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Profiling of *WDR36* Missense Variants in German Patients with Glaucoma

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Purpose. Mutations in *WDR36* were recently reported in patients with adult-onset primary open-angle glaucoma (POAG). In this study, the prevalence of *WDR36* variants was investigated in patients with glaucoma who were of German descent with diverse age of onset and intraocular pressure levels.

Methods. Recruited were 399 unrelated patients with glaucoma and 376 healthy subjects of comparable age and origin, who had had repeated normal findings in ophthalmic examinations. The frequency of observed variants was obtained by direct sequencing of the entire *WDR36* coding region.

Results. A total of 44 *WDR36* allelic variants were detected, including 14 nonsynonymous amino acid alterations, of which 7 are novel (P31T, Y97C, D126N, T403A, H411Y, H411L, and P487R) and 7 have been reported (L25P, D33E, A163V, H212P, A449T, D658G and I264V). Of these 14 variants, 6 were classified as polymorphisms as they were detected in patients and control individuals at similar frequencies. Eight variants present in 15 patients (3.7%) but only 1 control individual (0.2%) were defined as putative disease-causing variants ($P = 0.0005$). Within this patient group, 12 (80%) presented with high and 3 (20%) with low intraocular pressure. Disease severity and age of onset showed a broad range.

Conclusions. The occurrence of several rare putative disease-causing variants in patients with glaucoma suggests that *WDR36* may be a minor disease-causing gene in glaucoma, at least in the German population. The large variability in *WDR36*, though, requires functional validation of these variants, once its function is characterized. (*Invest Ophthalmol Vis Sci.* 2008;49:270–274) DOI:10.1167/iovs.07-0500

Glaucoma refers to a group of clinically and genetically heterogeneous ophthalmologic disorders leading to visual impairment and blindness. The characteristic clinical sign is cupping of the optic nerve head with subsequent retinal nerve fibers loss, usually associated with elevated intraocular pressure. The disease affects more than 67 million people worldwide.[1] Epidemiologic studies have repeatedly confirmed that primary open-angle glaucoma (POAG), the most common adult form of the disease, is one of the main causes of blindness (8%) in European populations.[2,3] The age of onset of glaucoma manifestation ranges from birth to late adulthood. Affected individuals are usually asymptomatic until the late stages of disease, when significant and irreversible optic nerve degeneration has already occurred.[4] As glaucoma-related visual loss is preventable in many cases and as the sensitivity of current diagnostic methods is suboptimal, there is an urgent need to diagnose glaucoma in its early stages.[5,6] Identification of the genes involved in the etiology of glaucoma provides a significant opportunity for presymptomatic diagnosis, improved prognosis, and better understanding of the etiology of this blinding condition.

Although many cases are sporadic, POAG shows familial clustering consistent with autosomal dominant inheritance and incomplete penetrance. Reduced penetrance and excess of sporadic cases is particularly seen in late-onset forms. Nevertheless, more than 11 (GLC1A-GLC1M) different POAG loci have been mapped so far.[7–12] During the past decade, two genes have been reported for POAG: myocilin (*MYOC*) on chromosome 1, long-arm region q24.3-q25.2, primarily mutated in juvenile-onset patients,[13] and optineurin (*OPTN*) on chromosome 10, short-arm region p14-p15, mainly mutated in individuals with normal-tension glaucoma (NTG).[14,15] Although investigators in several studies have consistently found mutations in *MYOC* in approximately 3% of cases including the German population (3.2%),[16] mutations in OPTN seem to be a rather infrequent cause of POAG or NTG.[17,18] In a recent study, a new POAG locus was identified on chromosome 5, region q22.1 (designated as GLC1G). Screening of the WD40-repeat 36 gene (*WDR36*) in 130 patients with an adult-onset form of glaucoma with high and low pressure identified mutations in approximately 5% of patients. Both familial and sporadic cases were affected.[19]

WD40-repeats are stretches of 40 amino acids that contain tryptophan (W) and aspartic acid (D). WD-repeat–containing proteins comprise a large family found in all eukaryotes and are implicated in a variety of functions ranging from signal transduction and transcription regulation to cell cycle control and apoptosis. The underlying common function of all WD-repeat proteins is coordinating multiprotein complex assemblies, where the repeating units serve as a rigid scaffold for protein interactions. Based on sequence similarity, *WDR36* was proposed to contain five[20] to eight[19] WD40 repeats. In addition, *WDR36* contains a C-terminal UTP21 domain that is specifically associated with WD40 repeats[21] as well as sequence stretches that are characteristic for AMP-binding or which exhibit structural similarity to the C-terminal part of cytochrome *cd1*[19] Expression of *WDR36* was shown in human ocular and non-ocular tissues as well as in embryonic and adult mouse tissues.[19] It has been suggested that *WDR36* may be involved in T-cell activation[20] and recently, T-cell-mediated responses have been hypothesized to participate in glaucoma-associated optic nerve degeneration.[22] However, the exact physiological function of the protein and its role in glaucoma pathogenesis remain unclear. The purpose of this study was to determine the

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prevalence of *WDR36* sequence variants in a well-characterized group of 399 unrelated German patients with POAG, NTG, or juvenile open-angle glaucoma (JOAG).

Material and Methods

Patients and Control Subjects

The study was approved by the ethics review board of the Medical Faculty of the University of Erlangen-Nuremberg and was in accordance with the tenets of the Declaration of Helsinki. All subjects gave informed consent before entering the study.

The group of patients with glaucoma consisted of 399 subjects of German (European) origin: 270 had primary open-angle glaucoma (high-pressure POAG), 47 had juvenile open-angle glaucoma (JOAG), and 82 had normal-tension open-angle glaucoma (NTG). All individuals underwent standardized clinical examinations for glaucoma at the Ophthalmologic Department of the University of Erlangen-Nuremberg, Erlangen. These comprised slitlamp biomicroscopy, gonioscopy, automated visual field testing (Octopus G1; Interzeag, Schlieren, Switzerland), fundus photography (Carl Zeiss Meditec, Oberkochen, Germany), optional laser scanning tomography (HRT I and II; Heidelberg Engineering, Heidelberg, Germany) of the disc and a 24-hour Goldmann-applanation intraocular pressure (IOP) tonometry profile with five measurements. Manifest high-tension POAG was defined as the presence of glaucomatous optic disc damage (in at least one eye), visual field defects in at least one eye, and intraocular pressure higher than 21 mm Hg in one eye without therapy. Causes of secondary glaucoma, such as primary melanin dispersion and pseudoexfoliation, were excluded. Glaucomatous optic nerve damage was defined as focal loss of neuroretinal rim or nerve fiber layer associated with a specific visual field defect. According to Jonas, stage 0 optic disc was defined as normal, stage I with vertical elongation of the cup and neuroretinal rim loss at the 12 and 6 o'clock positions, stage II with focal rim loss, stage III and IV with advanced rim loss, and stage V, as absolute optic disc atrophy. Disc area was measured with HRT or estimated with a Goldmann lens and slitlamp (Haag-Streit, Köniz, Switzerland).[23] A pathologic visual field was defined by a pathologic Bebie curve, three adjacent test points with more than 5 dB sensitivity loss or at least one point with a more than 15-dB loss. Patients who showed glaucomatous changes of the optic disc and visual field but no IOP elevation over 21 mm Hg after a 24-hour IOP-measurement (sitting and supine body position) without therapy received a diagnosis of NTG. Patients were classified as having JOAG when age at onset in the index case was below 40 years and no other ocular reason for open-angle glaucoma was visible. In total, 178 (44.4%) patients had a family history of glaucoma. All patients were also screened for myocilin mutations, as determined by direct sequencing of all coding regions of *MYOC*. Mutations were identified in 18 (4.5%) patients also included in the present study, of whom one carried a *WDR36* variant (described later). Detailed results on *MYOC* screening will be described elsewhere (Pasutto et al., manuscript in preparation). A subset of 96 patients tested with the same methods was negative for *OPTN* mutations. As *OPTN* mutations are very rare, the entire cohort was not screened.

The 376 control subjects were all of German origin and were recruited from the same geographic regions as the patients. In addition, the age- and sex-matched control subjects underwent ophthalmic examination. Thus, at the time of examination and inclusion in this study the age ranged from 51 to 92 years (mean, 73.9 ± 6.4). They had IOP below 20 mm Hg, no glaucomatous disc damage, and no family history of glaucoma. Visual acuity was at least 0.8, and the media were clear for examination.

Mutation Screening

Genomic DNA was prepared from peripheral blood samples by a standard salting-out protocol. Individual coding exons of the WDR36 gene including flanking intronic/untranslated region (UTR) sequences were amplified by polymerase chain reaction (PCR) by the appropriate amplification protocols. Primer sequences were selected with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/) and are available on request. Purified PCR fragments were sequenced (Big Dye Termination chemistry ver. 3.1; Applied Biosystems, Weiterstadt, Germany) on a capillary automated sequencer (model 3730 Genetic Analyzer; Applied Biosystems). Each variant was confirmed by a second independent analysis. GenBank Accession NM_139281 was used as cDNA reference sequence and NT_034772 as genomic reference sequence (http://www.ncbi.nlm.nih.gov/ National Center for Biotechnology Information, Bethesda, MD). We used Q8NI36 (WD36_HUMAN) from the Swiss-Prot/Trembl database (http://www.sanger.ac.uk, Sanger Centre, Hinxton, UK) as the reference protein sequence. Evolutionary conservation of nonsynonymous variants was investigated with protein sequence alignment generated by ClustalW (http://www.ebi.ac.uk/clustalw/ European Molecular Biology Laboratory, Heidelberg, Germany) and compared with that presented by the Ensembl Database (http://www.ensembl.org).[24]

Results

Direct sequence analysis of *WDR36* in 399 unrelated patients with glaucoma identified 44 allelic variants, 14 of which cause amino acid substitution (Table 1). Seven of these are novel (P31T, Y97C, D126N, T403A, H411Y, H411L, and P487R), whereas six variants (L25P, D33E, A163V, H212P, A449T, and D658G) have been reported.[19,25]

These nonsynonymous variants are located in the amino-terminal region, as well as in the WD-40 repeat domains (Fig. 1A). The latter mostly affect positions evolutionary conserved among orthologous in mouse, rat, zebra fish, and puffer fish (Fig. 1B). Variations L25P, P31T, and D33E could not be unambiguously aligned because of the lack of sequence conservation of the N-terminal region.

Mutations that were defined as disease-causing[19] were found in 1.8% (7/399) of the patients and in 2.1% (8/376) of the control individuals. Sequence variants reported to be potential disease-susceptibility mutations[19] were detected in 4.7% (19/399) of the patients and 4.8% (18/376) of control subjects (Table 1). One variant that had not been classified (D33E)[25] was seen in eight (2.0%) patients and one (0.3%) control individual. The seven variants not reported before were seen in seven patients only. One previously reported nonsynonymous SNP, I264V, is a common sequence variant and was found in patients and controls at a similar frequency (Table 1). Altogether, the nonsynonymous variants (excluding the common I264V variant) were detected in a total of 41 (10.2%) patients compared with 27 (7.2%) control subjects ($P = 0.1619$; Fisher exact test). However, owing to our data (Table 1) and to recent WDR36 screenings reported by other groups,[25–28] the nonsynonymous variants L25P, A163V, H212P, A449T, and D658G are rather addressed as polymorphisms due to frequent detection in healthy subjects. Consequently, when these five putative polymorphisms were excluded from our statistical analysis, the remaining eight nonsynonymous amino acid alterations were detected in 15 patients (3.7%) and 1 control subject (0.2%; $P = 0.0005$).

Six synonymous amino acid changes, one of which was novel (R430R), and 24 additional intronic variants were seen in patients and controls at comparable frequency (Table 1). Based on their positions we judged these synonymous changes and the intronic variants unlikely to affect correct splicing and therefore to be polymorphisms thus excluding them from further analysis (Table 1).

In the group of 41 unrelated patients with glaucoma carrying the nonsynonymous amino acid changes, we could not detect a significant correlation between the presence of a specific *WDR36* variation (either defined as polymorphism or putative disease-causing variant) and a particular clinical aspect or diagnostic parameter (Table 2).

TABLE 1. *WDR36* Sequence Variants in Patients and Control Individuals

Exon/Intron	Alleles	Db SNPs	AA Substitution	Protein Domain	Patients	Controls
Nonsynonymous						
1	c.74T>C		L25P*	Unknown	8/399	2/376
1	c.91C>A		P31T	Unknown	1/399	0/376
1	c.99C>G		D33E	Unknown	8/399	1/376
1	c.290A>G		Y97C	WD40	1/399	0/376
3	c.377G>A		D126N	WD40	1/399	0/376
4	c.488C>T		A163V*	WD40	7/399	5/376
5	c.635AT>CC		H212P*	WD40	4/399	11/376
7	c.790A>G	rs11241095	I264V	WD40	204/399	48/94
10	c.1207A>G		T403A	WD40	1/399	0/376
10	c.1231C>T		H411Y	WD40	1/399	0/376
10	c.1232A>T		H411L	WD40	1/399	0/376
11	c.1345G>A		A449T†	WD40	3/399	3/376
12	c.1460C>G		P487R	WD40	1/399	0/376
17	c.1973A>G		D658G†	WD40	4/399	5/376
Synonymous						
3	c.402C>T		G134G	WD40	6/399	14/376
3	c.423T>C	rs17132775	Y141Y	WD40	2/399	0/376
5	c.591G>A		Q197Q	WD40	3/399	2/376
11	c.1290T>C		R430R	WD40	1/399	0/376
18	c.2142C>G	rs17624563	V714V	None	53/307	13/94
19	c.2181A>T	rs13186912	V727V	Utp21	172/307	52/94
Intronic						
5′UTR	IVS0-75C>T		—	—	195/399	218/376
5′UTR	IVS0-32T>C		—	—	1/399	0/376
IVS1	IVS1-130C>G	rs17623144	—	—	123/307	ND
IVS1	IVS1-38T>A		—	—	1/307	ND
IVS2	IVS2-66T>C		—	—	1/307	0/376
IVS3	IVS3-47G>C		—	—	6/399	2/376
IVS4	IVS4-27A>G		—	—	2/399	1/376
IVS5	IVS5+30C>T	rs10038177	—	—	219/399	221/376
IVS6	IVS5-25C>T		—	—	2/307	ND
IVS8	IVS8+36C>T		—	—	1/307	ND
IVS9	IVS9-81T>C		—	—	2/307	1/376
IVS12	IVS12+90C>T	rs10043631	—	—	287/399	284/376
IVS12	IVS12-39G>A		—	—	1/307	ND
IVS13	IVS13+89G>A		—	—	145/307	ND
IVS13	IVS13+91A>G		—	—	1/307	ND
IVS16	IVS16+41C>T		—	—	6/307	ND
IVS16	IVS16-30A>G	rs17553936	—	—	222/399	216/376
IVS21	IVS21+60G>C	rs2290680	—	—	52/307	ND
IVS21	IVS21-75G>A		—	—	21/307	ND
IVS21	IVS21-23A>G		—	—	11/307	ND
IVS21	IVS21-8T>G	rs10041326	—	—	1/307	ND
IVS22	IVS22-65T>C		—	—	1/307	ND
IVS23	IVS23+7A>T		—	—	1/307	ND
IVS23	IVS23+17A>C		—	—	16/307	ND

ND, not determined.
* Previously designated as disease-susceptible.
† Previously designated as disease-causing.

DISCUSSION

We report the largest variation screening for *WDR36* in patients with glaucoma to date. We identified 13 rare nonsynonymous amino acid variants, of which 7 were novel and 6 had been described. Five of these described variants (L25P, A163V, H212P, A449T and D658G) were found in similar frequencies in patients with glaucoma and control subjects (6.5% and 69%, respectively) and failed to cosegregate with the disease in their respective families (data not shown). As glaucoma has an extremely variable age of onset, we cannot exclude that in some of these healthy subjects ($n = 26$, age ranges from 62 to 83, mean age 69.9 ± 5.6) glaucoma may develop later in life, or it may never manifest in some patients. On the other hand, the absence of a significant overall difference between patients and controls questions the previous assumption[19] that these variants in *WDR36* can cause glaucoma. Studies in an Australian, an Iowa and a French-Canadian population reported a single and three *WDR36* variants, respectively, to be at similar or even higher frequency in controls,[26–28] supporting a neutral role for them.

Another recent study that screened a smaller cohort of 118 patients with glaucoma in the United States[25] also reported several families with three of these *WDR36* variants that failed to segregate with the disease. This differs from the initial report of cosegregation in one family showing linkage to the GLC1G locus. Whereas *WDR36* is located at this locus, the data cannot exclude the casual cosegregation in this family due to linkage disequilibrium. Thus, another gene located in close proximity at this locus could be the causative gene. This notion is supported by the increasing number of reports identifying families linked to the GLC1G locus but lacking a *WDR36* mutation[29–31] and by a new study that maps the glaucoma locus GLC1M next to GLG1G.[32]

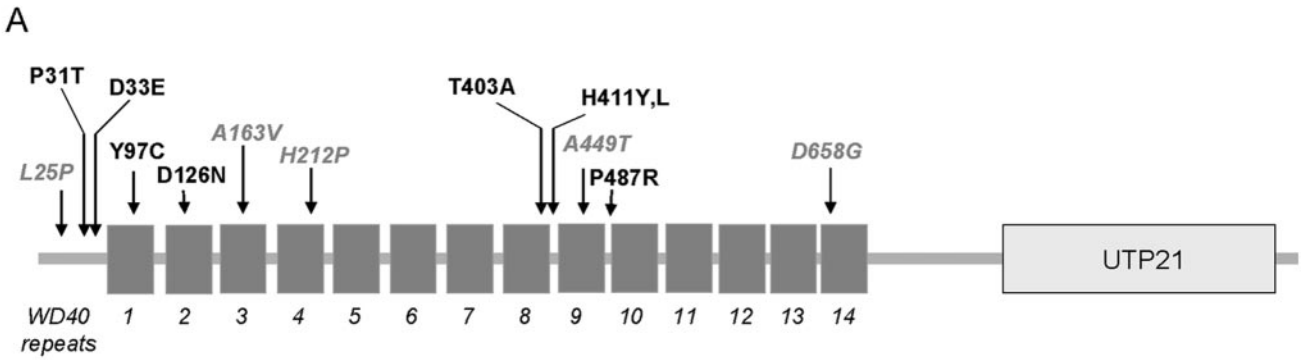

FIGURE 1. Evolutionary conservation of nonsynonymous WDR36 amino acid variants and location on protein domain structure. (**A**) Multiple amino acid sequence alignment shows evolutionary conservation of seven WDR36 variants among different species. *Gray*: residues affected by mutations. (**B**) Our protein modeling predicts WDR36 to contain 14 WD40 repeats. (Structure prediction of WDR36 was performed by using the consensus structure prediction available via the BioInfo Meta-Server, http://bioinfo.pl/meta/ BioInfoBank Institute, Poznan, Poland.) All eight putative disease-causing variants identified in this study are shown (*black*) above the predicted domain organization. Six variants were located in the proposed WD-40 repeats, whereas none was identified in the C-terminal region of the protein.

Seven rare variants were seen, each in one patient, but not in any of the control individuals, whereas one variant (D33E) was found in six patients and only one control subject 75 years of age. Altogether these variants were found more frequently in patients than in controls (3.7% and 0.2%, respectively). The occurrence of different rare variants is characteristic of highly heterogeneous diseases such as glaucoma, as rare mutations have been also reported for *MYOC*. Moreover, since six of eight of these mutations are located within a WD40 domain, it is likely that their alterations directly interfere with the function of the protein. Validation as bona fide mutations would require experimental verification in functional assays, which at the moment are difficult to perform given the unknown function of *WDR36*.

In any case these rare variants would represent only a minor cause of open-angle glaucoma. This conclusion is

TABLE 2. Phenotypic Composition of Patients with *WDR36* Variations

Missense Mutations	GLC Type	Age at Diagnosis (y)		Max IOP (mmHg)		Optic Disc (Jonas)	Mean Defect (dB) Median	Corrected Loss Variance (dB^2)		Disc Area (mm^2)		Chamber Angle
		Mean	SD	Mean	SD			Mean	SD	Mean	SD	
L25P	5 POAG, 1 NTG, 2 JOAG	45.4	18	30	8.7	I, II, IV	7.65 (3.7–18.6)	14.1	10.1	2.4	0.6	3–4
P31T	1 POAG	77		28		V (blind)	15.8	—				3
D33E	5 POAG, 2 NTG, 1 JOAG	45.8	14.2	29.3	5.3	II, III, IV	11.1 (5.2–14.7)	68.5	31.3	2.7	0.6	3–4
Y97C	1 POAG	53		25		II	6.45	61.7				4
D126N	1 NTG	65		21		I						
A163V	7 POAG	55.6	8.6	29.5	4.8	II, III, IV	16.1 (2.0–20.8)	54.1	18.2	2.4	0.6	3–4
H212P	3 POAG, 1 NTG	64.7	10.5	30.5	10.7	II, III, IV	10.5 (4.2–17.1)	56.5	52.4	3.2	1.7	2–4
T403A	1 JOAG	29		40		V (blind)	13.8	86.3		2.5		4
H411Y	1 POAG	61		26		IV	3.8 (4.3–2.5)	37.3	19.7	2.8		4
H411L	1 POAG	66		22		II		3.5				
A449T	2 POAG, 1 NTG	53.6	12.6	26.8	16	0, I, II, V	5.3 (1.9–11.8)	29.0	38.0	3.0	0.2	4
P487R	1 POAG	60		28		III	12.7	135.8		2.99		3
D658G	2 POAG, 1 NTG, 1 JOAG	43	22.6	37.7	21.4	II, III, IV	13.9 (3.6–24.2)	79.2	9.9	2.5	2.4	3–4

The width of the chamber angle is according to Shaffer. Missing data were not available for evaluation.

supported by the recent report by Weisschuh et al.,[33] who reported a frequency of 3.6% (4/112) of rare mutation carriers in a smaller cohort of 112 German patients with NTG, which is very similar to the frequency found in our NTG subgroup (3.7%, 3/82; Table 2, and the Material and Methods section). In addition, our data suggest that these variants in *WDR36* are not characteristic of any particular group of patients with glaucoma and none seems to correlate with a particular clinical aspect or disease severity (Table 2). For example, amino acid change D33E was found in eight patients with age at onset ranging from 14 to 72 years and both normal and high ocular tension (20–40 mm Hg). Overall, in patients carrying a variant, the age of onset ranged from juvenile (14 years) to late adulthood (77 years), and the maximum intraocular ocular pressures varied from 16 to 50 mm Hg, thus indicating that *WDR36* variants are equally present in all three types of open-angle glaucoma (4.2% JOAG, 3.7% NTG and 3.7% POAG patients, Table 2). The degree of disc atrophy ranged from mild cupping to progressed loss of neuroretinal rim of the optic disc, resulting in wide variety of mild and severe visual field loss. Also the disc size ranged from small discs with 1.6 mm^2 to large discs with 5.0 mm^2. The chamber angle in the eye was wide open in all patients.[34] Thus, we conclude that *WDR36* variations are not restricted to a specific type of glaucoma.

In summary, our findings indicate that sequence variants in *WDR36* are only rare causes of unrelated glaucoma in German population. Clearly, investigation of additional families and populations, extensive functional studies, as well identification of *WDR36* binding partners are essential for further understanding the role of *WDR36* in the pathophysiology of glaucoma.

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