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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.

Key Words: myocilin, denaturing high-performance liquid chromatography, primary open-angle glaucoma, Japanese, mutation

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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.

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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			

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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	Frequency 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.

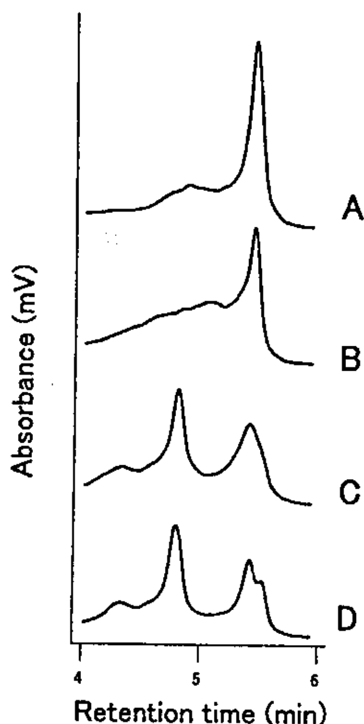

FIGURE 1. DHPLC tracing of the Exon3C region. (A) Normal pattern, control. (B) Pattern shows a broad shoulder, Thr448Pro mutation. (C and D) Patterns show characteristic double peaks indicative of sequence variations. (C) Pro481Ser. (D) Ala488Ala.

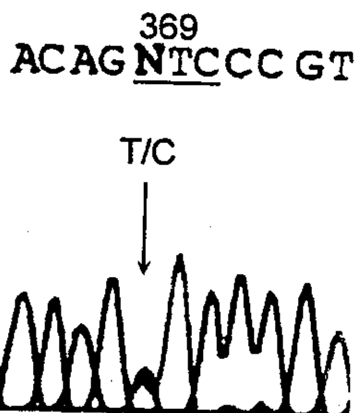

FIGURE 2. Direct sequencing of a POAG patient sample showing Phe369Leu, resulting from a heterozygous transition mutation 1105T>C.

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	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.

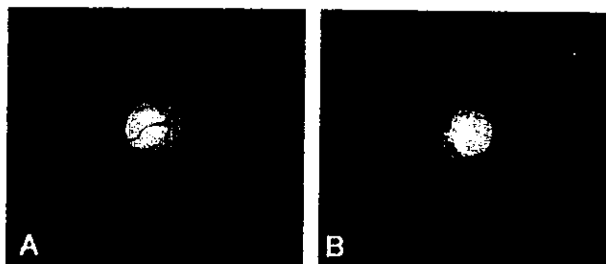

FIGURE 4. Appearance of optic disc of the proband with the Phe369Leu mutation. (A) Right eye. (B) Left eye. Nerve fiber layer defects in the superior and inferior arcades in the left eye were observed.

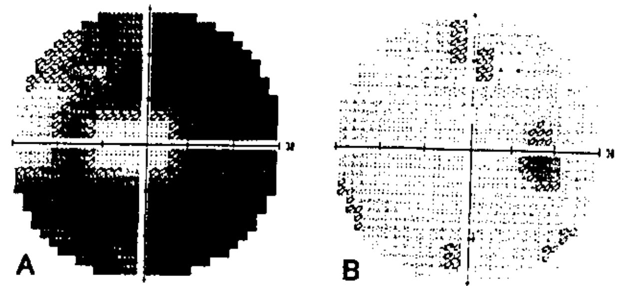

FIGURE 5. Humphrey 30-2 visual fields of the proband with the Phe369Leu mutation. The values of mean deviation and corrected pattern standard deviation were −23.71 dB, 13.22 dB in the left eye (A) and −4.60 dB, 1.96 dB in the right eye (B).

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

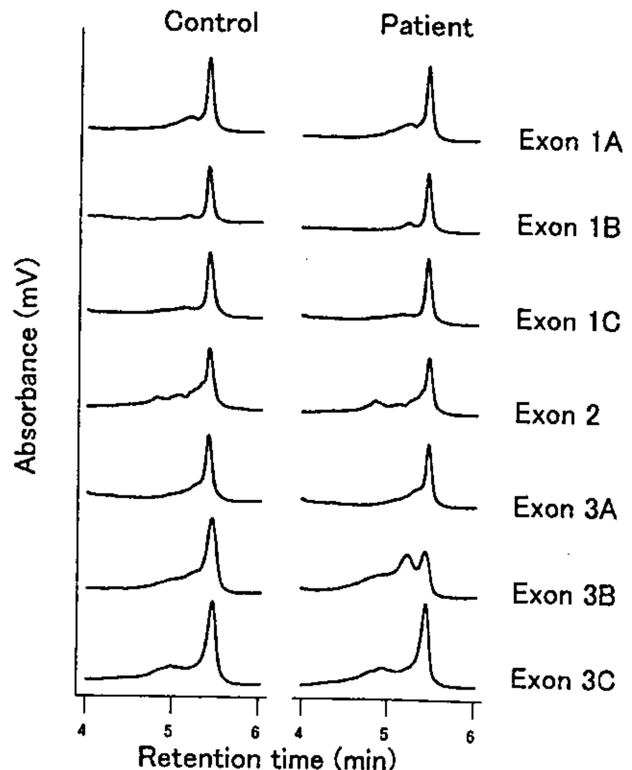

FIGURE 6. A DHPLC tracing for 7 exonic regions in a patient with Phe369Leu. Analysis was performed by 96-well plate PCR followed by DHPLC on patient having a Phe369Leu mutation. An abnormal tracing for the exon3B region is indicative of sequence variation.

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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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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 (Ampli*Taq* 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: CGCCCGCCGCGCCCCGCGCCCGCC.

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′-AGGCAATAGGTTTTGAGGGCCAT-3′, and the reverse primer, 5′-GTAGTGGGCCCTGCACCTTCT-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′-AAGTCGAGTATGGGGACCCCCCGTTAA-3′, and the reverse primer, 5′-CCCCAGTGTGTGGCCATATCTTCTT-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′-GCTGAGAAGATGAAG-GAAAAGTC-3′, and the reverse primer, 5′-CCTCTACATGGCCCT-GTCCT-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 (%)				Genotype Frequency (%)			Genotype Frequency (%)			Allele Frequency (%)		
		G/G	G/A	A/A	P*	G/G	G/A + A/A	P*	G/G + G/A	A/A	P†	G	A	P*
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)	
		T/T	T/A	A/A	P*	T/T	T/A + A/A	P*	T/T + T/A	A/A	P†	T	A	P*
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.[15] 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.

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

Phenotype	n	Genotype Frequency (%) 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)	

Phenotype	n	Genotype Frequency (%) 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)	

Phenotype	n	Genotype Frequency (%) 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.

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.[40] Alternatively, the c.412G→A polymorphism may be linked to another unknown gene that lies near the *OPTN* gene.[41]

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

	−857C→T C/C(%)					C/T + T/T (%)			
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**
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)		P^*
		G/G	G/A + A/A	
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)		P^*
		T/T	T/A + A/A	
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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Early-Onset Macular Degeneration with Drusen in a Cynomolgus Monkey (*Macaca fascicularis*) Pedigree: Exclusion of 13 Candidate Genes and Loci

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Purpose. To describe hereditary macular degeneration observed in the cynomolgus monkey (*Macaca fascicularis*), which shares phenotypic features with age-related macular degeneration in humans, and to test the involvement of candidate gene loci by mutation screening and linkage analysis.

Methods. Ophthalmic examinations with fundus photography, fluorescein angiography (FA), indocyanine green angiography (IA), electroretinography (ERG), and histologic studies were performed on both affected and unaffected monkeys in the pedigree. The monkey orthologues of the human *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* genes were cloned and screened for mutations by single-strand conformation polymorphism (SSCP) analysis or denaturing high-performance liquid chromatography (DHPLC) and direct sequencing in six affected and five unaffected monkeys from the pedigree and in six unrelated, unaffected monkeys. Subsequently, 13 human macular degeneration loci including these five genes were analyzed to test for linkage with the disease. Nineteen affected and seven unaffected monkeys in the pedigree were analyzed by using human microsatellite markers linked to the 13 loci.

Results. Yellowish white spots were observed in the macula and fovea centralis, and in some cases the spots scattered to the peripheral retina along the blood vessels. FA showed hyperfluorescence corresponding to the dots except in the foveola. No anomalies were found by IA and ERG. Histologic studies demonstrated that the spots were drusen. Mutation analysis of the *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* genes identified a few sequence variants, but none of them segregated with the disease. Linkage analysis with markers linked to these five genes and an additional eight human macular degeneration loci failed to establish linkage. Haplotype analysis excluded the involvement of the 13 candidate loci for harboring the gene associated with macular degeneration in the monkeys.

Conclusions. Significant homology was identified between monkey and human orthologues of the five macular degeneration genes. Thirteen loci associated with macular degeneration in humans or harboring macular degeneration genes were excluded as causal of early-onset macular degeneration in the monkeys. It is likely that none of these loci, but rather a novel gene, is involved in causing the observed phenotype in this monkey pedigree. (*Invest Ophthalmol Vis Sci.* 2005;46:683–691) DOI:10.1167/iovs.04-1031

The inherited macular dystrophies comprise a heterogeneous group of blinding disorders characterized by central visual loss and atrophy of the macula and underlying retinal pigment epithelium (RPE).[1] The complexity of the molecular basis of monogenic macular disease is being elucidated through identification of many of the disease-causing genes.[2-8] Because of limitations associated with studies in humans, nonhuman species with phenotypes similar to human macular degeneration have been used as model systems to study these diseases. Rodent models generated by altering the genes homologous to the disease-causing genes in humans are most extensively used in such studies; however, rodents do not have a defined macula and, hence, the clinical symptoms observed in humans with macular degeneration cannot be fully replicated.[9-11] Because the macula is found only in primates and birds, a monkey model of macular degeneration would be extremely valuable for studies elucidating the mechanism and etiology underlying these diseases. A primate model for macular degeneration is much needed to develop sensitive diagnostic techniques and potential therapeutic strategies to cure or prevent the disease. Furthermore, such models are of particular value if their genetic basis is understood.

Macular degeneration in monkeys was first described by Stafford in 1974.[12] He reported that 31 (6.6%) of eyes of elderly monkeys showed pigmentary disorders and/or drusen-like spots. In 1978, El-Mofty et al.[13] reported a high incidence (50%) of maculopathy in a closed rhesus monkey colony at the

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Caribbean Primate Research Center of the University of Puerto Rico. The latest report from the center states that specific maternal lineages have a statistically significant higher prevalence of drusen.[14] Although they suspected the involvement of hereditary factors, genetic analysis of the macaque population has not been reported.

We have reported a high incidence of macular degeneration in one of the cynomolgus monkey (*Macaca fascicularis*) colonies at the Tsukuba Primate Center.[15,16] This macular degeneration originated from one affected male monkey, which showed phenotypic characterization of macular degeneration. The disease affects the central retina specifically, with yellowish white dots in the macula and lipofuscin deposits in the RPE, consistent with the phenotype observed in the early stages of age-related macular degeneration (AMD). These symptoms appear at the age of ~2 years and progress slowly throughout life. Mating experiments have demonstrated that this familial macular degeneration is segregating as an autosomal dominant trait.[17]

AMD is currently considered a multifactorial disorder involving both environmental and genetic factors. Recent studies have substantiated the evidence for AMD as a complex genetic disorder in which one or more genes contribute to an individual's susceptibility to the development of the disease.[18-20] To date, full-genome scan studies have indicated that some regions of the genome harbor AMD-predisposing genes.[21,22] However, most genes associated with susceptibility to AMD have not been identified, presumably because of a complex pattern of inheritance, late age of onset, and difficulties in obtaining large pedigrees for standard linkage analysis. Genes implicated in monogenic macular dystrophies that occur earlier in life with a clear pattern of inheritance have been considered as good candidates for susceptibility to AMD.[23-26] To date, 15 macular degeneration genes have been linked or cloned for human macular degeneration (RetNet; http://www.sph.uth.tmc.edu/Retnet/home.htm; provided in the public domain by University of Texas Houston Health Science Center, Houston, TX). However, with the exception of *ABCA4*, none of these genes has shown a convincing association with AMD.

Because the monkey macular degeneration model we present here shares phenotypic similarities with the early stages of AMD, the identification of the gene involved in this monkey pedigree may provide critical clues to the understanding of the mechanism of AMD. In this study, monkey orthologues of the human genes responsible for Stargardt macular degeneration 1 (*ABCA4*),[2] Best macular degeneration (*VMD2*),[3,7] Doyn honeycomb dystrophy (*EFEMP1*),[4] Sorsby fundus dystrophy (*TIMP3*),[5] and Stargardt macular degeneration 3 (*ELOVL4*)[6,8] were cloned and screened for mutations in the affected monkeys. Subsequently, 13 human macular degeneration loci, including these five genes, were analyzed to test for linkage with the disease in the pedigree. During this process, we evaluated the nature and utility of human microsatellite markers in the cynomolgus monkey for linkage studies. This article also describes the gene structure and evolutionary conservation of the five human macular degeneration genes in the cynomolgus monkey.

Materials and Methods

Maintenance of Monkeys

The cynomolgus monkeys in the pedigree with macular degeneration were reared at the Tsukuba Primate Center for Medical Science (National Institute of Infectious Diseases; Tokyo, Japan). All monkeys were treated in accordance with the rules for care and management of animals at the Tsukuba Primate Center[27] under the Guiding Principles for Animal Experiments using Non-Human Primates formulated and enforced by the Primate Society of Japan (1986). All experimental procedures were approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases of Japan. These animal protocols fulfill the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Clinical Studies

Fundus photographs, fluorescein angiography (FA), and indocyanine green angiography (IA) were performed with a fundus camera (TRC50; Topcon, Tokyo, Japan) in animals under anesthesia. Electroretinography (ERG) was recorded in four affected and six normal monkeys with a white/color LED stimulator and contact lens electrode (LS-W; Mayo, Aichi, Japan). After 20 minutes of dark adaptation, rod ERG, combined ERG, and oscillatory responses were recorded, and single-flash cone response and 30-Hz flicker ERG were recorded after 10 minutes of light adaptation. The stimulus and recording conditions conformed to the standards for clinical electroretinography recommended by the International Society for Clinical Electrophysiology of Vision.[28]

Genomic DNA and RNA Isolation

Peripheral blood was collected from 19 affected and 11 unaffected monkeys from the pedigree (Fig. 1, asterisks, pound signs) and an

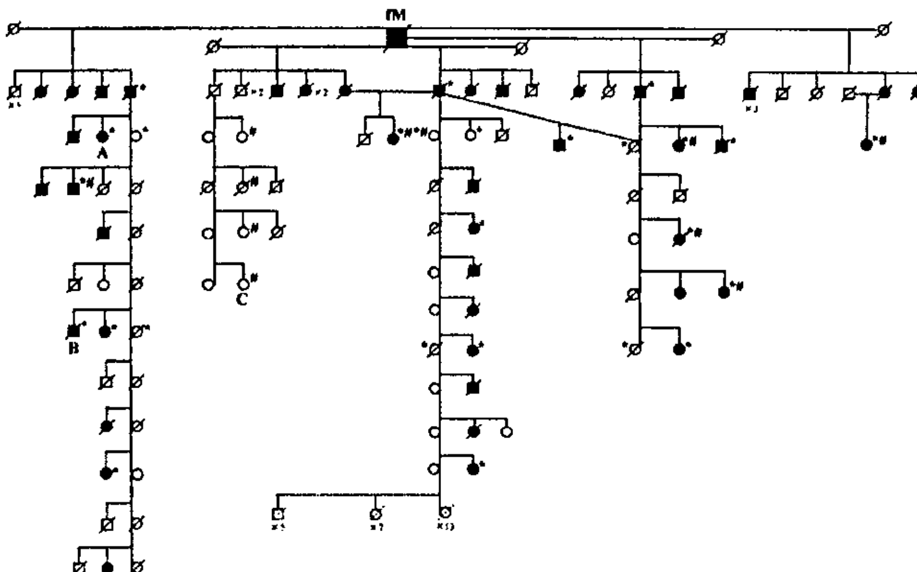

Figure 1. Edited version of the monkey pedigree with macular degeneration: fM, the founder breeding male monkey with typical macular degeneration, is shown with five healthy mates arrayed horizontally. The first-generation offspring are also arrayed horizontally. The breeding members from each branch of the first generation offspring are arrayed vertically with their mates and progeny. Monkeys used for *linkage analysis and #mutation screening are marked.

Table 1. Primer Sets Used for Cloning of the Monkey Homologues

Gene	Amplified Region	Amplified Name	Forward Primer	Position	Name	Reverse Primer	Position	Size (kb)
VMD2	Exon 1	P1F	GACCAGAAACCAGGACTGTTGA	Intron	P1R	GAACTCGCCATATAGCAGCTT	Exon 2	2.1
	Exon 2	P2F	GCTCTGACCAGGGTCTCTGA	Intron	P3R	CCGCACCTTTCCCTGAACTA	Intron	4.5
	Exon 3	P3F	CTAGACCTGGGGACAGTCTCA	Intron	P3R	CCGCACCTTTCCCTGAACTA	Intron	0.3
	Exon 4-5	P4F	CACGGAAGAACAACAGCTGA	Exon 3	P5R	ACACCAGTGGGATACTAATCCAG	Exon 6	2.3
	Exon 6	P6F	GCCAGGAATGGACCATGAGTA	Intron	P6R	GAGCCACTTAGCCTCTAGGTGA	Intron	0.3
	Exon 7-8	P7F	CCTGGAGCATCCTGATTTCA	Intron	P8R	TGAGGCCTCCCTACAGAACA	Intron	2.3
	Exon 9	P9F	TGGCAGAGCAGCTCATCA	Exon 8	P9R	AGCTTCCAGGCCTTGTTG	Exon 10	3.0
	Exon 10	P10F	AAGGGAGAAGGCCAGGTGTT	Intron	P10R	TTTCCTGTAGTGCTTGGGTACTA	Intron	1.2
	Exon 11	P11F	TGCCCTCCTACTGCAACATT	Intron	P11R	ATGCAATGGAGTGTGCATTA	Intron	1.1
EFEMP1	Exon 1	P1F	TTCTAGAACCCTCTGGTCTCTGA	Intron	P1R	CCCTTTCTTAACAGCAAGCTAAC	Intron	0.9
	Exon 2	P2F	GATTGGAAGTTGAGTATGGTGGA	Intron	P2R	CATTCTAGGGATAATGTGGTACCAA	Intron	1.3
	Exon 3-4	P3F	AAGATGGTACTGGGCAACTGTAC	Intron	P4R	ACATCTGTAGAGTAGCTTGACAGCA	Intron	1.4
	Exon 5	P5F	CTACACAGGCTAGAGGAATATGATCA	Intron	P5R	GACACAGGATTTAAGTAACTTGCTCA	Intron	1.3
	Exon 6-7	P6F	CACTGAATGGCATGAACATTG	Intron	P7R	TAGAACAGAATTCCCATGGGTAA	Intron	1.6
	Exon 8	P8F	AATAGGACAAGAAGCCAGATCTCT	Intron	P8R	TTCCTGGTTAAAACTAAATACCTAACA	Intron	0.4
	Exon 9-10	P9F	AACAGATGAACAATAGGTGCTTGA	Intron	P10R	TATCTATCTGGCAGTGTTACCAAGA	Intron	0.9
	Exon 11	P11F	GTATTAGACAAGGGATAAGAGCCAA	Intron	P11R	CAGAGGTTATGCATATATGCTGTGA	Intron	1.7
TIMP3	Exon 1	P1F	CCCAGCGCTATATCACTCG	Intron	P1R	AGCCACTGTGAGTTTCCTCTG	Intron	0.7
	Exon 2	P2F	CAATGGCTCTAACAGGAGAAGTAG	Intron	P2R	CTTGACCAAGGTCTCATGGTTA	Intron	0.8
	Exon 3-4	P3F	TCCAGTTCCAGCTGCATTG	Intron	P4R	AGTTAGTGTCCAAGGGAAGCT	Exon 5	2.6
	Exon 5	P5F	ATGTACCGAGGCTTCACCAA	Exon 3	P5R	AGGTGAGCTAAACACTATTCTGGA	Intron	3.5

additional six unrelated normal monkeys, and genomic DNA was extracted (QIAamp DNA Blood Maxi Kit; Qiagen, Valencia, CA). A normal monkey outside the pedigree was killed for bilateral eye enucleation, and enucleated eyes were immersed and stored in RNA-stabilization solution (RNAlater; Ambion, Austin, TX) at −80°C until RNA isolation. After thawing on ice, the eyeballs were dissected to separate the neural retina and choroid followed by extraction of total RNA.

Histologic Studies

An affected 14-year-old male monkey (Fig. 1, monkey B) was killed for histologic studies. Enucleated eyes were fixed in 10% neutralized formaldehyde solution at 4°C overnight, dehydrated, and embedded in paraffin. Four-micrometer-thick sections were prepared and stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS). Serial sections were used for immunohistochemical analysis with anti-complement 5 (C5) antibody. After pretreatment with 0.4 mg/mL proteinase K in phosphate-buffered saline (PBS) for 5 minutes and blocking with 5% skim milk in PBS for 20 minutes at room temperature, the sections were incubated with rabbit anti-human C5 polyclonal antibody (Dako, Glostrup, Denmark) diluted to 1:200 dilution in PBS for 2 hours at room temperature. Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR), diluted to 1:200 in PBS, was used as the secondary antibody. The negative control experiments were performed using normal rabbit immunoglobulin fraction (Dako) instead of anti-C5 antibody.

Characterization of the Genomic Organization and cDNA Sequence of the Monkey *ABCA4*, *VMD2*, *EFEMP1*, and *TIMP3* Genes

Gene-specific primers of the human macular degeneration genes *ABCA4*, *VMD2*, *EFEMP1*, and *TIMP3* were designed based on the human genomic DNA sequence to amplify exons of monkey genes (Table 1). Amplified products were directly sequenced. For all genes except *ABCA4*, the 5′/3′-rapid amplification of cDNA ends (5′/3′-RACE) was performed using total RNA isolated from the monkey retina. Amplification of partial cDNAs by both 5′- and 3′-RACE was designed to generate overlapping PCR products to obtain a full-length cDNA sequence. Primers were initially designed based on the exonic sequences obtained by genomic sequence (Table 2). RACE products were subcloned into the pCRII cloning vector (TA Cloning Kit Dual Promoter; Invitrogen, Carlsbad, CA) and sequenced directly. The obtained nucleotide sequence data have been submitted to GenBank, and assigned accession numbers: *TIMP3*: AY207381-207385, AH012631; *EFEMP1*: AY312407-312415, AH012997; *VMD2*: AY357925-357936, AH013172; *ELOVL4*: AF461182-461187, AH012403; *ABCA4*; AY793687 (http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

Mutation Analysis

Coding regions and adjacent intronic sequences of the monkey *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* genes were analyzed for sequence variants by single-strand conformation polymorphism (SSCP) or denaturing (D)HPLC (for the *ABCA4* gene) analysis in parallel with direct sequencing. Genomic DNA from six affected and five unaffected monkeys from the pedigree (Fig. 1, pound signs) and six unrelated normal subjects were used for mutation analysis. Primers located in the intronic regions were designed to amplify coding sequences of individual genes (Table 3). Large exons were divided into smaller segments to obtain amplification products suitable for SSCP analysis. The purified amplicons were analyzed by SSCP or DHPLC analysis, as previously described.[29,30] All the samples were also analyzed by bidirectional sequencing with the PCR primers. Exons 2, 7, and 10 of the *VMD2* gene were screened for sequence variants only by direct sequencing.

Table 2. Primers for 5′-3′-RACE

Gene	5′-RACE	Position	3′-RACE	Position
VMD2	GTATACACCAGTGGGATA	Exon 6	AGAGCAACAGCTGATGTTTGAGAA	Exon 3
EFEMP1	GGATGGTACATTCATCTA	Exon 7	GATCCTGTGAGACAGCAATGCA	Exon 3
TIMP3	ATCATCTGGGAAGAGTTA	Exon 5	GATGAAGATGTACCGAGGCTTCA	Exon 2-3

TABLE 3. Primer Sets Used for Mutation Screening

Gene	Exon No.	Length (bp)	Name	Forward Primer	Name	Reverse Primer	Size (bp)
ABCA4	1	66	01F	TCTTCGTGTGGTCATTAGC	01R	ACCCCACACTTCCAACCTG	152
	2	94	02F	AAGTCCTACTGCACACATGG	02R	CTAGACAAAAGGCCCAGACC	266
	3	142	03F	TTCCCAAAAAGGCCAACTC	03R	CACGCACGTGTGCATTTCAG	301
	4	139	04F	GCTATTTCCTTATTAATGAGGC	04R	GGGAAATGATGCTTGAGAGC	212
	5	128	05F	CCCTTCAACACCCTGTTCTT	05R	TTCTTGCCTTTCTCAGGCTGG	237
	6	198	06F	GTATTCCCAGGTTCTGTGG	06R	TACCCCAGGAATCACCTTG	330
	7	88	07F	AGCATATAGGAGATCAGACTG	07R	GGCATAAGAGGGGTAAATGG	241
	8	238	08F	GAGCATTGGCCTCACAGCAG	08R	CCCCAGGTTTGGTTTCACC	397
	9	139	09F	AGACATGTGATGTGGATACAC	09R	GTGGGAGGTCCAGGGTACAC	271
	10	117	10F	AACACTAAGTGATAGGGGCAGAA	10R	GGCCTGCTTGTTGTATTTTGAT	344
	11	198	11F	AGCTCACTCGCTCTTTAGGG	11R	TTCAAGACCACTTGACTTGC	406
	12	206	12F	TGGGACAGCAGCCCTTATC	12R	CCAAATGTAATTTCCCACTGAC	362
	13	177	13F	AATGAGTTCCGAGTCACCCTG	13R	CCCATTAGCGTGTCATGG	308
	14	223	14F	TCCATCTGGGCTTTGTTCTC	14R	AATCCAGGCACATGAACAGG	407
	15	222	15F	AGACAGTAACTAACAGGCTCGTG	15R	GGACTGCTACAGACCCTTCC	386
	16	205	16F	CTGTTGCATTGGATAAAAGGC	16R	GATGAATGGAGAGGGCTGG	330
	17	65	17F	CTGCGGTAAGGTAGGATAGGG	17R	CACACCGTTTACATAGAGGGC	232
	18	90	18F	CAGCTCCCGGTGGTAGAGTA	18R	CCCTTGCCATGAGATGTTTT	222
	19	175	19F	TGGGGCCATGTAATTAGGC	19R	TGGGAAAGAGTAGACAGCCG	322
	20	132	20F	GCATGTTGCTAAAGGCCATC	20R	TATCTCTGCCTGTGCCCAG	293
	21	140	21F	GTAAGATCAGCTGCTGGAAG	21R	GAAGCTCTCCTGCTCCAAGC	301
	22	138	22F	CCCTCCACAGTCCCTTAACTC	22R	GAGAGTGGGGACCACAGGTA	244
	23	194	23F	TTTTGCAACTATGTAGCCAGGA	23R	AGCCTGTGTGAGTAGCCATG	384
	24	85	24F	GCATCAGGGAGAGGCTGTC	24R	CCCAGCAATATTGGGAGATG	212
	25	206	IVS24F	GTAAGGACTGGACGGGCCATACTTGG	IVS24R	TCCAGCTCTCTGAAAAGGCTGGCATA	2 kb
			IVS25F	AAAGCTGGTGGAGTGCATTGGTCAAG	IVS25R	CCTGAATCAGAATCCTCCGTGACCTTC	500
	26	49	26F	TCCCATTATGAAGCAATACC	26R	ACCCAGCCCCTTAGACTTTC	228
	27	266	IVS26F	GGATTCTGATTCAGGACCTCTGTTTGC	IVS26R	CTGCGGATGGTGTGTTGGAATCTCTT	2 kb
			IVS27F	TCCCAGAGAGAAGGCTGGACAGACAC	IVS27R	CCCATATATCCAGGGGTGAAGGGTCA	1 kb
	28	125	28F	TGCACGCGCACGTGTGAC	28R	TGAAGGTCCCAGTGAAGTGGG	291
	29	99	29F	CAGCAGCTATCCAGTAAAGG	29R	AACGCCTGCCATCTTGAAC	263
	30	187	30F	GTTGGGCACAATTTCTTATGC	30R	ACTCAGGAGATACCAGGGAC	347
	31	95	IVS30F	GAGAAGCTCACCATGCTGCCAGAGT	IVS30R	GAGATGTTCCTGTCCGTCAGGTCTTG	2 kb
			IVS31F	CGCAGCACGGAAATTCTACAAGACCT	IVS31R	CCTCTGTTCATTGACCCAGAATTTGCT	700
	32	33	32F	ACGGCACTGCTGTACTTGTG	32R	TCAACATGGCTGTGAGGTGT	182
	33	106	IVS32F	GAGCAAATTCTGGGTCAATGAACAGAGG	IVS32R	CGCTTAAAAACCCAACAAGTGCTTCC	1.2 kb
			IVS33F	AGGTATGGAGGAATTTCCATTGGAGGA	IVS33R	CTTTAGAGGCCTCTCTAGTGATAGG	300
	34	75	34F	AAACCGTCTTGTTTGTTTGTTT	34R	AGGAGGGAGGGAATTCAATG	208
	35	170	IVS34F	GGCCCTATCACTAGAGAGGCCTCTAAAG	IVS34R	GGTTGGCTAATGACGGTGATTCCATAC	550
			IVS35F	CATGCCCTGGTCAGCTTTCTCAATGT	IVS35R	GAGAAAATCACGCAGATGGCAACCAC	2 kb
	36	178	36F	TGTAAGGCCTTCCCAAAGC	36R	TGGTCCTTCAGAGCACACAC	346
	37	116	37F	CATTTTGCAGAGCTGGCAGC	37R	CTTCTGTCAGGAGATGATCC	260
	38	158	38F	GGAGTGCATTATATCCAGACG	38R	CCTGGCTCTGCTTGACCAAC	302
	39	125	39F	TGCTGTCCTGTGAGAGCATC	39R	CTTCCAGCCCAACAAGGTC	344
	40	130	IVS39F	CTGCTCATTGTCTTCCCCCACTTCTG	IVS39R	CAGCAGGGTCAGGAGGAAGTACACCA	700
			IVS40F	GTGAGGAGCACTCTGCAAATCCGTTC	IVS40R	AGATGAGGAAAAGGGGTCAGGATTGG	3.5 kb
	41	121	41F	GAAGAGAGGTCCCATGGAAAGG	41R	GCTTGCATAAGCATATCAATTG	299
	42	63	42F	CTCCTAAACCATCCTTTGCTC	42R	AGGCAGGCACAAGAGCTG	214
	43	107	43F	GGTCTCTAGGGCCAGGCTA	43R	CACATCTTTCAGGGCCTCAG	271
	44	142	44F	GAAGCTTCTCCAGCCCTAGC	44R	TGCACTCTCATGAAACAGGC	277
	45	135	IVS44F	ACATCTTTACCTTTATGCCCGGCTTCG	IVS44R	AATGAGTGCGATGGCTGTGGAGAGTT	4 kb
			IVS45F	TTAAGAGCCTGGGCCTGACTGTCTACG	IVS45R	GAATCTCTTGCCTGTGGGATGTGAGG	1 kb
	46	104	48F	GAAGCAGTAATCAGAAGGGC	46R	GCCTCACATTCTTCCATGCTG	257
	47	93	47F	TCACATCCCACAGGCAAGAG	47R	TTCCAAGTGTCAATGGAGAAC	258
	48	250	48F	ATTACCTTAGGCCCAACCAC	48R	ACACTGGGTGTTCTGGACC	365
	49	87	49F	GGTGTAGGGTGGTGTTTTCC	49R	ACTGCCTCAAGCTGTGGACT	187
VMD2	2*	152	P2F	GCTCTGACCAGGGTCTCTGA	P3R	CCGCACCTTTCCCTGAACTA	4.5 kb
	3	95	P3F	CTAGACCTGGGGACAGTCTCA	P3R	CCGCACCTTTCCCTGAACTA	325
	4	234	MP4aF	TGGGAGACAGAACCCTTGGA	MP4aF	GTCCTTGCCTTCCACGAA	302
			MP4bF	TGGTGGAACCAGTACGAGAA	MP4bF	TCCACCCATCTTCCATTGTT	286
	5	155	MP5F	AAAGGAGTGCTGAGGTTCCTATA	MP5R	CTTGTTTCCTGTGAACCACAA	330
	6	78	P6F	GCCAGGAATGGACCATGAGTA	P6R	GAGCCACTTAGCCTCTAGGTGA	292
	7*	153	P7F	CCTGGAGCATCCTGATTTCA	P8R	TGAGGCCTCCCTACAGAACA	2.3 kb
	8	81	MP8F	GCATCATGTGGTGTGGAAAT	P8R	TGAGGCCTCCCTACAGAACA	270
	9	152	MP9F	CAAGTCATCAGGCACGTACAA	MP9R	CTAGGCAGACCCCTGCTACTA	286
	10*	639	P10F	AAGGGAGAAGGCCAGGTGTT	P10R	TTTCCTGTAGTGCTTGGGTACTA	1.2 kb
	11	19	P11F	TGCCCTCCTACTGCAACATT	MP11R	AAGTAGTCCTGGACTGCTGATTT	270
EFEMP1	2	81	MP2F	CCGCAGCAGATACTAAATATCAG	MP2R	CCGCTGAACCGTACTTATTTC	173
	3	49	MP3F	CTTAGGGAATGGACACACCAA	MP3R	ACAGAAGGCCAAAGATCACAT	155

(*continues*)