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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[11] secondary LHON mutations[11] are more frequently found in European LHON patients than in unaffected Europeans, and they are polymorphisms 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 were 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.

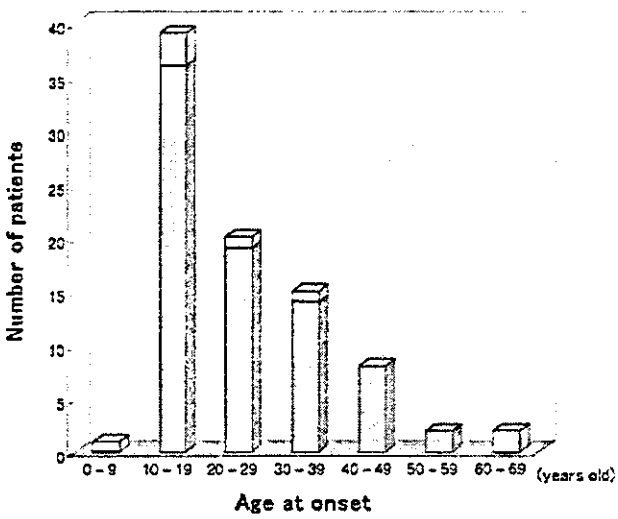

Figure 1. Distribution of ages at onset in 87 patients with Leber's hereditary optic neuropathy (LHON). ⊏, male; ⊏, female.

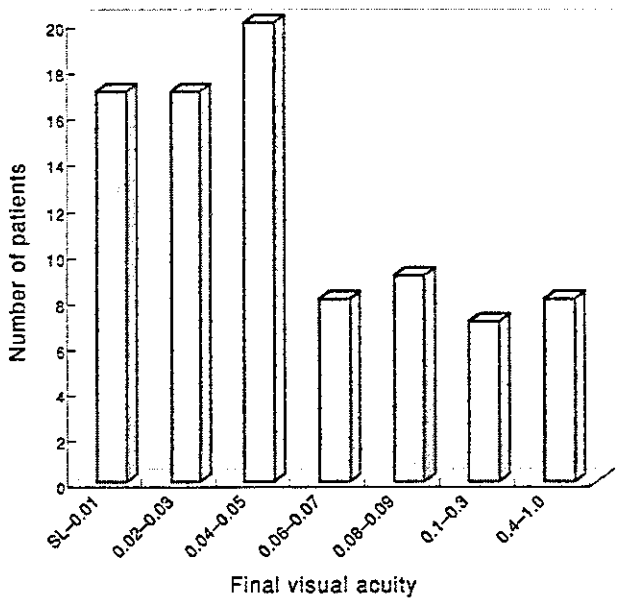

Figure 2. Distribution of the final visual acuity in 86 eyes from 86 patients with LHON. The visual acuity of the eye with the better acuity is plotted.

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 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 genes, 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 test 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 genotypes (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 genotypes 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 genotypes (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* genotypes

	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

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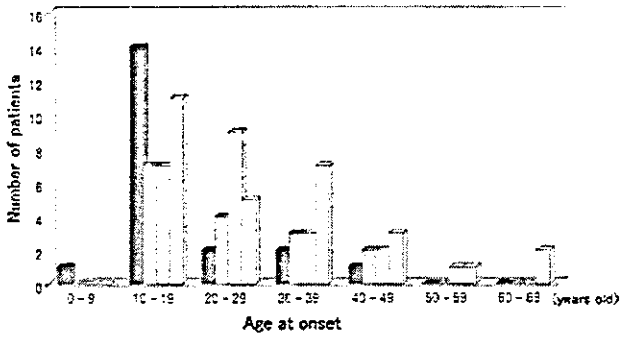

Figure 3. Distribution of the ages at onset among LHON patients with four different combinations of genotypes. ■, Group A. *TP53* (Arg/Arg) and *EPHX1* (His/His); □, Group B. *TP53* (Arg/Arg) and *EPHX1* (except His/His); □, Group C. *TP53* (except Arg/Arg) and *EPHX1* (His/His); □, Group D. *TP53* (except 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 genotype 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*/I8-83G→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]

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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

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

AGT T174M	Genotype Frequency TT	TM	MM	*P**	AGT M235T	Genotype Frequency 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)	

REN I8-83G→A	Genotype Frequency GG	GA	AA	*P*	ACE I/D	Genotype Frequency 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)	

CMA −1903A→G	Genotype Frequency AA	AG	GG	*P*	CYP11B2 −344T→C	Genotype Frequency 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)	

AGTR1 −521C→T	Genotype Frequency CC	CT	TT	*P*	AGTR1 −713T→G	Genotype Frequency 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)	

AGTR1 1166A→C	Genotype Frequency 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)	

AGTR2 (Female) 3123C→A	Genotype Frequency CC	CA	AA	*P*	AGTR2 (Male) 3123C→A	Genotype Frequency 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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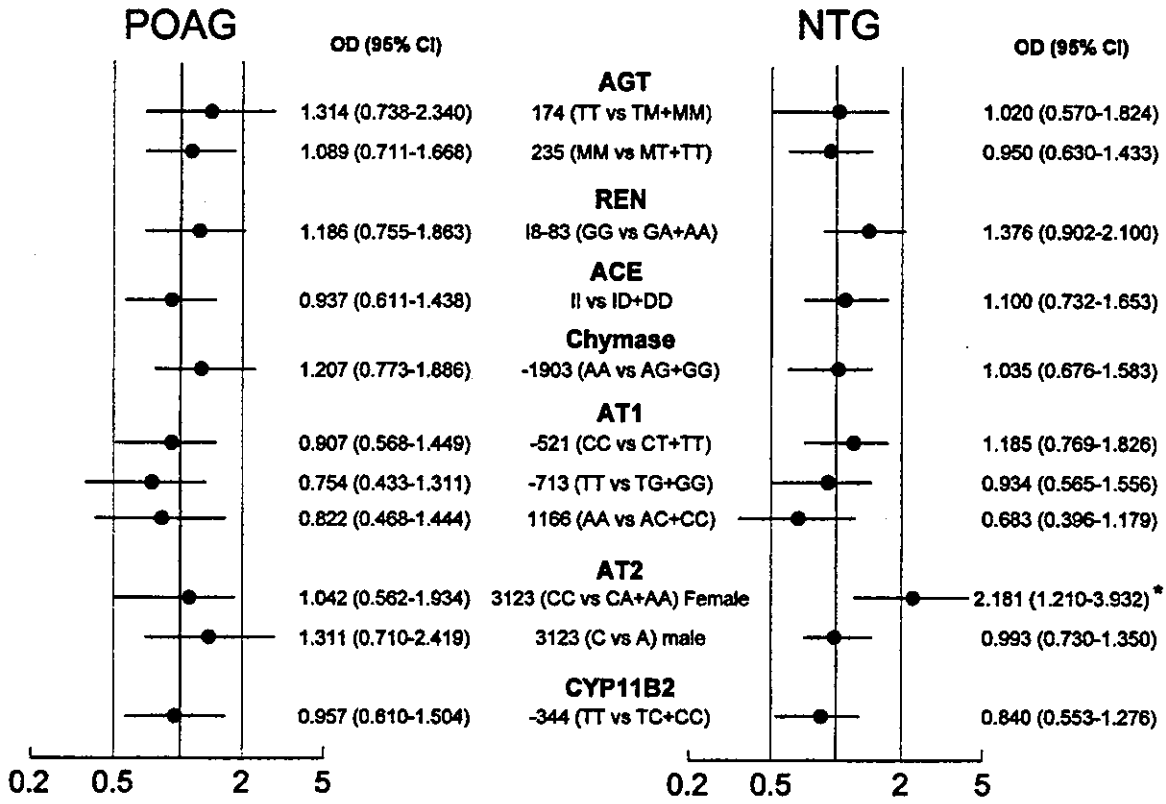

FIGURE 1. Genotypic ORs for glaucoma and 95% CI in 10 polymorphisms in the RAA system, assuming a dominant genetic model (major homozygote versus others). T, threonine; M, methionine, respectively. *P = 0.0095.

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.

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TABLE 5. Comparison of Clinical Characteristics of Female Patients with NTG, According to ACE (I/D) and *AGTR2* Genotypes (3123C→A)

Clinical Characteristics at Diagnosis	ACE II		ID + DD		P
	CC	CA + AA	CC	CA + AA	
Age (y)	63.6 ± 10.9 (n = 15)	57.0 ± 11.2 (n = 47)	56.2 ± 14.1 (n = 23)	58.5 ± 12.0 (n = 51)	0.313
IOP (mm Hg)	16.0 ± 2.2 (n = 16)	16.5 ± 2.6 (n = 43)	16.1 ± 2.7 (n = 20)	16.5 ± 2.2 (n = 49)	0.75
Visual field score	2.47 ± 0.51 (n = 17)	2.64 ± 0.53 (n = 47)	3.13 ± 0.76 (n = 23)	2.65 ± 0.59 (n = 52)	0.012†

† $P < 0.05$ by Kruskal-Wallis test.

anterior chamber routes results in a significant increase in IOP in rats.[40,41] In humans, systemic ATII receptor blockers lower the IOP.[21,22]

The RAA system contains at least seven genes. Initially, we selected candidate polymorphisms in association with glaucoma as follows: (1) polymorphisms associated with cardiovascular diseases in the Japanese population, because the frequency of polymorphisms varies among races; (2) heterozygosity of polymorphisms >0.1 in Japanese; and (3) polymorphisms associated with the function of the gene, if possible, or polymorphisms located in the promotor region. We did not select polymorphisms that are rare in Japanese. Our study, designed to detect the involvement of 10 SNPs of the RAA system in glaucoma, showed that the *AGTR2* polymorphism was associated with NTG. Other gene polymorphisms in the RAA system were not associated with POAG or NTG. It is uncertain whether the −713T→G polymorphism in the *AGTR1* gene is actually associated with POAG, because neither a dominant model nor a recessive model of this polymorphism showed any significant difference in the genotype frequency. However, as the frequency of the GG genotype was higher in patients with POAG (3.2%) than in control subjects (0.4%), further studies are needed to confirm this finding or to identify other functional variants of the *AGTR1* gene.

We found a gender-specific association between the *AGTR2*/3123C→A polymorphism and NTG. Women with NTG who had the CA+AA genotype (i.e., A carriers) were significantly more likely to develop NTG than those with the CC genotype (non-A carriers; $P = 0.0095$). Although there was no difference between three clinical features and genotypes of the *AGTR2*/3123C→A, only the visual field score was significantly worse ($P = 0.012$) in the female patients with NTG with the CC genotype than those with the CA+AA genotype if they were D carriers of the *ACE* gene. These results indicate that the effect of the *AGTR2* polymorphism on the progression of visual field defects in NTG may depend on the *ACE* I/D polymorphism. As for that polymorphism, the D allele was associated with increased plasma ACE concentration, which appears to result in increased ATII formation in the plasma.[42] Genetic interaction may be essential for the development or the susceptibility to diseases.[43–46] As the IOP at diagnosis in female patients with NTG was not associated with this effect, the progression of visual field defects may be independent of IOP in the RAS in these patients.

Although the gender-specific association cannot be readily explained, some previous studies have shown a similar gender-specific tendency or association between this polymorphism and hypertension[47] and hypertrophic cardiomyopathy.[48] However, the pattern of frequencies of the genotypes in hypertensive patients differed from that in patients with NTG. Women with the AA genotype were significantly more likely to have hypertension than those with the CC+CA genotype, in this Japanese group ($P = 0.0058$).[47]

Because the *AGTR2*/3123C→A polymorphism is located in the 3′ noncoding region of the gene, the amino acid sequence of the receptor is not altered. The *AGTR2*/3123C→A polymor-

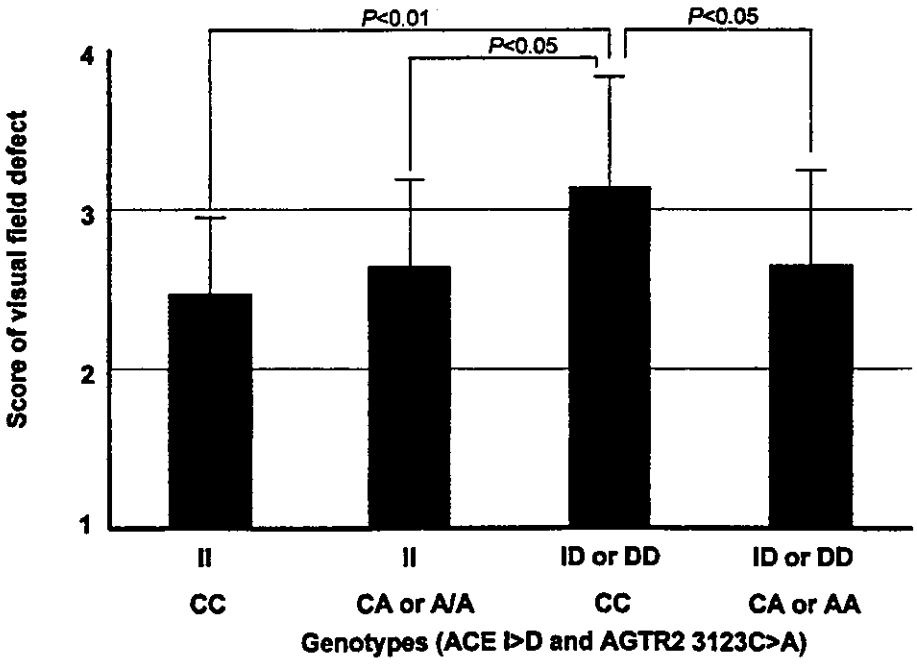

FIGURE 2. Comparison of visual field scores of female patients with NTG, according to *ACE* (I/D) and *AT2* genotypes (3123C→A). Probabilities were obtained by the Scheffé multi-comparison test.

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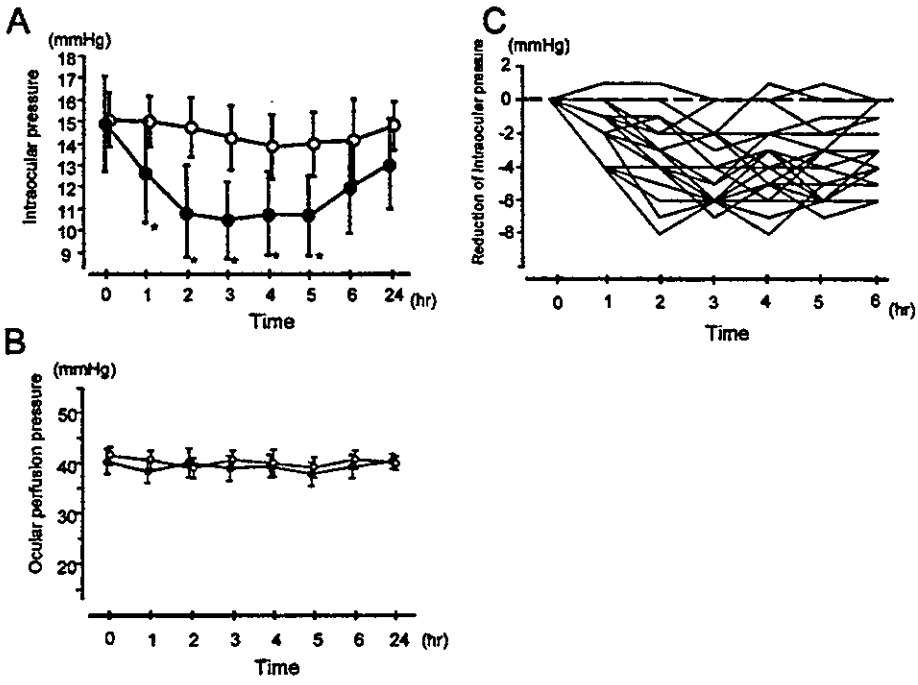

FIGURE 3. Variations in IOP and OPP after oral administration of the angiotensin II receptor blocker candesartan cilexetil (●) or a placebo (○). (A) IOP variations (mean ± SD). ANOVA with the Bonferroni correction, *$P < 0.0001$. (B) OPP variations (mean ± SD). (C) Reduction of IOP variations in 20 subjects.

phism may be in linkage disequilibrium with an unidentified functional variant of the *AGTR2* gene. Alternatively, the polymorphism may be in linkage disequilibrium with a nearby gene responsible for associations with the clinical end points. Further study is necessary to identify the new functional polymorphisms associated with the *AGTR2*/3123C→A polymorphism.

Of interest, the *AGTR2* polymorphism was associated with NTG only in women, whereas the *AGTR1* polymorphisms were likely to be associated with POAG. Accordingly, different pathogenetic mechanisms appear to exist in these two diseases, although clinically they are considered to represent parts of a continuum. AGTR1 mediates the vasopressive and aldosterone-secreting effects of ATII. Furthermore, AGTR1 may mediate aqueous humor dynamics and therefore affect IOP,[49] which is strongly supported by the lowering of IOP by systemic use of an ARB.[21,22] However, the function of *AGTR2* is unknown. This receptor is apparently involved in the morphogenesis of the central nervous system and the urinary tract. Allelic variants of *AGTR2* have been associated with mental retardation,[50] and there is also a strong association between allelic variants and increased incidence of congenital anomalies of the kidney and lower urinary tract.[51] Yamada et al.[52] hypothesized that *AGTR2* mediates programmed cell death (apoptosis) which is considered to play an important role in developmental biology.

The effect of the ARB losartan potassium on IOP has demonstrated that drug administration significantly reduces IOP in normal subjects who do or do not have hypertension and in patients with POAG with or without hypertension.[21] The total outflow facility increased significantly in all subjects, and SBP decreased only in hypertensive patients. These results suggest that the mechanism is not mediated by a decrease in blood

TABLE 6. Effects of Angiotensin II Receptor Blocker on IOP in Association with Genotypes of the Angiotensin II Receptor Genes

Polymorphisms	Genotype	Eyes (*n*)	Maximum Reduction of IOP (mm Hg)	*P**
AGTR1 −713T→G	TT	18	4.9 ± 1.8	
	TG	2	5.0 ± 4.2	0.898
	GG	0	0	
AGTR1 −521C→T	CC	18	4.9 ± 1.8	
	CT	1	2	0.117†
	TT	1	8	
AGTR1 1166A→C	AA	18	5.1 ± 2.0	
	AC	2	5.2 ± 1.6	0.405
	CC	0	0	
AGTR2 3123C→A	C (male)	9	5.0 ± 1.1	0.014‡
	A (male)	4	2.3 ± 0.5	
	CC (female)	3	7.0 ± 1.0	
	CA (female)	4	6.0 ± 1.6	0.354
	AA (female)	0	0	

* Probabilities by Mann-Whitney *U* test.
† Probabilities by Kruskal-Wallis test.
‡ $P < 0.05$.

pressure, but rather is more specific, confirming the role of the RAS in the regulation of IOP.[21] We studied the effect of another ARB, candesartan cilexetil, on IOP and demonstrated a reduction in IOP for 5 hours after administration.

Miller et al.[53] demonstrated a relationship between the *AT1R*/1166A→C polymorphism and the renal hemodynamic response to losartan potassium in a Canadian group. In our study, we examined a relationship between the presence of three *AGTR1* polymorphisms or of one *AGTR2* polymorphism and the degree of reduction of IOP by candesartan cilexetil. No relationship was observed for the three *AGTR1* polymorphisms and IOP reduction. For the *AGTR2*/3123C→A polymorphism, however, nine men with the C allele (5.0 ± 1.1 mm Hg, $P = 0.014$) had a significantly greater reduction in IOP than did four men with the A allele (2.3 ± 0.5 mm Hg). Further studies are needed to determine the genetic locus responsible for this effect.

In conclusion, the polymorphisms of the angiotensin II receptor gene in the RAS may be a major genetic risk factor for the development or progression of glaucoma in the Japanese population. The RAS-related genetic background influencing susceptibility may differ between patients with POAG and those with NTG.

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APPENDIX

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