$p<0.001$ で，多群間で統計学的に有意差がみられた．Fisher ptotected least significant difference(Fisher PLSD)検定で，すべての時間において内服前より有意に眼圧は低下していた($p<0.01$)．眼圧下降は内服後3時間で最大となり，24時間下降効果は維持された．血圧(拡張期および収縮期)や脈拍数に有意の変化はみられなかった．図25は38名の個々の眼圧変化を示した図であるが，AT1遺伝子多型と眼圧効果には関連はなかった．各個々により薬剤効果に差があることがわかる．

アンギオテンシンII受容体ブロッカーは高血圧でない人にはそれほど血圧下降を来さないことが報告されている．したがって，ダイアモックス®が何らかの理由で内服できず，高血圧を合併していない緑内障患者では使用できる可能性がある．現在点眼薬(オルメサルタンメドキソミル)の開発も行われている(フェーズ2)．今回の結果は，緑内障患者で高血圧治療薬としてアンギオテンシンII受容体ブロッカーを内服している患者の眼圧は，実際の眼圧よりも数mmHgほど低いことを念頭に入れておく必要があることを示唆している．問診の時にアンギオテンシン転換酵素(ACE)阻害剤やアンギオテンシンII受容体ブロッカーの内服の有無を知っておく必要がある．

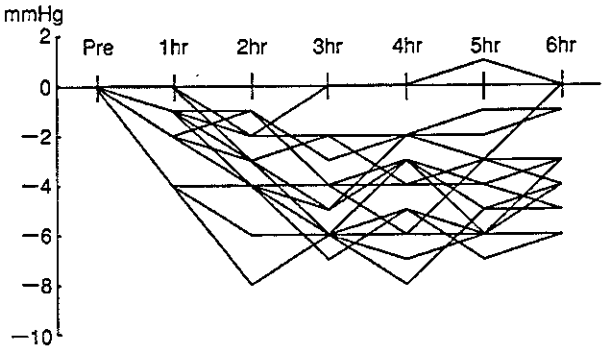

図25　アンギオテンシンII受容体ブロッカー(カンデサルタン)内服による眼圧下降幅．
38名の個々の眼圧下降幅を示す．

VI　遺伝子変異検出パネルの作成

将来の遺伝子診療や個別化医療に備え，Invader法を用いた緑内障遺伝子のミオシリン変異診断パネルのプロトタイプを作成した(http://biotech.nikkeibp.co.jp/news/detail.jsp?newsid=SPC2003063023256)．現在解析できる変異はIle360Asn，Ala363Thr，Gly367Arg，Pro370LeuおよびThr448Proである．

今後は，緑内障発症または進行に関するリスク診断を行う感受性遺伝子多型検出パネルの作成を目指している．例えば，ある人が危険因子を持っているとして，数が多ければそれだけ発症や進行のリスクが高くなることが考えられる．図26は予想図であるが，持っている数によりその危険度は異なるため，将来的には多数症例を検討することにより，リスクの高い範囲を決定できると考えられる．しかしながら，緑内障発症や進行の危険度は危険因子の数だけの問題ではなく，その質(または種類)にもよるので，危険因子の質にもリスク度の順番を決定して行くことが重要と考える．

VII　遺伝子診断の今後の展望

図27は第107回日本眼科学会総会の特別講演で塚原

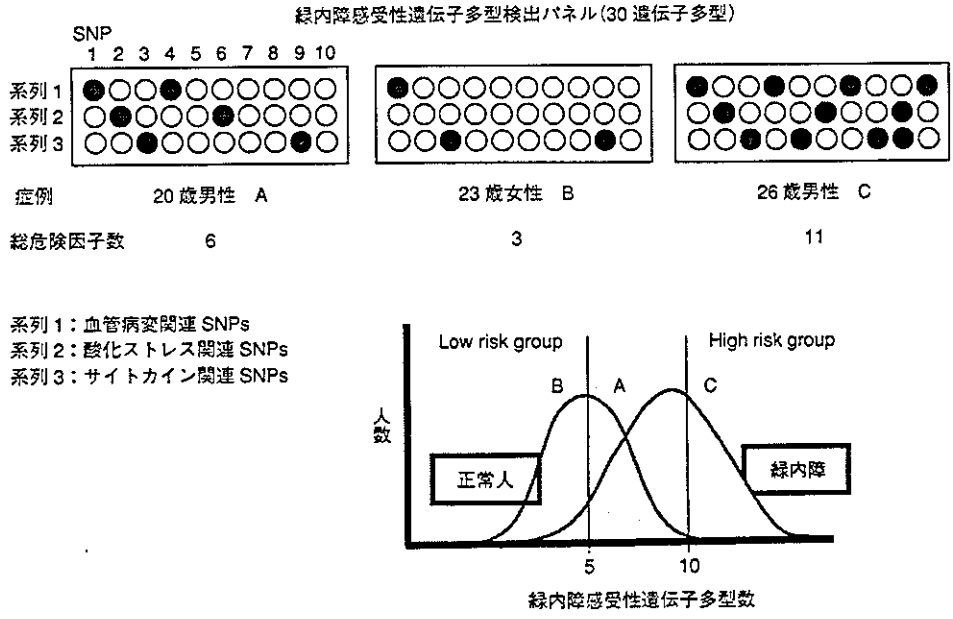

図26　緑内障感受性遺伝子多型検出パネルと緑内障発症リスクの予想．
診断パネルにより危険因子数を検出し(上段)，それに基づき危険度を推定する(下段)．

一次予防：発症危険因子の同定 ⟶ 緑内障感受性遺伝子多型
➡ 危険因子の除去
生活、環境改善(生活習慣病)

二次予防：早期発見、早期治療 ⟶ 緑内障遺伝子変異
➡ 啓蒙活動
スクリーニングシステム

三次予防：進行危険因子の同定 ⟶ 緑内障感受性遺伝子多型
➡ 危険因子の除去
有効な治療法の開発(眼圧下降剤、神経保護剤)

図 27 緑内障による失明予防戦略.
塚原重雄：21世紀の緑内障診療の現状と未来. 日眼会誌 107：745, 2003より改変した. 各個人における危険因子としての遺伝子多型を明らかにすることで, 個別化医療への道が開ける.

重雄名誉教授が述べられている「21世紀の緑内障診療の現状と未来」を参考にゲノム医療における緑内障による失明予防戦略を作成したものである.

一次予防として, 緑内障の発症に関連する危険因子が同定できれば, その因子を除去することにより発症を抑えるか, 発症を遅くすることが可能となる. 最近, スタチン(HMG-CoA還元酵素阻害剤)や他のコレステロールを下げる内服薬を長期に使用することにより緑内障発症の危険を下げることが報告[61]された. 特に, コレステロールが高い人ではより有効であった. スタチンはコレステロールを低下させるだけでなく, 血管保護作用(血管内皮のNO合成酵素の発現増加やAT1受容体作用の抑制)が報告されている. 各個人における危険因子を診断することができれば, それに応じた治療が行われる(個別化医療). 二次予防としては, 早期発見, 早期治療であるが, 緑内障の患者はその20%が医療機関を受診しているに過ぎないことから, 積極的な啓蒙活動を行うことが重要と思われる. 家族歴は高い危険因子なので, 家系内に緑内障を発症している人は, 緑内障遺伝子診断を行うことで, 発症前にその確定診断を受けることができ, 早期治療が可能となる.

三次予防としては, 緑内障を発症した患者の進行を遅らせることであるが, 各個人において, 進行の危険因子がわかれば, それに応じた治療が行われる. 例えば, エンドセリンは緑内障との関連が示唆されているが, エンドセリン関連の感受性遺伝子多型が悪化に関連するとすれば, その危険因子を持った患者はエンドセリンを抑える薬剤の投与が有効である可能性がある[62)63)].

21世紀のゲノム医療の時代における遺伝子診断の意義は, 多因子疾患におけるリスク診断である. 緑内障感受性遺伝子多型の検索は, 各個人のゲノムの多様性, すなわち緑内障になりやすい体質の差や進行しやすい体質の差を検出することになり, その情報は個別化医療にむけて発症の危険性や予防, 予後予測, および将来的には薬剤などの治療法の選択および新しい治療薬の開発につながることが期待される. また, 今回, アンギオテンシンII受容体ブロッカーによる眼圧下降効果に関連する遺伝子多型は発見できなかったが, レニン・アンギオテンシン系多型と関連する緑内障患者はアンギオテンシンII受容体ブロッカーが有効である可能がある. したがって, 今後は既存の内服薬でもスタチンやアンギオテンシンII受容体ブロッカーのように, 緑内障予防や治療に関連する内服薬を個別化医療の中で緑内障治療薬として使用できる可能性が考えられる.

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本論文で記載した遺伝子異常に関する検査(レーベル病におけるミトコンドリアDNA G 3460 A変異，G 11778 A変異，T 14484 C変異の有無とヘテロプラスミー定量検査および開放隅角緑内障におけるミオシリン遺伝子 Ile 360 Asn 変異，Ala 363 Thr 変異，Gly 367 Arg 変異，Pro 370 Leu 変異，Thr 448 Pro 変異)は下記の検査受託会社で提供されている．読者の便宜のために参考までに記載する(株式会社ビー・エム・エル(http：//uwb01.bml.co.jp/test/index.html)．ただし，著者はこの会社と営利的あるいはそれに類似した関係はない．

Comment：大庭 紀雄

第108回日本眼科学会宿題報告「眼科検査診断法：個別化医療の時代にむけての遺伝子診断」は，検査や診断の領域にも新しい時代がやってきたことを感じさせる．今や，evidence-based medicineに準じて医療を標準化しcritical pathwayを活用して効果的に提供する時代であるが，同じ外因に曝されて発症しても病状は個体ごとにさまざまであるし，同じライフスタイルで過ごしても生活習慣病を患う個体もあれば患わない個体もある，同じ疾病を患っても薬物に反応する個体もあれば反応しない個体もある，同じ疾病の有病率が人種や民族によって著しく異なる，といったことは日常茶飯事に経験することである．患者ごとにきめ細かに対応しようという個別化医療は，いわばready-madeからorder-madeへのパラダイムの変革である．こうしたtailored-medicineを進めるために欠かせないのが集団や個人の遺伝情報である．分子生物学の黎明から半世紀，臨床に応用されて成功を収めたのは，Nathansの先天色覚異常(1986年)，Wallaceのレーベル遺伝性視神経症(1988年)，Dryjaの網膜色素変性(1990年)の三つを先駆けとする単一遺伝子病原因遺伝子の発見であった．メンデル遺伝病の全貌が明らかにされて遺伝子診断学が完成するのも遠くはないであろう．一方，新課題の個別化医療の標的は多因子性遺伝病polygenic disease(multifactorial disease)あるいは生活習慣病というカテゴリーのcommon diseaseである．対象を例示すれば，アトピー性眼症，翼状片，加齢白内障，各種緑内障，高血圧，糖尿病，加齢黄斑変性，網膜剝離と重要疾患が目白押しである．こうした広義の生活習慣病はa prioriに，多数の遺伝要因と多数の環境要因とが複雑に絡み合って成立すると想定されるのだが，ヒト白血球抗原(HLA)との関連性が確認されているベーチェット病や原田病を例外として，実体的知識はほとんどない．遺伝要因を把握するための方略として今のところ有力視されているのは，箇々の疾患で関連すると考えられる候補遺伝子の多型(polymorphism)と罹病性(disease susceptibility)との関係性(association)を把握することである．この報告では，原発開放隅角緑内障201例・正常眼圧緑内障234例・健常コントロール236例という多数を試料として候補遺伝子38種類の多型頻度を調査している．個別化医療は今のところスローガンや掛け声が先行し，実践を促すにはデータ不足ではあるが，精力的に取り組まれているから今後迅速に進むのではないだろうか．そして，数千も数万もの遺伝子多型を簡単に調べる分析技術，疾病との関連性を検定するための統計分析技術が開発されるであろう．その日に備えて，患者と健常対照の良質の試料をできるだけ多く集めて保存しておくことが大切だと思う．

Analysis of Porcine Optineurin and Myocilin Expression in Trabecular Meshwork Cells and Astrocytes from Optic Nerve Head

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PURPOSE. To determine the cDNA sequences and analyze the expression of porcine optineurin and myocilin in trabecular meshwork cells (TMCs) and astrocytes from the optic nerve head under normal and experimental conditions.

METHODS. Both porcine optineurin and myocilin were cloned to determine the cDNA sequences. Porcine TMCs and astrocytes were isolated and treated with dexamethasone (500 nM) for 2 weeks, incubated under hypoxic conditions (7% O_2) for 72 hours, or exposed to 33 mm Hg hydrostatic pressure for 72 hours. A 10% mechanical stretch for 24 hours was also performed on TMCs. The expression level of the optineurin and myocilin transcripts was analyzed by real-time quantitative PCR.

RESULTS. The sequences of porcine optineurin and myocilin cDNA were determined, and the expression of both genes was confirmed in both TMCs and astrocytes. Amino acid sequences of porcine optineurin and myocilin were homologous to those of humans by 84% and 82%, respectively, and shared protein motifs and modification sites. The expression of myocilin mRNA by TMCs and astrocytes was increased by 8.0- and 5.5-fold, respectively, after exposure to dexamethasone. In contrast, the expression of optineurin was suppressed to 68% in TMCs and 48% in astrocytes after exposure to dexamethasone. A significant reduction of myocilin expression was observed after 72 hours of incubation under hypoxic conditions in both types of cells, whereas optineurin was not affected. Hydrostatic pressure for 72 hours and mechanical stretching for 24 hours had minimal affects on gene expression of both optineurin and myocilin.

CONCLUSIONS. The high homology of porcine optineurin and myocilin to the comparable human genes indicates that pigs can be used to study changes in gene expression in hypertensive eyes. The alterations in expression of myocilin but not of optineurin under stress suggest that different mechanisms in the phenotype of glaucoma associated with the two genes are involved in development of glaucoma. (*Invest Ophthalmol Vis Sci.* 2004;45:2652–2659) DOI:10.1167/iovs.03-0572

Characteristic degeneration and excavation of the optic nerve head are found in glaucomatous eyes. These changes are considered to be due to ocular hypertension with the intraocular pressure (IOP) continuously more than 21 mm Hg. In contrast, there are patients with normal ocular tension who show glaucomatous changes in the optic nerve head. These patients, in whom there is no evidence of an elevation of IOP at any time, are said to have normal-tension glaucoma (NTG).

Currently three genes—myocilin (*MYOC*),[1,2] cytochrome P4501B1 (*CYP1B1*),[3,4] and optineurin (*OPTN*)[5]—are associated with glaucoma. Optineurin is the most recent gene to be identified and is responsible for 16.7% of families with hereditary NTG.[5] It has been identified and studied by different groups under various names: NRP, NF-κB essential modulator (NEMO)-related protein[6]; FIP-2, adenovirus E3-14-kDa interacting protein 2[7]; Huntingtin interacting protein L (HYPL)[8]; and transcription factor IIIA interacting protein (TFIIIA-INTP).[9] Optineurin is homologous to NEMO, a structural and regulatory subunit of the high molecular weight kinase complex (IKK) that is responsible for the phosphorylation of NF-κB inhibitors.[6]

Some of the functions of optineurin are known. They include inhibition of the tumor necrosis factor (TNF)-α pathway,[7] interaction with transcription factor IIIA,[9] and mediation of the interaction of Huntingtin and Rab8 for regulation of membrane trafficking and cellular morphogenesis.[8] Optineurin is induced by TNF-α and binds to an inhibitor of TNF-α and the E3-14.7-kDa protein.[7]

The optineurin protein contains two leucine zippers (LZs); an N-terminal LZ responsible for the association with Rab8, and a C-terminal LZ required for Huntingtin. The gene is mapped to 10p14 and contains 16 exons encoding a 66-kDa protein. It contains two putative bZIP transcription factor motifs, a C2H2 type zinc finger, and two LZ domains.

Recently, Vittitow and Borrás[10] reported that elevated IOP, and exposure to TNF-α and dexamethasone (DEX) led to an upregulation of optineurin expression in an organ culture system. However, it is still unclear how mutations of the optineurin gene lead to glaucoma.

Another gene associated with glaucoma is myocilin, which is found in 36% of juvenile-onset POAG and 4% of adult-onset POAG.[11–15] Myocilin is a 57-kDa protein that contains motifs homologous to the olfactomedin domain where nearly all mutations in patients with POAG have been identified.[1,11–15]

Pigs and miniature pigs are readily available and have been used for a wide variety of medical studies, including tissue transplantation.[16,17] Because their eyes are similar in size and

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anatomy to human eyes,[18] pigs have often been used to study the aqueous outflow system and the regulation of IOP.

The purpose of this study was to clone both the porcine optineurin and myocilin genes to determine their cDNA sequences, and then to use the sequences to determine the transcriptional response of isolated porcine trabecular meshwork cells (TMCs) and astrocytes from the optic nerve head after exposure to dexamethasone (DEX), increased hydrostatic pressure, hypoxia, and mechanical stretching.

Materials and Methods

Cell Cultures

Pig eyes were obtained within 3 hours of death from a local abattoir. The eyes were disinfected in 0.2% povidone iodine for 10 minutes followed by soaking in 70% alcohol for 30 seconds. The eyes were washed several times in phosphate-buffered saline (PBS) and cut into halves along the equator.

After the lens and iris were removed from the anterior half, the trabecular tissue was trimmed from the cornea at the Schwalbe's line and then from the sclera, as described.[19,20] The optic nerve head was separated from the sclera and surrounding tissues. The prelaminar region was dissected from the optic nerve head and cut into three or four pieces.[21,22] The trabecular and prelaminar tissues were placed separately in 35-mm plastic Petri dishes in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco, Grand Island, NY) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% antibiotic-antimycotic (Invitrogen-Gibco).

The tissues were incubated for 1 to 2 weeks at 37°C in humidified 5% CO_2 and 95% air until cells migrated from the tissue onto the surface of the culture dish. Cells were isolated, and fourth-passage cells were obtained for experimental use. The cells that migrated from the optic nerve head were confirmed to be astrocytes by immunostaining with anti-glial fibrillary acidic protein (GFAP), a protein marker for astrocytes (Sigma-Aldrich).

Cloning of Porcine Optineurin cDNA

mRNA was isolated from cultured TMCs using mRNA isolation kits (MicroPoly(A)Pure; Ambion, St. Austin, TX). Primers (sense primer, 5′-ATGTCCCATCAACCTCTGAGCT-3′, antisense primer 5′-TGTCCTCGGCTCCTCTTTGAAA-3′) were designed to include the conserved sequences for human, mouse (Discovery System; Celera, Gaithersburg, MD), and rat to amplify the open reading frame of porcine optineurin mRNA using a commercial system (Superscript One-Step RT-PCR System; Invitrogen-Gibco), according to the manufacturer's protocol.

The PCR products were cloned into a TA cloning vector (pDrive; Qiagen, Valencia, CA) using a PCR Cloning Kit (Qiagen), and the inserts were sequenced using a fluorescent dideoxynucleotide automated sequencer (CEQ2000XL DNA Analysis System; Beckman-Coulter, Fullerton, CA). The missing 3′ and 5′ ends of the cDNAs were amplified using the 3′ and 5′ rapid amplification of cDNA ends (RACE) method (Marathon cDNA Amplification Kit; BD Biosciences-Clontech, Palo Alto, CA). The full-length cDNA sequence of porcine optineurin can be obtained from GenBank under accession number AF513722 (http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

Cloning of Porcine Myocilin cDNA

The same mRNA used for optineurin cDNA cloning was used for myocilin cDNA amplification. The sense primer, 5′-ATGCCAGCTS(G/C)TCCAGCTGCT-3′, and antisense primer, 5′-GACCATGTTGAAGTTGTCCCA-3′, were designed to include the conserved sequence of human, mouse, rat, and bovine myocilin and to amplify the open reading frame of porcine myocilin mRNA, using the RT-PCR system (Superscript One-Step RT-PCR System; Invitrogen-Gibco). The PCR products were cloned into a TA cloning vector (TA Cloning Kit; Invitrogen, San Diego, CA), and the inserts were sequenced. The missing 3′ and 5′ ends of the cDNA were amplified using the RACE method (Marathon cDNA Amplification Kit; BD Biosciences-Clontech). The full-length cDNA sequence of porcine myocilin can be obtained from GenBank under accession number AF350447.

Sequence Analysis of Porcine Optineurin and Myocilin

Amino acid sequences of both optineurin and myocilin were analyzed for domain structure and potential protein modification sites. The PROSITE scanning tool[23] (http://www.nhri.org.tw/prosite/; provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland) was used to scan the optineurin protein sequence for the occurrence of patterns and profiles stored in the PROSITE database. Potential glycosylation and phosphorylation sites were predicted by the program developed by Hansen et al.,[24] and Blom et al.,[25] respectively. Sequence homology was determined by a sequence-analysis program (Omiga 2.0; Accelrys, San Diego, CA).

Stress Experiments for Optineurin and Myocilin

All stress experiments were performed using fourth-passage TMCs and astrocytes from three different porcine eyes. For the DEX treatment, DEX stock solution (50 mM DEX/dimethyl sulfoxide) was added to cultured TMCs and astrocytes at a final concentration of 500 nM. The culture medium was replaced every 3 days and maintained for 2 weeks. For control, cultured cells were treated with dimethyl sulfoxide alone.

To examine the effect of hypoxia, both types of cultured cells were incubated in 7.0% O_2 and 5% CO_2, in a multiple gas incubator (model 9200; Wakenyaku, Kyoto, Japan) for 12, 24, 48, or 72 hours. Control cells were incubated for the same times in 5% CO_2 and 95% air in a standard CO_2 incubator.

To examine the effects of hydrostatic pressure, we exposed both types of cultured cells to a hydrostatic pressure of 33 mm Hg above atmospheric pressure for 12, 24, 48, or 72 hours in a CO_2 incubator, using the system illustrated in Figure 1. The culture flasks were filled with the medium and capped with a silicon stopper to prevent leakage. The height of the reservoir containing the medium was adjusted to control the pressure in the flask. For gas exchange, the medium was circulated with a peristaltic pump (Eyela, Tokyo, Japan), and the pressure was monitored with a pressure gauge (model PG-208; Copal Electronics, Tokyo, Japan). Control cells were exposed to hydrostatic pressure of 3 mm Hg above atmospheric pressure for 12, 24, 48, and 72 hours.

To examine the effects of mechanical stretching, cultured porcine TMCs were transferred onto a 10-cm^2 collagen-coated silicon chamber (S.Tec, Osaka, Japan). The silicon chamber had a 100-μm-thick transparent bottom, and the side walls were 1.5-mm thick to prevent narrowing at the bottom center. The silicon chamber was then attached to a stretching apparatus for a 10% linear stretch for 24 hours in a standard CO_2 incubator. Control cells were plated onto a collagen-coated silicon chamber without the stretching for the same amount of time.

Optineurin and Myocilin Transcript Analysis

Total RNA was isolated from cultured cells exposed to stimuli or stresses (RNAzol B; Tel-Test, Friendswood, TX). The total RNA was reverse transcribed (Superscript First Strand Synthesis System for RT-PCR; Invitrogen-Gibco) according to the manufacturer's protocol. Real-time quantitative PCR was performed to determine the optineurin, myocilin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript with a sequence-detection system (GeneAmp 5700; Applied Biosystems, Inc. [ABI], Foster City, CA). PCR reactions were performed in 50 μL of reaction mixture containing 25 μL master PCR mix (SYBR Green PCR Master Mix; ABI), 5 pM primer pairs, and 1 μL cDNA samples. To measure myocilin transcript, 4 μL cDNA samples was used because of lower expression. The 18S ribosomal RNA gene was used as

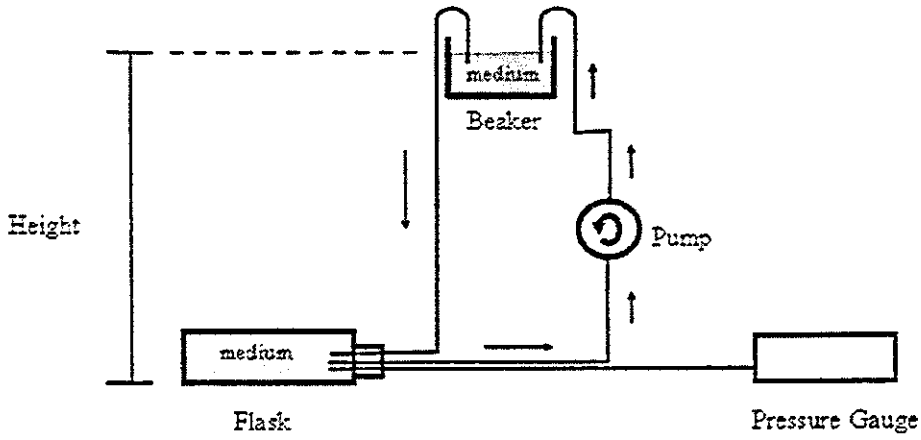


FIGURE 1. System used for hydrostatic pressure experiments. The culture flask was filled with medium and capped with a silicon stopper. The height of the reservoir with the medium was adjusted to maintain the pressure in the flask. For gas exchange, the medium was circulated by a peristaltic pump, and 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′-GGTCATTCCGGCAGTGAA-GAA-3′ and antisense primer 5′-ACGCCGTACTTGCCAGTGATT-3′ were used.

For amplification of optineurin cDNA, sense primer 5′-GACCCA-CAACAGGCTTCTTCA-3′ and antisense primer 5′-TCTGCCATTTC-CAGCTTTCC-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.

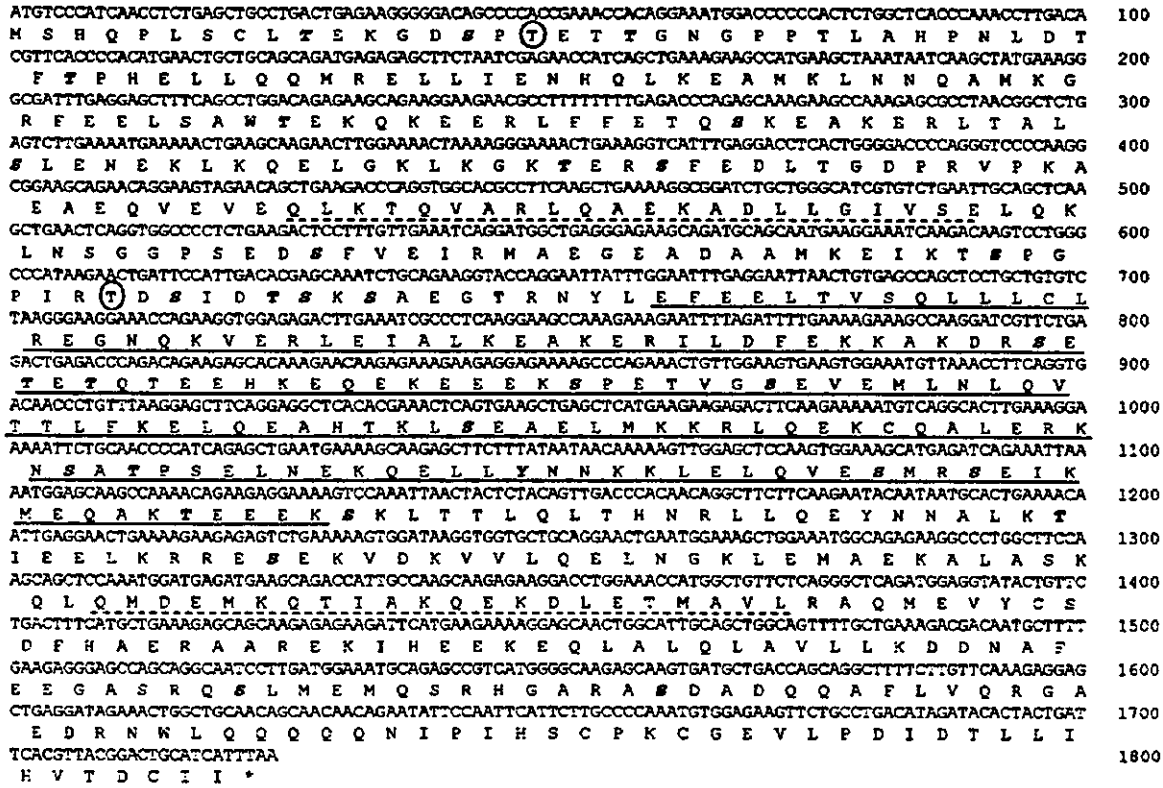
DISCUSSION

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



B

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.

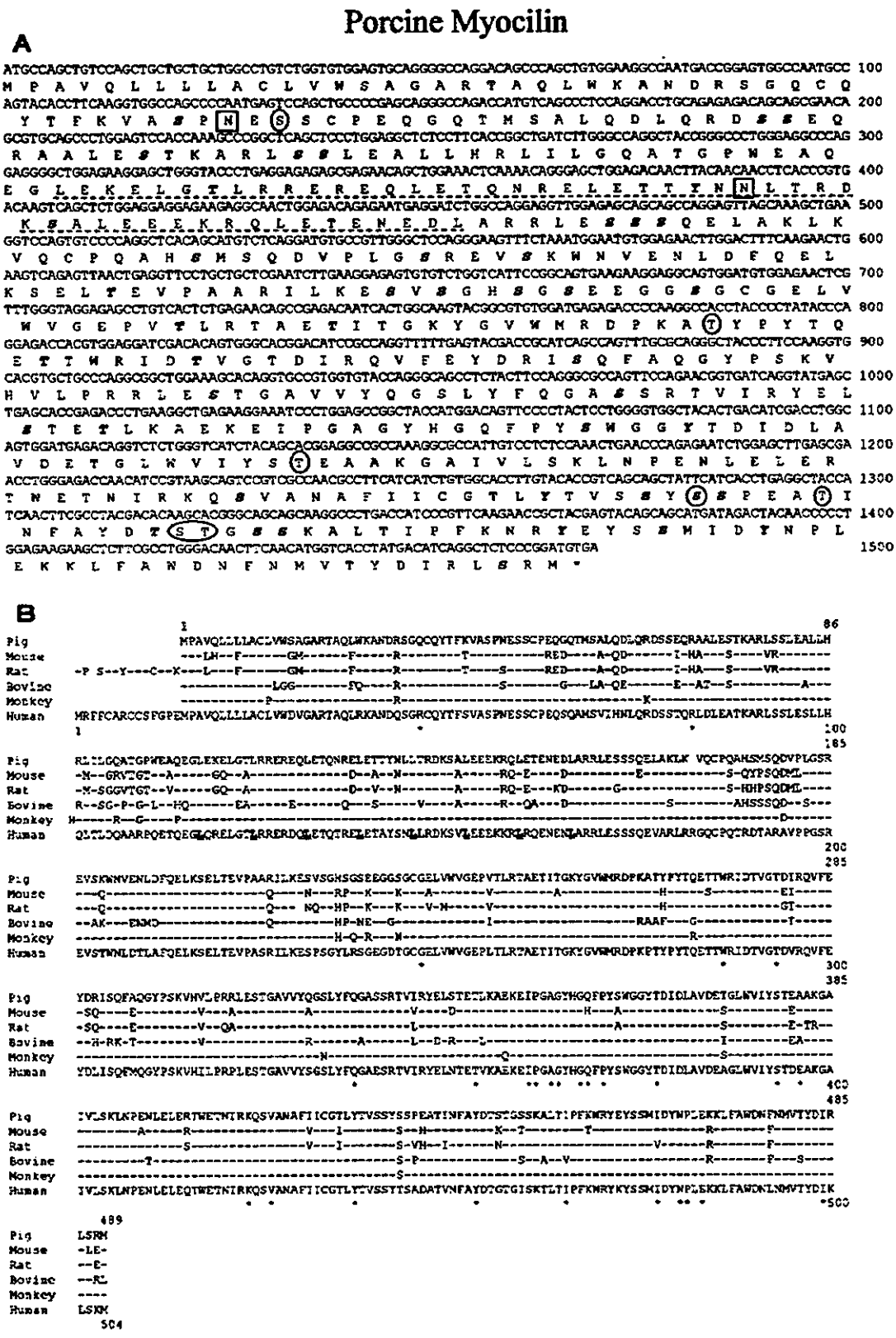


FIGURE 3. Nucleotide sequence and deduced amino acid sequence of porcine myocilin and comparison of porcine myocilin amino acid sequences with those of other species. (A) Porcine myocilin is composed of 489 amino acids. *Circles*: O-glycosylation sites; *squares*: predicted N-glycosylation sites; *dashed 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.

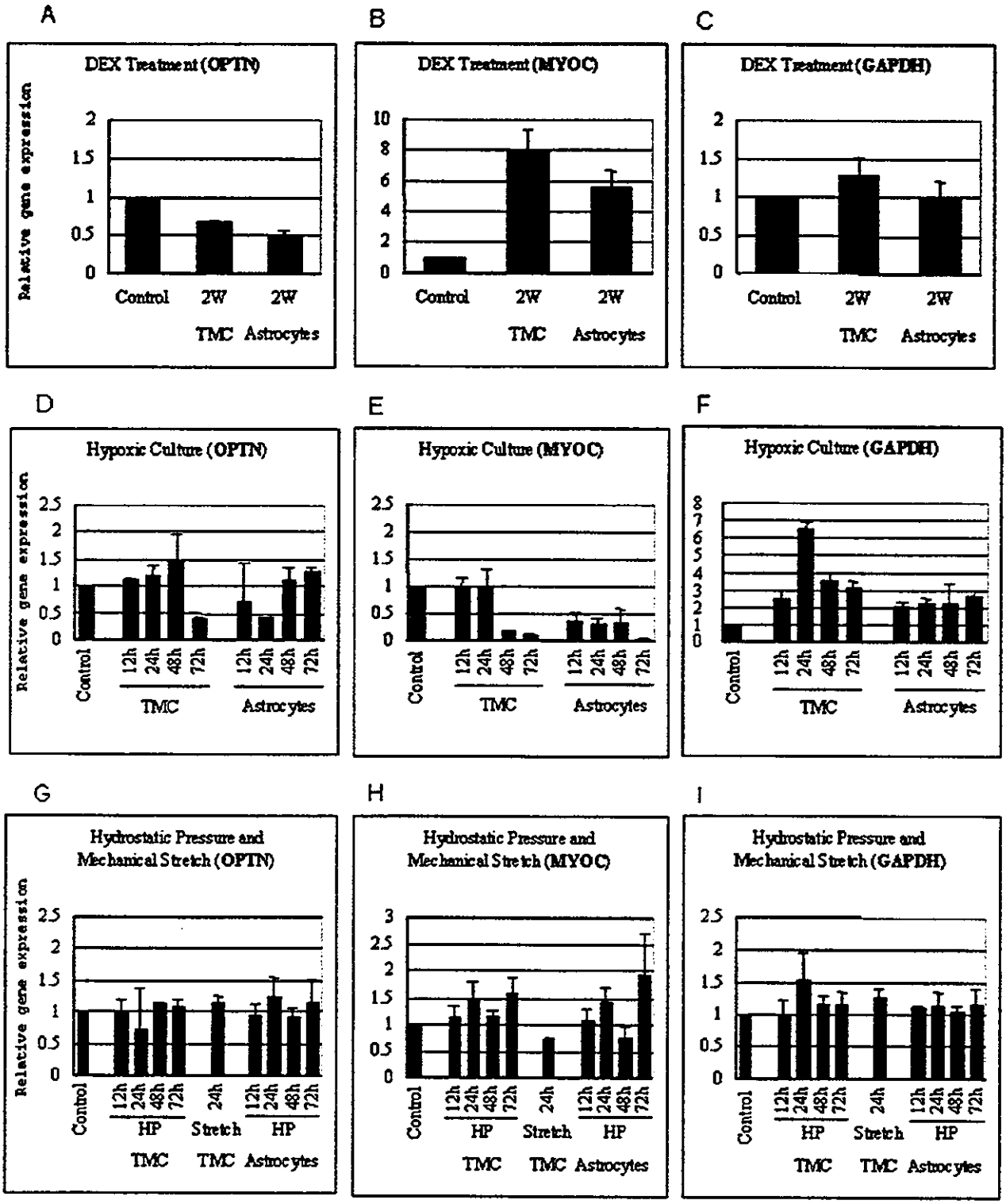


FIGURE 4. Quantitative PCR analysis of optineurin and myocilin under various conditions. The relative gene expression of optineurin, myocilin, and GAPDH is shown for each stimulus or stress condition. Expression level for control cells are shown as 1. (A–C) DEX treatment (500 nM for 2 weeks); (D–F) hypoxia (7.0% O_2); (G–I) hydrostatic pressure (33 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-

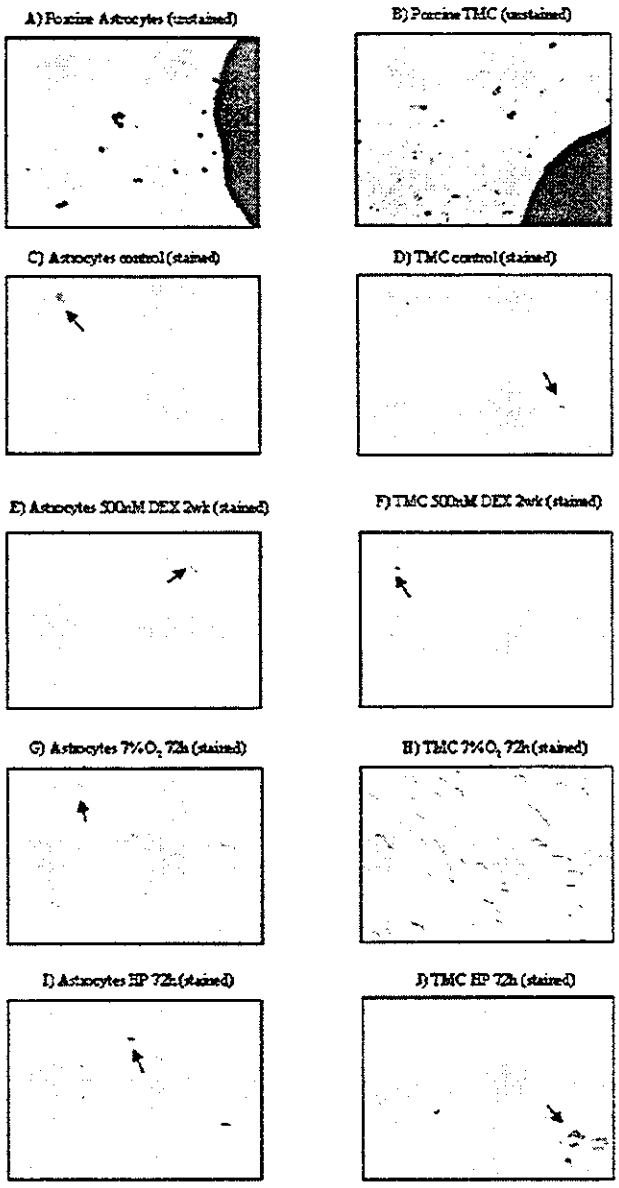


FIGURE 5. Trypan blue staining of TMCs and astrocytes in the various experimental conditions. Trypan blue staining was performed on primary porcine cells to determine cell death caused by stress and stimulation. The stain was added to the culture medium and incubated for 5 minutes before observation. 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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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.

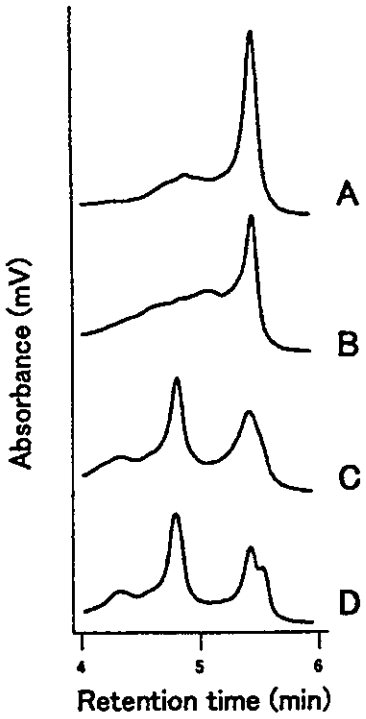

FIGURE 1. DHPLC tracing of the Exon3C region. (A) Normal pattern, control. (B) Pattern shows a broad shoulder, Thr448Pro mutation. (C and D) Patterns show characteristic double peaks indicative of sequence variations. (C) Pro481Ser. (D) Ala488Ala.

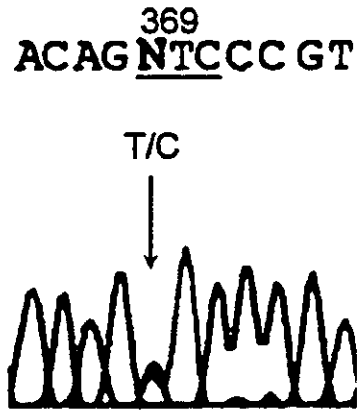

FIGURE 2. Direct sequencing of a POAG patient sample showing Phe369Leu, resulting from a heterozygous transition mutation 1105T>C.

	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.

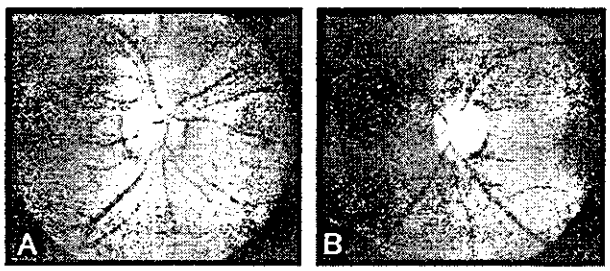

FIGURE 4. Appearance of optic disc of the proband with the Phe369Leu mutation. (A) Right eye. (B) Left eye. Nerve fiber layer defects in the superior and inferior arcades in the left eye were observed.

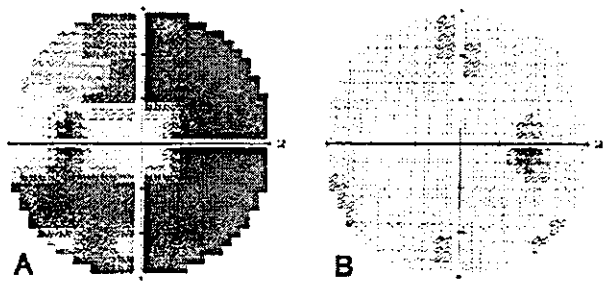

FIGURE 5. Humphrey 30-2 visual fields of the proband with the Phe369Leu mutation. The values of mean deviation and corrected pattern standard deviation were −23.71 dB, 13.22 dB in the left eye (A) and −4.60 dB, 1.96 dB in the right eye (B).

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

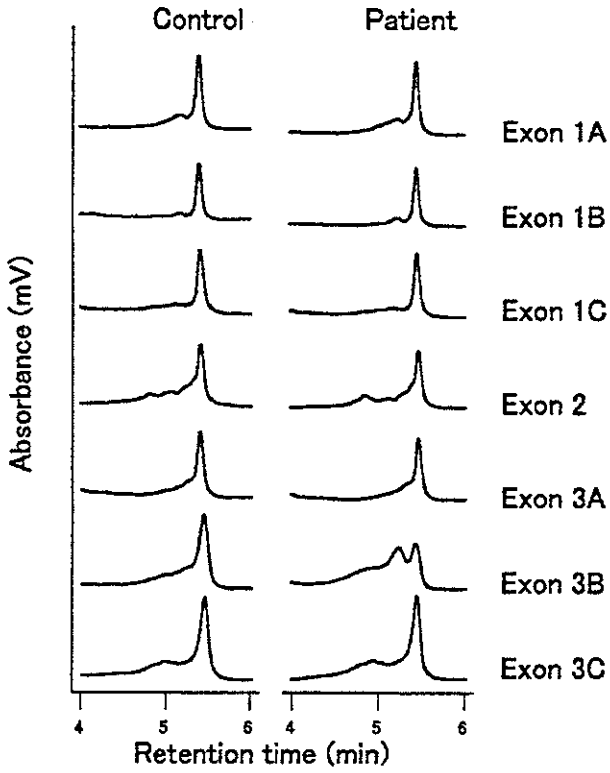

FIGURE 6. A DHPLC tracing for 7 exonic regions in a patient with Phe369Leu. Analysis was performed by 96-well plate PCR followed by DHPLC on patient having a Phe369Leu mutation. An abnormal tracing for the exon3B region is indicative of sequence variation.

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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