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Identification of a novel adult-onset primary open-angle glaucoma (*POAG*) gene on 5q22.1

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Glaucoma is a leading cause of blindness in virtually every country. Development of an accurate diagnostic test for presymptomatic detection of individuals at risk is an urgent requisition for this condition. Herein, we report mapping of a new adult-onset primary open-angle glaucoma (POAG) locus on 5q22.1 (*GLC1G*) and identification of its defective gene. Mutation screening of seven candidate genes from the *GLC1G* critical region (~2 Mb between D5S1466 and D5S2051) identified only one significant alteration in the *WDR36* (WD40-repeat 36) gene. This mutation (i.e. D658G) was segregated in all affected members of our first *GLC1G*-linked family but it was absent in 476 normal control chromosomes. Further screening of *WDR36* in a total of 130 POAG families revealed 24 DNA variations. Overall, four mutations (N355S, A449T, R529Q and D658G) were identified in 17 (5.02–6.92%) unrelated POAG subjects, 11 with high-pressure and six with low-pressure glaucoma. These mutations were absent in a minimum of 200 normal control chromosomes and, further they were conserved between *WDR36* orthologues in mouse, rat, dog, chimp and human. *WDR36* is a novel gene with 23 exons, which encodes for 951 amino acids and a protein with multiple G-beta WD40 repeats. By northern blotting, two distinct mRNA transcripts of 5.9 and 2.5 kb were observed in human heart, placenta, liver, skeletal muscle, kidney and pancreas. *WDR36* gene expression in lens, iris, sclera, ciliary muscles, ciliary body, trabecular meshwork, retina and optic nerve were established by RT–PCR. In mouse, two transcripts of 3.5 and 2.9 kb showed analogous expression patterns to human. mRNA expressions were detected in 7-, 11-, 15- and 17-day-old developing mouse embryos. In summary, *WDR36* is a novel causative gene for adult-onset POAG at the *GLC1G* locus. Specific ocular expressions and observed mutations are consistent with *WDR36* role in etiology of both high- and low-pressure glaucoma.

INTRODUCTION

Glaucoma is a group of ocular disorders characterized by a specific pattern of optic nerve and visual field defects. This condition is one of the two leading causes of blindness, affecting over 67 million people worldwide (1). Open-angle glaucoma is usually asymptomatic until the late stages of the disease, by which time significant and irreversible optic nerve damage has already taken place (2,3). As the sensitivity of current diagnostic techniques is suboptimal, the diagnosis of glaucoma is usually made once an irreversible damage has already occurred. As glaucoma related visual loss is preventable in many cases, there is an urgent need to diagnose glaucoma at its early stages and to institute appropriate neuroprotective management of the ganglion cells (4,5). Mapping, cloning and identification of novel mutations involved in the etiology of glaucoma provide a significant opportunity for presymptomatic diagnosis, improved prognosis and better understanding of the etiology of this blinding condition.

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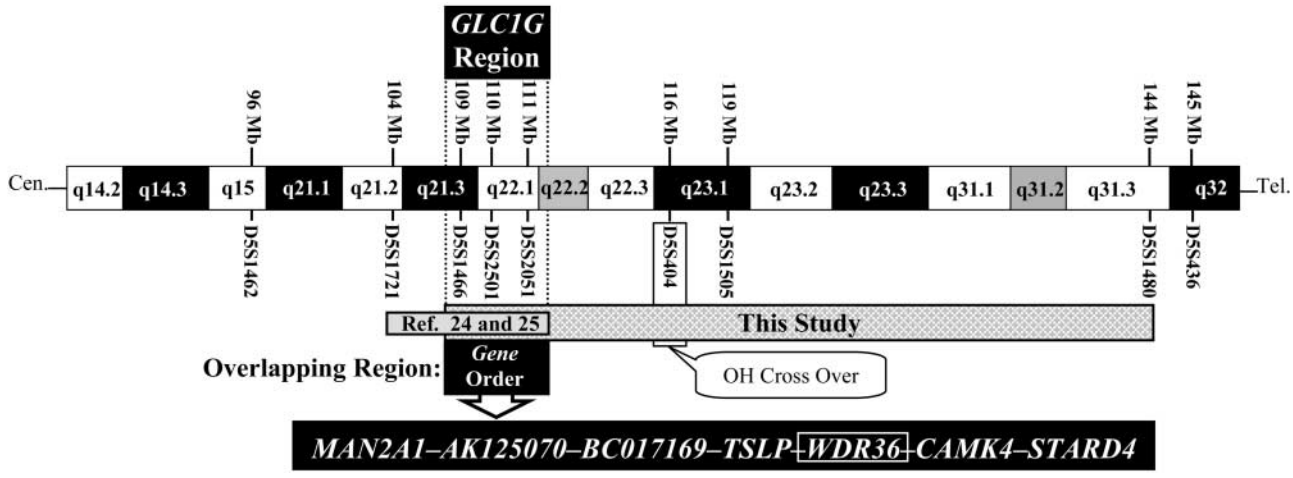

Figure 1. Partial genetic and physical map of chromosome 5q. Cytogenetic banding and map positions (in Megabases) for a selected group of DNA markers are shown. This study identified two flanking markers of D5S1466 (109 Mb from the top of chromosome 5) and D5S1480 (144 Mb) and a further cross over with D5S404 (116 Mb) in a subject with OH. Another study by Samples *et al.* (24) and Kramer *et al.* (25) identified a region between D5S1721 (104 Mb) and D5S2051 (111 Mb). An overlapping region of ~2 Mb between D5S1466 and D5S2051 defined the *GLC1G* locus. Order of seven known genes in this overlapping region that were fully sequenced in our study are also shown. D5S2501 maps between *AK125070* and *BC017169.*

Adult-onset primary open-angle glaucoma (POAG; MIM no. 137760) is the commonest form of this ocular group, usually manifesting itself after the age of 40 years (6,7). The prevalence of POAG is ~1–2% over age 50 in white populations (8) and four to five times greater in black populations of the same age (9). Clinical diagnosis of all groups is based on characteristic changes of the optic nerve head and visual field, which are usually accompanied by increased intraocular pressure (IOP) (10). Family history is an important risk factor for POAG, and genetic study of families shows that dominant genes are predominantly involved in this condition (7,11,12).

During the last decade, a total of six genetic loci (13–18) and two genes have been reported for POAG: myocilin (*MYOC*) is primarily mutated in juvenile-onset subjects (19), whereas optineurin (*OPTN*) is mainly mutated in low pressure POAG individuals (20,21). Here, we report chromosomal mapping of a new POAG locus on 5q22.1 (designated as *GLC1G*) and identification of its causative gene, *WDR36* (WD40-repeat 36), from within this region. We further present the first expression analysis of this gene in a variety of human ocular and non-ocular tissues. For an evolutionary comparative analysis, we further determined expression of the homologue of this gene (*Wdr36*) in various stages of mouse embryonic developments as well as a group of tissues from the adult animals.

RESULTS

Positional mapping of a new locus (*GLC1G*) for adult-onset POAG

Initially, genome scans of two large POAG families (i.e. POAG-002 and POAG-527) with 20 affected and nine glaucoma suspects were used to obtain a provisional hint of linkage to the 5q33–q35 region (22). Extended saturation mapping established critical recombination in two of the affected individuals in POAG-002 and, therefore, excluded genetic linkage of this family to this region of 5q. However, additional genotyping in POAG-527 revealed consistent sharing of an extended affected haplotype from 5q21.3 (D5S1466) to 5q35.2 (D5S498) in all seven affected members of this family (23). Therefore, in light of this new linkage information and due to a discrepancy that currently exists between genetic linkage and physical map position of several DNA markers from this region of chromosome 5 (including D5S1466), the upper physical boundary for this new POAG locus is now 5q21.3 and not 5q33 as initially envisaged (22).

After a hint of linkage was observed with the 5q region, seven additional POAG families with a total of 194 individuals and 31 living affected subjects were genotyped. Only two of these families were consistent with linkage to the same 5q region. Further saturation mapping in these families confined this new linkage to an interval of ~35 Mb (Fig. 1) that is flanked by D5S1466 (5q21.3) and D5S1480 (5q31.3). Additional recombination in a subject with ocular hypertension (OH) and for DNA marker of D5S404 (5q23.1) suggested that the identified genetic linkage may be limited to ~7 Mb (Fig. 1). In a parallel study, a group of 638 individuals (including more than 400 affected subjects) in 139 POAG families were being screened in our laboratory for the entire genome. As this screening also covered this newly identified region on 5q, a number of new families showed potential linkage to this region. Altogether, a total of seven families were consistent with linkage to this region of 5q. Table 1 shows individual and multi-point LOD score values for three closely linked DNA markers of D5S1462, D5S2501 and D5S1505 (Fig. 1). This newly identified POAG locus has now been designated as *GLC1G* by HUGO Gene Nomenclature Committee (HGNC). Recently, Samples *et al.* (24) and Kramer *et al.* (25) reported linkage in a single adult-onset POAG family and, within a 6.6 Mb region (Fig. 1) that is flanked by D5S1721 (5q21.2) and D5S2051 (5q22.1). Taken together, these two overlapping linkage data defined the critical region

Table 1. Two-point and multi-point LOD score values for three closely *GLC1G*-linked markers

DNA markers	Recombination fractions (cM)										Z_{max}	θ_{max}
	0.00	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45		
D5S1462	$-\infty$	2.25	2.22	1.99	1.68	1.33	0.96	0.60	0.29	0.08	2.27	0.07
D5S2501	5.41	4.93	4.39	3.81	3.19	2.54	1.87	1.20	0.60	0.15	5.41	0.00
D5S1505	2.45	2.32	2.10	1.82	1.50	1.16	0.82	0.50	0.24	0.06	2.45	0.00
Multi-point	4.90	4.70	4.44	4.05	3.61	3.13	2.61	2.07	1.51	0.96	4.90	0.00

of the *GLC1G* locus between D5S1466 (5q21.3) and D5S2051 (5q22.1) and within a region of ~2 Mb (Fig. 1). This newly identified *GLC1G* locus provided an opportunity for rapid mutation screening and identification of the defective gene at this locus.

GLC1G candidate genes screening

The critical region of the *GLC1G* locus contains seven known genes (*MAN2A1*, *AK125070*, *BC017169*, *TSLP*, *WDR36*, *CAMK4* and *STARD4*) and at least three other predicted genes (LOC153778, LOC441101 and LOC441102). For each of the seven known genes (Fig. 1), a series of genomic primers were made and their respective coding exons were PCR amplified and screened for mutation by direct DNA sequencing. Analysis of genomic DNA from at least two affected subjects of the original *GLC1G*-linked family of POAG-527 revealed four sequence alterations in only two genes. Three of these variations (i.e. IVS6−5A>G, S337S and A413V in *CAMK4*) were polymorphisms and only one sequence change in *WDR36* proven to be significant. The observed variation in this gene was a single heterozygous DNA alteration in exon 17 (c.1973A>G; G*A*C>G*G*C) that is predicted to change aspartic acid (acidic) to glycine (neutral and polar) at amino acid 658 (D658G). This mutation (Fig. 2D) was observed in seven affected (with mean age of 63.7 years) and nine asymptomatic gene carriers (with mean age of 40.4 years) but it was absent in nine normal members of this family (with mean age of 45.3 years) and another six married-in normal spouses (Fig. 2E). Screening of a total of 476 normal control chromosomes by restriction enzyme digestion (*Bgl*I) showed that this mutation is absent in all the normal subjects tested. Direct sequencing of *WDR36* in another 129 unrelated affected glaucoma probands (including the six *GLC1G*-linked families) identified additional 23 DNA alterations (Table 2). Three of these alterations (N355S, A449T and R529Q) (Fig. 2A–C) were considered disease causing, as they were not present in any of the public databases and in a group of at least 200 tested chromosomes from normal control subjects. We directly sequenced the genomic DNA of at least one affected subject from each of the original seven linked families. However, two of these families did not show any DNA variations in the coding regions of the *WDR36* gene. Therefore, it is possible that other mutations interfering with the mRNA splicing machinery, mutations within the promoter of this gene or presence of other DNA rearrangements such as insertion, deletion or duplication is responsible for the POAG phenotype in these two families. Alternatively, it is possible that another POAG locus is located within our identified genetic linkage and, therefore, mutations in another gene are responsible for these two families.

Expression study of *WDR36* at RNA level

As a first step in determining the role of *WDR36* in glaucoma pathogenesis, we studied expression of this gene in different human ocular and non-ocular tissues by northern blot and reverse transcriptase–polymerase chain reaction (RT–PCR). Northern blotting of several human non-ocular mRNA samples revealed two different transcripts (i.e. 5.9 and 2.5 kb) that are highly expressed in heart, placenta, liver, skeletal muscle and pancreas (Fig. 3A). Using RT–PCR, similar *WDR36* expressions were also detected in heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (Fig. 3B). *WDR36* expression was also detected in a number of ocular tissues including lens, iris, sclera, ciliary muscles, ciliary body, trabecular meshwork, retina and optic nerve (Fig. 3C). As a comparative study, we also determined mRNA expression patterns for the orthologue of this gene (*Wdr36*) in mouse. Northern blotting of *Wdr36* in a panel of adult mouse tissues revealed analogous expression patterns to human with only one exception; in skeletal muscle, the expression was stronger in human (Fig. 4A). Using RT–PCR, high levels of *Wdr36* expression were detected in mouse heart, brain, liver, skeletal muscle, kidney, testis and lower expressions in spleen and lung (Fig. 4B). Study of *Wdr36* during various stages of mouse embryonic developments showed that this gene is detectable in 7-day-old embryos (Fig. 4C).

DISCUSSION

This article describes mapping of a new locus for POAG (*GLC1G*) on the 5q22.1 region and identification of its disease-causing gene, *WDR36* (GenBank accession no. NM_139281). As *WDR36* is a novel gene, using northern blot analysis we determined the transcript size of this gene in two commercially available human and mouse mRNA blots, and using RT–PCR, we established expression of this gene in human ocular and non-ocular as well as in embryonic and adult mouse tissues.

WDR36 has recently been identified as one of the genes that is uniquely involved in T-cell activation and highly co-regulated with interleukin 2 (IL2). This gene encodes for a T-cell activation protein with a minimum of eight WD40 repeats (26) and, therefore, it is also recognized as T-cell activation WD repeat protein (TA-WDRP). This gene contains 23

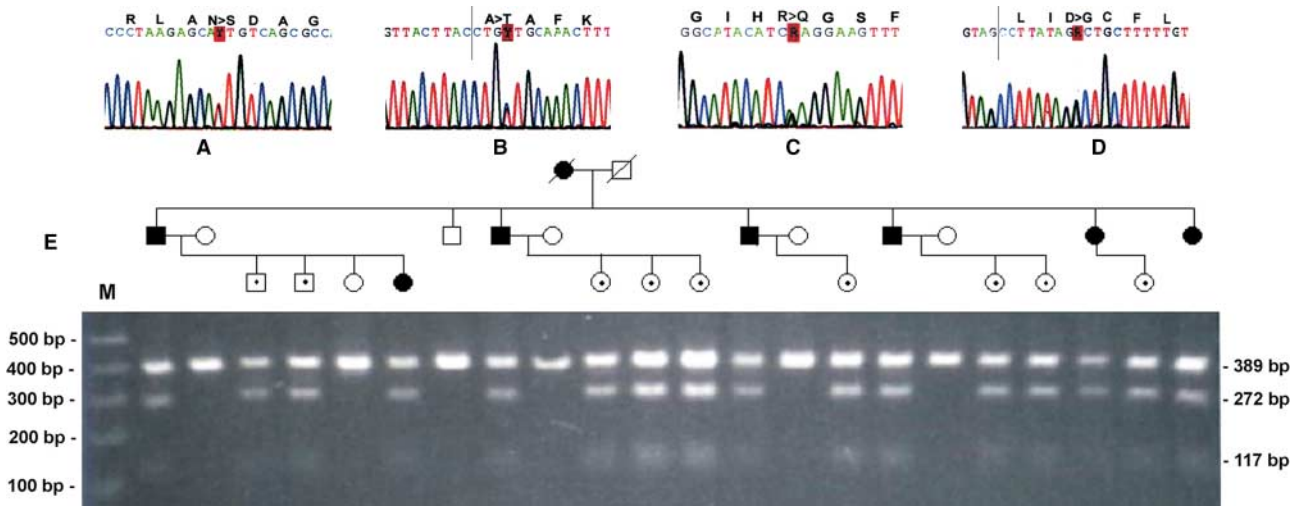

Figure 2. Predicted POAG-causing mutations in the *WDR36* gene. (**A**, **B**) Partial reverse sequence for N355S (A*A*T>A*G*T) and A449T (*G*CA>*A*CA); (**C**, **D**) Partial forward electropherogram for R529Q (C*G*A>C*A*A) and D658G (G*A*C>G*G*C). Vertical line identifies the intron–exon boundaries for A449T and D658G sequences. (**E**) Segregation of D658G mutation in our original *GLC1G*-linked family of POAG-527. *Bgl*I restriction digestion of a 389 bp PCR fragment produces two products of 272 and 117 bp only in presence of the mutant allele. For simplicity, only seven affected (filled-in), nine gene carriers (dot inside their symbols), two normal subjects and four available spouses are shown. All other normal subjects with no D658G mutation in the third and fourth generations were omitted.

exons and encodes for a 951 amino acid protein. The encoded protein consists of at least four known motifs: a guanine nucleotide binding protein (or G)-beta WD40 repeat, an AMP-dependent synthetase and ligase, a cytochrome cd1-nitrite reductase-like (C-terminal heme d1) and an Utp21-specific WD40 associated putative domain.

Mutation analysis of *WDR36* in 130 unrelated affected individuals revealed a total of 24 allelic variants, of which 12 were amino acid coding and 12 involved intronic alterations (Table 2). Comparative sequence alignments of this gene among human, chimp, dog, rat and mouse revealed that 11 of these amino acids (except V727) have been fully conserved among these species (Fig. 5). Evolutionary conservation of the four identified disease-causing mutations (N355S, A449T, R529Q and D658G) in at least five different species and their absence in a minimum of 200 human normal control chromosomes imply that these four amino acids perform a fundamentally important role in the organization of WDR36 protein.

Although one of the *WDR36* disease-causing mutations identified in this study (A449T) is not part of a known protein motif, the other three are located in separate G-beta WD40 repeats (Fig. 5). The N355S mutation in exon 8 maps to the fourth WD40 repeat (covering amino acids 321–361), the R529Q in exon 13 maps to the sixth WD40 repeat (amino acids 525–567) and D658G in exon 17 maps to the eighth WD40 repeat (amino acids 653–692). The G proteins are a family of membrane-associated proteins that act as intermediaries in transduction of the signals generated by transmembrane receptors. The G-beta subunit is required for membrane anchoring and receptor recognition. Structurally, the G-beta consists of eight tandem repeats of about 40 residues, each containing a central Trp-Asp dipeptide (thus named WD40 repeats). Therefore, mutations affecting the structure of these WD40 repeats may interfere with interaction of WDR36 with other proteins. This repetitive WD40 segment is also present in over 250 proteins that are encoded by a variety of genes mapping to every one of the human chromosomes. None of the WDR36 detected amino acid alterations in this study was located in the AMP-binding domain (amino acids 553–564) and only one single nucleotide polymorphism or SNP (V727V) was identified from within the Utp21 domain (amino acids 724–948).

The D658G mutation (Figs 2D and Fig. 5) that is located within the eighth WD40 repeat also maps to the C-terminal part of the cytochrome heme cd1 (cyt cd1) domain (amino acids 109–318 and 537–686). This 'cyt cd1' is part of a bi-functional enzyme with cytochrome oxidase activity (27). It is interesting that we have previously identified another member of the cytochrome P450 family (*CYP1B1*) that is involved in the etiology of primary congenital glaucoma (28,29). Therefore, there might be a functional association between *WDR36* and *CYP1B1*, as mutations in these two genes are responsible for two different forms of glaucoma.

As D658G mutation was originally observed in five out of 130 unrelated and familial POAG subjects, we further tested for the presence of this one particular mutation in an additional 540 unrelated affected individuals from different POAG subgroups. This mutation creates a new recognition site (*Bgl*I) thus providing a rapid method for its screening (Fig. 2E). In total, 13 out of 670 (1.94%) subjects tested were found to be heterozygous for D658G (seven with high- and six with low-pressure glaucoma). Originally, we sequenced the entire of *WDR36* gene in a total of 130 familial POAG cases and identified three mutations of N355S, A449T and R529Q in four families (3.08%) and the common mutation of D658G in five families. Altogether, nine out of 130 (6.92%) families that were fully sequenced showed mutations in the *WDR36* gene. When additional 540 unrelated affected subjects (268 familial and 272 sporadic) were only tested for presence of

Table 2. Distribution of 24 sequence variations identified in the *WDR36* gene

Exon or intron position	cDNA change[a]	Predicted protein change	Protein domain affected	Observed mutations/total no. of POAG families (%)	Observed mutations/total no. of normal chromosomes (%)	*P*-values[b]
Predicted disease-causing mutations						
EX-8	c.1064A>G	N355S	WD40 Domain 4	1/130 (0.77)	0/200 (0.00)	0.828
EX-11	c.1345G>A	A449T	None	2/130 (1.54)	0/200 (0.00)	0.301
EX-13	c.1586G>A	R529Q	WD40 Domain 6	1/130 (0.77)	0/200 (0.00)	0.828
EX-17	c.1973A>G	D658G	WD40 Domain 8: Cyt_cd1-Hem 2	13/670 (1.94)[c]	0/476 (0.00)	0.006
Sum of all predicted disease-causing mutations				(5.02)[d]	(0.00)	
Potential disease-susceptibility mutations						
EX-1	c.74T>C	L25P	None	5/130 (3.85)	2/428 (0.47)	0.010
EX-4	c.488C>T	A163V	WD40 Domain 1: Cyt_cd1-Hem 1	4/130 (3.08)	1/422 (0.24)	0.014
EX-5	c.646−647TA>CC	Y216P	Cyt_cd1-Hem 1	6/130 (4.62)	3/200 (1.50)	0.176
Sum of all potential disease-susceptibility mutations				(11.55)	(2.21)	
Amino acid polymorphisms						
EX-3	c.402C>T	G134G	Cyt_cd1-Hem 1	6/130 (4.62)	100% Linkage disequilibrium with Y216P	
EX-7	c.790A>G	I264V	WD40 Domain 2: Cyt_cd1-Hem 1	67/130 (51.54)	(SNP: *rs11241095*)	
ED-17	c.2011A>G	M671V	WD40 Domain 8: Cyt_cd1-Hem 2	1/130 (0.77)	(SNP: *rs11956837*)	
EX-18	c.2142C>G	V714V	None	22/130 (16.92)	95% Linkage disequilibrium with IVS18 + 216C>T; (SNP: *rs17624563*)	
EX-19	c.2181A>T	V727V	Utp21	73/130 (56.15)	(SNP: *rs13186912*)	
Intronic polymorphisms						
IVS4	IVS4−27A>G	N/A		1/130 (0.77)	Present in normal population	
IVS4	IVS4−139A>T	N/A		1/130 (0.77)	Present in normal population	
IVS5	IVS5+30C>T	N/A		1/130 (0.77)	(SNP: *rs10038177*)	
IVS7	IVS7+105A>G	N/A		2/130 (1.54)	Present in normal population	
IVS7	IVS7−39T>G	N/A		1/130 (0.77)	0/90 (0.00)	
IVS8	IVS8+92G>A	N/A		1/130 (0.77)	0/90 (0.00)	
IVS12	IVS12+90C>T	N/A		86/130 (66.15)	(SNP: *rs10043631*)	
IVS13	IVS13+89G>A	N/A		24/130 (18.46)	Present in normal population	
IVS14	IVS14+89C>A	N/A		71/130 (54.62)	(SNP: *rs13161853*)	
IVS16	IVS16−30A>G	N/A		57/130 (43.85)	Present in normal population	
IVS18	IVS18+216C>T	N/A		21/130 (16.15)	Present in normal population	
IVS21	IVS21+60G>C	N/A		13/130 (10.00)	(SNP: *rs2290680*)	

[a]Based on GenBank accession no: NM_139281.
[b]χ^2-test using Yates correction.
[c]Five D658G mutations identified in the same group of 130 POAG families (3.85%) plus another eight mutations in additional 540 unrelated subjects (i.e. five out of 268 familial and three out of 272 sporadic cases) that were tested only for this one particular mutation. Altogether, 13 D658G mutations were observed in 670 (1.94%) unrelated familial (10/398 or 2.51%) and sporadic (3/272 or 1.10%) cases of POAG.
[d]This figure is 6.92% (nine out of 130) for the same 130 POAG families that were fully sequenced for *WDR36*.

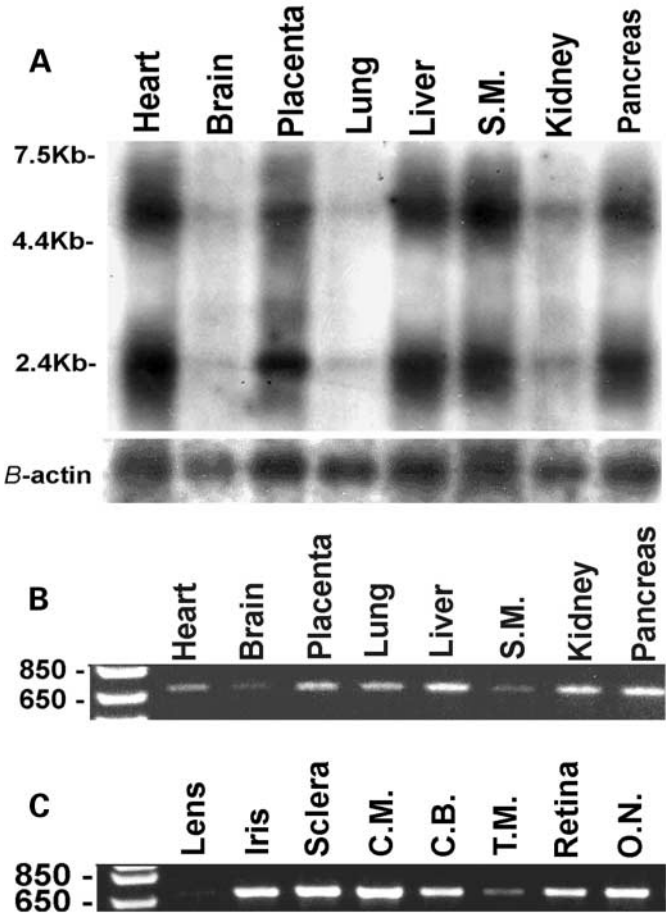

Figure 3. *WDR36* gene expression in various human tissues. (**A**) Northern blotting of non-ocular tissues revealed two transcripts of ~5.9 and 2.5 kb (top panel). Beta-actin used as a standard control. Heart, placental, liver, smooth muscle and pancreas exhibited the strongest signals. (**B**) mRNA expression patterns of *WDR36* in various non-ocular tissues by RT–PCR. Placental, lung, liver, kidney and pancreas produced the strongest signals. (**C**) RT–PCR of *WDR36* in a group of human ocular tissues showing a strong gene expression in iris, sclera, ciliary muscle, ciliary body, retina and optic nerve.

D658G, this mutation was observed in another five familial and three sporadic cases. Altogether, D658G was observed in 2.51% of familial (10 out of 398) and 1.10% of sporadic (three out of 272) cases, thus providing a combined frequency of 1.94% (13 out of 670) for this one mutation alone. When this is added to the frequency of 3.08% obtained from the other three mutations, a minimum mutation rate of 5.02% is obtained for this gene. After the entire of *WDR36* gene in the earlier-mentioned 540 familial and sporadic cases is fully sequenced, it is anticipated that additional mutations will be identified thus, altering the mutation frequency of 5.02–6.92% as presented here. In summary, we identified four different *WDR36* mutations in 17 subjects with either high-pressure (65% of subjects) or low-pressure (35% of subjects) POAG. This observation indicates that the *WDR36* gene is involved in etiology of both types of glaucoma and IOP, as traditionally used to group these into two separate clinical entities, may not be supported by this study and by recent molecular delineation of this group of optic neuropathies (20,21).

We also identified three other amino acid alterations of L25P, A163V and Y216P in a group of 15 POAG subjects (Table 2). As these three alterations were absent in all of the public databases, we screened for their presence in 428, 422 and 200 normal control chromosomes, respectively. As the ratio of their presence in the POAG group was significantly higher than that obtained for the normal group (11.55 versus 2.21%, respectively), we considered these as potential disease-susceptibility alterations (Tables 2 and Fig. 5). The role of these amino acid variations in the etiology of POAG requires further investigation.

We also observed three amino acid polymorphisms in our POAG or normal control subjects (I264V, M671V and V727V). In addition to these, the *Ensembl* database lists two other polymorphisms (S90I and A149A) that were not observed in our study. The two other silent amino acid polymorphisms (G134G and V714V) identified in this study were also present in the public EST databases. It is interesting that during this study (Table 2), we also observed 100% linkage disequilibrium between G134G and Y216P, and 95% between V714V and IVS18+216C>T. Of the 12 intronic alterations that were identified in this study, only four have previously been deposited in the SNP databases (Table 2). Therefore, we screened a total of 90 normal control chromosomes for the presence of these previously unreported intronic alterations. As shown in Table 2, only two of these changes (i.e. IVS7−39T>G and IVS8+92G>A) were absent in the normal control chromosomes but the significance of their presence in the POAG subjects is not clear at this point and requires further investigation.

As there is limited *in silico* expression data for *WDR36*, we studied the mRNA transcripts of this gene in various mouse and human tissues. Using northern analysis two different transcript sizes were detected in human and mouse (Figs 3 and 4). However, it is not clear at this point whether these two transcripts were produced by alternative splicing or by use of two different promoters. In humans, intense hybridization was observed in heart, placenta, liver, skeletal muscle, kidney and pancreas (Fig. 3A and B). In mouse, heart, liver and kidney produced the highest signal (Fig. 4A and B). Using RT–PCR, we determined expression of *WDR36* in lens, iris, sclera, ciliary muscles, ciliary body, trabecular meshwork, retina and optic nerve (Fig. 3C). In mouse, expressions were detected in 7-, 11-, 15- and 17-day-old developing embryos (Fig. 4C).

Although the *in vivo* function of *WDR36* is yet unknown, identification of this gene as an adult-onset POAG gene provides an opportunity to find and to study biochemical pathways that are expected to be involved in the pathogenesis of this group of optic neuropathies. WDR36 contains multiple G-beta WD40 repeats that are also present in a large family of proteins with diverse functions. As this WD40 repeat motif is also involved in protein–protein interaction and as WDR36 is now implicated in the etiology of POAG, this finding provides an opportunity to search for WDR36-interacting proteins aiming to identify other proteins/genes that have direct functional effect on the pathophysiology of this blinding condition. Additionally, although different mutations in *WDR36* were observed in both high- and low-pressure glaucoma, molecular screening of this gene may provide a useful

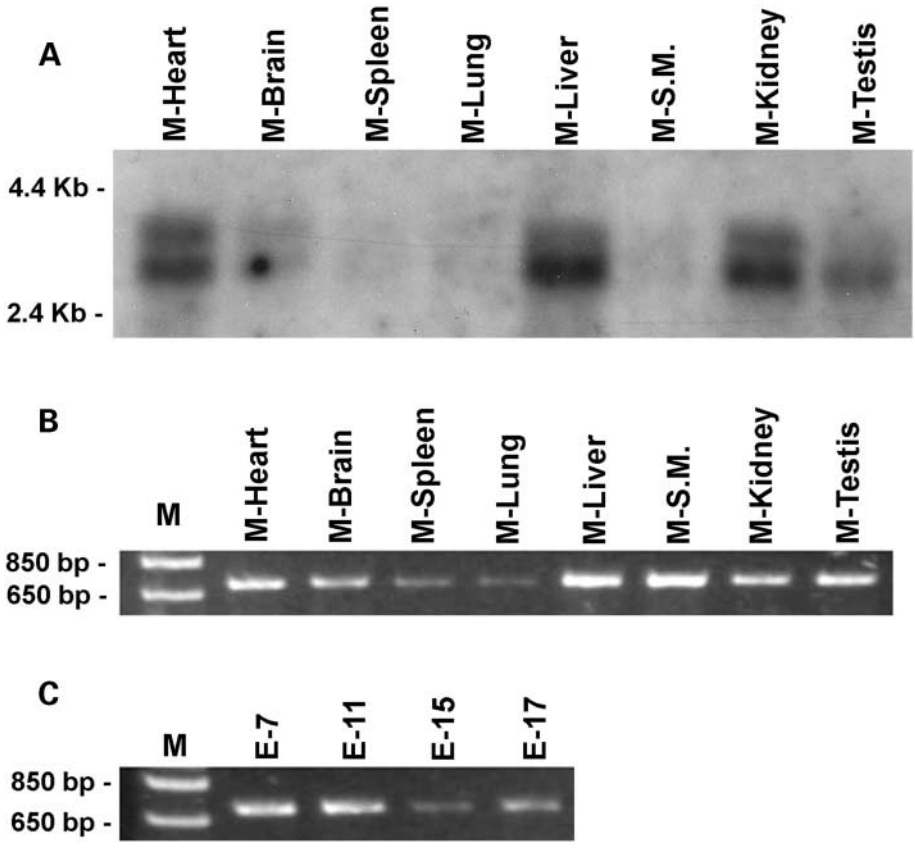

Figure 4. *Wdr36* gene expression in mouse. (**A**) Northern blot analysis exhibited two transcripts of ~3.5 and 2.9 kb. Strongest signals observed in heart, liver, kidney and testis. (**B**) mRNA gene expression in various adult mouse tissues. Similar to northern blotting, spleen and lung had the weakest signals. (**C**) RT–PCR of *Wdr36* in mouse embryo at different gestational age. The earliest gene expression was at the embryonic day 7.

tool for presymptomatic detection of individuals at risk in families or in the elderly population.

MATERIALS AND METHODS

Family ascertainment

The clinical status of all affected POAG members of studied families have previously been confirmed by their respective caring ophthalmologists. The diagnoses of glaucoma agreed with the standard criteria and included abnormal cup/disc ratio and glaucomatous visual field loss with or without increased IOP. The original *GLC1G*-linked family of POAG-527 has a total of 108 members in five generations, of whom 54 (seven affected, five glaucoma suspect and 42 unaffected) participated in this study. The other large family of POAG-002 that was originally used in our genome scan but subsequently proven to be unlinked to the *GLC1G* locus has a total of 99 members in four generations, 40 of whom have been sampled previously (13 affected, four OH and 23 unaffected). The clinical status of all other unrelated familial and sporadic POAG subjects screened during this study have previously been confirmed. The University of Connecticut Health Center Institutional Review Board has approved this study for inclusion of Human Subjects in Research.

Genome-wide scan

A genome-wide scan was carried out with two different sets of fluorescently end-labeled primers (ABI and CHLC-Weber linkage mapping sets). The amplified PCR products were loaded onto a 96-well gel and run on an ABI-377 automated DNA sequencer. Computer assisted data collection and genotyping were performed with the ABI GENESCAN analysis and ABI GENOTYPER computer software. The obtained genotypic data were analyzed and further used for additional linkage analysis and saturation mapping.

Saturation mapping and refinement of the *GLC1G* critical region

All genotypes either from the ABI or from the CHLC-Weber florescent linkage mapping sets were automatically transferred into an in-house database management system (DMS) program. Pedigrees and their genotypic data for each of the 22 chromosomes were subsequently exported to the CYRILLIC program and haplotypes were constructed for each pedigree and for each chromosome. Co-inheritance of affected-bearing haplotypes with the POAG phenotype was inspected manually for each of the studied families. Using additional short tandem repeat polymorphism (STRP) markers potential regions of interest were saturated in these

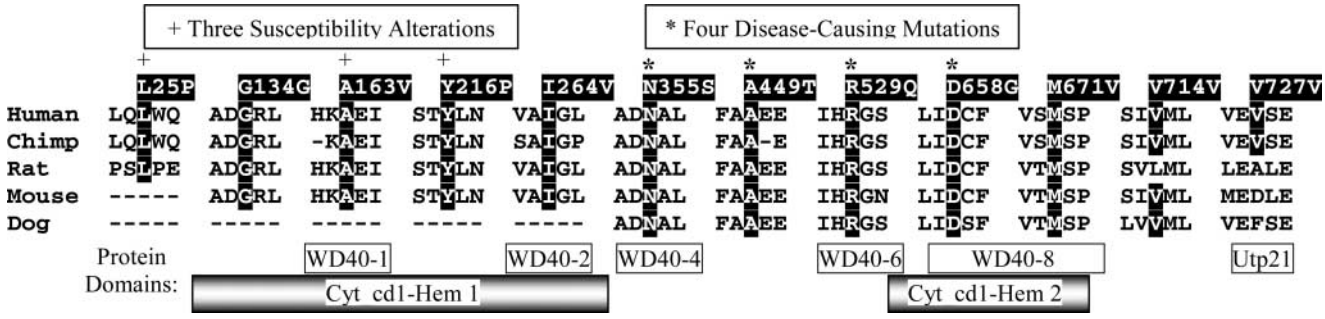

Figure 5. Partial amino acid alignment and evolutionary conservation of 12 *WDR36* variations in human and four other species.

families. After a hint of linkage was observed on chromosome 5q, fluorescent genotyping and saturation mapping of other families with new STRP markers were carried out in order to reduce the identified interval even further. The two-point and multi-point LOD scores were calculated with the MLINK and LINKMAP components of the LINKAGE program, respectively (30).

Mutation screening

After linkage to the *GLC1G* locus was established and its candidate region was reduced to a region of ~2 Mb, all the seven known genes mapping to the critical region (Fig. 1) were selected for mutation screening. Primers were designed to flank intron–exon boundaries of the selected genes and PCR amplification performed using genomic DNA of affected individuals (primer sequences are available on request). Direct sequencing carried out with ABI-Big Dye Terminator Cycle sequencing kit and run on an ABI-3100 Genetic Analyzer and DNA Sequencer.

RNA analysis

Under a dissecting microscope, the lens, iris, ciliary body, ciliary muscle, trabecular meshwork, sclera, retina and optic nerve were micro-dissected and immediately used for RNA extraction. The TRIzol reagent method (Invitrogen) was used to isolate RNA from these ocular tissues. For RT–PCR, cDNA were made from purified RNAs, using random hexamer primers and Superscript III reverse transcriptase (Invitrogen). Furthermore, expression of the *WDR36* gene in human and mouse tissues were investigated by northern hybridization. Radiolabeled probes were prepared from a 691 and 693 bp PCR fragment amplified from human and mouse cDNA, respectively. Expression of this gene was studied by use of the same probe and a premade blot (Clontech) containing poly(A)+ mRNA (2 μg/lane) from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas in human and heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis in mouse.

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