Defining the Pathogenicity of *Optineurin* in Juvenile Open-Angle Glaucoma

Colin E. Willoughby,[1,2] Louie Loh Yen Chan,[1,2] Sarah Herd,[1,2] Gail Billingsley,[1,2] Nima Noordeh,[1,2] Alex V. Levin,[1] Yvonne Buys,[3] Graham Trope,[3] Mansoor Sarfarazi,[4] and Elise Héon[1,2]

PURPOSE. Juvenile open-angle glaucoma (JOAG) differs from primary open-angle glaucoma in that it is usually a more severe phenotype and has an earlier age of onset. Optineurin was recently associated with a variant of POAG that is characterized by intraocular pressure within normal limits: normal-tension glaucoma. The present study tested whether *OPTN* sequence changes play a role in early-onset glaucoma characterized by elevated intraocular pressure.

METHODS. Sixty-six patients with JOAG characterized by high intraocular pressure were screened for mutations. Mutational analysis was performed with a combination of restriction enzyme digestion, single-strand conformation polymorphism, and direct sequencing. The effects of select changes on exon splicing were assessed using bioinformatic modeling approaches and RT-PCR.

RESULTS. Ten sequence changes were identified, of which H486R was strongly suggestive of pathogenicity. H486R represents the first reported *OPTN* mutation associated with JOAG. Also, L41L is proposed to confer an increased susceptibility to the development of JOAG. Most of the other sequence changes observed were not thought to be biologically significant. The frequency of the previously reported M98K allele was not increased in the JOAG population studied but showed the previously reported skewed distribution in the POAG study population. The changes identified were not shown to affect the splicing machinery.

CONCLUSIONS. The results of this work support the hypothesis that mutations in *OPTN* are not specifically associated with low-pressure glaucoma, but can play a role in JOAG. (*Invest Ophthalmol Vis Sci.* 2004;45:3122–3130) DOI:10.1167/iovs.04-0107

Glaucoma refers to a genetically heterogeneous neurodegenerative disease in which selective loss of retinal ganglion cells is central to the disease process, leading to specific visual field defects and degeneration of the optic nerve head.[1–3] Glaucoma is the leading cause of irreversible blindness in the world, affecting approximately 67 million people worldwide in all age groups and populations, of which 6.7 million are bilaterally blind.[4] The heritable nature of the disease offers the potential for molecular genetic analyses to be used to determine the underlying mechanisms, and this knowledge is central to improving clinical outcomes.[5–9]

Primary open-angle glaucoma (POAG; Mendelian Inheritance in Man [MIM] 137760) occurs after the age of 40 and is usually characterized by increased intraocular pressure (IOP > 21 mm Hg). However, optic nerve damage can occur despite an IOP in the normal range and is then referred to as normal-tension glaucoma (NTG; IOP < 21 mm Hg; MIM 606657).[10] Juvenile open-angle glaucoma (JOAG; MIM 137750) is arbitrarily defined herein as high-tension glaucoma with an early age of onset (5–40 years) and usually a severe phenotype. NTG normally does not occur in patients younger than 40 years. The high IOPs in JOAG are often resistant to medical therapy and are associated with severe visual impairment without successful intervention.[11,12] In some cases, JOAG and POAG have been documented to be allelic variants, but the understanding of these genotype–phenotype correlations has not been fully elucidated.[13] Six disease loci have been associated with POAG: GLC1A,[14] GLC1B,[15] GLC1C,[16] GLC1D,[17] GLC1E,[18] and GLC1F.[7] Causative mutations were identified in two POAG genes: myocilin (*MYOC*; GLC1A, MIM 601652)[14,19] and optineurin (*OPTN*; GLC1E, MIM 602432).[18,20] Mutations in *CYP1B1* have mostly been associated with congenital glaucoma. Recent studies estimate that *MYOC* mutations are found in 3% to 5% of cases of sporadic POAG[9] and in up to 13% of cases of JOAG.[13] In a previous study, we suggested that *CYP1B1* also acts as a genetic modifier of *MYOC* mutations,[13] but the molecular interactions of mutant MYOC protein and its role in the pathophysiology of glaucoma are still unclear.

Mutations in *OPTN* were initially reported in 16.7% of families with hereditary POAG, with most of them having NTG.[18,20] The role of *OPTN* in high-pressure glaucoma (POAG) has been a source of controversy.[20–25] The role of *OPTN* in JOAG has not been explored. *OPTN*, which stands for *opt*ic *n*europathy–inducing protein, contains 13 conserved coding exons (Fig. 1).[20] Three different *OPTN* isoforms generated by alternative splicing have been reported, all having the same predicted open reading frame.[20,26] Optineurin codes for a conserved protein, which contains numerous predicted transcription factor motifs, including two putative bZIP motifs, several leucine-zipper domains, coiled-coil motifs, and a C-terminal C2H2-type zinc finger domain.[20,26] *OPTN* is expressed in various human ocular tissues, such as trabecular meshwork, nonpigmented ciliary epithelium, retina, and brain and in other nonocular tissues.[20,26] The role of *OPTN* in normal ocular physiology and glaucoma is currently unclear. We assessed a

FIGURE 1. Schematic representation of *OPTN* with annotations of the sequence alterations identified in patients with JOAG.

cohort of patients affected with JOAG for mutations in *OPTN*, to determine whether it plays a role in high-pressure glaucoma.

METHODS

Patient and Sample Recruitment

This project was approved by the institutional Research Ethics Board of The Hospital for Sick Children, and informed consent was obtained in accordance with the tenets of the Declaration of Helsinki. The primary patient population consisted of individuals with JOAG who were recruited from eye clinics of the Greater Toronto Area through the Glaucoma Genetic Study Project. Criteria for inclusion included age of diagnosis less than 40 years, elevated IOP > 22 mm Hg, visual field loss, and/or optic nerve head cupping characteristic of glaucoma. Normal ethnically and age-matched control subjects were also recruited. A secondary, previously recruited study population affected with POAG[27] was studied in parallel, to investigate further some genotype–phenotype correlations. POAG had the same diagnostic criteria as JOAG, except for an age of onset more than 40 years. POAG subjects with IOP < 21 mm Hg were subclassified as NTG. Individuals with secondary glaucoma due to trauma, uveitis, steroid use, pigment dispersion, or anterior-segment developmental anomalies were excluded. No participant had any other associated ocular abnormalities or relevant systemic disease. Blood samples (20 mL) were collected for DNA extraction using standard protocols.[28] RNA was extracted using the manufacturer's protocol (Invitrogen Canada Inc., Burlington, Ontario, Canada). In selected cases, DNA was extracted from buccal swabs using previously described protocols.[13]

Genomic DNA Analysis

The primers and protocol used for DNA analysis are available on request.

Mutational Analysis of *OPTN* and *MYOC*. Mutational screening was performed with a combination of single-strand conformation polymorphism (SSCP) analysis,[29] followed by bidirectional direct-cycle sequencing and restriction endonuclease digestion, when indicated, according to previously described protocols.[13,30] All patients with *OPTN* sequence alterations were screened for *MYOC* mutations in the same fashion as for *OPTN*, using a combination of SSCP analysis and direct-cycle sequencing.

Restriction Enzyme Digestion Assays for c.603T→A (M98K) and c.433G→A (L41L) OPTN Sequence Changes. *Stu*1 restriction endonuclease digestion was used to screen for the *OPTN* sequence change c.603T→A (M98K), with primers specific to exon 5B. To detect the L41L (c.433G→A) change, primers specific to exon 4a and *Pst*1 restriction endonuclease digestion were used.

Amplification Refractory Mutation System Assay for *MYOC* Gly252Arg Sequence Change. Controls were screened for the *MYOC* mutation Gly252Arg using an amplification refractory mutation system (ARMS; data not shown).[31] The forward primer had a 3′ nucleotide mismatch designed to amplify the Gly252Arg mutation at 451G→A (5′-GGAGAACTAGTTTGGATAA-3′) as well as a second mismatch 4 bp from the 3′ end to prevent read-through of the wild-type allele. Primers for the polymorphic marker D9S1785 were multiplexed,

as a positive control for PCR, with the ARMS primer and the reverse paired primer.

Statistical Analyses. The Fisher exact test was used to determine the significance of detected differences in allele and genotype frequencies among the three groups of study subjects (control, JOAG, and POAG).

Bioinformatics Analysis

Sequence Alignment. Genomic sequencing results were analyzed through pair-wise sequence alignments using the National Center for Biotechnology (NCBI, Bethesda, MD) BLAST 2 sequences program (URLs are provided at the end of the article). Multiple sequence alignments of nucleotide and protein sequences of optineurin from various species were performed using the sequence-alignment program ClustalX (version 1.8) and were formatted using the GeneDoc program.

Scoring of Splice Site Strength. The putative strength of the donor and acceptor splice sites was assessed using the Splice Site Score Calculator, a Web-based algorithm based on nucleotide weight tables.[32] The potential for sequence changes to introduce a cryptic splice site was assessed with SpliceSiteFinder.

Evaluation of Putative Exon Splicing Enhancers. Putative exon splicing enhancers (ESEs) in the *OPTN* coding region were mapped using the Web-based ESEfinder algorithm.[33] ESEfinder is a predictive analysis derived from functional in vivo and in vitro systematic evolution of ligands by exponential enrichment (SELEX) and statistical analysis.[33] ESE motif matrix score calculations are available for four SR family proteins: SRp40, SRp55, SF2/ASF, and SC35. Scores higher than the default threshold level are considered significant predictions for putative ESE motifs.[33]

RNA Secondary Structure Modeling. The MFOLD Web browser was used to predict the energetically most stable RNA secondary structures. MFOLD is a dynamic programming algorithm incorporating a nearest-neighbor, thermodynamic, minimum free-energy method and RNA sequence covariation analysis.[34] Wild-type and mutant mRNA secondary structures were modeled and compared for all detected *OPTN* sequence changes observed.

Transcript Analysis

Total RNA was extracted from whole blood and EBV-transformed lymphoblasts from individuals carrying the L41L sequence change and from normal control subjects. Total RNA was extracted using red blood cell lysis solution (Purogene RNA Isolation Kit D-50K; Gentra, Minneapolis, MN) and an RNA extraction kit (TRIzol; Invitrogen), according to the manufacturer's instructions, and was reverse-transcribed using random hexamer primers and reverse transcriptase (Superscript II; Invitrogen), according to the manufacturer's instructions. Primers of interest were designed from the *OPTN* isoform 1 cDNA (GenBank AF420371) using the Primer3 program and were synthesized commercially (ACGT Corp., Toronto, Ontario, Canada). Fragments of interest were sequenced using standard protocols. RT-PCR products were assessed using 13% polyacrylamide gel electrophoresis and semiquantitative RT-PCR with FAM-labeled primers with fragment sequence analysis (ALF sequencer; Pharmacia Biotech, Piscataway, NJ).[35,36] This analysis was repeated 10 times. Exon-bridging primers were used to detect alternatively spliced *OPTN* isoforms and potential aberrantly spliced isoforms, based on bioinformatics modeling. Nonspecific priming on other constitutive isoforms was prevented by base pair mismatching at the primer 3′ end.[37] PCR products were run on 2% agarose gel and stained with ethidium bromide.

RESULTS

Genomic DNA Analysis

Samples of sixty-six unrelated mixed ethnicity probands with JOAG were screened for mutations in *OPTN*. The patient cohort included 41 males and 25 females with an average age of

TABLE 1. Detailed Genotyping Data for Patients with Observed *OPTN* Sequence Changes

	Mixed	English	Chinese	English Pakistan	European Afro-Caribbean Malaysian Mixed	Afro-Caribbean Jewish/Scottish/ African Somalian	ND	ND	Spanish/ English	Chinese
Exon*	4	4	4	4	5	6	7	9	14	16
Codon change	T34T	L41L	T49T	IVS4+16	M98K	A134A	IVS6-5	IVS8-51	H486R	R545Q
Nucleotide change	G→A	G→A	C→T	C→T	T→A	C→A	T→C	T→C	A→G	G→A
JOAG subjects	38 G/G	65 G/G	65 C/C	65 C/C	60 T/T	64 C/C	49 T/T	33 T/T	65 A/A	65 G/G
	22 G/A	1 G/A	1 C/T	1 C/T	6 T/A	2 C/A	14 T/C	27 T/C	1 A/G	1 G/A
	6 A/A	0 A/A	0 C/C	0 T/T	0 A/A	0 A/A	3 C/C	6 C/C	0 G/G	0 A/A
JOAG totals†	66	66	66	66	66	66	66	66	66	66
Control subjects	65 G/G	165 G/G	165 C/C	164 C/C	97 T/T	108 C/C	65 T/T	54 T/T	101 A/A	181 G/G
	34 G/A	**0 G/A**	2 C/T	1 C/T	3 T/A	1 C/A	35 T/C	35 T/C	**0 A/G**	3 G/A
	8 A/A	0 A/A	0 T/T	0 T/T	1 A/A	0 A/A	2 C/C	13 C/C	0 G/G	0 A/A
Control totals†	107	165	167	165	101	109	102	102	101	184
POAG subjects	26 G/G	88 G/G	88 C/C	52 C/C	41 T/T	56 C/C	31 T/T	21 T/T	108 A/A	48 G/G
	19 G/A	1 G/A	1 C/T	0 C/A	8 T/A	1 C/A	8 T/C	18 T/C	0 A/G	0 G/A
	4 A/A	0 A/A	0 T/T	0 A/A	0 A/A	0 A/A	1 C/C	6 C/C	0 G/G	0 A/A
POAG totals†	49	89	89	52	49	57	40	45	108	48

Nucleotides are numbered from GenBank AF420371. ND, not determined.
* Exons where sequence changes were observed.
† Total number of individuals screened.

diagnosis of 28.7 years of age (range, 8–40 years). The mixed ethnicity of the study population included individuals of predominantly European and English-Canadian (57.5%) ancestry, as well as Afro-Caribbean (12%), Chinese (4.5%), Filipino, and East Indian individuals. A total of 10 *OPTN* sequence alterations were identified in subjects with glaucoma (JOAG and POAG) and control individuals (Table 1). The detailed genotype data and respective allele frequencies are provided in Table 1. Three of the observed changes were missense, four were silent or synonymous changes, and three involved intronic regions (Table 1). In patients with observed *OPTN* sequence changes, the following previously published *MYOC* sequence changes were also detected: Gly252Arg, Arg76Lys, Gln368STOP, Thr325Thr, and Lys398Arg (Table 2).[9,12]

Genotype–Phenotype Correlations

Three observed *OPTN* sequence alterations were initially thought to bear functional importance in patients with JOAG: c.1767A→G (H486R), c.1944G→A (R545Q), and c.433G→A (L41L). The c.1767A→G nucleotide change (H486R) in exon 14 of *OPTN* was seen in one patient with JOAG (1/66), but was not present in 101 control individuals or in 107 patients with POAG. The H486R proband had JOAG diagnosed at age 17, with advanced disease in the right eye, as shown by the visual

field analysis and optic disc photographs (Fig. 2). IOPs were elevated on diagnosis at 35 mm Hg in the right eye and 33 mm Hg in the left eye and the cup-disc ratios were 0.9 in the right eye and 0.55 in the left eye (normal cup-disc ratio, 0.2–0.5). She is currently maintained on medical therapy. No other family member was available. No *MYOC* change was identified.

The R545Q change in exon 16 of *OPTN* has been reported as a disease-causing mutation in POAG[20] in a Japanese patient (Sarfarazi M, personal communication, May 2003). We observed this change in one Chinese patient with JOAG from Hong Kong, with an age at diagnosis of 29 years. Her father, R545Q/− (mt/wt), had JOAG with an age at diagnosis of 38 years and required bilateral trabeculectomy to control his glaucoma. The R545Q sequence change was not seen in 170 mixed ethnicity control individuals. However, it was seen in 3 of 16 Chinese control individuals. The R545Q proband and her father were also shown to have a previously reported *MYOC* mutation, Gly252Arg, which segregated with the disease phenotype in the family and was not seen in 100 control individuals, by ARMS assay (data not shown).

The silent or synonymous *OPTN* change c.433G→A (L41L) was seen as a heterozygous sequence change in patients with JOAG (1/66; 1.5%) or POAG (1/88; 1.1%), but not in control individuals (0/165 individuals). The JOAG proband (D665)

TABLE 2. Summary of the Observed *MYOC* Sequence Changes in JOAG Patients with an *OPTN* Alteration

OPTN Nucleotide Change	*OPTN* Codon Change	JOAG Mutated Allele Frequencies	Associated *MYOC* Amino Acid Changes
c.412G→A	T34T	28 (42.4)	None
c.433G→A	L41L	1 (1.5)	gln368stop/lys398arg, arg76lys/arg76lys
c.457C→T	T49T	1 (1.5)	None
Intron 4	IVS4+16 C→T	1 (1.5)	None
c.603T→A	M98K	6 (9.1)	None
c.712C→A	A134A	2 (3)	arg76lys/− thr325thr/−
Intron 6	IVS6-5 T→C	17 (25.8)	None
Intron 8	IVS8-51 T→C	33 (50)	None
c.1767A→G	H486R	1 (1.5)	None
c.1944G→A	R545Q	1 (1.5)	gly252arg/−

Data are the number of subjects showing the change in 66 total subjects, with the percentage of the total group in parentheses. *OPTN* nucleotides are numbered from GenBank AF420371. −, wild type.
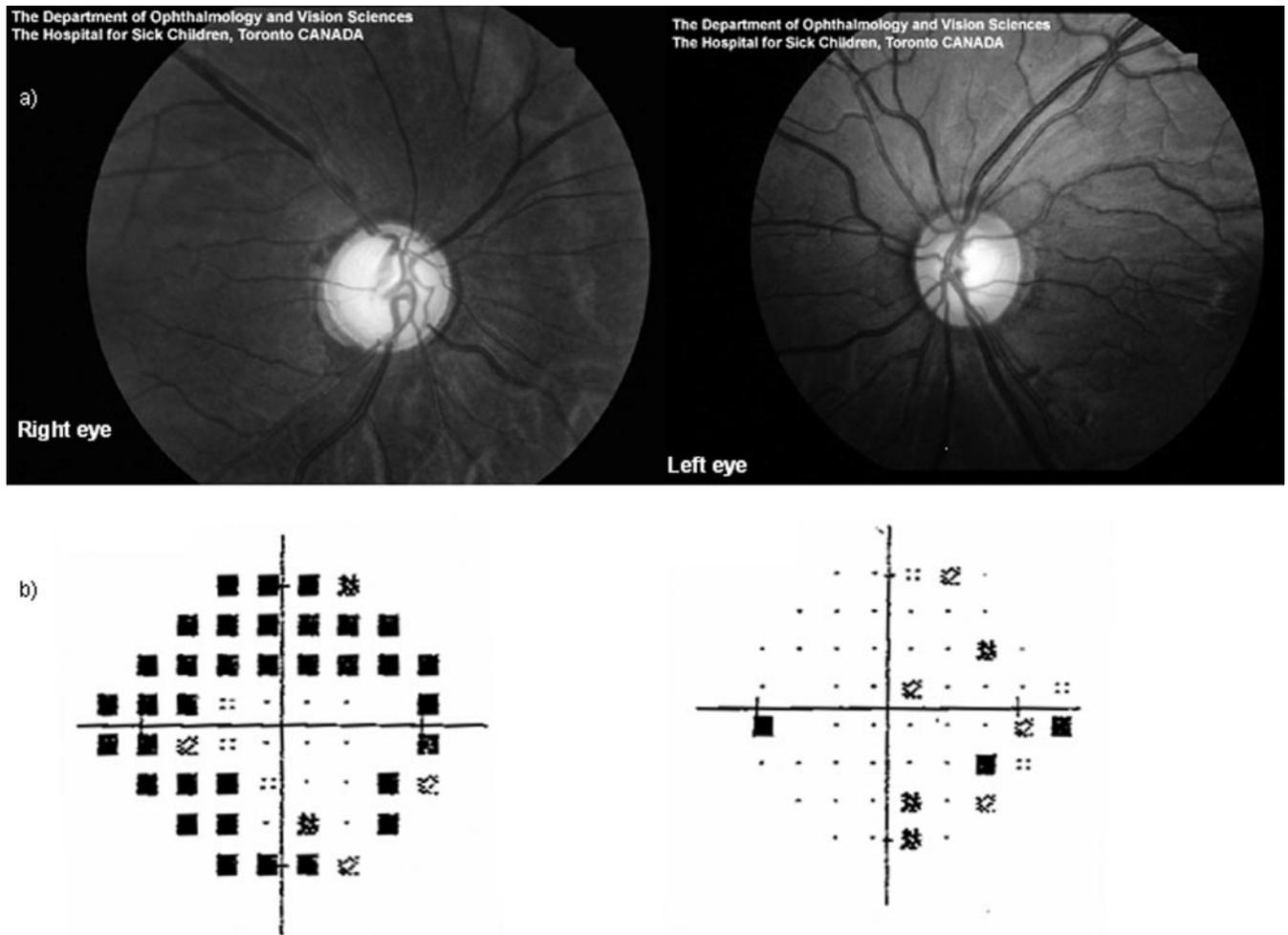
FIGURE 2. Phenotype documentation of an 18-year-old patient with an H486R change. (**A**) Optic disc imaging and corresponding (**B**) static perimetry results. The high cup-disc ratio in the right eye is characteristic of advanced glaucoma. The corresponding visual field loss, demonstrated by perimetry, is associated with the optic disc abnormalities and is represented by the *shaded* or *filled* (more severe) *squares* (**B**).

with the L41L variant was of English-Canadian ancestry and had a positive family history of glaucoma (Fig. 3). Two *MYOC* changes were also detected in this JOAG patient (D665): the

commonest disease-causing sequence change Gln368STOP and a previously published polymorphism Lys398Arg.[9,12] The proband D665 (*OPTN*: L41L; *MYOC*: Gln368STOP/Lys398Arg) pre-

FIGURE 3. Pedigree of a family in which *OPTN* L41L segregates with *MYOC* Glu368STOP. *Filled symbols*: affected status for JOAG; *unfilled symbols*: unaffected or unknown clinical status for glaucoma; *slashed symbols*: deceased individuals. *Hashed symbols*: POAG with *oblique lines*: MYOC mutation or *horizontal lines*: no identified sequence change (phenocopy). The results of *OPTN* and *MYOC* mutational analysis are shown. Dx, age at diagnosis.
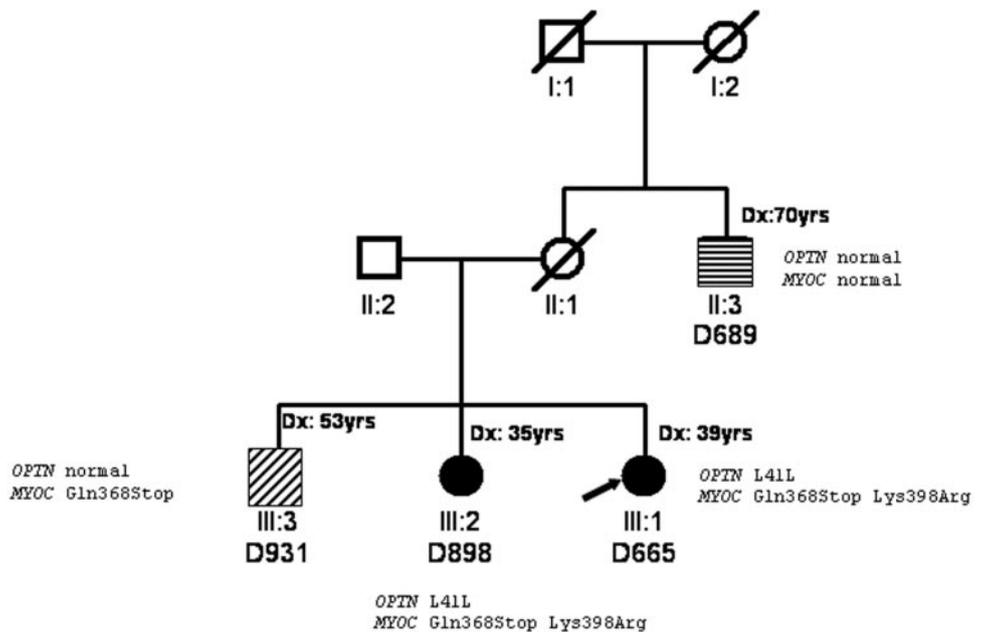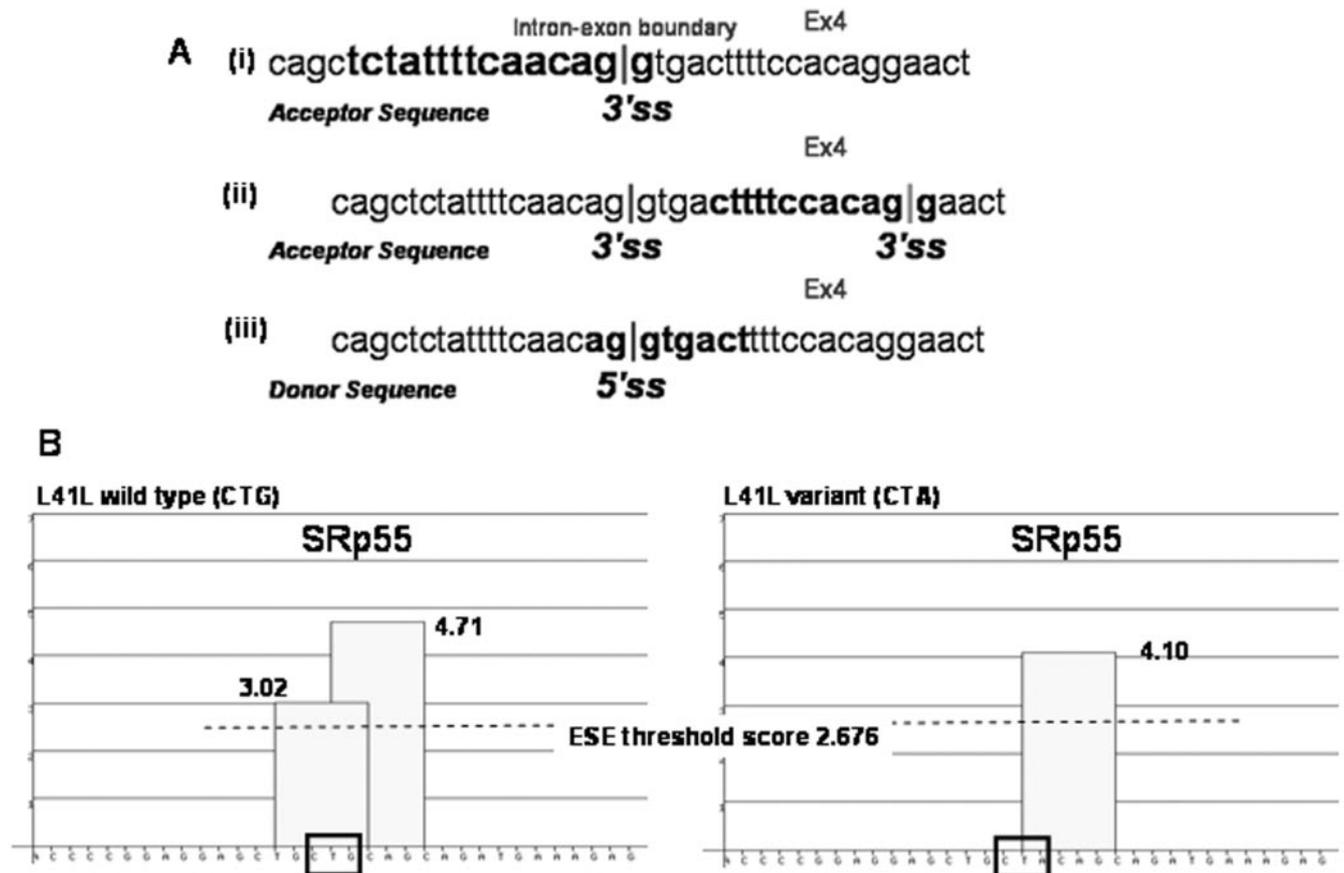
FIGURE 4. ESE prediction modeling of L41L. (**A**) Ambiguity of donor and acceptor splice sites for the 5′ end of exon 4 *OPTN* (i and iii), with differential acceptor splice site usage by the different *OPTN* isoforms. Isoforms 2 and 3 use a site 15 bp downstream from the isoform 1 acceptor splice site (i and ii). (**B**) Effect of synonymous sequence change L41L (CTG→CTA) on potential ESE sites in exon 4 for the SRp55 protein. The exonic sequence is represented on the *x*-axis, with only ESE scores above the threshold shown. The height of the bars indicates the motif scores (as given on the *y*-axis) and the width of the bars indicate the ESE motif length.

sented with elevated IOP (30 mm Hg in both eyes) at age 39, for which she underwent bilateral glaucoma filtration surgery and remains on topical antiglaucoma monotherapy. The proband's sister D898 (*OPTN*: L41L; *MYOC*: Gln368STOP/Lys398Arg) presented with glaucoma at age 35, had bilateral glaucoma filtration surgery for uncontrolled glaucoma, and remains on topical antiglaucoma triple therapy. The proband's brother D931 (*MYOC*: Gln368STOP/Lys398Arg) had a later disease onset at age 53, with IOPs on presentation of 34 mm Hg right and 38 mm Hg left with significant disc cupping in the left eye (cup-disc ratio, 0.8; data not shown). His IOP was controlled with laser trabeculoplasty and topical antiglaucoma monotherapy. The proband's maternal uncle (D689) had glaucoma diagnosed at age 70 with a moderate elevation in IOP, 27 mm Hg in the right eye and 24 mm Hg in the left, and with IOP controlled by topical medication alone. No *OPTN* or *MYOC* sequence changes were detected in him (D689). L41L was also seen in an unrelated patient with POAG who was an 83-year-old female of English-Canadian ancestry with disease onset at age 68. The glaucoma was controlled with dual topical antiglaucoma therapy after laser trabeculoplasty. This patient was also homozygous for a published polymorphism in exon 1 of *MYOC*, Arg76Lys, not considered functionally important.[12]

Bioinformatics Analysis

Exons with weak splice site consensus sequences are vulnerable to aberrant splicing. *OPTN* exons with sequence changes were studied and "weakly defined" exons were identified using a splice site scoring algorithm. A number of exons studied had suboptimal splice site scores when compared with the consensus donor and acceptor splice sites (see Supplemental Data at www.iovs.org/cgi/content/full/45/9/3122/DC1). For example, the 5′ end of exon 4, a constitutive exon that harbors the initiation codon, has an expected 3′ acceptor consensus sequence (splice site score: 94.7) but also represents a 5′ donor consensus sequence (AG|GTGACT; splice site score: 82.5; Fig. 4). None of the *OPTN* sequence changes was predicted to introduce a cryptic splice site. L41L altered the potential ESE sites in exon 4 of *OPTN*, which has an unusual 5′ exon boundary, resulting in the loss of a SRp55 site (Fig. 4B) but the gain of a SRp40 and SC35 site. M98K (c.603T→A) and H486R (c.1767A→G) both introduced new ESE sites, with M98K reducing the native ESE scores.

Using MFOLD, the H486R missense mutation was the only one predicted to alter the mRNA secondary structure and thermal stability (Fig. 5).

Transcript Analysis

Exons 1 to 6 of the *OPTN* cDNA were amplified (Ex1-6a) to assess the potential splicing effects of L41L. The three published isoforms (isoforms 1, 2, and 3) were clearly observed in all individuals: 781, 560, and 712 bp long, respectively (Fig. 6A). Two additional PCR fragments (350 and 650 bp) were detected in all the affected and unaffected individuals screened and herein are referred to as isoform 4 (350 bp) and 5 (650 bp; Figs. 6A, 7). By sequencing, we showed that the novel isoform 4 corresponds to the entire skipping of the 203-bp exon 5 of *OPTN*. Gel purification of the ~650-bp band was difficult due

A. H486R wildtype

B. H486R mutant
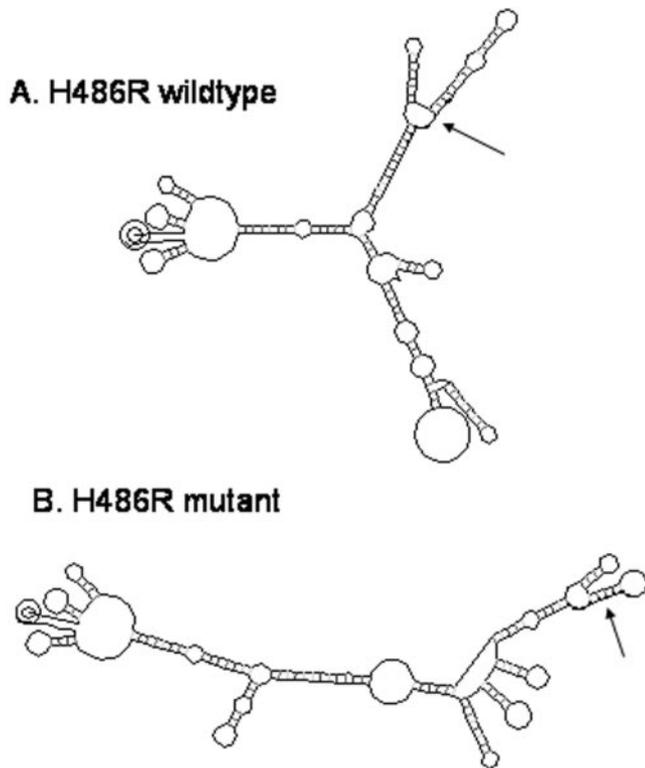
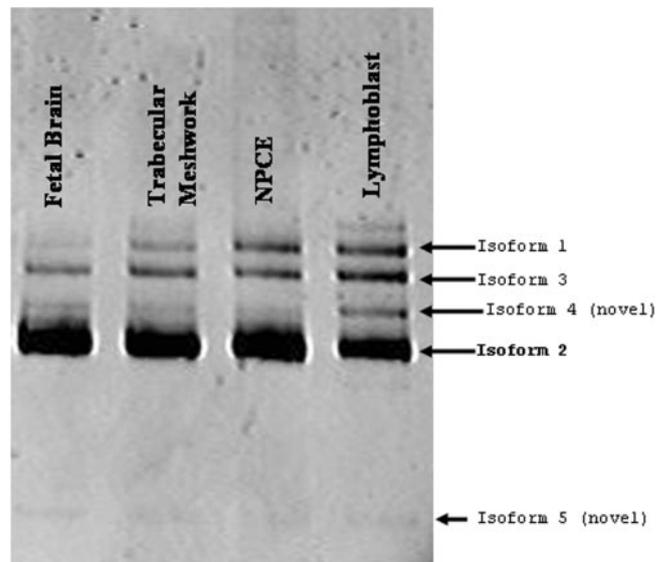**FIGURE 5.** Predicted changes in RNA structure resulting from the H486R change. The mutation H486R alters the RNA secondary structure when modeled with MFOLD. *Arrow*: sequence containing the c.1767A→G nucleotide change resulting in H486R and the wild-type RNA structure (**A**) is compared with the mutant (**B**).

FIGURE 7. Tissue specificity of different *OPTN* isoforms. Amplification of cDNA from fetal brain, trabecular meshwork, nonpigmented ciliary epithelium (NPCE) and lymphoblast for OPTN exons 1 through 6 showed tissue-specific expression patterns. In fetal brain and trabecular meshwork, the expression of isoform 1 (GenBank AF420371) and the novel isoform 4 is significantly reduced, and isoform 2 (GenBank AF420372) is the most highly expressed *OPTN* isoform.

to its size proximity with the other fragments (Fig. 6A). PCR amplification of a smaller amplicon (exons 4–5) showed two fragments in all the samples (Fig. 6B), representing the expected 224-bp amplicon and an aberrant 318-bp amplicon, which was seen as the 650-bp band in the previously described

FIGURE 6. Different OPTN isoforms from human leukocytes. Results of RT-PCR with primers specific for exons 1 to 6 of *OPTN* showing the three previously published isoforms: isoform 1 (781 bp; GenBank AF420371), isoform 2 (560 bp; GenBank AF420372), and isoform 3 (712 bp; AF420373); and two novel isoforms: isoform 4 (650 bp in **A** and 318 bp in **B**) and isoform 5 (350 bp). The 224-bp fragment in (**B**) represents an amplicon containing exons 4 and 5 generated from isoforms 1 to 3.

PCR. Sequencing demonstrated that this novel *OPTN* isoform (the 650- and 318-bp bands, Fig. 6), which was also present in controls and patients, uses an alternative intron 4 acceptor (3′) splice site (data not shown). The splice site score for this variant intron 4 acceptor splice site (87.1) is actually higher than that of the normal acceptor site (84.0) for intron 4. These two novel splicing patterns introduce premature termination codons (PTCs).

L41L was not seen in control subjects and was predicted, using bioinformatics modeling of ESE site involvement and splice site strength, to alter splicing. Analysis of samples carrying the L41L mutation failed to detect obvious exon skipping of exon 4 (L41L), by using RT-PCR, allele (mutation), and exon-specific PCR with FAM-labeled primers and relative quantification or exon-bridging primers (data not shown).

Amplification of fetal brain, human trabecular meshwork and nonpigmented ciliary epithelium cDNA showed a decreased number of isoforms compared with amplification from leukocytes (Fig. 7), suggesting tissue-specific expression patterns. In fetal brain and trabecular meshwork, the expression of isoform 1 (with all exons) was significantly reduced as was that of isoform 4.

DISCUSSION

We report for the first time, to our knowledge, the association of *OPTN* mutations with an early and high-pressure form of glaucoma (JOAG). We also propose that sequence variants in *OPTN* may influence the expression of MYOC sequence changes to modify the phenotype.

H486R represents a novel JOAG-disease causing sequence change in exon 14 of *OPTN* and the first reported mutation in this patient population. H486R was recently reported in an individual with NTG,[22] supporting our hypothesis that *OPTN* sequence changes are not necessarily low-pressure specific. H486R results in a conservative substitution between two basically charged, polar amino acids: histidine replaced with arginine. Structurally, histidine has a bulkier imidazole side-chain and also is highly conserved across many species, includ-

ing humans, mouse, rat, and macaque (data not shown). H486R is located in the C terminus of OPTN which interacts with the adenovirus E3-14.7K protein, an inhibitor of TNF-α cytolysis.[26] There have been studies suggesting that TNF-α–mediated apoptosis is involved in the neurodegenerative process of glaucoma.[38]

The c.1944G→A nucleotide change (R545Q) in exon 16 of *OPTN* was seen in 3 (18.75%) of 16 Chinese control subjects from Toronto and was recently reported in 14 (9.8%) of 142 Chinese control individuals from Hong Kong[2] and in 5 (2.55%) of 196 Japanese control individuals,[24] supporting the conclusion that R545Q is a common polymorphism in some Asian populations. The *MYOC* mutation Gly252Arg, identified in our family with R545Q, appears to represent a pathogenic mutation and has been reported.[39,40]

The *OPTN* M98K change (c.603T→A) was reported as a susceptibility allele for POAG[20] with more effect on NTG (Child A, et al. *IOVS* 2003;44:ARVO E-Abstract 1109). In our study population, the M98K polymorphism may confer a susceptibility risk to POAG, but does not appear to predispose to JOAG. M98K was seen in 6 (9.1%) of 66 JOAG patients, 8 (16.3%) of 49 POAG patients, and 3 (3.9%) of 101 control subjects, with the POAG M98K carrier frequency significantly different from the control ($P = 0.02$). Only one of our eight POAG patients with the M98K allele had NTG. Recent in vitro work using a quartz-crystal microbalance investigated the effect of OPTN amino acid substitutions in vitro on Rab8 interactions. The observed reduction in the expected protein-protein affinity in M98K constructs also further supports the potential functional importance of M98K (Iwata T, et al. *IOVS* 2003;44:ARVO E-Abstract 1114).

The phenotypic variability in glaucoma including the age at onset, severity, rate of progression, and IOP has been well documented.[12,30] Some of this variability may be seen for the same sequence change and may be due to an identifiable genetic factor or factors not yet identified influencing the expression of mutated alleles.[9,30,41–43] In our study, we report this finding with the association of *OPTN* mutation H486R with severe JOAG, whereas it was previously associated with later-onset disease and NTG.[2] Variable disease phenotypes can result from a combination of allelic heterogeneity, environmental influences, and modifier gene effects.[44–47] In monogenic disorders, such as cystic fibrosis, in which mutations in a single gene account for all cases of the disease, the influence of genetic modifiers may be more readily identifiable.[44,48,49] In genetically heterogeneous diseases such as glaucoma, although more difficult to prove, the effect of genetic modifiers has been suggested to alter phenotypic expression.[13] We have reported the modifier effect of a mutant *CYP1B1* allele (R368H) on the phenotypic expression of a *MYOC* mutation (Gly399Val), leading to a more severe and earlier disease.[13] Modifier genes may affect the phenotypic spectrum of the target or "disease" gene by altering gene transcription and expression and protein–protein interactions and by affecting the functional interaction of genes or proteins in similar biological pathways.[44,47,50] In the present study we documented the potential modifying effect of *OPTN* on *MYOC* mutations, suggesting that these genes may act through a common pathway.

Support for such a modifying effect is suggested from Figure 3 which shows that individuals who had the silent *OPTN* change c.433G→A (L41L) with *MYOC* Gln368STOP had a more severe phenotype than did those without the L41L change. Marked phenotypic variations for the Gln368STOP genotype have been documented, making counseling challenging.[43,51–53] This case suggests that *OPTN* L41L may act as a phenotypic modifier of the *MYOC* Gln368STOP mutation, and OPTN-MYOC interactions may explain some of the phenotypic variability associated with this and other *MYOC* mutations.

There is a growing body of evidence supporting the role of the splicing machinery as a potentially potent modifier of the phenotypic outcome of a genotype.[54,55] Accurate splicing is paramount to eukaryotic cell function but exon–intron boundaries are defined by weakly conserved intronic *cis*-elements (the 5′ or donor splice site, the 3′ or acceptor splice site, and the branch site), and require additional elements, termed ESEs and exonic splicing silencers (ESSs), to define the exon.[56–58] Splicing disruptions can play a modifier role that influences phenotypic variability, such as tissue–organ specificity, disease severity, and age of onset.[59] The extent of splicing as a significant disease-causing mechanism is underestimated,[54,58,60] as it is thought that at least 15% of disease-causing point mutations (missense/nonsense) can cause RNA splicing defects.[58,61] Traditionally, translationally silent or synonymous changes were considered as coding single-nucleotide polymorphisms (cSNPs), with little if any functional significance. However, these silent changes have the potential to alter the efficiency and specificity of alternative splicing and so contribute to phenotypic variability.[58] We have evaluated the pathogenicity of synonymous sequence variations such as L41L, because this was seen in two patients with glaucoma but was absent from control individuals (0/165). When combining these data with results of Sarfarazi et al., in which L41L was detected in 4 of 523 patients with POAG/NTG but was not seen in 108 additional control individuals (Sarfarazi M, personal communication, May 2003), L41L was detected in 6 of 678 patients with glaucoma (0.9% of POAG/JOAG) and was absent from control subjects (0/273). The G nucleotide involved is highly conserved among the human, macaque, rat, mouse, and bovine sequences (data not shown). Using several experimental approaches, we were unable to detect aberrant or skipped transcript arising from the c.433G→A (L41L) change. Mutant, alternatively spliced transcripts can be rare, as ESS and ESE disruptions are not an all-or-nothing disease mechanism. The degree of exon skipping and relative levels of mutant determine disease severity, but may also be tissue specific.[54] This situation has recently been reported, for the ryanodine receptor (*RYR1*) gene,[62] in which the mutant aberrantly spliced transcript shows a tissue-specific expression and is only detected in skeletal muscle resulting in a congenital myopathy.[63] Ocular tissue from our patients was not available to test for tissue-specific defects in *OPTN* expression in affected individuals harboring *OPTN* sequence variations. However, preliminary work assessing the differential expression of OPTN using fetal brain cDNA, trabecular meshwork, nonpigmented ciliary epithelium, and leukocyte cDNA showed a difference in the expressed isoforms. Differences in the relative levels and activities of splicing factors (SR proteins and hnRNPs) may regulate tissue-specific differences in splicing patterns and alter phenotypic severity.[54,63–66]

We identified two novel *OPTN* splice variants that were reproducibly detected in mRNA derived from peripheral blood lymphocytes and lymphoblastoid cell lines of both affected patients and control subjects. These novel transcripts (isoforms 4 and 5; Fig. 6) result in premature termination codons (PTCs) and would be potential targets for nonsense mediated decay (NMD) and so incapable of generating functional protein. Although, we are unsure of the significance of these aberrant transcripts, it has been proposed that such regulated, unproductive splicing and translation are mechanisms that regulate protein expression.[67]

In summary, *OPTN* plays a role in early-onset glaucoma and may have a modifying effect on *MYOC* expression. Although the mechanisms involved in glaucoma-related visual loss are unclear, they do not appear include only IOP. Silent changes should not systematically be considered polymorphic, because they may have pathogenic potential. Further investigation of

potential partners that interact with *OPTN* is critical to the better understanding of the phenotype variability observed.

Electronic-Database Information

URLs for data presented herein are as follows and are open-access.

ClustalX (version 1.8): ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/ (for nucleotide and protein multiple sequence alignments)

ESEfinder program: http://exon.cshl.org/ESE/index.html (for exon splicing enhancer prediction)

GeneDoc: www.psc.edu/biomed/genedoc (for visualization and formatting of ClustalX files)

MFOLD: http://www.bioinfo.rpi.edu/applications/mfold

NCBI BLAST2 sequences program: http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html

NEBcutter program (New England Biolabs, Beverly, MA): http://tools.neb.com/NEBcutter

NCBI GenBank http://www.ncbi.nlm.nih.gov/GenBank/ (for *OPTN* mRNA sequences for human (accession no. AF420371), macaques (AB063036 or AB056409), mouse (AY071834), and rat (AB069907) and partial cDNA sequences for bovine (AV605192 and AV605191), and porcine (AU059594): http://www.ncbi.nlm.nih.gov/SNP/index.html

Primer3 program: http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/

Splice Site Score Calculator and SpliceSiteFinder: http://www.genet.sickkids.on.ca/~ali/splicesitescore.html and http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html (for scoring splice sites and detecting cryptic splice sites).

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