WDR36 Rare Variants Present Exclusively in Glaucoma or OHT Patients

Seven rare variants exclusively present in cases were detected in 8 (2.9%) patients (Table 2). Four of these variants were novel, and one of them, c.-577G>A, was also identified in an OHT patient. Although individually none of these mutations was associated with the disease, collectively their frequency was significantly higher in cases than in controls (*P* = 0.001). Three of them were nonsynonymous, and the rest were located in noncoding regulatory regions: two affected the pro-

moter and 3'UTR, and the other two were located in terminal intronic sequences. To evaluate the pathogenicity of this group of DNA variants initially, we analyzed the degree of evolutionary conservation. The coding mutations and one of the noncoding nucleotide changes (*16T>C) affected highly conserved amino acid residues or nucleotides, respectively (Fig. 2). It is interesting that mutation *16T>C forms part of a predicted target sequence for two microRNAs (miRNAs), mir-9 and mir-224 (Fig. 2B). In addition, we identified three mutations present exclusively in two OHT patients (p.S664L, c.IVS3-72T>G, c.IVS9+86delT) (Table 2). Two of these mutations (c.IVS3-72T>G and c.IVS9+86delT) were novel. Finally, the c.-70A>G variant located in the 5' UTR, was present only in an NTG subject and has not been reported previously. These four mutations were not counted in the POAG group.

WDR36 SNPs

Twelve *WDR36* SNPs, distributed along the gene in regulatory and coding sequences, were also detected in POAG1 patients (Fig. 1). Three linkage disequilibrium (LD) blocks were estab-

TABLE 2. Frequency of the Rare *WDR36* Sequence Variations Identified in the Study

Region	DNA Change* (SNP)	Predicted Amino Acid Change	POAG Patients† (n = 268)	Controls‡ (n = 380)	OHT Patients‡ (n = 40)
Nonsynonymous					
Exon 1	c.74T>C (ss295490444)	L25P	6	21	1
Exon 1	c.91C>A (ss295490445)	P31T	0	3	1
Exon 1	c.99C>G (rs35629723)	D33E	1	1	0
Exon 1	<u>c.290A>C</u>	<u>Y97S</u>	1	0	0
Exon 5	c.752AT>CC	H212P	4	10	1
Exon 11	c.1345G>A (rs35703638)	A449T	1	7	0
Exon 12	<u>c.1468T>A (ss295490455)</u>	<u>L490M</u>	1	0	0
Exon 13	c.1586G>A (rs116529882)	R529Q	0	3	0
Exon 13	c.1589G>C	G530A	0	1	0
Exon 17	c.1973A>G (rs34595252)	D658G	4	7	0
Exon 17	c.1901C>T	S664L	0	0	1
Exon 17	<u>c.1994C>T (ss295490456)</u>	<u>A665V</u>	0	1	0
Exon 17	c.2011A>G (rs11956837)	M671V	0	1	0
Exon 19	<u>c.2111C>G (ss295490460)</u>	<u>S743W</u>	1	0	0
Synonymous					
Exon 3	c.402C>T‡	G134G	0	1	0
Exon 5	c.591G>A	Q197Q	0	6	0
Promoters and UTRs					
Promoter	<u>c.-578C>T (ss295490439)</u>		1	3	0
Promoter	<u>c.-577G>A (rs10050834)</u>		1	0	1¶
5'UTR	<u>c.-350G>A (ss295490441)</u>		6	6	0
5'UTR	<u>c.-107C>G (ss295490442)</u>		1	1	0
5'UTR	<u>c.-70A>G§ (ss295490442)</u>		1	0	0
3'UTR	<u>*16T>C (ss295490461)</u>		1	0	0
Introns					
IVS3	<u>c.IVS3-72T>G (ss295490447)</u>		0	0	1#
IVS3	<u>c.IVS3-47G>C (ss295490448)</u>		1	0	0
IVS3	<u>c.IVS3-14insT (ss295490449)</u>		1	0	0
IVS7	<u>c.IVS7+95A>G (ss295490450)</u>		0	1	0
IVS7	c.IVS7-39T>G		0	1	1¶
IVS8	c.IVS8+92G>A		3	8	1
IVS8	<u>c.IVS8-90A>G (ss295490451)</u>		0	1	0
IVS8	<u>c.IVS8-78T>C (ss295490452)</u>		0	1	0
IVS9	<u>c.IVS9+86delT (ss295490453)</u>		0	0	1#
IVS9	<u>c.IVS9-40T>C (ss295490454)</u>		3	4	0
IVS17	<u>c.IVS17+18G>A (ss295490457)</u>		0	1	0
IVS17	<u>c.IVS17+130A>G (ss295490458)</u>		0	1	0
IVS18	<u>c.IVS18-83A>G (ss295490459)</u>		3	2	0
IVS21	c.IVS21-75G>A‡		3	4	0
IVS21	c.IVS21-23A>G‡		1	8	0

Mutations in bold are novel, and those underlined are present in patients and absent in controls.

* Based on cDNA and genomic sequences NM_139281 and NT_034772, respectively.

† All mutations were detected in the heterozygous state.

‡ Variants genotyped in 100 POAG and 100 controls.

§ Variant identified in an NTG patient.

|| Variants present simultaneously in the same POAG patients (G4, GLC67, G73).

¶ Variants identified in the same OHT patient (G149).

Variants identified in the same OHT patient (G114).

lished based on pairwise $r^2 > 0.8$ (Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>). SNPs -640G>A, p.I264V, and c.IVS18+217C>T were selected as tag SNPs for each block. Then we analyzed the association with POAG of the six *WDR36* SNPs that were not in LD. All the SNPs were in Hardy-Weinberg equilibrium, indicating that the genotyping process was correct. Only SNP c.-235A>G showed statistically significant differences in allele and genotype frequencies between POAG1 patients and controls (Supplementary Tables S2 and S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>). Allele A and genotype AA were more frequent in POAG1 patients than in controls. However,

after stringent Bonferroni correction of *P* values for multiple comparisons, the differences were not significant.

Haplotype Analysis

Six SNPs (-640G>A, -235A>G, -192C>T, c.IVS5+30C>T, p.I264V, and c.IVS18+217C>T) were used to infer haplotypes in POAG1 cases and controls using haploview, PowerMarker, and SNPstats programs. Three common haplotypes with frequencies >7% in at least 1 of the 2 groups were predicted (Table 3). Haplotype H2 (A-A-C-T-G-C), which includes allele A of SNP c.-235A>G, was significantly more frequent in POAG1 patients than in controls (23.5% vs. 17.0%; *P* = 0.012) (Table 3).

FIGURE 2. Multiple amino acid (A) and nucleotide sequence (B) alignment of *WDR36* from different species in the regions where variants identified only in patients are located. Alignments were generated by ClustalW. Arrowheads: residues affected by mutations. Asterisks: amino acid positions at which all the query sequences are identical. Two dots (:): amino acid positions at which all the analyzed sequences have amino acids that are chemically similar. One dot (.): amino acid positions with a weak chemical similarity. Black circles: novel mutations. Mutation p.S664L was identified in an OHT patient (■). Prediction of miRNA binding sites shown in (B) was carried out with MicroSNPer.³⁴ Seed-complementary sequences are shown over a gray background.

Diploypes were also estimated for all subjects using PowerMarker (Table 4). Two diploypes containing the risk haplotype H2 (H1/H2 and H2/H2) were also predicted with significantly larger frequency in POAG1 patients than in controls. Another interesting finding was that in POAG1 patients, the OR was higher for the homozygous H2/H2 diploype than for the heterozygous H1/H2 haplotype (2.83 vs. 1.79, respectively; Table 4).

Genotype-Phenotype Correlation

To investigate whether rare *WDR36* sequence variants were associated with a specific clinical outcome, we compared different clinical parameters between POAG1 patients who carried the mutations found exclusively in cases (Table 5) with those of the rest of patients. POAG was diagnosed in this group of carriers at a younger age than in the rest of patients (65.8 vs. 57.5 years), although the difference was not statistically significant, probably because of the reduced size of the group (Supplementary Table S4, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>). IOP could not be compared readily because despite the availability of this value at diagnosis in the group of carriers, it was not available for most of the other patients (Supplementary Table S4, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>). The mean IOP, measured under treatment, in the second group of patients was normal, indicating that they were well controlled. C/D ratios were similar in both patient groups. Clinical features of carriers of mutations identified both in POAG1 cases and controls are shown in Supplementary Table S5 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>). We also analyzed age at diagnosis, IOP, and optic cupping in the patients carrying the most frequent variants (p.L25P, p.H212P, p.D658G and c.350G>A). No significant differences were found between these carriers and the rest of the patients (Supplementary Table S6, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>).

P53 Polymorphisms

The association of two common *P53* polymorphisms (p.R72P and c.97-147ins16bp) with POAG was also initially analyzed in POAG1 cases and controls. They were in Hardy-Weinberg equilibrium, indicating that the results were not biased by genotyping errors. We found that allele P (C) and genotype RP (GC) of p.R72P SNP were significantly more frequent in glaucoma patients than in controls, even after conservative Bonferroni correction ($P = 0.042$ and $P = 0.048$, respectively; Table 6). We analyzed the association with POAG using different models of inheritance with SNPstats. According to the lowest values of Akaike's information criterion and Bayesian information criterion, the model that best fit the data was the dominant model (Table 6). Subjects who were either homozygous or heterozygous for the susceptibility allele P had a 1.5-fold increased risk for glaucoma (95% CI, 1.12-2.18). The c.97-147ins16bp *P53* polymorphism was analyzed in a subgroup of 175 POAG1 patients and 227 controls. Allele and genotype frequencies did not differ significantly between the two groups (Table 6). With both these *P53* polymorphisms, we inferred four haplotypes in all the subjects but did not observe any statistically significant difference in either allele or genotype frequencies (Supplementary Table S7, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>).

Genetic Interaction between *WDR36* and *P53* Polymorphisms in POAG

We next examined whether polymorphisms of the two genes in concert may confer susceptibility to POAG as a result of epistasis. As an initial step we performed logistic regression analysis of the interaction between individual *WDR36* polymorphisms and the p.R72P *P53* SNP using SNPstats. Three of the two-locus genotypes were significantly overrepresented in the POAG1 group (Supplementary Table S8, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-DCSupplemental>). Each of these genotypes was present in >9% of subjects and were characterized by the presence of the *P53* risk genotype RP (GC) in combination with any of the following three

TABLE 3. Frequencies of the WDR36 Inferred Haplotypes in Cases and Controls

Haplotype	POAG1 (%) (n = 249)	Controls (%) (n = 317)	POAG1 vs. Control		POAG2 vs. Control		POAG Combined (%) (n = 455)		POAG Combined vs. Control	
			P (P*)	OR (95% CI)	P (P*)	OR (95% CI)	POAG2 (%) (n = 204)	POAG Combined (%) (n = 455)	P (P*)	OR (95% CI)
H1 (G-A-T-C-A-C)	42.50	37.60	0.082 (NS)	NS	NS	NS	39.60	41.30	NS	NS
H2 (A-A-C-T-G-C)	23.50	17.00	0.004 (0.012)	1.50 (1.10–2.03)	0.004 (0.012)	1.54 (1.12–2.12)	24.00	23.80	0.0008 (0.0024)	1.53 (1.17–1.99)
H3 (G-A-C-C-A-C)	13.50	12.40	NS	NS	NS	NS	14.10	13.80	NS	NS
Rest of haplotypes	20.50	33.00	—	NS	—	NS	22.30	21.10	—	NS

Haplotypes were constructed with polymorphisms –640G>A, –235A>G, –192C>T, c.IVS5+30C>T, p.L264V, and c.IVS18+217C>T. Only individual haplotypes inferred with a probability >0.7 and with frequencies larger than 7% in at least one of the groups are shown. NS, not significant.

* Bonferroni corrected (P > 0.1).

TABLE 4. Frequencies of the WDR36 Inferred Diplotypes in Cases and Controls

Diplotype	POAG1 (%) (n = 249)	Controls (%) (n = 317)	POAG1 vs. Control		POAG2 (%) (n = 204)		POAG2 vs. Control		POAG Combined (%) (n = 455)		POAG Combined vs. Control	
			P* (P†)	OR (95% CI)	P* (P†)	OR (95% CI)	P* (P†)	OR (95% CI)	P* (P†)	OR (95% CI)	P* (P†)	OR (95% CI)
H1/H1	18.88	15.14	NS	1.30 (0.82–2.08)	19.61	1.37 (0.84–2.23)	NS	1.37 (0.84–2.23)	17.80	1.21 (0.81–1.83)	NS	1.21 (0.81–1.83)
H1/H2	20.88	12.93	0.01 (0.04)	1.79 (1.11–2.87)	18.14	1.49 (0.89–2.49)	NS	1.49 (0.89–2.49)	19.56	0.01 (0.04)	0.01 (0.04)	1.64 (1.08–2.51)
H1/H3	11.24	9.46	NS	1.21 (0.68–2.17)	6.86	0.70 (0.38–1.28)	NS	0.70 (0.38–1.28)	9.23	NS	NS	0.97 (0.58–1.65)
H2/H2	8.43	3.15	0.006 (0.02)	2.83 (1.24–6.85)	4.41	1.42 (0.50–3.95)	NS	1.42 (0.50–3.95)	5.93	0.052 (NS)	0.052 (NS)	1.94 (0.89–4.55)
Rest of diplotypes	40.56	59.31	—	—	50.98	—	—	—	47.47	—	—	—

Individual diplotypes inferred with a probability >0.7 and with frequencies higher than 5% in at least one of the groups are shown. Only diplotypes with frequencies larger than 7% in at least 1 of the 2 groups were analyzed. NS, not significant.

* Pearson's χ^2 test of individual diplotypes grouping all others together.

† Bonferroni corrected.

TABLE 5. Clinical Characteristics of POAG Patients Carrying Rare WDR36 Variants Not Identified in Controls

Patient	WDR36 Variant	Age (y) Diagnosis	IOP at Diagnosis (mm Hg) OD/OS	Current IOP (mm Hg) OD/OS	Visual Field Alteration OD/OS	C/D Ratio OD/OS	Treatment (no. drugs) OD/OS
HAV48	p.Y97S	67/61	24/40	10/9	N/M	0.1/0.9	1/0 (surgery)
HAV3	p.L490M	77/64	38/32	12/18	Ammaurosis/M	1.0/0.5	0 (surgery†)/0 (surgery)
GLC64	p.S743W	68/60	24/26	4/17	NA/NA	NA/0.6	1/1
GLC16	c.-577G>A (Promoter)	50/45	30/30	18/23	E/S	0.1/0.1	1/1
HAV12	c.IVS3-47G>C	86/82	22/36	6/7	NA/NA	0.3/0.8	NA/NA
HAV29	c.IVS3-14insT	68/60	NA/NA	15/15	E/M	0.9/0.9	3/3
G149	c.-577G>A, c.IVS7-39T>G	46/35	25/24	20/20	E/N	0.3/0.4	1/1
HAV15	*16T>C (3'UTR)	70/53	24/20	15/16	S/S	0.8/0.8	1/1
G16 (OHT)	p.S664L	76/NA	NA/NA	20/20	N/N	0.2/0.4	2/2
G114 (OHT)	c.IVS3-72T>G, IVS9+86delT	45/34	22/26	12/13	N/N	0.2/0.2	1/1
GLC75 (NTG)	c.-70A>G (5'UTR)	39/39	16/17	12/14	M/E	0.2±0.1§	1/1

Data of two OHT patients and one NTG patient are also included. NA, not available.

† Two trabeculectomies + endocyclophotocoagulation.

‡ Thinning of supertemporal neuroretinal rim and decreased retinal nerve fiber layer.

§ Reduction of the inferior neuroretinal rim. Mutations indicated in bold are novel. Patients were classified as having normal (N), early (E), moderate (M), or severe (S) visual field alteration, according to the classification by Hodapp et al.⁵⁵

WDR36 SNPs: c.-235A>G, p.I264V, or c.IVS18+217C>T. Odds ratios, which were similar to or larger than the value obtained for each individual SNP, ranged from 1.60 to 2.22.

We also studied the interaction of the WDR36 haplotypes and diplotypes with the P53 risk genotype RP. Only five of the two-locus genotypes constituted by a combination of WDR36 haplotypes and the P53 SNP genotypes presented frequencies higher than 7% in at least one of the groups of cases or controls (Table 7). The two-locus genotype H2/RP, which combines two of the previously mentioned genetic susceptibility factors, was significantly more frequent in POAG1 patients than in controls (9.40% vs. 4.18%). Interestingly, glaucoma susceptibility associated with this haplotype increased 1.6-fold in combination with the genotype RP (OR, 2.47; 95% CI, 1.28–4.78). On the other hand, carrying the H1/H2 diplotype in combination with the risk genotype RP (9.64% in POAG1 patients vs. 3.47% in controls; Table 8) also increased 1.6 times the susceptibility to POAG (OR, 2.97; 95% CI, 1.36–6.84; Bonferroni-corrected, $P = 0.016$). Last, the two-locus genotype H2H2/RP was not included in the analysis because its estimated frequency was below the established 7% frequency threshold. However, and as anticipated, we observed that it was six times more frequent in cases than in controls (2.01% in POAG1 cases vs. 0.32% in controls).

Replication of the Association Analysis

To replicate SNP and haplotype associations, we recruited a second sample of 211 POAG patients (POAG2), as indicated in Materials and Methods. Their demographic features were similar to those of POAG1 and control subjects (Table 1). In both this and the combined sample (POAG1+POAG2, $n = 479$), we analyzed the six WDR36 tag SNPs and the P53 p.R72P polymorphism. Overall, we observed similar allele and genotype frequencies of individual WDR36 SNPs in the three samples, and we confirmed the absence of individual associations after Bonferroni correction (Supplementary Tables S2 and S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-/DCSupplemental>). Interestingly, the role of the WDR36 H2 haplotype as a risk factor was confirmed in both the POAG2 and the combined sample ($P = 0.0024$; OR, 1.53; Table 3). On the other hand, the association of diplotype H1/H2 with glaucoma was positive in the combined sample, whereas that of H2/H2 was not confirmed (Table 4). Allele and genotype frequencies of the P53 p.R72P SNP in POAG2 were also similar to those of POAG1, although the differences with the control group did not reach statistical significance (Table 6), probably because of the smaller sample size of POAG2 compared with POAG1. Importantly, the association of both allele P and genotype RP with glaucoma was replicated in the combined POAG group ($n = 479$; Table 6).

Finally, we attempted to verify the interaction between the two genes in glaucoma. Except for SNP I264V (two-locus genotype AG/RP), whose interaction was also confirmed in POAG2 patients, and SNP c.IVS5+30C>T, whose association was not replicated in either of the two groups (Supplementary Table S8, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-/DCSupplemental>), positive interactions of individual WDR36 SNPs with the P53 risk genotype RP were replicated only in the combined POAG sample. Similarly, the two-locus genotypes H2/RP and H1H2/RP were associated with glaucoma only in the combined group of patients (Supplementary Table S8, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7489/-/DCSupplemental>). These data support a moderate interaction between the two genes.

TABLE 6. Allele and Genotype Frequencies of P53 p.R72P [c.215G>C (rs1042522)] and c.97-147ins16bp (rs17878362) Polymorphisms in POAG and Control Subjects

	POAG1 vs. Control			POAG2 vs. Control			POAG Combined vs. Control			
	POAG1 (%) (n = 268)	Control (%) (n = 380)	<i>P</i> (<i>P</i> †) [AIC/BIC]	OR (95% CI)	POAG2 (%) (n = 211)	<i>P</i> (<i>P</i> †) [AIC/BIC]	OR (95% CI)	POAG Combined (%) (n = 479)	<i>P</i> (<i>P</i> †) [AIC/BIC]	OR (95% CI)
<i>p.R72P [c.215G>C (rs1042522)]</i>										
Allele										
R (G)	75.20	80.82	0.021 (0.042)	1.00	76.10	0.052 (NS)	1.00	75.80	0.014 (0.028)	1.00
P (C)	24.80	19.18		1.39 (1.05–1.83)	23.90		1.32 (0.98–1.78)	24.20		1.34 (1.06–1.71)
Genotype										
Codominant model										
R/R (G/G)	55.47	66.19	0.024 (0.048) [826.2/839.5]	1.00	59.5	NS [746/758.9]	1.00	57.30	0.035 (NS) (1117.3/1131.5)	1.00
R/P (C/G)	39.45	29.26		1.61 (1.14–2.27)	33.8		1.28 (0.89–1.86)	36.9		1.46 (1.08–1.97)
P/P (C/C)	5.08	4.55		1.33 (0.62–2.85)	6.7		1.63 (0.77–3.45)	5.8		1.47 (0.77–2.80)
Dominant model										
R/R (G/G)	55.47	66.19	0.007 (0.014) [824.5/833.3]	1.00	59.5	NS [744.3/753]	1.00	57.3	0.0095 (NS) (1115.3/1124.7)	1.00
R/P+P/P (GC+C/C)	44.53	33.81		1.57 (1.12–2.18)	40.5		1.33 (0.94–1.90)	42.7		1.46 (1.10–1.94)
Recessive model										
R/R+RP (G/G+G/C)	95.50	94.90	NS [831.6/840.4]	1.00	93.30	NS [745.7/754.3]	1.00	94.2	NS (1121.4/1130.8)	1.00
P/P (C/C)	4.50	5.10		1.12 (0.53–2.38)	6.70		1.50 (0.72–3.14)	5.8		1.29 (0.68–2.44)
Overdominant model										
R/R+PP (G/G+C/C)	70.70	60.50	0.005 (0.010) [824.8/833.6]	1.00	66.20	NS [745.7/754.3]	1.00	63.10	0.021 (0.042) (1116.8/1125)	1.00
R/P (G/C)	29.30	39.50		1.58 (1.12–2.21)	33.80		1.23 (0.86–1.78)	36.90		1.41 (1.05–1.90)
<i>c.97–147ins16pb (rs17878362)</i>										
Allele										
No Ins	(n = 175)	(n = 227)	NS	NS						
Ins16pb	88.00	85.68								
Ins16pb	12.00	14.32								
Genotype										
Ins16pb/Ins16pb	1.14	2.20								
Ins16pb/No Ins	21.71	24.23	NS	NS						
No Ins/No Ins	77.14	73.57								

AIC, Akaike's information criterion; BIC, Bayesian information criterion; NS, not significant. † Bonferroni corrected.

TABLE 7. Interaction between the WDR36 Haplotypes and the P53 SNP p.R72P [c.215G>C (rs1042522)]

WDR36 Haplotype/P53 p.R72P Genotype	POAG1 (%) (n = 250)	Controls (%) (n = 323)	POAG1 vs. Control OR (95% CI)	POAG2 (%) (n = 204)	POAG2 vs. Control OR (95% CI)	POAG Combined (%) (n = 455)	POAG Combined vs. Control OR (95% CI)
H1/RR	22.20	24.61	1.00	26.90	1.00	25.70	1.00
H1/RP	17.00	10.99	1.89 (0.92–3.87)	13.70	1.27 (0.74–2.30)	16.70	1.42 (0.76–2.67)
H2/RR	13.80	12.23	1.19 (0.81–1.74)	13.20	1.26 (0.83–1.90)	14.06	1.22 (0.87–1.70)
H2/RP	9.40	4.18	2.47 (1.28–4.78)	7.35	1.40 (0.69–2.84)	9.23	1.90 (1.07–3.40)
H3/RR	7.60	8.51	0.88 (0.54–1.43)	4.44	0.69 (0.41–1.17)	6.59	0.79 (0.52–1.20)
Rest	30.00	39.48	—	34.41	—	27.72	—

Haplotypes were constructed with polymorphisms -640G>A, -235A>G, -192C>T, c.IVS5+30C>T, p.I264V, and c.IVS18+217C>T. Only individual haplotypes inferred with a probability >0.7 and with frequencies larger than 7% in at least one of the groups are shown.

TABLE 8. Interactions between the WDR36 Diploypes and the P53 SNP p.R72P [c.215G>C (rs1042522)]

WDR36 Diploype/P53 p.R72P Genotype	POAG1 (%) (n = 250)	Controls (%) (n = 323)	POAG1 vs. Control		POAG2 vs. Control		POAG Combined (%) (n = 455)	POAG Combined vs. Control	
			P* (P†)	OR (95% CI)	P* (P†)	OR (95% CI)		P* (P†)	OR (95% CI)
H1/H1/RR	9.64	10.41	NS	NS	NS	NS	10.32	NS	NS
H1/H1/RP	7.63	3.79	NS	NS	NS	NS	6.15	NS	NS
H1/H2/RR	9.24	9.15	NS	NS	NS	NS	9.67	NS	NS
H1/H2/RP	9.64	3.47	0.004 (0.02)	2.97 (1.36–6.84)	NS	1.62 (0.71–4.41)	7.91	0.009 (0.045)	2.23 (1.17–5.17)
H1/H3/RR	7.23	6.31	NS	NS	NS	NS	5.71	NS	NS
Rest of haplotypes	56.62	66.87	—	—	—	—	60.24	—	—

Only haplotypes inferred with a probability >0.7 and diploypes with estimated frequencies larger than 7% in at least one of the groups were considered for the analysis. NS, not significant.
* Pearson's χ^2 test of individual diploypes grouping all others together.
† Bonferroni corrected.

DISCUSSION

Role of Rare Variants of *WDR36* in POAG

We identified a large number of *WDR36* rare variants, approximately half of which had not been reported previously and had primarily affected noncoding regulatory regions. On an individual basis, none of the rare variants was associated with POAG. However, eight patients (2.9%) carried eight heterozygous variants that were not detected in the control group. Considered together, they are significantly more frequent in POAG cases than in controls, indicating that these variants could predispose to the disease in a low number of glaucoma patients. Alternatively, we cannot rule out the possibility that all or some of the sequence variations are in linkage disequilibrium with unidentified causative alleles. In accordance with these data, rare *WDR36* alleles, present in 3% to 5% of POAG patients and absent in controls, have been reported in most previous studies.^{10–12} Higher prevalence for these low-frequency variants have also been reported in Japanese patients (9%)¹³ and Caucasian patients from the United States (17%).¹⁴ In addition, we have identified one *WDR36* rare variant in an NTG patient and four variants in three OHT patients. We did not find any clear clinical correlation associated with these mutations. Three of the rare nucleotide changes identified only in patients are predicted to produce missense mutations (p.Y97S, p.L490M, and p.S743W), and the remaining five are located in regulatory regions (promoter, terminal intron sequences, and UTRs). Some data support their classification as putative disease-causing mutations. For instance, missense mutations affect highly conserved amino acids, and the physicochemical properties of the mutated amino acids differ significantly from those of the wild-type residue. Therefore, these changes have the potential to impair protein both structurally and functionally. On the other hand, the mutations located in regulatory regions could alter normal levels of the *WDR36* protein by affecting the synthesis, processing, stability, or translation of the RNA encoding this polypeptide. Mutation *16T>C is embedded in a putative target sequence for mir-9 and mir-224. It is particularly interesting that this mutation affects the seed-complementary sequence of mir-224, which is considered the most critical sequence for selecting miRNA targets. In addition, mir-224 has been reported to be involved in apoptosis regulation.³⁶ This kind of regulatory mutations, which may subtly affect gene function, could underlie the complex nature of diseases such as glaucoma. Nevertheless, we cannot rule out that changes in DNA are in LD with unknown causative variants. Functional studies of these mutations and replication in other populations are required to confirm their role as glaucoma risk factors.

Three *WDR36* mutations identified in this study in both POAG1 patients and controls (p.L25P, p.D33E, p.H212P) have been functionally evaluated in a yeast model system using protein Utp21, which is structurally homologous to *WDR36*.²⁷ This study suggests that mutation p.L25P can be pathogenic in the correct genetic background, but no evidence of pathogenicity was found for the other two mutations. We studied the segregation of mutations p.L25P (GLC63), p.H212P (GLC1, GLC60), and c.IVS8+92G>A and c.IVS9–40T>C (both present in patient GLC67). None of them showed consistent segregation with glaucoma (data not shown), supporting that they are individually insufficient to cause POAG. However, and alternatively, we cannot completely rule out that they are not involved in the disease.

Three of the rare variants (p.A449T, p.R529Q, p.D658G) were originally classified as disease-causing mutations.¹⁰ However, and in accordance with other studies, we found these

mutations at similar frequencies in cases and controls, which supports that they play a neutral role in glaucoma.^{11,12,15}

Role of Common Variants of *WDR36* and *P53* in POAG Susceptibility

Our data show that common *WDR36* variants may also be involved in glaucoma. Although individual SNPs were not associated with glaucoma after conservative Bonferroni correction for multiple testing, we found an association of haplotype H2 with glaucoma, which was replicated not only in the second group of patients, but also in the combined sample. Glaucoma susceptibility of H2 homozygotes was not confirmed, possibly because of its relatively low frequency, insufficient sample size, or both. One of the SNPs (IVS5+30C>T) that formed part of risk haplotype H2 has been reported to be associated with glaucoma in patients from China³⁷ or the United States.¹⁴ Nevertheless, it was not associated with POAG in this study on an individual basis, which is in accordance with a previous study carried out in German patients.¹¹

We found a significant association between the minor allele P (C) of the *P53* polymorphism p.R72P and glaucoma. According to a dominant model, subjects who carried at least one copy of this allele showed increased susceptibility to POAG. These associations were replicated in only the combined sample of cases, suggesting a weak effect of the SNP on glaucoma risk. Larger sample sizes could be required to reach statistical significance. Our data also reveal that neither a second *P53* polymorphism analyzed in this study (c.97–147ins16pb) nor the haplotypes composed of the two polymorphisms were associated with glaucoma. The weak contribution of the *P53* SNP to glaucoma could probably explain its controversial role in this disease, at least in part. In agreement with our data, allele P has been reported to be associated with glaucoma in patients from Taiwan.²¹ However, later studies failed to find an association of this polymorphism with the disease in Indian,²⁰ Australian,²² Japanese,²⁵ Turkish,²⁴ or Brazilian²⁶ populations, or they reported that mayor allele R (G) is the risk variant in Caucasians from the United States²³ and NTG patients from Hong Kong.³⁸ The 16-bp insertion polymorphism was not associated with glaucoma in four of the aforementioned populations,^{20,22,23,25} although the R/no Ins haplotype frequency was found significantly increased in patients from the United States²³ and the United Kingdom.³⁹

Our cases and controls were ethnically matched. The *P53* genotype frequencies in controls (66.19% RR, 29.26% RP, and 4.55% PP; Table 6) were almost identical with those previously reported in a group of 567 Spanish healthy controls (67.2% RR, 28.7% RP, 4.1% PP)⁴⁰ and similar to those described in the HapMap for Europeans. In our study, this polymorphism followed the Hardy-Weinberg equilibrium, thus supporting that the genotyping was correct. Therefore, biases caused by population stratification or genotyping errors are unlikely. The p.R72P variants are functionally distinct in their ability to induce apoptosis in cells in culture.⁴¹ The increased susceptibility to glaucoma conferred by allele P cannot be directly explained on the grounds of these data because the R variant induces apoptosis more efficiently than the P variant, at least in cell lines. Alternatively, it has been proposed that allele P could induce the instability of ocular ganglion cells, failing to protect them from apoptosis.²¹ Last, and to add further complexity, it should be taken into account that the final outcome of p.R72P variants in retinal ganglion cells may be modulated by epistasis of apoptosis-related genes.

Genetic Interaction between *WDR36* and *P53*

According to the aforementioned idea, the *wdr36* protein has been reported to interact with the *p53* stress-response path-

way, which suggests that an alteration of the genes involved in this pathway may modify WDR36-associated glaucoma.^{17,27} Thus, we also investigated the possible genetic interaction between the two genes. Individually considered, none of the WDR36 SNPs were associated with glaucoma; interestingly, however, p.I264V showed a positive association in combination with the P53 RP genotype (two-locus genotype AG/RP). Moreover, glaucoma risk associated with either haplotype H2 or diplotype H1/H2 slightly increased in those subjects who also carried the P53 RP genotype. This interaction was replicated when we increased the sample size in the combined glaucoma group. These data indicate the existence of a weak genetic interaction between the two genes that must be confirmed in other populations.

Our OR values are in accordance with those reported for most individual genetic susceptibility factors involved in complex diseases. These values are small and frequently range from 1.5 to 2.0,⁴² suggesting that, as in other common diseases, genetic susceptibility to POAG is probably determined by the combination, interaction, or both of multiple gene variants that, when considered individually, are associated with modest effect sizes.⁴² Increasing evidence shows that epistasis or gene-gene interactions play a role in susceptibility to common diseases. Indeed, complex gene-gene interactions have been proposed to be more important than the independent effect of individual susceptibility genes.^{43,44} Our results indicate that moderate epistatic WDR36-P53 interactions are a component of the complex genetic architecture of POAG, which can predispose to the disease by altering the apoptotic pathway in retinal ganglion cells, thus supporting that these epistatic interactions are involved in POAG susceptibility rather than in causation. Lack of consideration of gene interactions and the modest phenotypic effect could explain, at least in part, the discrepant results obtained in previous studies, which analyzed individually the contribution of either WDR36 or P53 variants to POAG susceptibility. Analysis of multiple gene variation will probably be required to comprehensively describe the genetic architecture of glaucoma.

In conclusion, this study supports that both rare and common WDR36 gene variants might play a role in glaucoma susceptibility and provides the first evidence for a genetic interaction between this gene and P53 in glaucoma.

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