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

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

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

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

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

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

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

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

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

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

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

Subjects and Methods

Patients and Control Subjects

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

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

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

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

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

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

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

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

DNA Extraction and PCR Conditions

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

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

DHPLC Analysis

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

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

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

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

GC-clamp: CGCCCGCCGCCGCCCGCCGC.

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_4IV (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_4IV, 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′-GCTGAGAAGATGAAGGAAAAGTC-3′, and the reverse primer, 5′-CCTCTACATGGCCCTGTCCT-3′. The reverse primer contained a one-nucleotide mismatch (italic), which allowed the use of the restriction enzyme. The C allele sequence was cut into two fragments (183 bp, 23 bp) by *Eco*NI, whereas the A allele sequence remained intact (206 bp). Transcriptional activity of the −863A allele was significantly greater than that of the −863C allele.[37]

Statistical Analyses

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

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

RESULTS

OPTN Variants in Japanese Subjects

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

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

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

Distribution of *OPTN* Variants in Japanese Subjects

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

Although the reason for the significant association of the c.412G→A polymorphism with patients with glaucoma is unknown, it may be linked to another unknown single-nucleotide polymorphism that exists in the promoter region and may alter the activity of the protein or may affect the stability or splicing accuracy of the mRNA.[40] Alternatively, the c.412G→A polymorphism may be linked to another unknown gene that lies near the *OPTN* gene.[41]

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

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

* P by χ^2 test.

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

Phenotype	−857C→T C/C(%)			Odds Ratio 95% CI	C/T + T/T (%)			Odds Ratio 95% CI
c.412G→A	G/G	G/A + A/A	P*		G/G	G/A + A/A	P*	
c.412G→A (Thr34Thr)								
POAG	92 (68.1)	43 (31.9)	0.204	1.40 (0.83–2.37)	33 (55.9)	26 (44.1)	0.006‡	2.86 (1.34–6.08)
NTG	97 (65.5)	51 (34.5)	0.077	1.58 (0.95–2.62)	51 (73.9)	18 (26.1)	0.531	1.28 (0.59–2.77)
Control	108 (75.0)	36 (25.0)			58 (78.4)	16 (21.6)		
Phenotype	−863C→A C/C (%)			Odds Ratio 95% CI	C/A + A/A (%)			Odds Ratio 95% CI
c.412G→A	G/G	G/A + A/A	P*		G/G	G/A + A/A	P*	
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)		
Phenotype	−857C→T C/C(%)			Odds Ratio 95% CI	C/T + T/T (%)			Odds Ratio 95% CI
c.603T→A	T/T	T/A + A/A	P*		T/T	T/A + A/A	P*	
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)		
Phenotype	−863C→A C/C (%)			Odds Ratio 95% CI	C/A + A/A (%)			Odds Ratio 95% CI
c.603T→A	T/T	T/A + A/A	P*		T/T	T/A + A/A	P*	
POAG	123 (87.2)	18 (12.8)	0.127	0.61 (0.33–1.15)	38 (71.7)	15 (28.3)	0.008‡	4.11 (1.37–12.27)
NTG	125 (78.6)	34 (21.4)	0.636	1.14 (0.66–1.97)	44 (75.9)	14 (24.1)	0.027†	3.31 (1.10–9.91)
Control	130 (80.7)	31 (19.3)			52 (91.2)	5 (8.8)		

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

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

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

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

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

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

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

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

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

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

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LABORATORY INVESTIGATION

Genetic Variants of *TP53* and *EPHX1* in Leber's Hereditary Optic Neuropathy and Their Relationship to Age at Onset

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Abstract

Purpose: To determine whether genetic polymorphisms of the genes for oxidative stress and apoptosis cause the clinical variability in patients with Leber's hereditary optic neuropathy (LHON).

Methods: Eighty-seven unrelated Japanese LHON patients carrying the 11778 mitochondrial mutation were studied at the Keio University Hospital. Their mean age (±SD) was 25.0 ± 13.0 years with a range of 3 to 65 years. Eleven polymorphisms in nine genes were studied: seven genes related to oxidative stress (*SOD2*, *GSTT1*, *GSTM1*, *EPHX1*, *NQO1*, *p22 PHOX*, and *NOS3*), and two genes related to apoptosis (*TP53* and *CD95*). Each genetic polymorphism was analyzed in relation to the age at onset and the final visual acuity.

Results: Among the oxidative stress-related polymorphisms, a significant association between Tyr113His in the *EPHX1* gene product and the age at onset of the disease was identified ($P = 0.026$). LHON patients who were homozygous for His113 developed the disease earlier than those without this polymorphism (21.9 vs. 27.9 years). Among the apoptosis-related polymorphisms, a significant association between Arg72Pro in the *TP53* gene product and the age at onset was identified ($P = 0.007$). LHON patients who were homozygous for Arg72 developed the disease earlier than those without this polymorphism (20.5 vs. 28.1 years). In addition, LHON patients with both polymorphisms developed the disease significantly earlier (17.5 years, $P = 0.011$). No associations were found between final visual acuity and the genetic polymorphisms examined.

Conclusion: Nuclear genetic polymorphisms related to oxidative stress or apoptosis may modify the age at onset of LHON. **Jpn J Ophthalmol** 2005;49:■■–■■ © Japanese Ophthalmological Society 2005

Key Words: *EPHX1*, Leber's hereditary optic neuropathy, polymorphism, *TP53*

Introduction

Leber's hereditary optic neuropathy (LHON) is a mitochondrial disease in which the retinal ganglion cells (RGCs) and the optic nerve fibers degenerate. This maternally transmitted eye disease mainly affects young men and causes permanent loss of central vision in the majority of cases.[1] More than 90% of LHON patients carry one of three mitochondrial DNA (mtDNA) mutations at nucleotide positions 3460, 11778, or 14484 in a complex I subunit,[2] most often in a homoplasmic state.

The major difference among LHON patients with one of these mtDNA mutations is in the clinical course. The 3460 and 14484 mutations are associated with a better visual prognosis than the 11778 mutation, which shows visual recovery rates of only 4% to 7%.[3–5] However, visual recovery has been documented in some patients with the 11778 mutation whose age at onset was in the low teens.[6,7] The recovery of vision appears to be more likely when visual deterioration begins at an early age, even in patients with the 11778 mutation.

The clinical variability of LHON patients, which includes age at onset, sex (male predilection), penetrance, and visual

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recovery, suggests that the disease most likely results from polygenic or multifactorial mechanisms, possibly involving environmental stress factors, X-chromosomal loci, or other mtDNA mutations.[8] However, attempts to identify a relevant locus on the X-chromosome have not been successful.[9,10] So-called secondary LHON mutations are more frequently found in European LHON patients than in unaffected Europeans, and they are linked to the European haplotype J. These polymorphisms are not strong autonomous risk factors.[11,12]

Therefore, primary mutations are the major risk factors for LHON, but additional etiologic factors that augment or modulate the pathogenic phenotypes appear to be necessary. Considerable evidence has accumulated that heavy alcohol and/or tobacco use increases the risk of optic neuropathy in LHON families with the 3460 and 14484 mutations, according to a case–control study,[13,14] although one study did not find this association.[15] However, smoking has been reported to be significantly associated with disease penetrance in one LHON pedigree with the 11778 mutation.[16] Possible secondary genetic interactions are complex and not firmly established.

Oxidative stress has been implicated in many disorders associated with mutations of mtDNA.[17] A recent investigation in an animal model identified reactive oxygen species (ROS) as likely factors in the pathogenesis of LHON.[18] Additionally, mtDNA LHON pathogenic mutations were found to predispose cells to Fas-dependent apoptotic death in vitro.[19] These findings imply that there must be some nuclear modifier genes involved in the development of LHON.

In our search for the causes of the clinical variability in LHON, we examined nuclear genetic polymorphisms related to oxidative stress and apoptosis in Japanese patients with the 11778 mutation.

Materials and Methods

Patients

We studied 87 unrelated Japanese patients with LHON carrying the 11778 mutation with homoplasmy who had been examined in the neuro-ophthalmology clinic at Keio University Hospital between 1980 and 2003.[5,7] Seventy-nine patients were men and eight were women. Their mtDNA mutation was confirmed by polymerase chain reaction followed by a restriction-enzyme assay, which revealed a concordant gain of the *Mae*III site.[5]

Our research was conducted in conformity with the tenets of the Declaration of Helsinki. Written informed consent was obtained after the nature and possible consequences of the study had been explained. Where applicable, the research was approved by the Keio Institutional Human Experimentation Committee.

The mean age (±SD) at the onset of visual loss in 87 LHON patients was 25.0 ± 13.0 years, with a range 3 to 65 years (Fig. 1): 24.5 ± 11.6 years for the men and 29.5 ± 23.5 years for the women. This difference in ages was not significant ($P = 0.820$, Mann-Whitney U test). The most recently determined visual acuity of 86 eyes of 86 patients is shown in Fig. 2. The better visual acuity in the two eyes was used for the analyses. The final visual acuity could not be obtained in one patient.

The visual function was evaluated by testing a subject's best-corrected visual acuity and visual fields by Goldmann kinetic perimetry or Humphrey static perimetry.

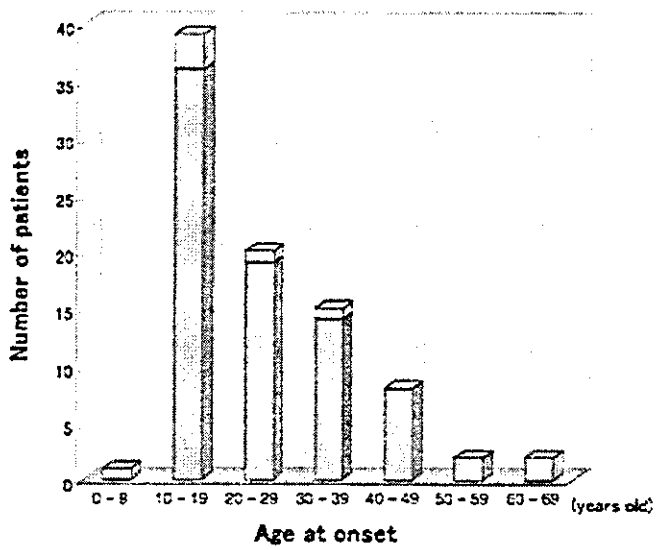

Figure 1. Distribution of ages at onset in 87 patients with Leber's hereditary optic neuropathy (LHON). □, male; □, female.

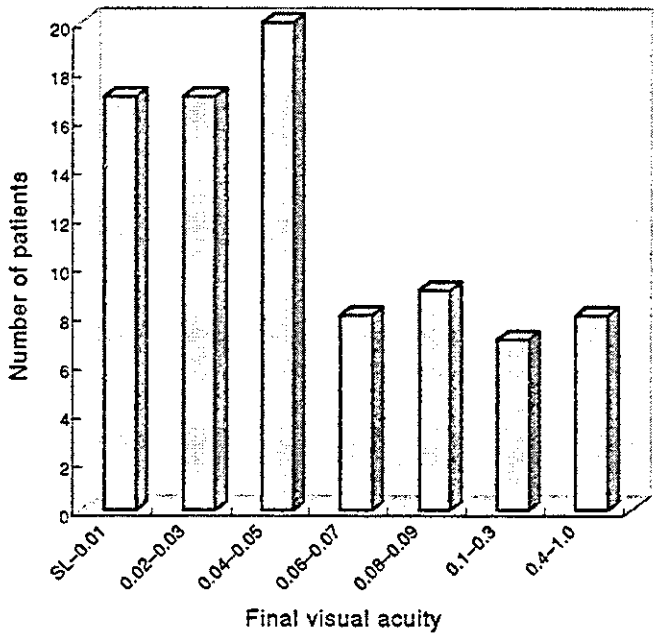

Figure 2. Distribution of the final visual acuity in 86 eyes from 86 patients with LHON. The visual acuity of the eye with the better acuity is plotted.

Genomic DNA Extraction and Genotyping

DNA was extracted from peripheral blood leukocytes by the sodium dodecyl sulfate-proteinase K and phenol/chloroform extraction method. Polymorphisms of the genes causing amino acid changes in enzymes related to oxidative stress examined were Val16Ala in manganese superoxide dismutase (*SOD2*),[20] positive or null in glutathione S-transferase T1 (*GSTT1*),[21] positive or null in glutathione S-transferase M1 (*GSTM1*),[21] Tyr113His and His138Arg in microsomal epoxide hydrolase (*EPHX1*),[22] Pro187Ser in NAD(P)H quinone oxidoreductase (*NQO1*),[23] His72Tyr in NADH/NADPH oxidase, p22 phox (p22 *PHOX*),[24] and −786T > C and Glu298Asp in endothelial nitric oxide synthase (*NOS3*).[25,26] Endothelium-derived nitric oxide (NO) plays a key role in the regulation of vascular tone and has vasoprotective effects by scavenging superoxide radicals.[27] Polymorphisms of two apoptosis-related gene products, Arg72Pro in p53 (*TP53*)[28] and −670A > G in Fas (*CD95*),[29] were examined.

Each polymorphism was identified using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques and is described by the associated change in the amino acid sequence of the gene product (protein). Each genetic polymorphism was analyzed in relation to the age at onset and final visual acuity.

Statistical Analyses

The mean age at onset and final visual acuity were compared with the genotypes by Mann-Whitney *U* test and Kruskal-Wallis test, as appropriate. The logarithm of the minimum angle of resolution (logMAR) was used for statistical analyses of the visual acuity measured with a Japanese standard visual acuity chart. The two-factor factorial analysis of variance (ANOVA) and the Scheffé test were also used as post hoc tests to examine the interactions and comparisons between polymorphisms of *TP53* (Arg72Pro) and *EPHX1* (Tyr113His). Statistical analyses were carried out with SPSS for Windows version 12.0 (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered to be significant.

Results

Of the 11 polymorphisms detected, two polymorphisms, a codon 72 substitution in *TP53* and a codon 113 substitution in *EPHX1*, were significantly associated with the age at onset of LHON (Table 1). The mean age at onset was earlier in LHON patients with the Arg/Arg genotype of *TP53* (20.5 years) than in patients with the Arg/Pro or Pro/Pro polymorphisms (28.1 years) ($P = 0.007$). In addition, the mean age at onset was earlier in LHON patients with the His/His genotype of *EPHX1* (21.9 years) than in patients with the Tyr/Tyr or Tyr/His genotypes (27.9 years) ($P = 0.026$). Two-factor factorial ANOVA results were significant ($P = 0.012$ for *TP53* and $P = 0.049$ for *EPHX1*), and although there were no significant interactions between these polymorphisms ($P = 0.577$), additivity was observed. Thus, patients with the Arg/Arg and His/His polymorphisms developed optic neuropathy significantly earlier (17.5 years, $P = 0.011$, Scheffé test; Table 2). The distribution of ages at onset among LHON patients with four different combinations of polymorphisms (Groups A to D) are shown in Fig. 3 and in Table 2. Among the 87 LHON patients, 6 were heavy smokers. However, the results were unchanged when they were omitted from the analysis (data not shown).

No significant association was found between the final visual acuity and any of the 11 polymorphisms.

Table 1. Age at onset in patients with Leber's hereditary optic neuropathy with different *TP53* or *EPHX1* genotypes

Genotype	Age at onset (years)	*P*
TP53 (Arg72Pro)		
Arg/Arg	20.5 ± 10.5 ($n = 36$)	0.007**
Arg/Pro + Pro/Pro	28.1 ± 13.8 ($n = 51$)	
Arg/Arg + Arg/Pro	24.7 ± 13.1 ($n = 78$)	0.499
Pro/Pro	27.1 ± 13.1 ($n = 9$)	
EPHX1 (Tyr113His)		
Tyr/Tyr	27.7 ± 15.2 ($n = 24$)	0.327
Tyr/His + His/His	24.0 ± 12.1 ($n = 63$)	
Tyr/Tyr + Tyr/His	27.9 ± 13.9 ($n = 45$)	0.026*
His/His	21.9 ± 11.3 ($n = 42$)	

Data are means ± SD.
* $P < 0.05$ (Mann-Whitney *U* test).
** $P < 0.01$ (Mann-Whitney *U* test).

Table 2. Age at onset in patients with Leber's hereditary optic neuropathy with different combinations of *TP53* and *EPHX1* polymorphisms

	TP53 (Arg72Pro)	
	Arg/Arg	others
EPHX1 (Tyr113His)		
His/His	17.5 ± 9.1 ($n = 20$; Group A)	25.9 ± 11.8 ($n = 22$; Group C)
others	24.4 ± 11.1 ($n = 16$; Group B)	29.8 ± 15.1 ($n = 29$; Group D)

Data are means ± SD (years).
There was a significant difference in age at onset between Groups A and D by Scheffé test ($P = 0.011$).

Discussion

LHON is characterized by degeneration of the RGC layer and the optic nerve without signs of marked inflammatory processes.[30] This optic neuropathy results in the loss of central vision due to the preferential death of the small nerve fibers of the papillomacular bundle, probably through an apoptotic pathway.[31]

LHON mutations in mtDNA have been hypothesized to generate ROS, which can directly damage RGCs and the

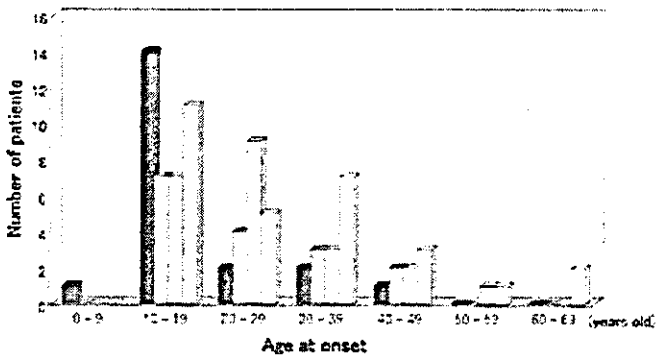

Figure 3. Distribution of the ages at onset among LHON patients with four different combinations of genotypes. ■, Group A, *TP53* (Arg/Arg) and *EPHX1* (His/His); □, Group B, *TP53* (Arg/Arg) and *EPHX1* (except His/His); □, Group C, *TP53* (except Arg/Arg) and *EPHX1* (His/His); □ Group D: *TP53* (except Arg/Arg) and *EPHX1* (except His/His).

optic nerve fibers, or initiate an apoptotic cascade.[31,32] ROS have been considered to be key factors in the pathogenesis of LHON based on studies of an animal model.[18] Because complex I activity and maximal respiration rates are mildly reduced in LHON patients with the G11778A mutation,[33] chronic accumulation of ROS or an increase of ROS production would be expected in patients with LHON.[34,35] The increased ROS in the retina and/or RGCs could then lead to premature death of optic nerve fibers resulting in blindness.

Impaired cellular defense against reactive intermediates in the oxidative metabolism of endogenous and exogenous compounds, oxidative stress, or antioxidant deficiency may promote the development of LHON. In this study, the relationship between *EPHX1* polymorphisms and the mean age at onset of the disease was associated with the Tyr113His polymorphism ($P = 0.026$), but not with the His139Arg polymorphism among LHON patients with the 11778 mutation. LHON patients who were homozygous for His113 developed the disease earlier than those without this polymorphism (21.9 vs. 27.9 years). The EPHX1 protein (microsomal epoxide hydrolase) catalyzes the hydrolysis of epoxides derived from the oxidative metabolism of endogenous or exogenous toxic components, such as an epoxide derivative of cigarette smoke components.[36] However, because no significant deleterious association was found between tobacco consumption and visual loss among LHON patients with the 11778 mutation in a case–control study,[14,15] other endogenous or exogenous toxic components leading to oxidative damage for neurons need to be considered.

Molecular and immunohistochemical studies have identified the EPHX1 protein in most neurons.[37] Hassett et al.[38] demonstrated by in vitro expression studies of cDNA that substitution of His113 for the more commonly occurring Tyr113 residue in exon 3 decreased the activity of EPHX1 protein by approximately 40%, and the substitution of Arg139 for the more commonly occurring His139 residue in exon 4 increased the activity of EPHX1 protein by approximately 25%. We suggest that the variant forms of the EPHX1 protein may alter the biodetoxification of cellular epoxides and subsequently affect levels of epoxide intermediates. These reactive intermediates may in turn cause oxidative damage to neurons affected by LHON.

Recently, cells bearing LHON-causing mutations were demonstrated to be sensitized to Fas-dependent apoptosis. The apparent site of activation of this apoptosis pathway was downstream from the Fas-receptor binding by its ligand, but upstream from the activation of caspase-3.[19] We found no association between the Fas (*CD95*) gene polymorphism and age at onset or visual recovery in our LHON cases.

Another apoptosis-related polymorphism, an Arg/Arg in codon 72 of *p53*, was identified to be significantly associated with early onset of the disease ($P = 0.007$). The p53 protein has two important functions: to delay or stop the progression of cells through the cell cycle, and to activate cell death. A common feature of the p53 response pathway is the activation of this protein by cellular damage. The p53 protein has a direct signaling role involving apoptosis in the mitochondria. Individual variations may exist in the apoptotic response, which is correlated with the polymorphism at codon 72 of *p53*. Bonafe et al.[39] reported that cultured cells from healthy subjects carrying the Arg/Arg polymorphism underwent more extensive apoptosis than cells from Arg/Pro subjects in response to the cytotoxic drug, cytosine arabinoside. Thus, naturally occurring genetic variability of the *p53* gene could partly explain individual differences in in vivo tests of the susceptibility of cells to a chemotherapeutic drug. The Arg72 variant was also reported to be more efficient than the Pro72 variant at inducing apoptosis, with one mechanism underlying this greater efficiency being the enhanced localization of the Arg72 variant to mitochondria in tumor cells.[40,41]

Interestingly, we found that patients with both Arg/Arg in the *TP53* and His/His in the *EPHX1* genotypes developed the disease significantly earlier (17.5 years, $P = 0.011$) than those without these genotypes. We suggest that there is an additive effect of the two genes that increases the risk of developing LHON earlier among Japanese LHON patients with the 11778 mutation. In the present study, however, we did not exam the causative relationship between the polymorphisms and disease penetrance. Whether these genotypes are truly associated with the onset of LHON remains uncertain, because we did not have sufficient numbers of asymptomatic individuals with the 11778 LHON mutation to perform a case–control association study.

Other gene polymorphisms examined in the present study, however, did not show an association between polymorphisms and age at onset or final visual acuity in our LHON patients. Polymorphisms in other genes associated with oxidative stress or apoptosis should be studied further to clarify clinical features in LHON patients.

In conclusion, our findings suggest that oxidative stress or apoptosis may modify the age at onset of LHON, a mitochondrial disease.

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AQ: 1

Genetic Polymorphisms in the Angiotensin II Receptor Gene and Their Association with Open-Angle Glaucoma in a Japanese Population

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PURPOSE. The local renin-angiotensin system (RAS) is present in the ciliary body and plays a role in regulating aqueous humor dynamics and thus intraocular pressure (IOP). The purpose of this study was to determine whether gene polymorphisms in the RAS increase the risk of development if glaucoma in the Japanese.

AQ: 2

METHODS. A case-control study was performed in 698 Japanese subjects: 190 patients with primary open-angle glaucoma (POAG), 268 patients with normal-tension glaucoma (NTG), and 240 normal subjects. Ten polymorphisms in seven genes—*AGT*/Thr174Met and *AGT*/Met235Thr; *REN*/18-83G→A; *ACE*/insertion(I)-deletion(D); *CMA*/−1930A→G; *AGTR1*/−731T→G, *AGTR1*/−521C→T, and *AGTR1*/1166A→C; *AGTR2*/3123C→A; and *CYP11B2*/−344T→C were examined. The age, IOP, and visual field defects, all at diagnosis, were examined to determine whether they were associated with the polymorphisms. The effects of oral angiotensin II receptor blocker (ARB) on IOP were examined in association with the *AGTR1* and *AGYT2* polymorphisms in 20 normal subjects.

AQ: 3

RESULTS. Of the 10 polymorphisms, the *AGTR2*/3123C→A polymorphisms had a significantly different distribution in female patients with NTG; the frequency of the CA+AA genotypes was significantly higher than in female control subjects ($P = 0.0095$ for CC versus CA+AA). Although no significant difference was seen in the clinical characteristics of female patients with NTG who carried the *AGTR2*/3123C→A genotype, patients with CC in the *AGTR2* gene had significantly worse visual field scores if they carried *ACE*/ID+DD (i.e., D carriers; $P = 0.012$). ARB significantly lowered IOP in normal subjects, but the male subjects with the *AGTR2*/3123A genotype had significantly less lowering of IOP than those with the C genotype ($P = 0.014$).

CONCLUSIONS. Angiotensin II receptor gene polymorphisms may be associated with the risk of glaucoma in the Japanese population. (*Invest Ophthalmol Vis Sci.* 2005;46:000-000) DOI:10.1167/iovs.04-1100

Open-angle glaucoma (OAG), the second most common cause of blindness worldwide, affects more than 100 million people, almost 2% of the global population older than 40 years.[1] The disease is characterized by an elevation of intraocular pressure (IOP) to >21 mm Hg, resulting in an excavation of the optic disc, which is associated with visual field changes. Patients with these findings have a diagnosis of primary open-angle glaucoma (POAG). Normal-tension glaucoma (NTG) is a form of OAG in which the typical glaucomatous cupping of the optic nerve head and visual field loss are present, but IOP does not exceed 21 mm Hg at any time.[2]

The risk factors for glaucoma include high IOP, advanced age, ethnicity, positive family history, myopia, presence of diabetes and/or hypertension, and specific genetic factors.[3-6] Although the exact pathogenesis of glaucomatous optic neuropathy remains uncertain, IOP is generally considered to be a major risk factor,[7] and thus, current treatments for glaucoma consist of interventions to lower IOP.[8] However, in some patients with glaucoma—for example, those with NTG or advanced POAG—the reduction of IOP does not prevent progression of the disease,[9,10] which indicates that factors other than an elevated IOP are involved in the progression of glaucoma.[11]

The association of glaucoma with various systemic vascular diseases including low systemic blood pressure, transient nocturnal decreases in blood pressure, hypertension, migraine, vasospasm, and diabetes have been reported.[6,11-13] The presence of optic disc hemorrhages in patients with NTG suggests that vascular insufficiency is probably involved in the development and progression of NTG.[13,14] Many patients with OAG have coexisting vascular disorders, and the most common is systemic hypertension, which occurs in 48% of the total OAG population.[15]

The renin-angiotensin-aldosterone (RAA) system is involved in vasoconstriction, regulation of electrolyte balance, and vascular remodeling. Local renin-angiotensin (RA) regulation is present in the eye.[16,17] Angiotensin II (ATII) is a potent vasoconstrictive agent, and recently two RAS components, angiotensin-converting enzyme (ACE) and ATII, have been

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TABLE 1. Demographic and Medical Characteristics among Patients with Glaucoma and Control Subjects

	POAG (n = 190)	NTG (n = 268)	Control (n = 240)
Demographic characteristics			
Age at diagnosis (mean ± SD), y	58.4 ± 12.0	56.1 ± 13.1	
Age at blood sampling (mean ± SD), y	65.3 ± 11.9	58.8 ± 13.4	69.7 ± 11.2
Familial history of glaucoma (n)	65/188 (34.6%)	88/264 (33.3%)	0/240 (0.0%)
Male/female (n)	108/82	129/139	113/127
IOP at diagnosis (mm Hg)	26.6 ± 6.1	16.5 ± 2.5	
Visual field score at diagnosis	3.09 ± 0.90	2.79 ± 0.69	
Medical characteristics (n)			
Hypertension	45/178 (25.3%)	53/260 (20.4%)	56/240 (23.3%)
Cardiovascular disease	8/178 (4.5%)	7/260 (2.7%)	15/240 (6.3%)
Lipid metabolism disorders	14/171 (8.2%)	16/257 (6.2%)	14/240 (5.8%)
Migraine	3/175 (1.7%)	11/260 (4.2%)	1/240 (0.4%)

identified in the human ciliary body and aqueous humor.[18,19] These findings suggest that the RA system (RAS) is probably involved in the regulation of aqueous humor dynamics and thus IOP. This interpretation is strongly supported by the observation that local or systemic ACE inhibitors[20] and ATII receptor blockers (ARBs) lower IOP.[21,22]

The purpose of this study was to determine whether single-nucleotide polymorphisms (SNPs) or insertion-deletion (I/D) polymorphisms in the seven RAA system genes are associated with OAG in the Japanese population. In addition, SNPs in the ATII receptor gene were studied to determine whether they are associated with the reduction of IOP after the oral administration of ARB.

SUBJECTS AND METHODS

Patients and Control Subjects

Blood samples were collected from 698 subjects at seven Japanese ophthalmologic institutions. The subjects included 190 patients with POAG, 268 patients with NTG, and 240 normal control subjects. None of the subjects was related to any other. The research procedures followed the tenets of the Declaration of Helsinki, and written informed consent was obtained after the nature and possible consequences of the study were explained. Where applicable, the research was approved by the local institutional human experimentation committee.

The clinical features recorded in 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; Table 1). The severity of the visual field defects was scored from 1 to 5.[23,24] Data obtained with different perimeters were combined using a five-point scale defined as follows: 1, no alteration; 2, early defect; 3, moderate defect; 4, severe defect; and 5, light perception only or no vision. Field defects were judged to be early, moderate, or severe, according to the classification of Kosaki et al.[25] and Hosoda et al.,[26] based on the results of Goldmann perimetry or the classification used by the Humphrey field analyzer.[27] The former classification is the most widely used in Japan.

The mean age at the time of blood sampling was 65.3 ± 11.9 (SD) years in the patients with POAG, 58.8 ± 13.4 years in the patients with NTG, and 69.7 ± 11.2 years in the normal subjects. The normal control subjects were selected to be significantly older than the patients with POAG ($P < 0.001$) and the patients with NTG ($P < 0.001$), to reduce the likelihood glaucoma developing in the control subjects at a later age (Table 1).

All patients underwent serial ophthalmic examinations, including IOP measurements by Goldmann applanation tonometry, Humphrey perimetric (30-2) or Goldmann perimetric measurements, gonioscopy, and optic disc examinations including fundus photographs. All the patients with glaucoma had the following characteristics: the presence of typical optic disc damage with glaucomatous cupping (cup-to-disc ratio, >0.7); loss of neuroretinal rim tissues of the optic disc; reproducible visual field defects compatible with the glaucomatous cupping; and open angles on gonioscopy. Among the patients with OAG, POAG was diagnosed if the patient had an IOP >21 mm Hg at any time during the follow-up period. Patients with exfoliative, pigmentary, or corticosteroid-induced glaucoma were excluded.

The patients with NTG had an untreated peak IOP ≤21 mm Hg at all times including the three baseline measurements and that obtained during the diurnal testing (every 3 hours from 6 hours to 24 hours); peak IOP, with or without medication, consistently at <22 mm Hg throughout the follow-up period; and the absence of a secondary cause of glaucomatous optic neuropathy, such as a previously elevated IOP after trauma, steroid use, or uveitis.

Control subjects were recruited from Japanese individuals who had no known eye abnormalities except cataracts. These 240 subjects were older than 40 years, with an IOP below 20 mm Hg, no glaucomatous disc changes, and no family history of glaucoma.

The medical characteristics of the patients with glaucoma and control subjects are shown in Table 1. The prevalence of patients with systemic hypertension in the POAG, NTG, and control groups varied from 20% to 25%, and the differences between the three groups were not significant ($P < 0.05$; by χ^2 test).

Genotyping

Ten polymorphisms in the RAA system were examined in each subject with or without glaucoma. Renin (*REN*) I8-83G→A,[28] angiotensin II receptor, type 1 (*AGTR1*) −731T→G, −521C→T, 1166A→C[29,30]; angiotensin II receptor, type 2 (*AGTR2*) 3123C→A,[31]; cytochrome P45011B2 (*CYP11B2*) −344T→C[32]; and chymase (*CMA*) −1903A→G,[29] were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

The biosynthesis of aldosterone is controlled by aldosterone synthase encoded by the *CYP11B2* gene and is regulated by the concentrations of angiotensin II and potassium. Chymase is a major angiotensin-II-forming enzyme in human hearts, and a chymase gene is associated with atherosclerosis.[33]

Polymorphisms in the *ACE* I/D were detected by PCR and agarose gel electrophoresis. To avoid false identification of the *ACE* I/D polymorphism, allele I was amplified specifically, according to the protocol of Lindpaintner et al.[34] Genomic DNA was isolated from peripheral blood lymphocytes by phenol-chloroform extraction. The primer sets and restriction enzymes used are listed in Table 2. Angiotensinogen (*AGT*) Thr174Met (T174M) and Met235Thr (M235T) were genotyped (Invader assay; Third Wave Diagnostics Molecular Diagnostics, Madison, WI).[35]

TABLE 2. Primer Pair Sequences Used for PCR Amplification and Restriction Enzymes of Polymorphic Sites in the RAS

Gene	Polymorphism	Primer Sequences	Annealing Temp (°C)	Product Size (bp)	Restriction Enzyme	Digested Products (bp)
REN	18-83G→A	TGAGGTTCGAGTCGGCCCCCT TCGCCAAACATGGCCACACAT	68	250	*Mbo*I	G: 250 A: 171 + 79
ACE	I/D 1st step	GCCCTGCAGGTGTCTGCAGCATGT GGATGGCTCTCCCCGCCTTGTCTC	63	D: 319 I: 597 D/D: no		
	I/D 2nd step	TGGCAGCACAGCGCCCGCCACTAC TCGCCAGCCCTCCCATGCCCATAA	67	product I: 335		
AGTR1	1166A→C	GAGGTTGAGTGACATGTTCGAAAC CGTCATCTGTCTAATGCAAAATGT	60	253	*Dde*I	A: 253 C: 155 + 98
	−521C→T	CGTGATGTCTTTATCTGGTTTTG CGAACTTTGGTAATACAGTTGTGG	60	270	*Ssp*I	C: 270 T: 144 + 126
	−713T→G	AAACTACAGTCACCCTACTCACCT TTCTTCACAAACTCTTCCAA	55	292	*Hinf*I	T: 170 + 122 G: 292
AGTR2	3123C→A	GGATTCAGATTTCTCTTTGAA GCATAGGAGTATGATTTAATC	53	340	*Alu*I	C: 340 A: 227 + 113
CYP11B2	−344C→T	CAGGAGGGATGAGCAGGCAGAGCACAG CTCACCCAGGAACCTGCTCTGGAAACATA	63	404	*Hae*III	C: 333 + 71 T: 404
CMA	−1903A→G	GGAAATGTGAGCAGATAGTGCAGTC AATCCGGAGCTGGAGAACTCTTGTC	51	285	*Bst*XI	A: 285 G: 195 + 90

Effect of Oral Angiotensin II Receptor Blocker on IOP in Normal Subjects and Its Association with SNPs in the *AGTR1* and *AGTR2* Genes

This part of the study was performed on 20 healthy volunteers (13 men and 7 woman; age range, 23–28 years) without systemic and eye diseases. In the morning (10:00 A.M.), each subject was given either 12 mg oral candesartan cilexetil (Blopress; Takeda Chemical Industries, Osaka, Japan) or a placebo, in a randomized, crossover, double-blind fashion.

The baseline heart rate, systolic-diastolic arterial pressure (SBP/DBP), and IOP were recorded. The subjects then received oral candesartan cilexetil or placebo, and measurements were repeated hourly for 6 hours and then after 24 hours. One month later, each subject received the alternative treatment. Only the right eye was measured and analyzed.

The ocular perfusion pressure (OPP)[36] is defined as the difference between the pressure in the arteries entering the tissue and the veins leaving it. The OPP can be approximated by the following formula, using the mean blood pressure (BPm) and IOP.

$$OPP = 2/3 \times BPm - IOP, \text{ where } BPm = DBP + 1/3 \times (SBP - DBP).$$

A search for polymorphisms in *AGTR1* and *AGTR2* was performed in the 20 subjects and the correlation determined between the changes in IOP. The research adhered to the tenets of the Declaration of Helsinki. 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 for analysis of DNA.

Statistical Analysis

The presence of the Hardy-Weinberg equilibrium was tested by the χ^2 test. The frequencies of the genotypes and alleles were compared between patients and control subjects by χ^2 analysis. Odds ratios (ORs) for a disease, assuming a dominant (major homozygote versus others) or a recessive genetic model (minor homozygote versus others), and the 95% confidence interval (CI) were calculated adjusting for age by logistic regression.

Multivariate analyses were performed with a logistic regression model to confirm the association between the three clinical variables and the genotype. To determine the combined effects of two polymorphisms, comparisons between groups were performed by Kruskal-Wallis test, followed by multiple comparisons testing using the Scheffé test. Statistical analysis was performed on computer (SPSS Inc., Chicago, IL). $P < 0.05$ was considered to be statistically significant.

Statistical analysis of the results after administration of ARB was also performed (StatView; SAS Institute, Cary, NC), using the repeated-measures ANOVA. ANOVA with Bonferroni correction was used for statistical analysis of each IOP. $P < 0.0004$ was considered to be statistically significant.

RESULTS

Genotype Distribution of Polymorphisms in the RAA System in Japanese Subjects

The distributions of the genotypes of candidate gene polymorphisms in patients with glaucoma and control subjects are shown in Table 3. All the genotype frequencies were consistent with the populations being in Hardy-Weinberg equilibrium. Of the 10 polymorphisms in the RAA system, two had a significantly different distribution of genotype frequencies: *AGTR1*/−713T→G for POAG ($P = 0.021$) and *AGTR2*/3123C→A for NTG ($P = 0.045$). The significant difference in the 3123C→A polymorphism was found only in female patients with NTG. T3

The genotypic ORs for POAG or NTG and 95% CI, assuming a dominant genetic model adjusted for age, are shown in Figure 1. For a dominant genotype model, the frequency of the CA+AA genotypes in the *AGTR2*/3123C→A polymorphism was significantly higher in female patients with NTG (71.2%) than in female control subjects (56.7%; $P = 0.0095$ for CC versus CA+AA; OR = 2.18; 95% CI = 1.21–3.93). This polymorphism was not associated with glaucoma in male subjects. In the recessive model, there was no significant difference in the genotype frequency in the 10 polymorphisms (data not shown). Although the *AGTR1*/-713T→G polymorphism had a significantly different distribution of genotype frequencies among the TT, TG, and GG in patients with POAG (Table 3), it was not significantly different in a dominant model or a recessive model. The frequency of GG genotype was higher in patients with POAG (3.2%) than in control subjects (0.4%, $P = 0.071$ for TT+TG versus GG). F1

Three clinical characteristics of the patients with glaucoma—age, IOP, and visual field score at diagnosis—were examined to determine whether they were associated with the 10 polymorphisms in the RAA system. The patients with glaucoma

TABLE 3. Genotype Distribution of Polymorphisms in the RAA System in Patients with glaucoma and Control Subjects

	Genotype Frequency					Genotype Frequency			
AGT T174M	TT	TM	MM	P*	AGT M235T	MM	TM	TT	P
POAG (n = 150)	115 (76.7)	34 (22.7)	1 (0.7)	0.3101	POAG (n = 171)	4 (2.3)	56 (32.8)	111 (64.9)	0.9185
NTG (n = 213)	178 (83.6)	32 (15.0)	3 (1.4)	0.1122	NTG (n = 206)	9 (4.4)	57 (27.6)	140 (68.0)	0.4626
Control (n = 210)	172 (81.9)	38 (18.1)	0 (0.0)		Control (n = 208)	5 (2.4)	64 (30.8)	139 (66.8)	
	Genotype Frequency					Genotype Frequency			
REN I8-83G→A	GG	GA	AA	P	ACE I/D	II	ID	DD	P
POAG (n = 190)	123 (64.7)	64 (33.7)	3 (1.6)	0.1085	POAG (n = 190)	87 (45.8)	82 (43.2)	21 (11.1)	0.7921
NTG (n = 268)	164 (61.2)	90 (33.6)	14 (5.2)	0.2837	NTG (n = 268)	111 (41.4)	133 (49.6)	24 (9.0)	0.5154
Control (n = 240)	163 (67.9)	66 (27.5)	11 (4.6)		Control (n = 240)	102 (42.5)	110 (45.8)	28 (11.7)	
	Genotype Frequency					Genotype Frequency			
CMA −1903A→G	AA	AG	GG	P	CYP11B2 −344T→C	CC	CT	TT	P
POAG (n = 190)	121 (63.7)	59 (31.1)	10 (5.3)	0.6083	POAG (n = 139)	62 (44.6)	60 (43.2)	17 (12.2)	0.9740
NTG (n = 268)	179 (66.8)	75 (28.0)	14 (5.2)	0.4634	NTG (n = 186)	89 (47.8)	81 (43.5)	16 (8.6)	0.3810
Control (n = 240)	157 (65.4)	75 (31.3)	8 (3.3)		Control (n = 170)	74 (43.5)	74 (43.5)	22 (12.9)	
	Genotype Frequency					Genotype Frequency			
AGTR1 −521C→T	CC	CT	TT	P	AGTR1 −713T→G	TT	TG	GG	P
POAG (n = 190)	135 (71.1)	44 (23.2)	11 (5.8)	0.2982	POAG (n = 190)	159 (83.7)	25 (13.2)	6 (3.2)	0.0206†
NTG (n = 268)	179 (66.8)	84 (31.3)	5 (1.9)	0.4388	NTG (n = 268)	219 (81.7)	49 (18.3)	0 (0.0)	0.5160
Control (n = 240)	165 (68.8)	67 (27.9)	8 (3.3)		Control (n = 240)	192 (80.0)	47 (19.6)	1 (0.4)	
	Genotype Frequency								
AGTR1 1166A→C	AA	AC	CC	P					
POAG (n = 190)	159 (83.7)	31 (16.3)	0 (0.0)	0.1968					
NTG (n = 268)	228 (85.1)	39 (14.6)	1 (0.4)	0.4343					
Control (n = 240)	197 (82.1)	40 (16.7)	3 (1.3)						
	Genotype Frequency					Genotype Frequency			
AGTR2 (Female) 3123C→A	CC	CA	AA	P	AGTR2 (Male) 3123C→A	C	A	P	
POAG (n = 82)	39 (47.6)	30 (36.6)	13 (15.9)	0.7562	POAG (n = 108)	62 (57.4)	46 (42.6)	0.4916	
NTG (n = 139)	40 (28.8)	75 (54.0)	24 (17.3)	0.045†	NTG (n = 129)	81 (62.8)	48 (37.2)	0.8925	
Control (n = 127)	55 (43.3)	53 (41.7)	19 (15.0)		Control (n = 113)	70 (61.9)	43 (38.1)		

Data are the number of subjects with the percentage of the total group in parentheses.
* $P < 0.05$; by χ^2 test.

did not show a significant association between clinical characteristics and 10 SNPs (data not shown, except in Table 4). T4

Clinical Characteristics of NTG Patients with the *AGTR2*/3123C→A and *ACE* I/D Polymorphisms

No significant association of clinical characteristics (age, IOP, and visual field score) was detected between female glaucoma patients with CC and those with CA+AA genotypes (Table 4). The visual field score had a tendency to be worse in patients with NTG with CC genotype than in those with CA+AA genotypes (P = 0.107).

However when combined with *ACE* (I/D) polymorphisms, female patients with NTG who carried CC in the *AGTR2* gene as well as ID+DD in the *ACE* gene had significantly worse visual field scores than did the patients with the other three combined genotypes (P = 0.012; Table 5, Fig. 2). This effect was not observed in patients with POAG (data not shown). T5 F2

Effect of an Oral Angiotensin II Receptor Blocker on IOP and Its Association with the *AGTR2* Genotype

The changes in IOP after oral candesartan cilexetil or placebo are shown in Figure 3A. IOP in the subjects who received the placebo was not altered significantly. However, as early as 1 hour after oral candesartan cilexetil, IOP had fallen significantly and remained low for 5 hours ($P < 0.0001$) compared with placebo. Candesartan cilexetil did not significantly affect perfusion pressure (Fig. 3B). No significant changes in SBP, DBP, and heart rate were detected after a single oral dose of candesartan cilexetil or placebo (data not shown). F3

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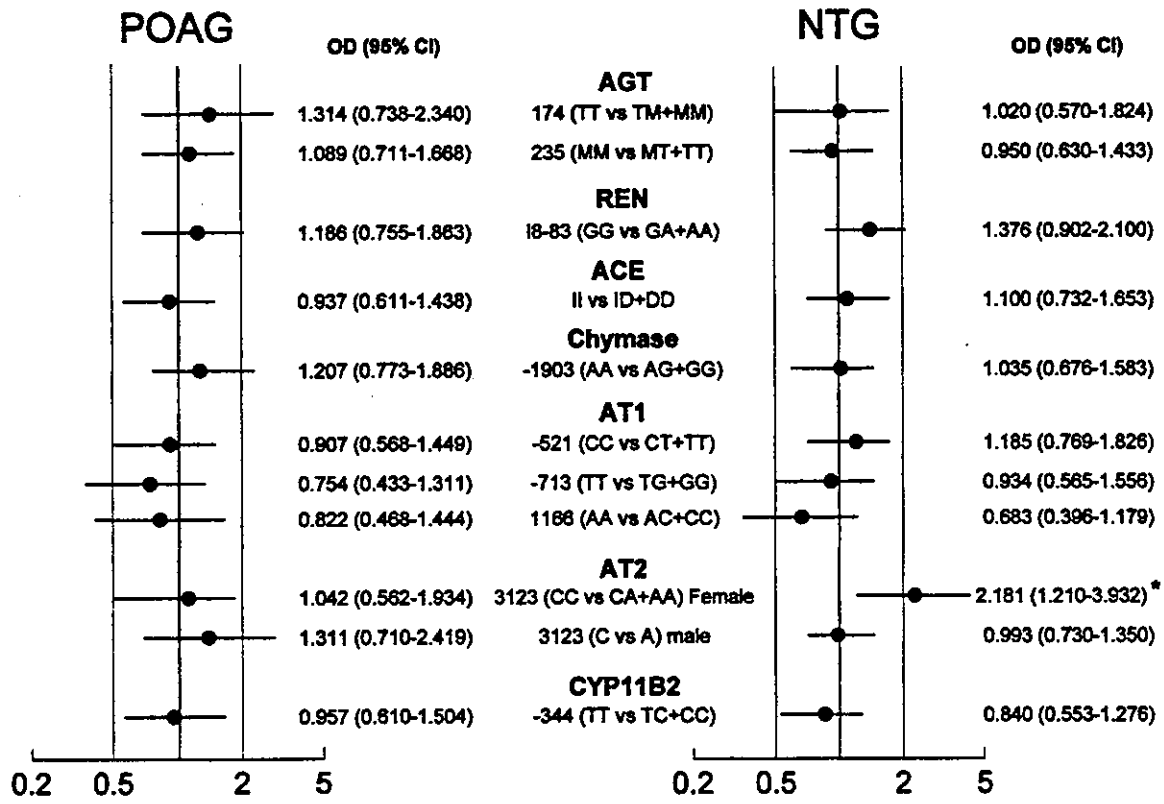

FIGURE 1. Genotypic ORs for glaucoma and 95% CI in 10 polymorphisms in the RAA system, assuming a dominant genetic model (major homozygote versus others). T, threonine; M, methionine, respectively. *$P = 0.0095$.

The changes in IOP after oral candesartan cilexetil in each of the 20 subjects are shown in Figure 3C. There was no significant association between the effects of candesartan cilexetil and the three SNPs in the *AGTR1* gene in the 20 control T6 subjects (Table 6). For the *AGTR2* genotype, however, four men with the A genotype showed a reduction of IOP by 2.3 ± 0.5 mm Hg, which was the same amount as that of subjects who received placebo and a significantly lesser decrease in IOP than in the nine men with the C genotype (5.0 ± 1.1 mm Hg, $P = 0.014$). No woman had the AA genotype in this study.

DISCUSSION

Although most cases of glaucoma are classified as POAG or NTG of unknown cause, multiple environmental and genetic factors are likely to be involved in the pathogenesis of glaucoma. SNPs can be used to detect linkage disequilibrium reliably between a marker genotype and a disease of multifactorial origin.[37] Using these markers, candidate genes of the RAA system, including *REN*, *AGT*, *ACE*, *AGTR1*, *AGTR2*, *CYP11B2*, and *CMA*, have been investigated in association studies concerning essential hypertension and other cardiovascular diseases.[28–32]

The RAS has been strongly implicated in the pathogenesis of essential hypertension, cardiovascular disease, progressive renal disease, and diabetic retinopathy.[38] The major biologically active product of the RAS is ATII, which is produced from AGT by the sequential action of renin and ACE or chymase. ATII, the final effecter in RAS activity, is both a powerful vasoconstrictor and a potent mediator of cellular proliferation and extracellular matrix protein synthesis and accumulation.[39] These effects contribute to progressive fibrotic disease in various organ systems. The effects of ATII are mainly receptor mediated at *AGTR1* and *AGTR2*.[39] Administration of ATII by intravenous or

TABLE 4. Comparison of Clinical Characteristics of Female Patients with Glaucoma According to *AGTR2* Genotypes

Phenotype Variable	CC	CA + AA	P*
POAG			
Age at diagnosis (y)	59.2 ± 11.6 ($n = 38$)	61.3 ± 11.1 ($n = 42$)	0.424
IOP at diagnosis (mm Hg)	25.3 ± 4.3 ($n = 35$)	26.6 ± 5.9 ($n = 39$)	0.243
Visual field score at diagnosis	3.15 ± 0.96 ($n = 39$)	2.98 ± 0.89 ($n = 43$)	0.729
NTG			
Age at diagnosis (y)	59.1 ± 13.3 ($n = 38$)	57.8 ± 11.6 ($n = 98$)	0.149
IOP at diagnosis (mm Hg)	16.0 ± 2.5 ($n = 36$)	16.5 ± 2.4 ($n = 92$)	0.32
Visual field score at diagnosis	2.85 ± 0.74 ($n = 40$)	2.64 ± 0.56 ($n = 98$)	0.107

* Probability for logistic regression analysis.