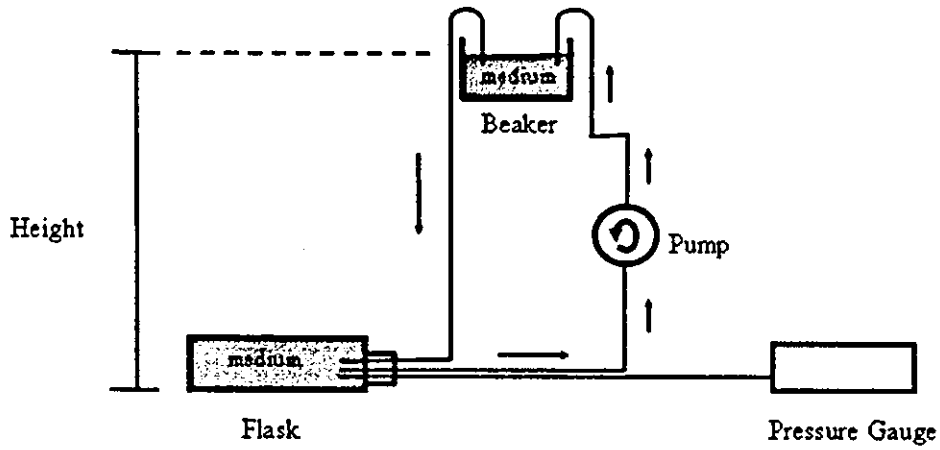

FIGURE 1. System used for hydrostatic pressure experiments. The culture flask was filled with medium and capped with a silicon stopper. The height of the reservoir with the medium was adjusted to maintain the pressure in the flask. For gas exchange, the medium was circulated by a peristaltic pump, and the pressure was monitored with the pressure gauge. For control, cultured cells were exposed to a hydrostatic pressure of 3 mm Hg above atmospheric pressure for 72 hours.

an internal control. All the primers used in these reactions were designed on computer (Primer Express software; ABI). For myocilin cDNA amplification, sense primer 5′-GGTCATTCCGGCAGTGAAGAA-3′ and antisense primer 5′-ACGCCGTACTTGCCAGTGATT-3′ were used.

For amplification of optineurin cDNA, sense primer 5′-GACCCACAACAGGCTTCTTCA-3′ and antisense primer 5′-TCTGCCATTTCCAGCTTTCC-3′ were used. For GAPDH cDNA amplification, sense primer 5′-TCATCAGGAATGCCTCCTGTAC-3′ and antisense primer 5′-ATGGCATGGACTGTGGTCATG-3′ were used. For 18S rRNA, sense primer 5′-GATCGAAGACGATCAGATACC-3′ and antisense primer 5′-CCAGACAAATCACTCCACC-3′ were used.

To confirm the specificity of PCR reaction, each PCR product was analyzed by agarose gel and subjected to a dissociation curve analysis, according to the manufacturer's instructions.

RESULTS

Cloning of Porcine Optineurin cDNA

The nucleotides and deduced amino acid sequences of porcine optineurin are shown in Figure 2A. A comparison of the predicted amino acid sequences of pig, human, mouse, and rat optineurin is shown in Figure 2B. The porcine optineurin is composed of 574 amino acids, and the homology of porcine optineurin to mouse, rat, and human was 71%, 72%, and 84%, respectively. Two LZ motifs, reported in the human optineurin, were also present in porcine optineurin at residues 143-164 and 437-458 (Fig. 2A, dashed underscore). A glutamic acid-rich region at residues 221-376 (Fig. 2A, solid underscore) and two potential O-glycosylation sites (Fig. 2A, circles) were found. Phosphorylation sites of 20 serine residues, 11 threonine residues, and 1 tyrosine residue were predicted (Fig. 2A, bold italic).

Cloning of the Porcine Myocilin cDNA

The nucleotides and deduced amino acid sequences of porcine myocilin are shown in Figure 3A. A comparison of the predicted amino acid sequences of pig, bovine, mouse, rat, monkey, and human myocilin is shown in Figure 3B. The porcine myocilin is composed of 489 amino acids, which is 14 amino acids shorter at the N terminus and lacks one more amino acid at codon 182 than the human myocilin has. The homology of porcine myocilin to mouse, rat, bovine, monkey, and human was 80%, 79%, 84%, 82%, and 82%, respectively. Porcine myocilin is the smallest myocilin described, and also contained a LZ motif at residues 103-152 (Fig. 3A, dashed underscore). Two predicted N-glycosylation sites and seven O-glycosylation sites were found (Fig. 3A, squares and circles, respectively). Phosphorylation sites of 31 serine residues, 8 threonine residues, and 5 tyrosine residue were predicted (Fig. 3A, bold italic). C-terminal olfactomedin-like domain was more conserved in all species than the N-terminal myosin-like domain.

DEX Treatment

After a 2-week exposure to 500 nM DEX, the expression of optineurin by TMCs and astrocytes was significantly decreased (to 67% and 48%, respectively), compared with that of untreated TMCs and astrocytes (Figs. 4A–C). The expression of porcine myocilin exposed to DEX increased by 8.02 ± 1.26-fold (mean ± SD) and 5.57 ± 1.05-fold in cultured TMCs and astrocytes, respectively. The expression of GAPDH was not altered in both types of cells by exposure to DEX.

Incubation in Hypoxic Conditions

In hypoxic conditions, porcine optineurin was relatively stable at all times points, whereas the expression of porcine myocilin decreased significantly (Figs. 4D–F). The expression of myocilin by astrocytes decreased by an average of 44% after 12 hours and declined to 4% after 72 hours of hypoxia compared with the control. For TMCs, a significant decrease in myocilin expression to 11% was observed after 72 hours compared with that of the control. We did not observe any cell death of both types of cells histologically at all time points (Fig. 5).

Incubation under Hydrostatic Pressure and with Mechanical Stretching

After 72 hours under hydrostatic pressure or 24 hours of mechanical stretching, optineurin and myocilin expression was unchanged in both TMCs and astrocytes.

DISCUSSION

Our results demonstrated that both optineurin and myocilin were expressed in porcine TMCs and astrocytes, and their amino acid sequences were homologous to human sequences by 84% and 82%, respectively. The protein motifs and protein modification sites were also shared with humans.

The response of optineurin and myocilin to DEX was different. Optineurin expression was decreased, whereas that of myocilin was increased. The increased expression of myocilin by TMCs confirmed earlier observations,[26–29] but we also detected an increase in astrocytes. Astrocytes are the major glial cells populating the optic nerve head and are probably responsible for the remodeling of the optic nerve head in glaucomatous eyes. Astrocytes are known to function as cellular support

Porcine Optineurin

A

```
ATGTCCCATCAACCTCTGAGCTGCCTGACTGAGAAGGGGGACAGCCCCACCGAAACCACAGGAAATGGACCCCCCACTCTGGCTCACCCAAACCTTGACA   100
M  S  H  Q  P  L  S  C  L  T  E  K  G  D  S  P  T  E  T  T  G  N  G  P  P  T  L  A  H  P  N  L  D  T
CGTTCACCCCACATGAACTGCTGCAGCAGATGAGAGAGCTTCTAATCGAGAACCATCAGCTGAAAGAAGCCATGAAGCTAAATAATCAAGCTATGAAAGG   200
  F  T  P  H  E  L  L  Q  Q  M  R  E  L  L  I  E  N  H  Q  L  K  E  A  M  K  L  N  N  Q  A  M  K  G
GCGATTTGAGGAGCTTTCAGCCTGGACAGAGAAGCAGAAGGAAGAACGCCTTTTTTTTGAGACCCAGAGCAAAGAAGCCAAAGAGCGCCTAACGGCTCTG   300
  R  F  E  E  L  S  A  W  T  E  K  Q  K  E  E  R  L  F  F  E  T  Q  S  K  E  A  K  E  R  L  T  A  L
AGTCTTGAAAATGAAAAACTGAAGCAAGAACTTGGAAAACTAAAAGGGAAAACTGAAAGGTCATTTGAGGACCTCACTGGGGACCCCAGGGTCCCCAAGG   400
S  L  E  N  E  K  L  K  Q  E  L  G  K  L  K  G  K  T  E  R  S  F  E  D  L  T  G  D  P  R  V  P  K  A
CGGAAGCAGAACAGGAAGTAGAACAGCTGAAGACCCAGGTGGCACGCCTTCAAGCTGAAAAGGCGGATCTGCTGGGCATCGTGTCTGAATTGCAGCTCAA   500
  E  A  E  Q  V  E  V  E  Q  L  K  T  Q  V  A  R  L  Q  A  E  K  A  D  L  L  G  I  V  S  E  L  Q  K
GCTGAACTCAGGTGGCCCCTCTGAAGACTCCTTTGTTGAAATCAGGATGGCTGAGGGAGAAGCAGATGCAGCAATGAAGGAAATCAAGACAAGTCCTGGG   600
  L  N  S  G  G  P  S  E  D  S  F  V  E  I  R  M  A  E  G  E  A  D  A  A  M  K  E  I  K  T  S  P  G
CCCATAAGAACTGATTCCATTGACACGAGCAAATCTGCAGAAGGTACCAGGAATTATTTGGAATTTGAGGAATTAACTGTGAGCCAGCTCCTGCTGTGTC   700
P  I  R  T  D  S  I  D  T  S  K  S  A  E  G  T  R  N  Y  L  E  F  E  E  L  T  V  S  Q  L  L  L  C  L
TAAGGGAAGGAAACCAGAAGGTGGAGAGACTTGAAATCGCCCTCAAGGAAGCCAAAGAAAGAATTTTAGATTTTGAAAAGAAAGCCAAGGATCGTTCTGA   800
  R  E  G  N  Q  K  V  E  R  L  E  I  A  L  K  E  A  K  E  R  I  L  D  F  E  K  K  A  K  D  R  S  E
GACTGAGACCCAGACAGAAGAGCACAAAGAACAAGAGAAAGAAGAGGAGAAAAGCCCAGAAACTGTTGGAAGTGAAGTGGAAATGTTAAACCTTCAGGTG   900
  T  E  T  Q  T  E  E  H  K  E  Q  E  K  E  E  E  K  S  P  E  T  V  G  S  E  V  E  M  L  N  L  Q  V
ACAACCCTGTTTAAGGAGCTTCAGGAGGCTCACACGAAACTCAGTGAAGCTGAGCTCATGAAGAAGAGACTTCAAGAAAAATGTCAGGCACTTGAAAGGA  1000
T  T  L  F  K  E  L  Q  E  A  H  T  K  L  S  E  A  E  L  M  K  K  R  L  Q  E  K  C  Q  A  L  E  R  K
AAAATTCTGCAACCCCATCAGAGCTGAATGAAAAGCAAGAGCTTCTTTATAATAACAAAAAGTTGGAGCTCCAAGTGGAAAGCATGAGATCAGAAATTAA  1100
  N  S  A  T  P  S  E  L  N  E  K  Q  E  L  L  Y  N  N  K  K  L  E  L  Q  V  E  S  M  R  S  E  I  K
AATGGAGCAAGCCAAAACAGAAGAGGAAAAGTCCAAATTAACTACTCTACAGTTGACCCACAACAGGCTTCTTCAAGAATACAATAATGCACTGAAAACA  1200
  M  E  Q  A  K  T  E  E  E  K  S  K  L  T  T  L  Q  L  T  H  N  R  L  L  Q  E  Y  N  N  A  L  K  T
ATTGAGGAACTGAAAAGAAGAGAGTCTGAAAAAGTGGATAAGGTGGTGCTGCAGGAACTGAATGGAAAGCTGGAAATGGCAGAGAAGGCCCTGGCTTCCA  1300
I  E  E  L  K  R  R  E  S  E  K  V  D  K  V  V  L  Q  E  L  N  G  K  L  E  M  A  E  K  A  L  A  S  K
AGCAGCTCCAAATGGATGAGATGAAGCAGACCATTGCCAAGCAAGAGAAGGACCTGGAAACCATGGCTGTTCTCAGGGCTCAGATGGAGGTATACTGTTC  1400
  Q  L  Q  M  D  E  M  K  Q  T  I  A  K  Q  E  K  D  L  E  T  M  A  V  L  R  A  Q  M  E  V  Y  C  S
TGACTTTCATGCTGAAAGAGCAGCAAGAGAGAAGATTCATGAAGAAAAGGAGCAACTGGCATTGCAGCTGGCAGTTTTGCTGAAAGACGACAATGCTTTT  1500
  D  F  H  A  E  R  A  A  R  E  K  I  H  E  E  K  E  Q  L  A  L  Q  L  A  V  L  L  K  D  D  N  A  F
GAAGAGGGAGCCAGCAGGCAATCCTTGATGGAAATGCAGAGCCGTCATGGGGCAAGAGCAAGTGATGCTGACCAGCAGGCTTTTCTTGTTCAAAGAGGAG  1600
E  E  G  A  S  R  Q  S  L  M  E  M  Q  S  R  H  G  A  R  A  S  D  A  D  Q  Q  A  F  L  V  Q  R  G  A
CTGAGGATAGAAACTGGCTGCAACAGCAACAACAGAATATTCCAATTCATTCTTGCCCCAAATGTGGAGAAGTTCTGCCTGACATAGATACACTACTGAT  1700
  E  D  R  N  W  L  Q  Q  Q  Q  Q  N  I  P  I  H  S  C  P  K  C  G  E  V  L  P  D  I  D  T  L  L  I
TCACGTTACGGACTGCATCATTTAA                                                                              1800
 H  V  T  D  C  I  I  *
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B

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                                                                                                      100
Pig    MSHQPLSCLTEKGDSPTETTGNGPPTLAHPNLDTFTPHELLQQMRELLIENHQLKEAMKLNNQAMKGRFEELSAWTEKQKEERLFFETQSKEAKERLTAL
Mouse ----------------C-------SNMV--S------------K---V-------------------------------------M----V----K--
Rat   ----------------SC--P----SNMV----------------K---V-----------------------------R-----QL--I---------K--
Human MSHQPLSCLTEKEDSPSESTGNGPPHLAHPNLDTFTPEELLQQMKELLTENHQLKEAMKLNNQAMKGRFEELSAWTEKQKEERQFFEIQSKEAKERLMAL
                                                                                                      100
                                                                                                      198
Pig    SLENEKLKQELGKLKGKTERSFEDLTGDPRVPKAEAEQEVEQLKTQ              VARLQAEKADLLGIVSELQLKLNSGGPSEDSFVEIRMAEGEA
Mouse TH---R--E----F-E-S-KPL-----GY-Y-R AL-E---K----VEQEVEHLK IQ-M--R-----------------------S----------T---T
Rat   -H---R--E------E-S--P---I--RCGF-RTDL----GATEEAGGAGSGASEDPGE-PSGLR-RT---HS-XTAAQAQLRRLLRRLLRGD--T----
Human SHENEKLKEELGKLKGKSERSSEDPTDDSRLPRAEAEQEKDQLRTQ              VVRLQAEKADLLGIVSELQLKLNSSGSSEDSFVEIRMAEGEA
                                                                                                      198
                                                                                                      282
Pig    DAAMKEIKTSPGPIRTDSIDTS       KSAEGTRNYLEFEELTVSQLLLCLREGNQKVERLEIALKEAKERILDFEKKAKDRSETETQTEEHKEQEKEE
Mouse EG----M-NC-T-T---P-SL-       NCT-DA-SCA-------------------------V--R------S------NGH-S--K--ARRADR-- -
Rat   EG----MRN-A--T-----IMG       -CT-DA-TXV-------------------------R------S------NGH-AI-----GSTQKE- -
Human EGSVKEIKHSPGSTRTVSTGTALSHYRRRSADGAKNYFEHEELTVSQLLLCLREGNQKVERLEVALKEAKERVSDFEKKTSNRSEIETQTEGSTEKENDE
                                                                                                      288
                                                                                                      382
Pig    EKSPETVGSEVEMLNLQVTTLFKELQEAHTKLSEAELMKKRLQEKCQALERKNSATPSELNEKQELLYNNKKLELQVESMRSEIKNEQAKTEEEKSKLTT
Mouse D-GQ-S--TAPPKD                    WHVPGMSP----------------------V-S--------------------------R-A-
Rat   D-D--S--I---T--V--AS---G-------------------------------------------V-S-W------------------------R-A-
Human EKGPETVGSEVEALNLQVTSLFKELQEAHTKLSEAELMKKRLQEKCQALERKNSAIPSELNEKQELVYPNKKLELQVESMLSEIKNEQAKTEDEKSKLTV
                                                                                                      388
                                                                                                      482
Pig    LQLTHNRLLQEYNNALKTIEELKRRESEKVDKVVLQELNGKLEMAEKALASKQLQMDEMKQTIAKQEKDLETMAVLRAQMEVYCSDFHAERAAREKIHEE
Mouse --A---K----H-K--------TKQQA-----ML----SE---L--Q---------------L----E-------------------------------
Rat   ------K----H-K--R-----TKQQAE-----Q----SE---L--Q---------------------E-------------------------------
Human LQMTHNKLLQEHNNALKTIEELTRKESEKVDRAVLKELSEKLELAEKALASKQLQMDEMKQTIAKQEEDLETHIILRAQMEVYCSDFHAERAAREKIHEE
                                                                                                      488
                                                                                                  574
Pig    KEQLALQLAVLLKDDNAFEEGASRQSLMEMQSRHGARASDADQQAFLVQRGAEDRNWLQQQQ QNIPIHSCPKCGEVLPDIDTLLIHVTDCII
Mouse ---------I---EN-DI---G---------C-----T--S---TY-F-------S-QHG--PRSI-----------------Q---M----
Rat   ---------I---EN-D--D-G---------C-----T--S----Y-F----K-MS-QHG--PRS-----------------Q---M----
Human KEQLALQLAVLLKENDAFEDGG RQSLMEMQSRHGARTSDSDQQAYLVQRGAEDRDWRQQR   NIPIHSCPKCGEVLPDIDTLQIHVMDCII
                                                                                                  577
```

FIGURE 2. Nucleotide sequence and deduced amino acid sequence of porcine optineurin and comparison of porcine optineurin amino acid sequences with those of other species. The coding region is defined by the positions of the initiation codon (ATG) and stop codon (TAA). (A) The porcine optineurin protein is composed of 574 amino acids. *Dashed underscore*: LZ motifs; *solid underscore*: glutamic acid-rich region; *circles*: O-glycosylation sites. (B) Only the amino acids that differ from porcine or human optineurin sequences are shown for mouse and rat. *Hyphens*: the same amino acid residues as human optineurin; *spaces*: the absence of amino acids corresponding to the same location in human optineurin; *asterisks*: positions of amino acids associated with glaucoma.

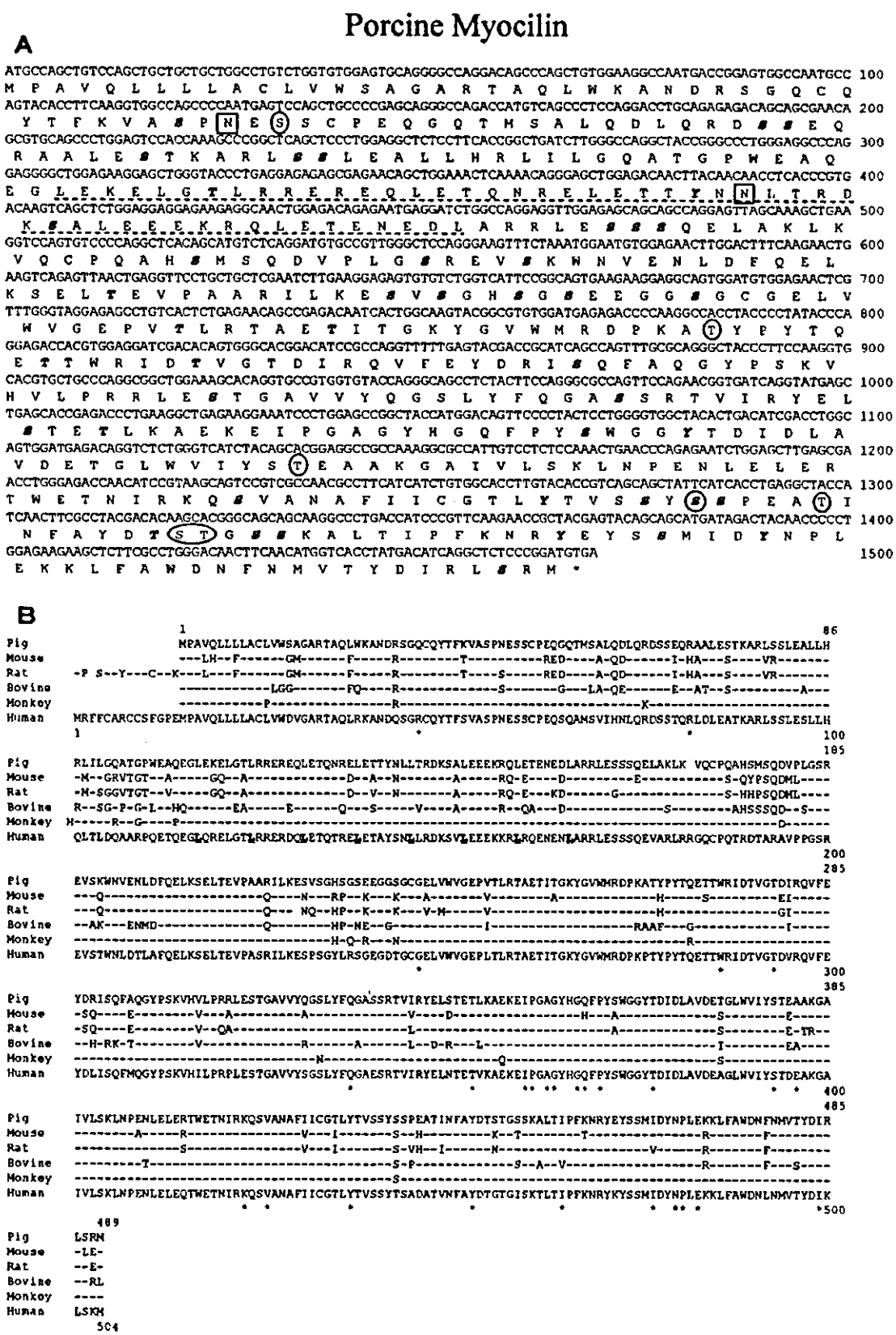

FIGURE 3. Nucleotide sequence and deduced amino acid sequence of porcine myocilin and comparison of porcine myocilin amino acid sequences with those of other species. (A) Porcine myocilin is composed of 489 amino acids. *Circles*: O-glycosylation sites; *squares*: predicted N-glycosylation sites; *dashed underscore*: LZ motif. (B) Only the amino acids that differ from pig or human myocilin sequence are shown for mouse, rat, bovine, and monkey. *Hyphens*: the same amino acid residues as human myocilin, *spaces*: absence of amino acids corresponding to the same location in human myocilin; *asterisks*: positions of amino acids associated with glaucoma.

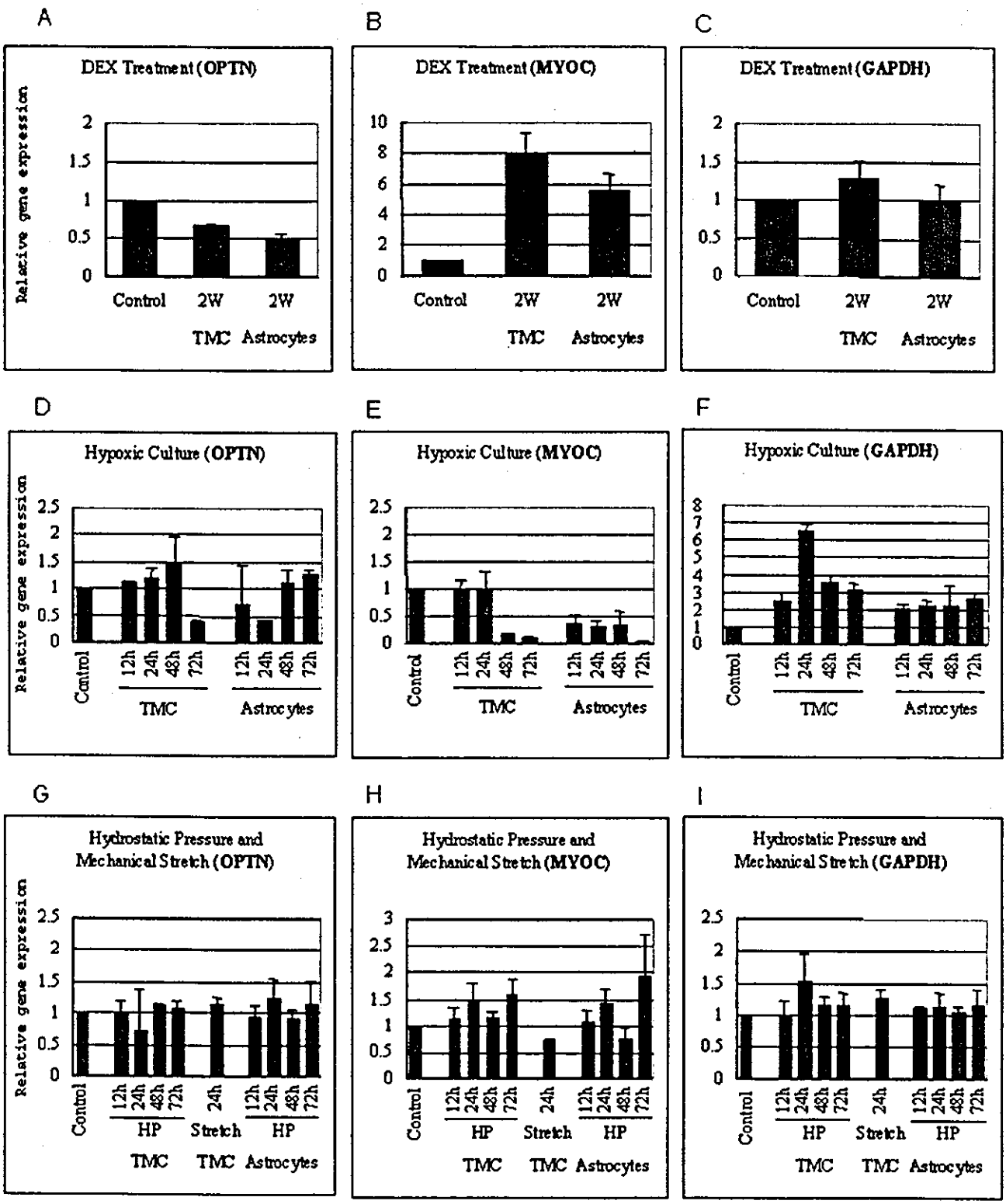

FIGURE 4. Quantitative PCR analysis of optineurin and myocilin under various conditions. The relative gene expression of optineurin, myocilin, and GAPDH is shown for each stimulus or stress condition. Expression level for control cells are shown as 1. (A–C) DEX treatment (500 nM for 2 weeks); (D–F) hypoxia (7.0% O_2); (G–I) hydrostatic pressure (55 mm Hg above atmospheric pressure), and mechanical stretching (a 10% linear stretch for 24 hours).

of the axons as well as synthesizers of various bioactive molecules including extracellular matrix proteins, transforming growth factor (TGF)-β, and platelet-derived growth factor (PDGF) (Tripathi BJ, et al. *IOVS* 1996;37:ARVO Abstract S411; Taylor AW, et al. *IOVS* 1995;36:ARVO Abstract S607; Lambert W, et al. *IOVS* 1997;38:ARVO Abstract S162).[30–33] In the glau-

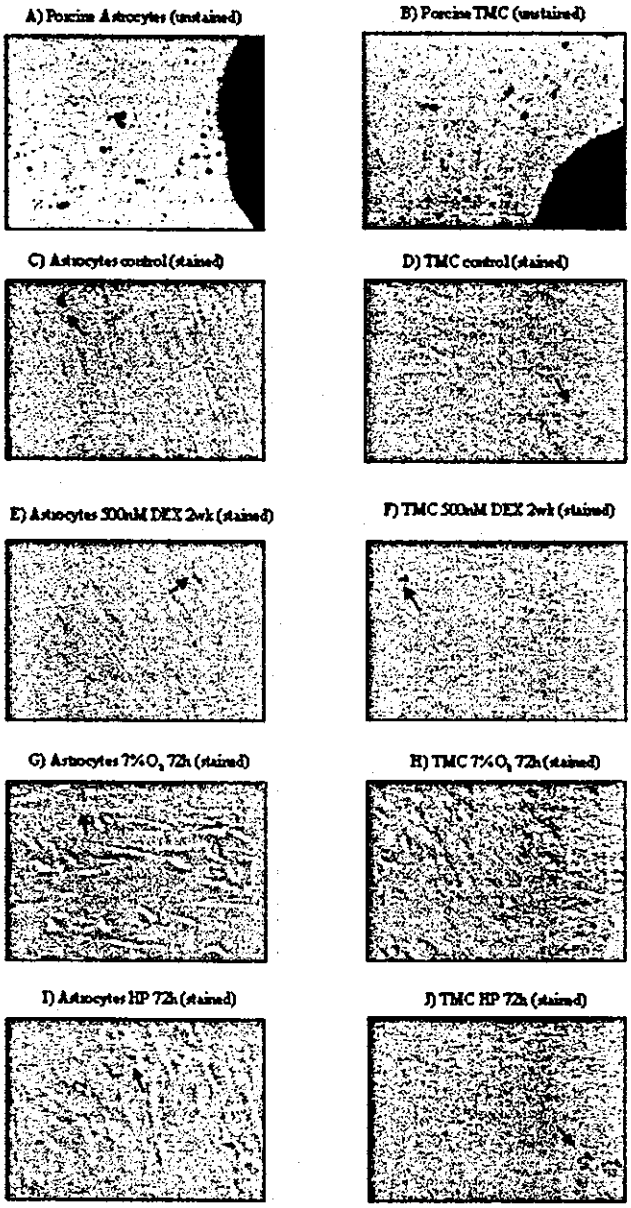

FIGURE 5. Trypan blue staining of TMCs and astrocytes in the various experimental conditions. Trypan blue staining was performed on primary porcine cells to determine cell death caused by stress and stimulation. The stain was added to the culture medium and incubated for 5 minutes before observation. Photographs were taken with a 2-M pixel digital camera (DMC2; Polaroid, Tokyo Japan). (A, B) Unstained primary porcine cells; (C–J) cells stained with TB. *Arrows*: trypan blue–stained dead cells. Magnification, ×100.

comatous process, remodeling of the extracellular matrix and reactive astrocytes induced after mechanical injury by increased IOP may play major roles in damaging the optic nerve axons.[30–33] Significant changes of myocilin expression in astrocytes may alter the normal function of the astrocytes to support the optic nerve head. The tissues in the optic nerve head are central to the pathologic course in glaucomatous eyes; thus, the effect of the fivefold elevation of myocilin transcript after DEX treatment in astrocytes should be further investigated.

Optineurin, in contrast, was significantly decreased in both astrocytes and TMCs after exposure to DEX. Optineurin has been shown to interact with the E3-14.7. kDa protein, one of the three protein encoded by human adenovirus C early region 3 (E3)[7] that use TNF-α or Fas ligand pathways to mediate apoptosis and inflammation. TNF-α plays a critical role in protecting cells from virus infection, which concurrently had been the target for virus. A downregulation of optineurin under DEX may result in the loss of the protective functions, especially in the optic nerve head.

Under hypoxic conditions that mimic the ocular hemodynamic condition in eyes with NTG the expression of myocilin was significantly reduced in both TMCs and astrocytes, whereas the expression of control GAPDH was increased more than twofold after 12 hours in both types of cells. Myocilin transcription was practically shut down in astrocytes after 72 hours, whereas the transcription of optineurin was not affected by the hypoxia. The significant changes of myocilin transcription were not due to cell death, as shown by trypan blue staining (Fig. 5).

A significant increase of GAPDH by hypoxia suggests that the transcriptional machinery is still active in TMCs under hypoxic conditions. In addition, the reduction of both OPTN and MYOC did not occur after 12 hours, which is enough time for the gas exchange in the culture medium of TMCs, suggesting that the transcriptional shut down was not triggered directly by hypoxia but by a factor(s) activated by hypoxia indirectly affecting the transcriptional regulation of both OPTN and MYOC.

Hydrostatic pressure had no affect on gene expression in both TMCs and astrocytes. Recently, Kamphuis and Schneemann[34] also reported no change of optineurin gene expression by pressure elevation in an anterior chamber perfusion model. Pressure elevation in a perfusion system is likely to stress the cells by compression and mechanical stretching. Data collected under our experimental conditions fully agree with the perfusion experiments by Kamphuis and Schneemann.[34] Vittitow and Borrás[10] have reported an increase of optineurin expression by 60% after 7 days of elevated pressure in a perfusion system. Quantification of gene expression by PCR followed by a gel scanner is usually difficult, with an accuracy within 50%, as described.[10] Their results are inconsistent with those of Kamphuis and Schneemann[34] and our results. Our results showed that the expression of myocilin is not affected by hydrostatic pressure or mechanical stretch, although Tamm et al.[27] had previously shown induction of myocilin by mechanical stretching in human TMCs. These results demonstrated that hydrostatic pressure of +33 mm Hg or a mechanical stretch of 10% is not sufficient to increase the myocilin gene expression in TMCs under elevated IOP.

In this study, optineurin and myocilin behaved differently in TMCs from astrocytes during changes of cellular environment by DEX treatment, hypoxia, hydrostatic pressure, or stretching. These results suggest that different mechanisms may be involved in the development of glaucoma by defects in these two genes.

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Variants in Optineurin Gene and Their Association with Tumor Necrosis Factor-α Polymorphisms in Japanese Patients with Glaucoma

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PURPOSE. To investigate sequence variations in the optineurin (*OPTN*) gene and their association with TNF-α polymorphisms in Japanese patients with glaucoma.

METHODS. The *OPTN* gene was analyzed in blood samples from 629 Japanese subjects. There were 194 patients with primary open-angle glaucoma (POAG), 217 with normal-tension glaucoma (NTG), and 218 with no eye disease (control subjects). The gene was screened for mutations by denaturing high-performance liquid chromatography. Genotyping of three polymorphisms of −308G→A, −857C→T, and −863C→A in the TNF-α promoter region was performed. The associations between the genotypes and age, intraocular pressure (IOP), and visual field defects at the time of diagnosis were examined.

RESULTS. A possible glaucoma-causing mutation, His26Asp, was identified in 1 of the 411 Japanese patients with glaucoma. A c.412G→A (Thr34Thr) polymorphism in the *OPTN* gene was significantly associated with POAG (genotype frequency, $P = 0.011$; allele frequency, $P = 0.003$). The frequency of TNF-α/−857T and optineurin/412A carriers was significantly higher ($P = 0.006$) in patients with POAG than in control subjects. Among the patients with POAG who were carriers of TNF-α/−857T, the optineurin/412A carriers had significantly worse ($P = 0.020$) visual field scores than the non-optineurin/412A ones. The frequency of TNF-α/−863A and optineurin/603A (or Lys98) carriers was significantly higher in patients with POAG ($P = 0.008$) or NTG ($P = 0.027$) than in control subjects. Among the patients with POAG who were carriers of TNF-α/−863A, the ones with optineurin/603A (or Lys98) had significantly worse ($P = 0.026$) visual field scores than did those with non-optineurin/603A (or Lys98).

CONCLUSIONS. These findings demonstrated that the *OPTN* gene is associated with POAG rather than NTG in the Japanese. Statistical analysis showed a possible interaction between polymorphisms in the *OPTN* and the TNF-α genes that would increase the risk for glaucoma. (*Invest Ophthalmol Vis Sci.* 2004;45:4359-4367) DOI:10.1167/iovs.03-1403

Primary open-angle glaucoma (POAG), the most common form of glaucoma, affects more than 100 million people, which is almost 2% of the world population >40 years of age.[1] This disease is second in importance as a cause of bilateral blindness.[2] Glaucoma includes a group of conditions that is characterized by progressive optic neuropathy and visual field changes corresponding to the excavation of the optic disc. These changes are usually associated with an elevation of intraocular pressure (IOP). Although the pathogenesis of the glaucomatous optic neuropathy is not well understood, elevated IOP is generally accepted to be a major risk factor for glaucomatous changes.[3]

In addition to high IOP, the risk factors for development of glaucoma include older age, race (more prevalent in blacks), positive family history, high myopia, and the presence of diabetes or hypertension.[4] Genetic factors also play a major role in the etiology of POAG,[5] and, to date, six chromosomal loci have been identified that are associated with POAG. The first gene to be characterized was the trabecular meshwork inducible glucocorticoid response (*TIGR*) gene on the long arm of chromosome 1. The *TIGR* gene was mapped to the glaucoma locus *GLC1A*.[6] The gene is now known as myocilin,[7] and mutations in the myocilin (*MYOC*) gene have been associated with juvenile-onset POAG as well as with adult-onset POAG in 3% to 5% of patients with glaucoma.[8-12]

Rezaie et al.[13] more recently identified a gene, *GLC1E*, that is associated with adult-onset POAG and normal-tension glaucoma (NTG) at a second locus. This gene was designated as optineurin (*OPTN;* GenBank accession number AF420371; http://www.ncbi.nlm.nih.gov/genbank; provided in the public

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domain by the National Center for Biotechnology Information, Bethesda, MD), and optineurin is located on chromosome 10 at p14 and has been identified by molecular genetic methods in a large family affected by NTG and adult-onset POAG.[14] Sequence alterations in the *OPTN* gene were found in 16.7% of families with hereditary POAG, including individuals with IOP <22 mm Hg.[13] However, other reports have indicated that alterations of the *OPTN* gene are only a rare cause of POAG or NTG.[15–20]

The expression of optineurin transcripts in two human cell lines is induced by tumor necrosis factor (TNF)-α in a time-dependent way.[21] Optineurin is also known to interact with adenovirus E3-14.7K protein,[21] Huntingtin,[22] NF-κB essential modulator (Nemo),[23] transcription factor IIIA,[24] and Rab8.[25] Because optineurin interferes with the protective effect of E3-14.7K protein against TNF-α–mediated cell death,[21] optineurin may be involved in the TNF-α–signaling pathway leading to apoptosis.

The purpose of this study was to determine the prevalence of mutations in the *OPTN* gene in Japanese patients with POAG or NTG. Denaturing high-performance liquid chromatography (DHPLC), an automated heteroduplex detection method with a proven sensitivity and specificity exceeding 95%, was used.[26,27] In addition, we investigated the distribution of TNF-α promoter polymorphisms in patients with glaucoma and normal control subjects to determine whether a significant association between optineurin polymorphism and TNF-α polymorphism is present in patients with POAG or NTG.

Subjects and Methods

Patients and Control Subjects

Six hundred twenty-nine blood samples were collected at seven institutions in Japan. There were 194 patients with POAG, 217 with NTG, and 218 normal control subjects; none of the subjects was related to others in this study. The patients whose age at diagnosis was <35 years and patients with more than −5.5 D of myopia were excluded. Patients with POAG with *MYOC* mutations were also excluded.

The procedures used in this research conformed to the tenets of the Declaration of Helsinki. Written, informed consent was obtained after the nature and possible consequences of the study were explained. When applicable, the research was approved by the appropriate institutional Human Experimentation Committee.

All patients received serial ophthalmic examinations, including IOP measurements by Goldmann applanation tonometry, Humphrey (30-2) or Goldmann perimetry, gonioscopy, and optic disc examination including fundus photography. In all patients, glaucoma was diagnosed according to the following criteria: presence of typical optic disc damage with glaucomatous cupping (cup-to-disc ratio, >0.7) and loss of neuroretinal rim; reproducible visual field defects compatible with the glaucomatous cupping; and open angles on gonioscopy.

Among the patients with open-angle glaucoma, POAG was diagnosed in those who had an IOP > 21 mm Hg at any time during the follow-up period. Patients with exfoliative glaucoma, pigmentary glaucoma, and corticosteroid-induced glaucoma were excluded.

Among the patients with open-angle glaucoma, NTG was diagnosed when the untreated peak IOP was ≤21 mm Hg at all times, including the three baseline measurements and during the diurnal testing (every 3 hours from 6 AM to 12 PM); when the peak IOP with or without medication after diagnosis was consistently <22 mm Hg throughout the follow-up period; and the when there was an absence of a secondary cause for glaucomatous optic neuropathy, such as a previously elevated IOP after trauma, a period of steroid administration, or uveitis.

The clinical characteristics that were recorded for the patients with glaucoma were age at diagnosis, untreated maximum IOP (defined as IOP at diagnosis), and visual field defects at the initial examination (defined as visual field defects at diagnosis). The severity of the visual field defects was scored from 1 to 5 according to previously reported criteria.[28,29] The data obtained by two types of perimetry were combined using a five-point scale: 1, no alterations; 2, early defects; 3, moderate defects; 4, severe defects; and 5, light perception only or no light perception. The first four groups on this severity scale followed Kozaki's classification based on Goldmann perimetry,[30,31] or the classification was based on results of visual field perimetry (Humphrey Field Analyzer; Carl Zeiss Meditec, Dublin, CA).[32] Kozaki's classification is widely used in Japan.

The mean age at diagnosis was 58.4 ± 12.0 years in the patients with POAG and 58.0 ± 11.6 years in the patients with NTG. The mean IOP at diagnosis was 26.7 ± 6.0 mm Hg in the patients with POAG and 16.5 ± 2.5 mm Hg in the patients with NTG. The mean visual field score at diagnosis was 3.1 ± 0.9 in POAG and 2.8 ± 0.7 in NTG. A positive family history was recorded in 61 (31.4%) of the 194 patients with POAG and 70 (32.3%) of the 217 patients with NTG. There were 110 (56.7%) men in the POAG group, 97 (44.7%) in the NTG group, and 92 (42.2%) in the control group.

The two hundred eighteen volunteers in the control group received the same examinations. If there was any doubt whether the subject had glaucoma, the subject was excluded. These volunteers were older than 40 years, had IOPs < 20 mm Hg, had normal optic discs, and had no family history of glaucoma. The mean age at the time of the blood sampling was 65.1 ± 12.0 years in POAG, 60.3 ± 12.4 years in NTG, and 70.6 ± 10.9 years in the control subjects. The mean age of the control subjects was significantly older than that of patients with POAG ($P < 0.001$) and the patients with NTG ($P < 0.001$). We purposely selected older control subjects to reduce the probability that a subset of them would eventually have glaucoma.

DNA Extraction and PCR Conditions

All the blood samples were analyzed at Keio University. Genomic DNA was isolated from peripheral blood lymphocytes by phenol-chloroform extraction. The 13 exonic coding regions of the *OPTN* gene were amplified by polymerase chain reaction (PCR), using the primer sets listed in Table 1. A 20-base GC-clamp was attached to some of the forward primers to detect mutations in the higher-melting-temperature domain by DHPLC analysis.[33]

In high-throughput analysis, samples from three patients were pooled. PCR was performed with a thermal cycler (iCycler; Bio-Rad, Hercules, CA) in a total volume of 20 μL containing 45 ng of genomic DNA, 2 μL 10× PCR buffer II (GeneAmp; Applied Biosystems, Inc. [ABI], Foster City, CA), 2 μL of dNTP mix (GeneAmp; ABI) with a 2.0 mM concentration of each dNTP, 2.4 μL of a 25-mM $MgCl_2$ solution; 4 pmol of each primer, and 0.1 U of DNA polymerase (AmpliTaq Gold; ABI). The PCR conditions were denaturation at 95°C for 9 minutes; 35 cycles at 95°C for 1 minute, 55°C to 60°C for 30 seconds (Table 1), and 72°C for 1 minute, 30 seconds; and a final extension step at 72°C for 7 minutes.

DHPLC Analysis

DHPLC analysis was then performed (Wave System; Transgenomic, Omaha, NE). For heteroduplex formation, products of each PCR (20 μL) were denatured at 95°C for 5 minutes and gradually cooled to 25°C. The annealed PCR products from the three mixed samples were automatically injected into the stationary phase of the DNASep cartridge (Transgenomic).

Buffer A was made up of 0.1 M triethylammonium acetate (TEAA; Transgenomic), and buffer B of 0.1 M TEAA and 25% acetonitrile. Analysis was performed at a flow rate of 0.9 mL/min and the Buffer B gradient increased by 2%/min for 4.5 minutes. Elution of DNA fragments from the cartridge was detected by absorbance at 260 nm. The temperatures used for the analysis were selected according to the sequences of the DNA fragments. The software (Wavemaker, ver. 4.1.44; Transgenomic) predicted the melting behavior of the DNA fragments at various temperatures. The predicted melting domains within the DNA fragment determined the temperatures for the DHPLC

TABLE 1. Primer Sequences, PCR Product Sizes, and PCR Annealing and DHPLC Analysis Temperatures

Exon		Primer Sequences (5′ to 3′)	PCR Product Size (bp)	PCR Tm (°C)	DHPLC Tm (°C)
4	F	CCAGTGGGTTTGTGGGACTCC	317	60	61.7
	R	AAAGGGATGGCATTTCTTGCA			
5	F	GTCCACTTTCCTGGTGTGTGACT	277	55	58.7
	R	CAACATCACAATGGATCG			
6	F	AGCCTTAGTTTGATCTGTTCATTCA	293	60	57.0, 62.5
	R	GTTTCATCTTTCCAGGGGAGGCT			
7	F	GC-clamp AATCCCTTGCATTTCTGTTTTT	188	55	60.4, 61.4, 62.4
	R	GTGACAAGCACCCAGTGACGA			
8	F	GC-clamp GGTTACTCTCTTCTTAGTCTTTGGA	320	57	54.6, 58.5
	R	GGGTGAACTGTATGGTATCTTAATT			
9	F	GC-clamp GCTATTTCTCTTAAAGCCAAAGAGA	242	55	57.4, 59.4
	R	CAGTGGCTGGACTACTCTCGT			
10	F	GC-clamp GTCAGATGATAATTGTACAGATAT	227	55	57.8, 59.8
	R	AATGTATATTTCAAAGGAGGATAAA			
11	F	CCACTGCGACGTAAAGGAGCA	286	60	57.5, 59.5
	R	CAAATCCGAATTCCAATCTGTATAA			
12	F	GC-clamp GGTTGGGAGGCAAGACTATAAGTT	233	60	55.5, 56.5
	R	TTCTGTTCATTACTAGGCTATGGAA			
13	F	CAGGCAGAATTATTTCAAAACCAT	264	60	60.5, 61.5
	R	CGAGAATACAGTCAGGGCTGG			
14	F	GCACTACCTCCTCATCGCATAAACA	260	60	56.7, 59.7
	R	GGCCATGCTGATGTGAGCTCT			
15	F	GC-clamp GGACTGTCTGCTCAGTGTTGTCA	282	60	56.0, 59.0, 62.0
	R	GGTGCCTTGATTTGGAATCCA			
16	F	GC-clamp CACAACTGCCTGCAAAATGGAACT	294	60	60.7, 61.7
	R	GAGGCAAAATATTTGAGTGAAAACA			

GC-clamp: CGCCCGCCGCCCGCCCGCCGC.

analysis (Table 1). When abnormal chromatographic patterns were detected in a pool of three samples, each of the three samples was reanalyzed individually in the DHPLC system (Wave System; Transgenomic). Then, the PCR product that showed an abnormal chromatographic pattern was sequenced. Once a correlation between abnormal chromatographic patterns and base changes was confirmed by direct-sequencing analysis, additional sequencing analyses were not performed when any of the known abnormal chromatographic patterns were observed in the DHPLC analysis.

Direct DNA Sequencing

To detect mutations by direct sequencing, the PCR products were first purified (QIAquick PCR purification kit; Qiagen, Valencia, CA) to remove unreacted primers and precursors. The sequencing reactions were then performed using dye termination chemistry (Prism BigDye Terminator, ver. 3.1 Cycle Sequencing Kit; ABI), according to the manufacturer's protocol. The data were collected by a gene analyzer (Prism 310; ABI) and analyzed by computer (PRISM Sequencing-Analysis Program, ver. 3.7; ABI).

Genotyping the *OPTN* c.412G→A (Thr34Thr) Polymorphism

The G-to-A substitution at position c.412 in exon 4 of the *OPTN* gene was detected by using the restriction enzyme *Hpy*CH$_4$IV (New England BioLabs, Beverly, MA), with the primers listed in Table 1 for the DHPLC analysis. The G allele sequence was cut into two fragments (188 bp, 129 bp) by *Hpy*CH$_4$IV, whereas the A allele sequence remained intact (317 bp). The polymorphism was confirmed by restriction enzyme assay and by the chromatographic pattern of DHPLC.

Genotyping the *OPTN* c.603T→A (Met98Lys) Polymorphism

The T-to-A substitution at position c.603 in exon 5 of the *OPTN* gene was detected by the restriction enzyme *Stu*I (TaKaRa, Shiga, Japan), using the same primers as for the DHPLC analysis (Table 1). The A allele sequence was cut into two fragments (175 bp, 102 bp) by *Stu*I, whereas the T allele sequence remained intact (277 bp). The polymorphism was confirmed by a restriction enzyme assay and the chromatographic pattern of DHPLC.

Genotyping the *OPTN* c.1944G→A (Arg545Gln) Polymorphism

The G-to-A substitution at position c.1944 in exon 16 of the *OPTN* gene was analyzed (Invader assay,[34] provided by the Research Department of R&D Center; BML, Saitama, Japan). The polymorphism was confirmed by this assay and by the chromatographic pattern of DHPLC.

Genotyping the TNF-α −308G→A Polymorphism

Genotyping the −308G→A polymorphism in the TNF-α promoter region was performed by using the restriction enzyme *Nco*I (New England BioLabs), with the forward primer, 5′-AGGCAATAGGTTTT-GAGGGCCAT-3′, and the reverse primer, 5′-GTAGTGGGCCCTGCAC-CTTCT-3′.[35] The forward primer contained a one-nucleotide mismatch (in italic), which allowed the use of the restriction enzyme. The G allele sequence was cut into two fragments (192 bp, 20 bp) by *Nco*I, whereas the A allele sequence remained intact (212 bp).

Genotyping the TNF-α −857C→T Polymorphism

Genotyping the −857C→T polymorphism in the TNF-α promoter region was performed by using the restriction enzyme *Hin*cII (TaKaRa), with the forward primer, 5′-AAGTCGAGTATGGGGAC-CCCCCGTTAA-3′, and the reverse primer, 5′-CCCCAGTGTGTGGC-CATATCTTCTT-3′.[36] The forward primer contained one nucleotide mismatch (italic), which allowed the use of the restriction enzyme. The C allele sequence was cut into two fragments (106 bp, 25 bp) by *Hin*cII, whereas the T allele sequence remained intact (131 bp). Transcriptional activity of the −857T allele was significantly greater than that of the −857C allele.[37]

Genotyping the TNF-α −863C→A Polymorphism

The −863C→A polymorphism in the TNF-α promoter region was genotyped by using the restriction enzyme *Eco*NI (New England

TABLE 2. *OPTN* Variants Observed in Patients with Glaucoma and Control Subjects

Location	Sequence Changes	Codon Changes	Frequency in Subjects (%)		
			POAG	NTG	Control
Exon 4	c.386C→G	His26Asp	1/194 (0.5)	0/217 (0)	0/218 (0)
Exon 4	c.449_451delCTC	Leu47del	0/194 (0)	0/217 (0)	1/218 (0.5)
Exon 5	c.603T→A	Met98Lys	33/194 (17.0)	48/217 (22.1)	36/218 (16.5)
Exon 16	c.1944G→A	Arg545Gln	11/194 (5.7)	15/217 (6.9)	11/218 (5.0)
Exon 4	c.412G→A	Thr34Thr	69/194 (35.6)	69/217 (31.8)	52/218 (23.9)
Exon 4	c.421G→A	Pro37Pro	0/194 (0)	1/217 (0.5)	0/218 (0)
Exon 4	c.457C→T	Thr49Thr	1/194 (0.5)	0/217 (0)	0/218 (0)
Exon 16	c.2023C→T	His571His	0/194 (0)	0/217 (0)	2/218 (1.0)
Intron 4	c.476 + 15C→A		0/194 (0)	1/217 (0.5)	0/218 (0)
Intron 6	c.863 − 10G→A*		NC	NC	NC
Intron 6	c.863 − 5C→T		NC	NC	NC
Intron 8	c.1089 + 20G→A		2/147 (1.4)	10/163 (6.1)	4/126 (3.2)
Intron 9	c.1192 + 19C→T		0/147 (0)	4/163 (2.5)	3/130 (2.3)
Intron 11	c.1458 + 28G→C		2/147 (1.4)	3/163 (1.8)	0/157 (0)
Intron 15	c.1922 + 10G→A		1/147 (0.7)	4/163 (2.5)	1/157 (0.6)
Intron 15	c.1922 + 12G→C		0/147 (0)	1/163 (0.6)	0/157 (0)
Intron 15	c.1923 − 48C→A*		NC	NC	NC

NC, not checked.

* Sequence variation was found by direct sequencing analysis.

BioLabs) with the forward primer, 5′-GCTGAGAAGATGAAGGAAAAGTG-3′, and the reverse primer, 5′-CCTCTACATGGCCCTGTCCT-3′. The reverse primer contained a one-nucleotide mismatch (italic), which allowed the use of the restriction enzyme. The C allele sequence was cut into two fragments (183 bp, 23 bp) by *Eco*NI, whereas the A allele sequence remained intact (206 bp). Transcriptional activity of the −863A allele was significantly greater than that of the −863C allele.[37]

Statistical Analyses

The frequencies of the genotypes and alleles in patients and control subjects were compared with the χ^2 test or the Fisher exact test. The odds ratio and 95% confidence intervals (CIs) were also calculated. The Hardy-Weinberg equilibrium for the observed frequencies was also calculated. Comparisons of the clinical characteristics between the two groups were performed using the Mann-Whitney test or Student's unpaired *t*-test when appropriate. Logarithmic transformation was performed on skewed-distribution clinical data, which were the IOP at diagnosis of POAG, visual field score at diagnosis of NTG, and POAG, to obtain a normal distribution for performing analysis of variance (ANOVA). One-way ANOVA was used to compare three clinical characteristics among patients with four different combinations of the TNF-α/−857C→T and optineurin/412G→A genotypes, or the TNF-α/−863C→A and optineurin/603T→A genotypes (see Table 6).

Statistical analyses were performed on computer (SPSS software; SPSS Inc., Chicago, IL). $P < 0.05$ was considered to be significant.

RESULTS

OPTN Variants in Japanese Subjects

Six hundred twenty-nine Japanese subjects were studied, and the results are presented in Table 2. Seventeen sequence changes were identified in the patients with glaucoma and control subjects. Among these, three were missense changes, one was a deletion of one amino acid residue, four were synonymous codon changes, and nine were changes in noncoding sequences. One possible disease-causing mutation, His26Asp, was identified in one POAG proband and was not present in the 218 normal Japanese control subjects. Her brother, aged 55, harbored the mutation and received a diagnosis of NTG. Her niece, aged 23, also had the mutation and showed cupping of the optic nerve head with a cup-to-disc ratio of 0.7, with no sign of visual field defect by perimetry (Humphrey 30-2 program; Carl Zeiss Meditec).

A deletion of Leu47 (3-bp deletion, CTC) was found in 1 control. A Met98Lys was identified in 33 patients with POAG, 48 patients with NTG, and 36 control subjects, and an Arg545Gln was identified in 11 patients with POAG, 15 patients with NTG, and 11 control subjects.

Four synonymous nucleotide substitutions, c.412G→A (Thr34Thr), c.421G→A (Pro37Pro), c.457C→T (Thr49Thr), and c.2023C→T (His571His), were found. The Thr34Thr substitution was present in 69 (35.6%) patients with POAG, 69 (31.8%) patients with NTG, and 52 (23.9%) control subjects, and the Pro37Pro was found in 1 patient with NTG. The Thr49Thr was identified in one patient with POAG, and the His571His was present in two control subjects.

Distribution of *OPTN* Variants in Japanese Subjects

The Thr34Thr (c.412G→A) polymorphism was significantly associated with POAG and NTG (Table 3). A significant association was found in patients with POAG ($P = 0.009$ in genotype frequency: G/G versus G/A+A/A, and $P = 0.003$ in allele frequency). No significant difference was detected between patients with glaucoma and control subjects in either genotype or allele frequency for the Met98Lys (c.603T→A) or the Arg545Gln (c.1944G→A) polymorphisms. However, the Met98Lys polymorphism had a higher tendency to be associated with NTG than with POAG. The observed genotype frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium.

Three clinical characteristics of the patients with glaucoma—age, IOP, and visual field score at diagnosis—were examined for association with the c.412G→A (Thr34Thr) or c.603T→A (Met98Lys) polymorphisms (Table 4). The patients with glaucoma did not show an association with the clinical characteristics with the c.412G→A polymorphism. Patients with POAG with the G/A+A/A genotype (or 412A carriers) tended to have more advanced visual field scores than those with the G/G genotype (or non-412A carriers; $P = 0.093$). Patients with POAG with the 603T→A polymorphism showed a weak association with age at diagnosis ($P = 0.046$).

TABLE 3. Genotype Distribution and Allele Frequency of Optineurin Gene Polymorphisms in Patients with Glaucoma and Controls Subjects

Phenotype	n	Genotype Frequency (%)			P*	Genotype Frequency (%)		P*	Genotype Frequency (%)		P†	Allele Frequency (%)		P*
		G/G	G/A	A/A		G/G	G/A + A/A		G/G + G/A	A/A		G	A	
c.412G→A (Thr34Thr)														
POAG	194	125 (64.4)	61 (31.4)	8 (4.1)	0.011‡	125 (64.4)	69 (35.6)	0.009§	186 (95.9)	8 (4.1)	0.051	311 (80.2)	77 (19.8)	0.003§
NTG	217	148 (68.2)	62 (28.6)	7 (3.2)	0.078	148 (68.2)	69 (31.8)	0.064	210 (96.8)	7 (3.2)	0.105	358 (82.5)	76 (17.5)	0.034‡
Control	218	166 (76.1)	50 (22.9)	2 (1.0)		166 (76.1)	52 (23.9)		216 (99.0)	2 (1.0)		382 (87.6)	54 (12.4)	
Phenotype	n	Genotype Frequency (%)			P*	Genotype Frequency (%)		P*	Genotype Frequency (%)		P†	Allele Frequency (%)		P*
		T/T	T/A	A/A		T/T	T/A + A/A		T/T + T/A	A/A		T	A	
c.603T→A (Met98Lys)														
POAG	194	161 (83.0)	32 (16.5)	1 (0.5)	0.990	161 (83.0)	33 (17.0)	0.893	193 (99.5)	1 (0.5)	0.990	354 (91.2)	34 (8.8)	0.888
NTG	217	169 (77.9)	43 (19.8)	5 (2.3)	0.133	169 (77.9)	48 (22.1)	0.139	212 (97.7)	5 (2.3)	0.122	381 (87.8)	53 (12.2)	0.071
Control	218	182 (83.5)	35 (16.0)	1 (0.5)		182 (83.5)	36 (16.5)		217 (99.5)	1 (0.5)		399 (91.5)	37 (8.5)	

* P by χ^2 test.
† P by Fisher exact test.
‡ $P < 0.05$.
§ $P < 0.01$.

Association between the *OPTN* and TNF-α Polymorphisms in Patients with Glaucoma

No significant difference in genotype or allele frequency was noted between patients and control subjects for the three polymorphisms of the 5′ flanking region of the TNF-α gene (Table 5). In addition, the patients with glaucoma did not show an association with the clinical characteristics for the three polymorphisms (data not shown). The observed genotype frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium.

However, among individuals with the C/T+T/T genotype (or −857T carriers) in the TNF-α gene, 44.1% of patients with POAG were G/A+A/A genotypes (or 412A carriers) of the *OPTN* gene compared with 21.6% of control subjects (Table 6). This difference in frequency was significant ($P = 0.006$). Among individuals with the C/A+A/A genotype (or −863A carriers) in the TNF-α gene, 603A carriers (or Lys98 carriers) in the *OPTN* gene were significantly associated with POAG as well as NTG ($P = 0.008$ and 0.027, respectively).

The clinical characteristics of these combined genotypes, such as age, IOP, and visual field score at diagnosis are shown in Table 7. The patients with POAG who were TNF-α/−857T and optineurin/412A carriers had significantly worse ($P = 0.020$) visual field scores than those who were TNF-α/−857T and non-optineurin/412A carriers. However, there was no significant difference in the three clinical features of patients with POAG among the four genotypes of combined −857T→A and c.412G→A polymorphisms (Table 6) by one-way ANOVA: $P = 0.823$ for age at diagnosis; $P = 0.692$ for IOP at diagnosis; and $P = 0.152$ for visual field score at diagnosis.

Patients with POAG who were TNF-α/−863A and optineurin/603A carriers had significantly worse ($P = 0.026$) visual field scores than those who were TNF-α/−863A and non-optineurin/603A carriers. However, there was no significant difference in the visual field score of patients with POAG among the four genotypes of combined −863C→A and c.603T→A polymorphisms (Table 6, one-way ANOVA: $P = 0.200$).

DISCUSSION

Rezaie et al.[13] detected two missense mutations, Glu50Lys and Arg545Gln, and one truncating mutation due to a 2-bp insertion (c.691_692 ins AG), in 9 (16.7%) of 54 families with hereditary POAG. Most of the family members presented with IOPs ≤ 21 mm Hg, and only 3 of the 23 affected members had a higher IOP (23, 26, and 40 mm Hg). These researchers also identified a risk-associated sequence change (Met98Lys) in 23 (13.6%) of 169 index cases and in 9 (2.1%) of 422 control subjects. This difference in the frequencies between patients and control subjects was significant.

In England, a Glu50Lys mutation was identified in 2 (1.5%) of 132 patients with NTG,[19] and in the Chinese population, two probable disease-causing mutations, Glu103Asp and His486Arg, were found in 2 (1.6%) of 119 patients with sporadic-occurring POAG.[18] However, the results of other studies suggested that alterations of the *OPTN* gene were rare causes of POAG or NTG.[15,17,20]

In our Japanese subjects, 17 sequence changes in the *OPTN* gene were identified. The missense mutation, His26Asp, in exon 4 was found in only one (0.24%) of 411 patients with open-angle glaucoma and not in 218 normal subjects. In Japan, this mutation has recently been reported in a patient with POAG.[38] Thus, His26Asp may be a disease-causing mutation. However, our results and those of two other studies on Japanese patients[16,20] suggested that *OPTN* gene mutations are rare

TABLE 4. Comparison of Clinical Characteristics of Patients with Glaucoma, According to *OPTN* Genotypes

	Phenotype Variable	G/G	G/A + A/A	P*
c.412G→A (Thr34Thr)				
POAG	Age at diagnosis (y)	58.1 ± 11.8 (*n* = 123)	58.8 ± 12.6 (*n* = 69)	0.663
	IOP at diagnosis (mm Hg)	27.0 ± 6.5 (*n* = 112)	26.1 ± 5.0 (*n* = 60)	0.360
	Visual field score at diagnosis	3.0 ± 0.9 (*n* = 125)	3.2 ± 0.9 (*n* = 69)	0.093
NTG	Age at diagnosis (y)	58.7 ± 11.7 (*n* = 148)	56.6 ± 11.2 (*n* = 69)	0.206
	IOP at diagnosis (mm Hg)	16.4 ± 2.6 (*n* = 139)	16.6 ± 2.2 (*n* = 67)	0.848
	Visual field score at diagnosis	2.8 ± 0.7 (*n* = 148)	2.7 ± 0.7 (*n* = 69)	0.135
	Phenotype Variable	**T/T**	**T/A + A/A**	**P***
c.603T→A (Met98Lys)				
POAG	Age at diagnosis (y)	57.6 ± 11.9 (*n* = 159)	62.2 ± 12.4 (*n* = 33)	0.046†
	IOP at diagnosis (mm Hg)	26.8 ± 5.8 (*n* = 143)	26.5 ± 7.1 (*n* = 29)	0.931
	Visual field score at diagnosis	3.1 ± 0.9 (*n* = 161)	3.2 ± 0.9 (*n* = 33)	0.280
NTG	Age at diagnosis (y)	58.4 ± 11.6 (*n* = 169)	56.6 ± 11.6 (*n* = 48)	0.304
	IOP at diagnosis (mm Hg)	16.4 ± 2.4 (*n* = 160)	16.8 ± 2.6 (*n* = 46)	0.270
	Visual field score at diagnosis	2.8 ± 0.7 (*n* = 169)	2.8 ± 0.6 (*n* = 48)	0.318

* *P* by Mann-Whitney test.
† $P < 0.05$.

as a glaucoma-causing gene in Japanese patients with POAG or NTG.

Our observations showed that the frequency of the A allele in c.603T→A (Met98Lys) was slightly higher in patients with NTG (A: 12.2%, $P = 0.071$) than in patients with POAG (A: 8.8%) or control subjects (A: 8.5%). The Met98Lys change was observed as 22.1% in NTG ($P = 0.139$), 17.0% in POAG ($P = 0.893$), and 16.5% in control subjects. In the United Kingdom, a significant association of Met98Lys with NTG but not POAG has been reported.[19] These results suggest that there may be genetic differences between the two phenotypes. Tang et al.[16] reported no significant difference in allele frequency between Japanese patients with POAG or NTG and control subjects for Thr34Thr, Met98Lys, or Arg545Gln. In contrast, Alward et al.[20] observed a significantly higher prevalence ($P = 0.01$) of the Met98Lys change in 51 (20.7%) of 247 Japanese patients with NTG, compared with 8 (9.0%) of 89 Japanese control subjects. However, the number of control subjects in their study was too few to perform a case–control association study.

In patients with POAG in France and Morocco, the Met98Lys frequency was similar to that of control subjects.[39] However, a Met98Lys variant was reported to be significantly associated with a lower initial IOP: There was a downward shift of the initial IOP in patients with POAG harboring Met98Lys.[39] In our study, a Met98Lys variant was not associated with a lower initial IOP, but was weakly ($P = 0.046$) associated with an older age at diagnosis in patients with POAG.

No significant difference in the frequency of the Arg545Gln variant was found between Japanese patients with glaucoma and control subjects. In a Chinese population, the Met98Lys and Arg545Gln variants were reported to have similar frequencies in patients with glaucoma and control subjects.[18] Arg545Gln is a common polymorphism in the Japanese and Chinese populations, but may be rare in whites.[20]

The distribution of c.412G→A (Thr34Thr) genotype in the *OPTN* gene differed significantly between POAG ($P = 0.011$) and control subjects in our Japanese population, with the A allele being significantly more frequent than the G allele (POAG, $P = 0.003$). This polymorphism is associated with POAG more than NTG ($P = 0.078$ in genotype frequency and $P = 0.034$ in allele frequency). This finding is new, although the c.412G→A polymorphism has been identified in the United States,[13,20] Finland,[15] Hong Kong,[18] and Japan.[16,20] Previous studies of this polymorphism in Japanese patients did not find an association with glaucoma.[16,20] Our Japanese subjects resided throughout the nation and consisted of a larger number of subjects, which may account for the differing results.

Although the reason for the significant association of the c.412G→A polymorphism with patients with glaucoma is unknown, it may be linked to another unknown single-nucleotide polymorphism that exists in the promoter region and may alter the activity of the protein or may affect the stability or splicing accuracy of the mRNA.[10] Alternatively, the c.412G→A polymorphism may be linked to another unknown gene that lies near the *OPTN* gene.[41]

TABLE 5. Genotype Distribution of Three Polymorphisms of the 5′ Flanking Region of the TNF-α Gene in Patients with Glaucoma and Controls Subjects

		Genotype Frequency (%)			
Phenotype	***n***	**G/G**	**G/A**	**A/A**	***P****
−308G→A					
POAG	194	192 (99.0)	2 (1.0)	0 (0)	0.442
NTG	217	211 (97.2)	6 (2.8)	0 (0)	1
Control	218	212 (97.2)	6 (2.8)	0 (0)	
		Genotype Frequency (%)			
Phenotype	***n***	**C/C**	**C/T**	**T/T**	***P****
−857C→T					
POAG	194	135 (69.5)	49 (25.3)	10 (5.2)	0.138
NTG	217	148 (68.2)	64 (29.5)	5 (2.3)	0.890
Control	218	144 (66.1)	69 (31.6)	5 (2.3)	
		Genotype Frequency (%)			
Phenotype	***n***	**C/C**	**C/A**	**A/A**	***P****
−863C→A					
POAG	194	141 (72.7)	46 (23.7)	7 (3.6)	0.056
NTG	217	159 (73.3)	55 (25.3)	3 (1.4)	0.606
Control	218	161 (73.8)	56 (25.7)	1 (0.5)	

* *P* by χ^2 test.

TABLE 6. Distribution of Optineurin Genotypes (c.412G→A and c.603T→A) According to TNF-α Genotypes (−857C→T and −863C→A) in Glaucoma Patients and Control Subjects

Phenotype	−857C→T C/C(%)			Odds Ratio 95% CI		C/T + T/T (%)			Odds Ratio 95% CI	
	c.412G→A	G/G	G/A + A/A	P*		G/G	G/A + A/A	P*	
c.412G→A (Thr34Thr)										
POAG		92 (68.1)	43 (31.9)	0.204	1.40 (0.83–2.37)	33 (55.9)	26 (44.1)	0.006‡	2.86 (1.34–6.08)	
NTG		97 (65.5)	51 (34.5)	0.077	1.58 (0.95–2.62)	51 (73.9)	18 (26.1)	0.531	1.28 (0.59–2.77)	
Control		108 (75.0)	36 (25.0)			58 (78.4)	16 (21.6)			
	−863C→A C/C (%)					C/A + A/A (%)				
Phenotype	c.412G→A	G/G	G/A + A/A	P*	Odds Ratio 95% CI	G/G	G/A + A/A	P*	Odds Ratio 95% CI	
POAG		91 (64.5)	50 (35.5)	0.017	1.84 (1.11–3.05)	34 (64.2)	19 (35.8)	0.280	1.56 (0.69–3.53)	
NTG		110 (69.2)	49 (30.8)	0.114	1.49 (0.91–2.46)	38 (65.5)	20 (34.5)	0.341	1.47 (0.66–3.28)	
Control		124 (77.0)	37 (23.0)			42 (73.7)	15 (26.3)			
	−857C→T C/C(%)					C/T + T/T (%)				
Phenotype	c.603T→A	T/T	T/A + A/A	P*	Odds Ratio 95% CI	T/T	T/A + A/A	P*	Odds Ratio 95% CI	
c.603T→A (Met98Lys)										
POAG		112 (83.0)	23 (17.0)	0.811	1.08 (0.57–2.03)	49 (83.1)	10 (16.9)	0.925	0.96 (0.39–2.37)	
NTG		111 (75.0)	37 (25.0)	0.056	1.75 (0.98–3.13)	58 (84.1)	11 (15.9)	0.795	0.89 (0.37–2.14)	
Control		121 (84.0)	23 (16.0)			61 (82.4)	13 (17.6)			
	−863C→A C/C (%)					C/A + A/A (%)				
Phenotype	c.603T→A	T/T	T/A + A/A	P*	Odds Ratio 95% CI	T/T	T/A + A/A	P*	Odds Ratio 95% CI	
POAG		123 (87.2)	18 (12.8)	0.127	0.61 (0.33–1.15)	38 (71.7)	15 (28.3)	0.008‡	4.11 (1.37–12.27)	
NTG		125 (78.6)	34 (21.4)	0.636	1.14 (0.66–1.97)	44 (75.9)	14 (24.1)	0.027†	3.31 (1.10–9.91)	
Control		130 (80.7)	31 (19.3)			52 (91.2)	5 (8.8)			

* P by χ^2 test.
† $P < 0.05$.
‡ $P < 0.01$.

Optineurin is induced by TNF-α and interacts with several proteins to regulate apoptosis, inflammation, and vasoconstriction. For example, optineurin interacts with adenoviral E3-14.7K protein which protects cells from the cytolytic activity of TNF-α.[21] Huntingtin is linked to the Rab8 protein through optineurin, which may regulate membrane traffic and cellular morphogenesis.[25] Vittitow and Borrás[42] studied the effect of glaucomatous insults on the expression of optineurin in a human anterior segment organ culture perfusion system under conditions mimicking physiologic pressure. Sustained elevated IOP, TNF-α exposure, and prolonged dexamethasone treatment significantly upregulated optineurin expression in the trabecular meshwork.

In glaucomatous eyes, the expression of TNF-α and TNF-α receptor-1 was upregulated in the retina and optic nerve head.[43,44] Yuan and Neufeld[45] reported that the expression of TNF-α and TNF-α receptor-1 appeared to parallel the progression of optic nerve degeneration. An association of TNF-α −308G→A polymorphism with POAG has been reported in the Chinese.[46] In this study, we examined three single-nucleotide polymorphisms, −308G→A, −857C→T, and −863C→A, in the TNF-α promoter region in a Japanese population. Transcriptional activity of the −857T allele or −863A allele was significantly greater than that of the −857C allele or −863C allele.[37] However, no significant difference in genotype or allele frequency was noted between patients and control subjects for the three single-nucleotide polymorphisms of the TNF-α gene. Especially, the −308G→A polymorphism is rare in the Japanese.[47]

The genotype frequency of the c.412G→A (Thr34Thr) polymorphism in the *OPTN* gene was significantly associated with POAG, and the frequency of 412A carriers was significantly greater in patients with POAG than in control subjects ($P = 0.009$). This association was influenced by TNF-α/−857C→T genotypes (Table 6). Among individuals with the C/T+T/T genotype (or −857T carriers) in the TNF-α gene, the frequency of optineurin/412A carriers was significantly greater in patients with POAG than in control subjects (odds ratio 2.86, $P = 0.006$). The visual field scores at diagnosis in patients with POAG were significantly worse in patients with optineurin/412A when they were TNF-α/−857T carriers ($P = 0.020$; Table 7), although we found no significant difference in the scores between the c.412G→A genotypes in the *OPTN* gene ($P = 0.093$, Table 4).

The same interactions were more clearly observed between the c.603T→A (Met98Lys) polymorphism in the *OPTN* gene and the −863C→A polymorphism in the TNF-α gene. Although there was no significant association between c.603T→A (Met98Lys) polymorphism and POAG or NTG, the frequency of optineurin/603A carriers was significantly greater in patients with POAG (odds ratio, 4.11; $P = 0.008$) than in control subjects and in patients with NTG (odds ratio, 3.31; $P = 0.027$) than in control subjects among individuals with the C/A+A/A genotype (or −863A carriers) in the TNF-α gene. The visual field scores at diagnosis in patients with POAG were significantly worse in patients with optineurin/603A (or Lys98) when they were TNF-α/−863A carriers ($P = 0.026$). However, there was no significant difference in visual field score at

TABLE 7. Comparison of Clinical Characteristics of Glaucoma Patients According to TNF-α Genotypes (−857T and −863A) and Optineurin Genotypes (c.412G→A and c.603T→A)

	(TNF-α Genotypes) (*OPTN* Genotypes)	C/T + T/T (−857T Carrier) G/G	C/T + T/T (−857T Carrier) G/A + A/A	*P**
c.412G→A (Thr34Thr)				
POAG	Age at diagnosis (y)	57.1 ± 10.7 (n = 32)	57.6 ± 13.1 (n = 26)	0.802
	IOP at diagnosis (mm Hg)	26.4 ± 6.1 (n = 30)	26.4 ± 5.5 (n = 20)	0.786
	Visual field score	2.9 ± 0.9 (n = 33)	3.3 ± 0.8 (n = 26)	0.020*
NTG	Age at diagnosis (y)	58.4 ± 11.1 (n = 51)	59.3 ± 10.5 (n = 18)	0.790
	IOP at diagnosis (mm Hg)	16.4 ± 2.6 (n = 46)	16.1 ± 2.3 (n = 17)	0.520
	Visual field score	2.8 ± 0.8 (n = 51)	2.6 ± 0.5 (n = 18)	0.335
	(TNF-α Genotypes) (*OPTN* Genotypes)	C/A + A/A (−863A Carrier) T/T	C/A + A/A (−863A Carrier) T/A + A/A	*P**
c.603T→A (Met98Lys)				
POAG	Age at diagnosis (y)	56.3 ± 10.5 (n = 38)	62.0 ± 13.8 (n = 15)	0.074
	IOP at diagnosis (mm Hg)	27.9 ± 6.5 (n = 36)	26.9 ± 8.7 (n = 14)	0.488
	Visual field score	3.0 ± 0.8 (n = 38)	3.5 ± 0.9 (n = 15)	0.026*
NTG	Age at diagnosis (y)	57.9 ± 11.4 (n = 44)	56.9 ± 11.9 (n = 14)	0.579
	IOP at diagnosis (mm Hg)	16.2 ± 2.4 (n = 40)	16.9 ± 2.4 (n = 14)	0.364
	Visual field score	2.9 ± 0.5 (n = 44)	2.7 ± 0.6 (n = 14)	0.296

* $P < 0.05$, Mann-Whitney test.

diagnosis in patients with POAG among the four different genotypes of combined TNF-α/−857T→A and optineurin/412G→A polymorphisms, or TNF-α/−863C→A and optineurin/603T→A polymorphisms in Table 6, by one-way ANOVA ($P = 0.152$ or $P = 0.200$, respectively). These results suggest an association between the visual field scores at diagnosis and combination of the TNF-α/−857C→T and optineurin/412G→A genotypes, or TNF-α/−863C→A and optineurin/603T→A genotypes.

In conclusion, the His26Asp mutation in the *OPTN* gene is a possible disease-causing mutation in Japanese patients with open-angle glaucoma. The c.412G→A polymorphism was significantly associated with POAG and NTG, and the c.603T→A (Met98Lys) polymorphism tended to be associated with NTG. Optineurin expression is directly induced by TNF-α. Genetic statistical analysis showed an interaction between single-nucleotide polymorphisms in the TNF-α gene (−857C→T and −863C→A) and those in the optineurin gene (c.412G→A and c.603 T→A), which increases the risk for the development and probably progression of glaucoma in patients with POAG.

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Novel *MYOC* Gene Mutation, Phe369Leu, in Japanese Patients with Primary Open-Angle Glaucoma Detected by Denaturing High-Performance Liquid Chromatography

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Purpose: To screen for mutations in the *MYOC* gene in Japanese patients with primary open-angle glaucoma (POAG) using denaturing high-performance liquid chromatography (DHPLC).

Patients and Methods: Blood samples were collected from 171 patients with POAG and 100 controls from seven institutions in Japan. For high-throughput analysis, seven exonic regions were amplified by polymerase chain reaction using DNA pooled from three patients; each DNA pool was then analyzed chromatographically. For analysis of a small number of samples, 7 exonic regions were amplified separately but simultaneously with annealing at 58°C in each patient and then chromatographed, using 7 wells of the same 96-well plate per sample. When chromatographic patterns were abnormal by either method, the PCR products of the individual samples were sequenced.

Results: Four glaucoma-causing mutations were identified in five POAG patients (2.9%). One missense mutation, Phe369Leu, is new; and three others, Ile360Asn, Ala363Thr, and Thr448Pro, have been reported in Japanese patients. Phe369Leu was associated with adult onset POAG.

Conclusions: Mutations in the *MYOC* gene were demonstrated chromatographically in 2.9% of our Japanese POAG patients. The use of pooled DNAs with DHPLC analysis is a time- and labor-saving technique. All mutations detected appear to be specific to Japanese patients.

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Primary open-angle glaucoma (POAG) is an important cause of blindness worldwide.[1] The disease produces characteristic visual field changes corresponding to excavation of the optic disc, usually in association with elevated intraocular pressure (IOP). Among the Japanese, 2 million people are estimated to have glaucoma, representing a prevalence of approximately 3.5% of individuals over 40 years of age (POAG, 0.58%; normal-tension glaucoma (NTG), 2.04%).[2]

Strong evidence indicates that genetic factors play a role in the pathogenesis of glaucoma,[3,4] and several chromosomal loci have been identified for POAG. A juvenile-onset form of POAG was first linked to the GLC1A locus on chromosome 1q21-1q31.[5] This region contains the trabecular meshwork inducible glucocorticoid response (*TIGR*) gene,[6] also known as the myocilin (*MYOC*) gene.[7] Over 50 different mutations associated with the development of glaucoma have been identified in the *MYOC* gene in multiple ethnic groups worldwide.[8–17] Mutations in the *MYOC* gene are associated with juvenile-onset POAG as well as with some cases of adult-onset POAG. The prevalence of *MYOC* mutations is 3 to 4% of unselected POAG patients; mutations have been found in 36% of juvenile-onset POAG probands and 4% of adult-onset POAG probands.[13] *MYOC* mutations have been found more frequently in familial POAG cases, and less frequently in sporadic cases.[17]

We have screened a large number of Japanese patients with POAG for mutations in the *MYOC* gene. The analysis of the large numbers of samples was accomplished by denaturing high-performance liquid chromatography (DHPLC) using an automated heteroduplex detection method with a proven sensitivity and specificity exceeding 95%.[18–21] Thus, this DHPLC method provides accurate detection of mutations. In addition, we developed a time-saving screening method for detecting mutations in the *MYOC* gene for a few samples, where PCR amplification and then DHPLC analysis are performed in the same 96-well plate.

MATERIALS AND METHODS

Patients

Blood samples were collected from 171 POAG patients and 100 normal subjects at 7 Japanese medical institutions. The samples were analyzed at Keio University. The subjects were unrelated, and their mean age at the time of examination was 55.1 ± 16.0 (±SD) years for the patients with POAG and 70.5 ± 10.6 years for the normal subjects. We purposely selected older control subjects to reduce the probability that a subset of them would develop glaucoma.

A detailed family history was obtained by interviews in 55 POAG patients (32.2%). There were 91 men (53.2%) in the POAG patients, and 41 men (41.0%) in the normal subjects.

This human research conformed to the tenets of the Declaration of Helsinki. A written informed consent was obtained after the nature and possible consequences of the study were explained. Where applicable, the research was approved by the Institutional Human Experimentation Committee.

A diagnosis of POAG was made if: the peak IOP was more than 22 mm Hg; the angle was normal (open); typical glaucomatous disc cupping associated with visual field changes were present; and other ocular, rhinologic, neurologic, or systemic disorders that can cause optic nerve damage, were absent. The 100 control samples were obtained from Japanese individuals who had IOPs below 20 mm Hg, no glaucomatous disc changes, and no family history of glaucoma.

DNA Extraction and Polymerase Chain Reaction Conditions

Genomic DNA was isolated from peripheral blood lymphocytes by standard methods. The 7 exonic regions of the *MYOC* gene were amplified by polymerase chain reaction (PCR) using the primer sets listed in Table 1. For high-throughput analysis of the patients, samples from 3 patients were pooled. The PCR reaction was performed with a thermal cycler (iCycler; Bio Rad, Hercules, CA) in a total volume of 25 μL. The PCR conditions were: denaturation at 95°C for 9 minutes; followed by 35 cycles at 95°C for 1 minute; 58°C for 30 seconds (Table 1); and 72°C for 1.5 minutes; a final extension step was then carried out at 72°C for 7 minutes. For heteroduplex formation, each PCR product (25 μL) was denatured at 95°C for 5 minutes and gradually cooled to 25°C.

For analyses of a few samples, each of 7 exonic regions was amplified simultaneously by PCR in a 96-well plate (96-well Multiplate, MLP-9601; MJ Research, Waltham, MA). Seven wells were used for each patient. Primer sets were designed to be effective using a single annealing temperature of 58°C (Table 1).

Denaturing High Performance Liquid Chromatography Analysis

For high-throughput analysis, a 25-μL volume of PCR products from the 3 patients was automatically injected into the chromatograph for analysis using the WAVE® System for DHPLC analysis (Transgenomic, Omaha, NE). The DHPLC melting temperatures are listed in Table 1. For analysis of a small number of samples, following 96-well-plate PCR, the plate was next placed in a WAVE® System programed to automatically analyze each well at 2 to 3 melting temperatures. Approximately 3 hours was sufficient time to analyze one individual's sample.

When abnormal chromatographic patterns were detected in the pooled samples by the high-throughput protocol, the sample was reanalyzed individually in the WAVE® System. The PCR product that showed the abnormal chromatographic pattern was then sequenced.

Direct DNA Sequencing

For direct sequencing, PCR products were purified with a QIA Quick PCR purification kit (Qiagen, Valencia, CA) to remove unused primers and precursors. The PCR products were directly sequenced with the same forward and reverse PCR amplification primers on an ABI310 automated sequencer using BigDye chemistry according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA).

TABLE 1. Primer Sequences, Product Size, and PCR Annealing and DHPLC Analysis Temperatures

Exon		Primer Sequences (5′ to 3′)	Product Size (bp)	PCR Tm (°C)	DHPLC Tm (°C)
1A	F	AGC ACA GCA GAG CTT TCC AGA GGA	302	58.0	61.9
	R	CTC CAG GTC TAA GCG TTG G			
1B	F	CAG GCC ATG TCA GTC ATC CA	298	58.0	61.2, 64.5
	R	TCT CAT TTT CTT GCC TTA GTC			
1C	F	GAA ACC CAA ACC AGA GAG	255	58.0	61.0, 63.5
	R	ATA TCA CCT GCT GAA CTC AGA GTC			
2A	F	CCT CAA CAT AGT CAA TCC TTG GGC	245	58.0	56.3, 59.3
	R	ACA TGA ATA AAG ACC ATG TGG GCA			
3A	F	GAT TAT GGA TTA AGT GGT GCT TCG	375	58.0	59.3, 61.3, 62.3
	R	TGT CTC GGT ATT CAG CTC AT			
3B	F	CAT ACT GCC TAG GCC ACT GGA	337	58.0	60.9, 61.4
	R	ATT GGC GAC TGA CTG CTT AC			
3C	F	GAA TCT GGA ACT CGA ACA AA	333	58.0	59.7, 61.7
	R	CTG AGC ATC TCC TTC TGC CAT			

RESULTS

Screening of Pools of DNA in One Hundred Seventy-One Patients

Four DHPLC tracing patterns in the Exon3C region are shown in Figure 1. The upper-most pattern (Fig. 1A) has a normal appearance, while the middle pattern (Fig. 1B) showed a broad shoulder, and the lower patterns (Fig. 1C and 1D) had a characteristic double-peak pattern indicative of sequence variations in this region. Sequencing analysis of samples B, C, and D revealed Thr448Pro, Pro481Ser, and Ala488Ala mutations (Table 2).

Four glaucoma-causing mutations were identified in 5 (2.9%) of 171 patients with POAG. In addition, 8 polymorphisms and 5 synonymous codon changes were identified (Table 2). One novel missense mutation, Phe369Leu, detected in exon 3 (Fig. 2) was not present in 100 normal Japanese subjects. The amino acid residue at position 369 is conserved as Phe in monkeys, bovines, pigs, rats, and mice (Fig. 3). The 3 other missense mutations, Ile360Asn,[22,23] Ala363Thr,[22] and Thr448Pro[24] have been reported in Japanese patients with POAG.

The patient with the Phe369Leu mutation was diagnosed with POAG at the age of 49 years and had an IOP of 45 mm Hg in the right eye and 57 mm Hg in the left eye. She had normal open angles on gonioscopy, and glaucomatous optic disc changes with a cup:disc ratio (C:D ratio) of 0.7 in the right eye and 0.8 in the left eye (Fig. 4). Her visual fields determined by a Humphrey Field Analyzer (central 30-2 threshold test), were at stage 0-1 in the right eye, and stage 5 in the left eye (Fig. 5) according to the modified Aulhorn-Greve classification. She was being treated with topical 2% carteolol hydrochloride, topical 0.12% isopropyl unoprostone, and 500 mg of oral acetazolamide. Although these medications were effective for the right eye, the IOP in the left eye remained uncontrolled, and trabeculectomy with mitomycin C was

TABLE 2. MYOC Mutations and Polymorphisms in Patients with POAG and Controls

	Exon	Sequence Change	Amino Acid Change	Frequency	
				Patients	Controls
Mutations	3	c.1079T>A	Ile360Asn	1/171	0/100
	3	c.1087G>A	Ala363Thr	2/171	0/100
	3	c.1105T>C	Phe369Leu*	1/171	0/100
	3	c.1342A>C	Thr448Pro	1/171	0/100
Polymorphisms	1	c.34G>C	Gly12Arg	1/171	2/100
	1	c.57G>T	Gln19His	1/171	1/100
	1	c.136C>T	Arg46Stop	1/171	1/100
	1	c.210C>T	Val70Val†	2/171	0/100
	1	c.227G>A	Arg76Lys	14/171	9/100
	1	c.369C>T	Thr123Thr	1/171	0/100
	1	c.473G>A	Arg158Gln	1/171	1/100
	2	c.611C>T	Thr204Met	0/171	1/100
	2	c.624C>G	Asp208Glu	5/171	2/100
	3	c.864C>T	Ile288Ile	1/171	0/100
	3	c.1110G>A	Pro370Pro	0/171	1/100
	3	c.1441C>T	Pro481Ser	1/171	0/100
	3	c.1464C>T	Ala488Ala	3/171	1/100

*Novel myocilin mutation.
†Novel myocilin polymorphism.

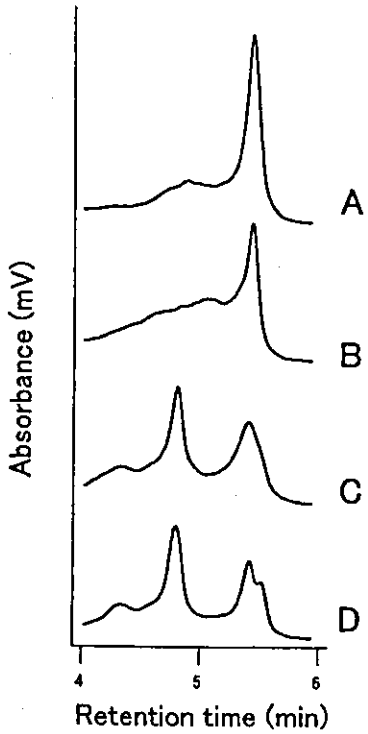

FIGURE 1. DHPLC tracing of the Exon3C region. (A) Normal pattern, control. (B) Pattern shows a broad shoulder, Thr448Pro mutation. (C and D) Patterns show characteristic double peaks indicative of sequence variations. (C) Pro481Ser. (D) Ala488Ala.

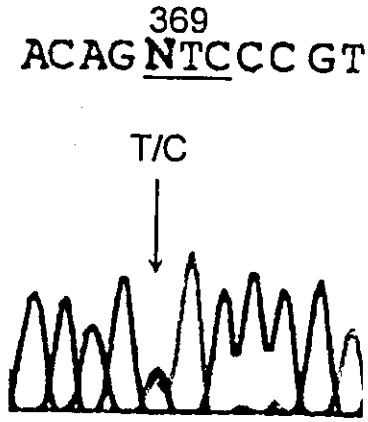

FIGURE 2. Direct sequencing of a POAG patient sample showing Phe369Leu, resulting from a heterozygous transition mutation 1105T>C.

	369
Human	YHGQ**F**PYSW
Monkey	YHGQ**F**PYSW
Bovine	YHGQ**F**PYSW
Pig	YHGQ**F**PYSW
Rat	YHGQ**F**PYAW
Mouse	YHGH**F**PYAW

FIGURE 3. Comparison of amino acids sequence alignment in 6 different myocilin proteins. Phe369Leu affected position is conserved in the 6 species.

performed. After the surgery, the IOP in the left eye has been held between 7 and 11 mm Hg without medication, and visual field defects have not developed.

The proband had one brother and one son. The 51-year-old brother did not have the Phe369Leu mutation, and did not show glaucomatous changes of the optic disc in both eyes. On the other hand, her 22-year-old son had the same mutation and had normal open angles and glaucomatous optic disc changes with a C:D ratio of 0.6 in the right eye and 0.8 in the left eye. However, his Humphrey visual field test showed no apparent glaucomatous changes, and his IOPs were in the low 20s in both eyes without medication. Considering this mutation might be associated with adult-onset glaucoma, he is considered to be at risk for developing visual field defects in the future.

Screening of Individual Patients by Plate Polymerase Chain Reaction Followed by Denaturing High-Performance Liquid Chromatography

A DHPLC tracing from a patient with POAG is shown in Figure 6. In the exon3B region, an abnormal tracing indicative of sequence variation can be seen, which proved to represent a Phe369Leu mutation on direct sequencing.

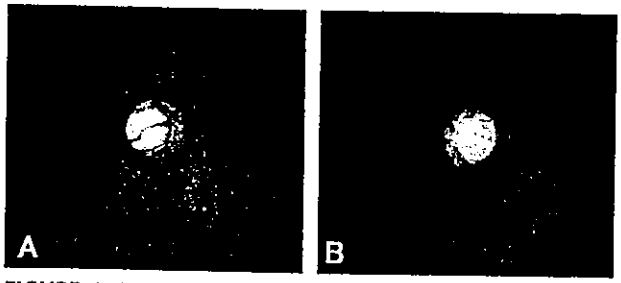

FIGURE 4. Appearance of optic disc of the proband with the Phe369Leu mutation. (A) Right eye. (B) Left eye. Nerve fiber layer defects in the superior and inferior arcades in the left eye were observed.

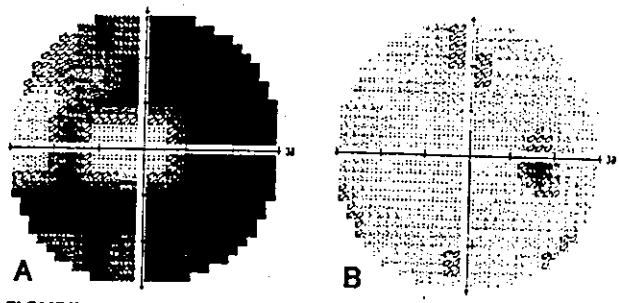

FIGURE 5. Humphrey 30-2 visual fields of the proband with the Phe369Leu mutation. The values of mean deviation and corrected pattern standard deviation were −23.71 dB, 13.22 dB in the left eye (A) and −4.60 dB, 1.96 dB in the right eye (B).

DISCUSSION

Family history of glaucoma is a risk factor for the development of POAG.[25] Glaucoma is a progressive disease and one of the most important causes of blindness throughout the world. Early diagnosis is critical because early treatment can postpone or prevent loss of vision.[26,27] Thus, information identifying a genetic risk of developing the disease would

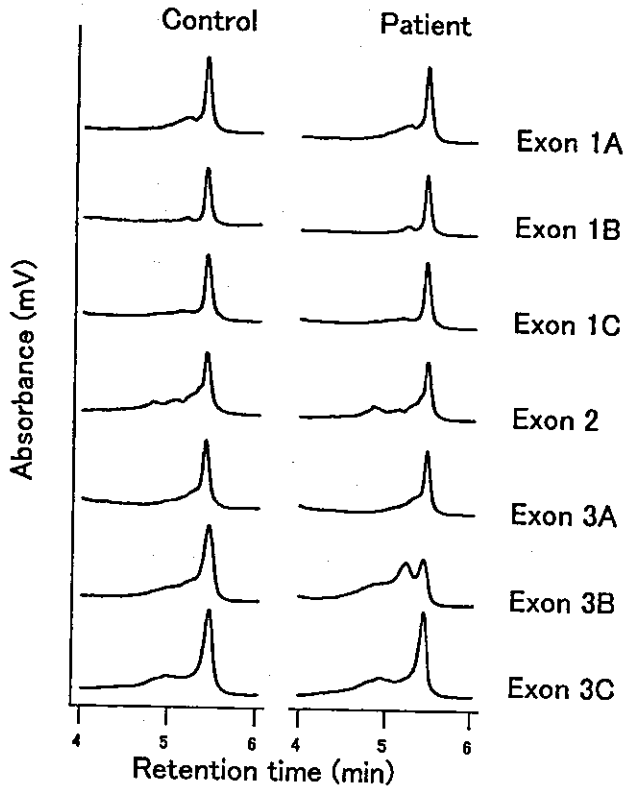

FIGURE 6. A DHPLC tracing for 7 exonic regions in a patient with Phe369Leu. Analysis was performed by 96-well plate PCR followed by DHPLC on patient having a Phe369Leu mutation. An abnormal tracing for the exon3B region is indicative of sequence variation.

permit individuals carrying glaucoma-causing mutations to undergo regular examinations to identify and treat POAG at an early stage.

To detect mutations in the *MYOC* gene, methods such as PCR-DGGE analysis,[28] PCR-SSCP analysis,[22] and direct sequencing[29] have been used in our laboratory. In recent years, automated DHPLC analysis based on a temperature-dependent resolution of heteroduplexes has shown very high sensitivity for detecting mutational changes in nucleotides.[20] Several advantages of DHPLC analysis in screening for nucleotide variations, including some in the *MYOC* gene, have been demonstrated in large population samples.[16,30–33] The superior performance of DHPLC (WAVE® System) over SSCP has been reported because of its high sensitivity and accuracy in detecting mutations, high degree of automation, and low cost of each sample for large-scale mutational screening.[18–21] Furthermore, the use of pooled DNAs with DHPLC analysis is time- and labor-saving.

We have detected *MYOC* mutations by PCR-SSCP in Japanese patients with POAG or NTG.[22] In this study, additional POAG patients were screened using DHPLC, and 4 missense mutations were identified. The prevalence of the *MYOC* mutations was 2.9% in our Japanese POAG patients. We also found one novel mutation, Phe369Leu. The amino acid residue at position 369 is conserved as Phe was found in 5 other species, viz., monkey, bovine, pig, rat, and mouse. Although the Pro481Ser variant was found in a POAG patient and not found in 100 normal subjects, this variant was previously reported in 1 of 100 normal Japanese subjects by Mabuchi et al.[34]

Two other missense mutations, Pro370Leu[35] and Gly367Arg,[36] have been reported in Japanese POAG patients as well as Caucasian patients with POAG, while the mutations of Ile360Asn,[22,23] Ala363Thr,[22] Phe369Leu, and Thr448Pro[24] may be specific to the Japanese. Patients with Gly367Arg, Pro370Leu, Ala363Thr, or Thr448Pro develop POAG before the age of 40 years, while patients with Ile360Asn and Phe369-Leu apparently can develop POAG after reaching that age.

We have also developed a plate-PCR method followed by DHPLC analysis that could analyze a small number of samples. Because all primer sets were designed for the same annealing temperature, PCR amplification was performed under uniform conditions. The same plate was then used for the WAVE® System analysis, which saves time.

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