Lattice Corneal Dystrophy Type IV (p.Leu527Arg) Is Caused by a Founder Mutation of the *TGFBI* Gene in a Single Japanese Ancestor

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Purpose. Lattice corneal dystrophy (LCD) type IV (LCD4) is a late-onset corneal dystrophy with amyloid deposition at the deep stromal layer of cornea. As with other corneal dystrophies, this LCD subtype is also caused by a mutation (p. Leu527Arg) of the transforming growth factor, β-induced (*TGFBI*) gene. Although LCD type I has been reported worldwide, LCD4 has been reported only in the Japanese population. In the present study, a haplotype analysis was performed to investigate whether this LCD subtype is caused by a founder mutation.

Methods. Genomic DNA samples were extracted from 13 unrelated patients with LCD4. As a control, genomic DNA samples from 96 normal volunteers were also analyzed. For the haplotype analysis, the samples were amplified by polymerase chain reaction (PCR), TA-cloned, isothermally amplified, and subjected to a 1-base primer extension assay against a mutation site (c.1580T>G) and six known single-nucleotide polymorphisms (SNPs; rs4669, rs2072239, rs7727725, rs17689879, rs6871571, and rs3792900), which are located adjacent to the mutation site.

Results. The haplotype analysis revealed that all the disease-carrying alleles from the 13 LCD4 patients shared an identical haplotype, whereas non-disease-carrying alleles from the normal volunteers and the LCD4 patients exhibited four haplotypes. There was a statistically significant difference in the haplotype distribution between the disease-carrying and the non-disease-carrying alleles.

Conclusions. The findings of this study strongly indicate that LCD4 was caused by a founder mutation of the *TGFBI* gene that occurred in a single Japanese ancestor. (*Invest Ophthalmol Vis Sci.* 2010;51:4523-4530) DOI:10.1167/iovs.10-5343

Cornea is one of the most transparent tissues in the body, and a substantial number of genes contribute to the attainment and maintenance of the specific properties of this tissue.[1,2] Recent advances in molecular biology have allowed us to understand corneal physiology and disease at the molecular level. One of the prominent events in this research area is the discovery of the transforming growth factor, β-induced (*TGFBI*) gene as a causative gene in five classic autosomal dominant corneal dystrophies.[3] Subsequently, other types of inherited corneal dystrophies, such as Meesmann corneal dystrophy (MECD)[4,5] and gelatinous droplike corneal dystrophy (GDLD),[6,7] have been reported.

Lattice corneal dystrophy (LCD) is characterized by stromal amyloid depositions that typically appear as a network or lattice. LCD type I (LCD1) is one of the five dominant *TGFBI*-related corneal dystrophies with characteristic latticelike refractile lines within the corneal stroma.[8] Other than this common LCD, several minor ones have been reported in the *TGFBI* gene (currently designated as Variant LCD in the IC3D classification) that are caused by different mutations.[9] LCD type IV (LCD4, a variant LCD) is one such corneal dystrophy first reported in 1998 as a late-onset LCD with characteristic amyloid depositions located at the deep stromal layer of cornea.[10] Although LCD1 has been reported world-wide,[3,11] LCD4 has been reported only in the Japanese population.[12-19] Thus, some researchers have theorized that LCD4 may be caused by a founder mutation that occurred in a Japanese ancestor.[20]

In this study, we performed a haplotype analysis on genomic DNA samples obtained from 13 patients with LCD4 to investigate this theory. We found that all the disease-carrying alleles of the investigated 13 LCD4 patients shared an identical haplotype around its causative mutation, whereas healthy alleles exhibited four haplotypes with no apparent preference. These data strongly suggest that all LCD4 mutations descend from a founder mutation that occurred in a single Japanese ancestor.

Materials and Methods

Human Samples

Peripheral blood was obtained from 13 patients from 13 unrelated families who had received a clinical diagnosis of LCD4. These 13 patients were 7 men and 6 women, ranging in age from 52 to 83 years (mean age, 69.8). Seven resided in Kyoto, one in Osaka, one in Mie, one in Niigata, one in Kanagawa, and two in Tokyo Prefecture (Fig. 1). Genomic DNA samples from 96 normal Japanese volunteers (48 men and 48 women) were obtained from a research-resource bank (Human Science Research Resource Bank, Osaka, Japan). Written informed consent was obtained from all patients after they were given a detailed explanation of the study protocols. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Committee for Ethical Issues at Kyoto Prefectural University of Medicine.

Mutation Analysis

Genomic DNA samples were extracted from the peripheral blood of all 13 LCD4 patients by using a commercially available, standard column-

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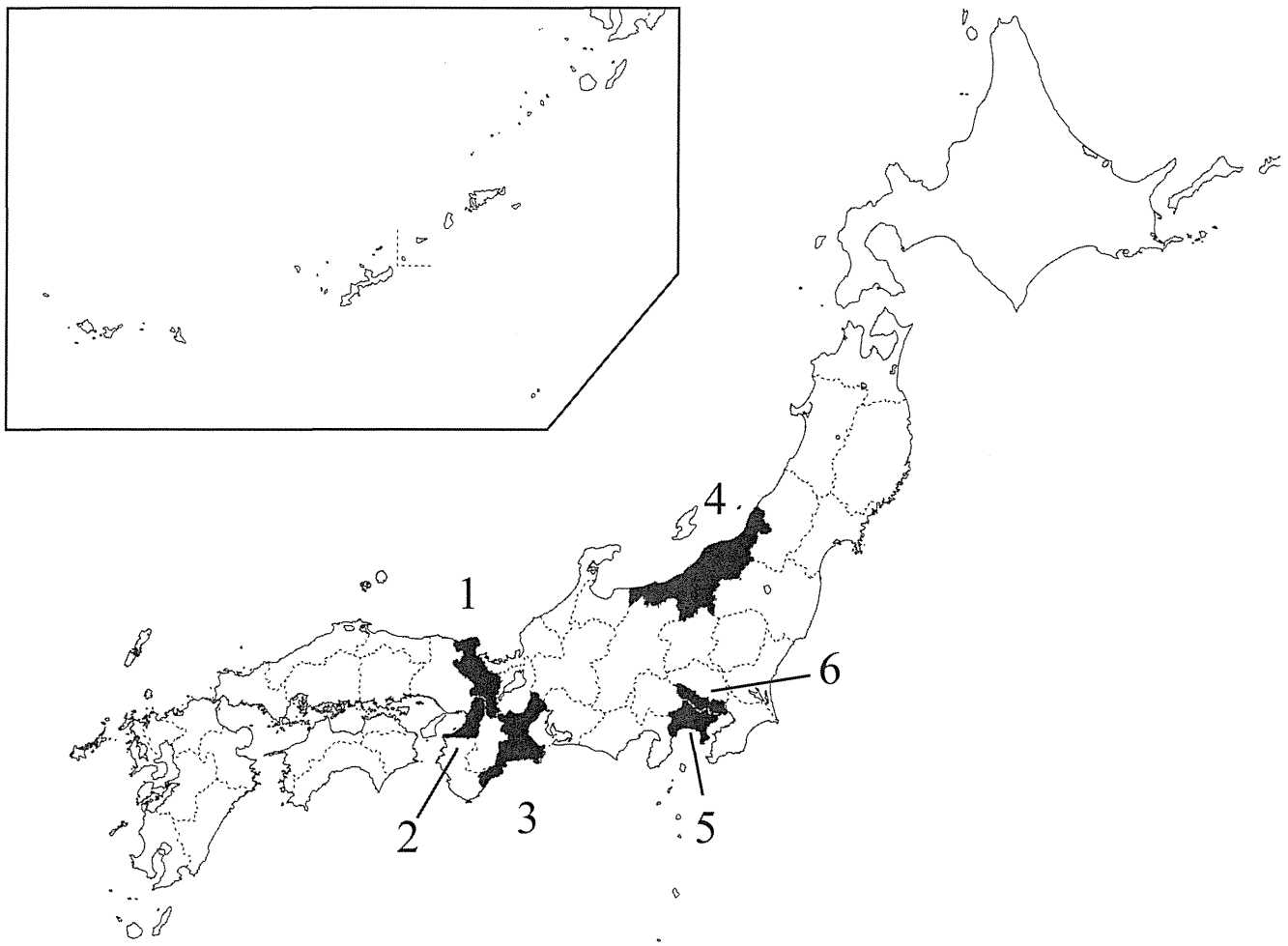

FIGURE 1. Geographic distribution of the residences of the 13 LCD4 patients. Prefectures of residence are shown in *black* and marked as 1, Kyoto; 2, Osaka; 3, Mie; 4, Niigata; 5, Kanagawa; and 6, Tokyo.

based kit (DNeasy Blood & Tissue Kit; Qiagen, GmbH, Hilden, Germany). The samples were then quantitated by the use of a spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE) and electrophoresed on a 1% agarose gel, to check their integrity. Next, the samples were amplified by polymerase chain reaction (PCR) with primer pairs (Table 1) against the mutation hot spots (exons 4, 11,

TABLE 1. List of the Oligomers

Oligomer	Target	Purpose	Direction	Sequence
Exon4_F	TGFBI	PCR	Forward	CCCCAGAGGCCATCCCTCCT
Exon4_R	TGFBI	PCR	Reverse	CCGGGCAGACGGAGGTCATC
Exon11_F	TGFBI	PCR	Forward	CTCGTGGGAGTATAACCAGT
Exon11_R	TGFBI	PCR	Reverse	TGGGCAGAAGCTCCACCCGG
Exon12_F	TGFBI	PCR	Forward	GACAGGTGACATTTTCTGTGT
Exon12_R	TGFBI	PCR	Reverse	GATCACTACTTTAGAAAAATG
Exon13_R	TGFBI	PCR	Reverse	GCTGCAACTTGAAGGTTGTG
TGFBI_27889	c.1580	1-Base primer extension	Forward	TGCCATCCAGTCTGCAGGAC
TGFBI_27929	rs4669	1-Base primer extension	Forward	TTTTTTTTTTGAAGGAGTCTACACAGTCTT
TGFBI_28010	rs2072239	1-Base primer extension	Forward	TTTTTTTTTTTTTTGTAAAGACCAACTTAAGTACAC
TGFBI_28238	rs7727725	1-Base primer extension	Forward	TTTTTTTTTTTTTTTTTTTTTTTTCAGGAACCAGGGAGGTCA
TGFBI_28641	rs17689879	1-Base primer extension	Forward	TTTTTTTTTTTTTTTTTTTTTTTTTTTTGGCAGGGGATCTAGTGGTTA
TGFBI_28700	rs6871571	1-Base primer extension	Forward	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCAGCCTGTGTTGGGAGGATT
TGFBI_29257	rs3792900	1-Base primer extension	Forward	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTGTTAGAGGTTGGTACAGG

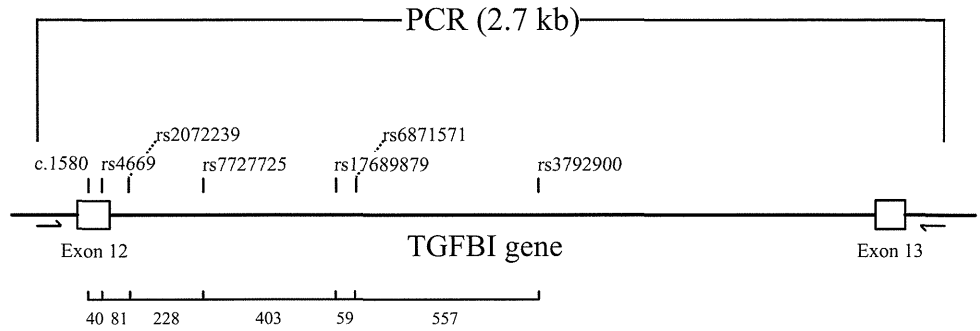

FIGURE 2. The genomic structure for the *TGFBI* gene with sites of mutation and SNPs investigated. *Arrows*: PCR primers that amplify a fragment containing all the SNP and mutation sites. The physical distance between two neighboring SNPs is also indicated in base lengths at the *bottom*.

and 12) of the *TGFBI* gene. The amplified products were then treated with a mixture of exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT; GE Health Care, Ltd., Little Chalfont, UK) to digest residual dNTP and primer. The amplified products were then subjected to sequencing reaction (BigDye Terminator ver. 3.1 Cycle Sequencing Kit; Applied Biosystems, Inc. [ABI], Foster City, CA), and the products were electrophoresed on an automated sequencer (3130xl Genetic Analyzer; ABI). The sequence data were analyzed through the use of commercially available alignment software (Variant Reporter; ABI).

Haplotype Analysis

Genomic DNA samples were amplified by PCR using a primer pair (exon12_F and exon13_R; Table 1) against a genomic region between exon 12 and 13 of the *TGFBI* gene, which harbors the site of the c.1580T>G mutation and six known SNPs (Fig. 2, Table 2). The amplified products were then electrophoresed on a 1% agarose gel, excised, purified with a commercially available column-based purification kit (Wizard SV Gel and PCR Clean-Up System; Promega, Madison, WI), and ligated to a TA-cloning vector (pGEM-T Easy Vector; Promega). The plasmid vector was transformed into chemically competent *Escherichia coli* cells (competent high JM-109; Toyobo Co., Ltd., Osaka, Japan) and seeded on a 1% LB agar plate supplemented with IPTG and X-gal for the standard blue-white selection. After 24 hours' incubation, 16 white colonies were picked from each sample and isothermally amplified overnight with a phi29 polymerase-based plasmid amplification kit (Illustra TempliPhi DNA Amplification Kit; GE Health Care). Each of the amplified products was then subjected to a 1-base primer extension assay (SNaPshot; ABI) with seven pooled primers (Table 1) against the sites of the mutation and the six known SNPs. After treatment with shrimp alkaline phosphatase, the assay products were electrophoresed on the automated sequencer, and the data were analyzed with the use of commercially available software (GeneMapper Software; ABI). Because artificial recombination presumably occurring during the PCR amplification and the bacterial transformation is not negligible in this analysis, a Perl-based program (HapTyper.pl) was created to estimate the most probable haplotype pair from the processed data for each sample.

Statistical Analysis

For the identification of the statistical significance in the haplotype distribution between the affected alleles and the nonaffected alleles, χ^2 and Fisher's exact tests were performed with commercially available statistical software (SAS ver. 9.1; SAS Institute Inc., Cary, NC). For the calculation of statistical power, R language (R Foundation, Vienna, Austria) was used.

RESULTS

The enrolled 13 LCD4 patients, except for 1 patient, exhibited similar corneal haze composed of isolated or fused refractile opacities, most of them being dotlike, and some being latticelike. Most important, these depositions were mainly located within the deep stromal layer, which seems to be specific to this disease and of great diagnostic value, as reported previously. Sequencing analysis revealed that all the 13 LCD4 patients enrolled in this study exhibited a substitution mutation (T to G; c.1580T>G), resulting in an amino acid transition from leucine to arginine (p.Leu527Arg; Fig. 3). Only one patient (59-year-old woman) was homozygous for the mutation site, and she exhibited a much more severe corneal phenotype than did other patients heterozygous for the mutation, such as another homozygous LCD4 patient detailed in a previous report.[19] One patient had a heterozygous substitution mutation from A to G at a different nucleotide position (c.1631A>G) that results in an amino acid transition from asparagine to serine (p.Asn544Ser), which has already been reported to be causative of another type of variant LCD.[17,20,21] In this patient, these two mutations were located on different alleles from one another, as determined by subsequence haplotype analysis (data not shown).

Haplotype analysis was performed to examine whether the combination of the six SNPs, which are close to the c.1580T>G mutation and hence show a strong linkage disequilibrium to that mutation site, was identical among the disease-

TABLE 2. List of SNPs within the Amplified Region

Region	rs ID	Heterozygosity	Sequence
Exon12	rs4669*	0.494	CCTCAACCGGGAAGGAGTCTACACAGTCTT (C/T) GCTCCCACAAATGAAGCCTTCCGAGCCCTG
Intron12	rs2072239*	0.294	TGAGGGATCACTACTTTAGAAAAATGGAGA (C/T) GTGTACTTAAGTTGGTCTTTACCCAAGAGT
Intron12	rs7727725*	0.494	GGAGGATGAGAGCAGGAACCAGGGAGGTCA (A/T) GAGCCTTGGACAAGGGCACAGAACAGCAGC
Intron12	rs17689879*	0.444	GAGGATGTTTGGCAGGGGATCTAGTGGTTA (C/T) GGGTGGCTAAGAAAAATGAGGAAGGTAAGA
Intron12	rs6871571*	0.494	GAGTATCTTGCAGCCTGTGTTGGGAGGATT (A/G) AATAGGATGCCACACACAGGGCCAGGCAGA
Intron12	rs58761304	N.D.	GCAGGAATGGGAGTTGCAGTGTTTAGCTCA (G/T) ATGCATGCCTGTGAGAGATGCTTCCACTCT
Intron12	rs3792900*	0.453	TGCATGGGATGTCCTTTCAATATCTCTAAC (A/G) CCTGTACCAACCTCTAACACTCTCTGTCCC
Intron12	rs45583534	0.023	ACTGATGTGGGCTGAAAGGAATGCTGAGAC (A/G) TGACGAGGAGAGATGCTGCGGAGGGAATAT
Intron12	rs41502049	0.076	GAAACATGAGTCATACTCACAGAGGAGTAT (C/G) GATTAACTCCTTCTCAGCAGCCAGGGAGCC
Intron12	rs45474493	0.011	AACCCAGAGGCCAACTGACTGCTGGGGCAG (A/T) TTTGTGGTCATGAACATGTGCTTTGTGTCC

The amplified region is illustrated in Figure 2.
* SNPs investigated.

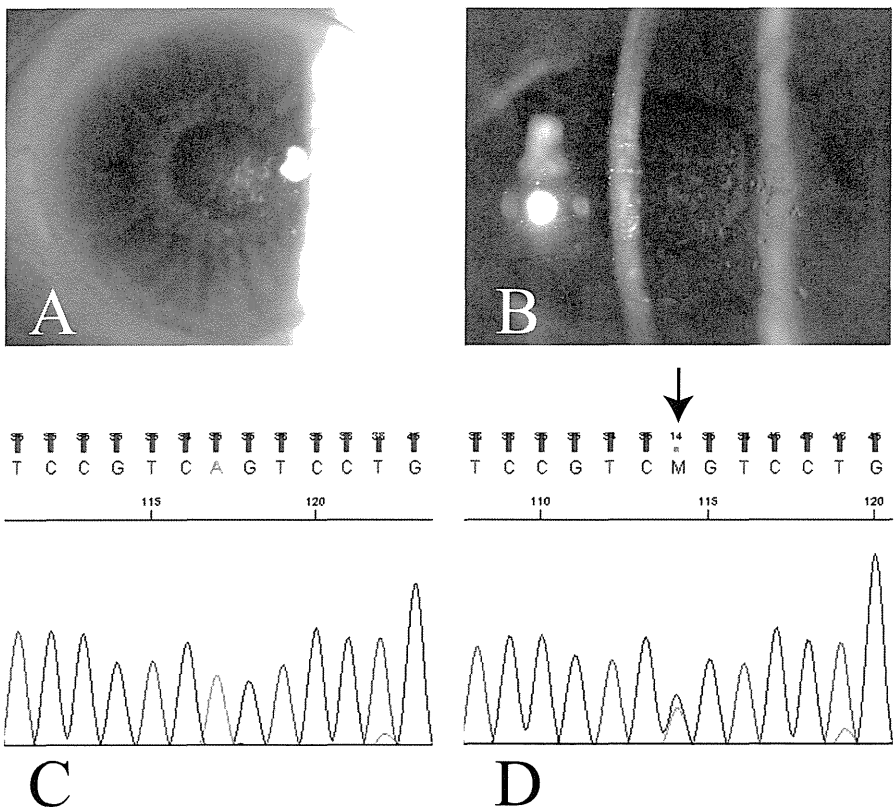

FIGURE 3. Results of the mutation analysis of the 13 LCD4 patients. Scleral scattering (**A**) and iris retroillumination (**B**) demonstrate the corneal opacity in LCD4. The depth of opacity was nearly at the level of Descemet's membrane. Compared with sequence data from normal volunteers (**C**), LCD4 patients carried a heterozygous (**D**) or homozygous T-to-G conversion at c.1580 nucleotide position (*arrow*).

carrying alleles of the 13 LCD4 patients. As expected, all the 14 (12 heterozygous plus 1 homozygous) disease-carrying alleles found in the patients demonstrated an identical SNP-haplotype (Table 3). Two of the 12 healthy alleles from the patients exhibited the same SNP-haplotype as found in the disease-carrying alleles. Three different haplotypes were also found in the healthy alleles. A random cross section of normal volunteers was also examined to investigate which haplotypes are

TABLE 3. Results of the Haplotype Analysis

Patient	Allele	c.1580	rs4669	rs2072239	rs7727725	rs17689879	rs6871571	rs3792900
LCD4_1	Healthy	T	C	G	A	C	G	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_2	Healthy	T	C	A	A	C	G	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_3	Healthy	T	T	G	T	C	A	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_4	Healthy	T	C	G	A	C	G	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_5	Healthy	T	C	A	A	C	G	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_6	Healthy	T	T	G	T	C	A	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_7	Healthy	T	T	G	T	T	A	T
	Disease-carrying	G	T	G	T	T	A	T
LCD4_8	Healthy	T	C	G	A	C	G	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_9	Healthy	T	C	A	A	C	G	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_10	Disease-carrying	G	T	G	T	T	A	T
	Disease-carrying	G	T	G	T	T	A	T
LCD4_11	Healthy	T	T	G	T	C	A	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_12	Healthy	T	T	G	T	T	A	T
	Disease-carrying	G	T	G	T	T	A	T
LCD4_13	Healthy	T	T	G	T	C	A	C
	Disease-carrying	G	T	G	T	T	A	T

c.1580 is the site of causative mutation in LCD4, where T means wild-type and G means mutated-type. All disease-carrying alleles shared an identical SNP haplotype (T-G-T-T-A-T). Note that patient LCD4_10 had c.1580T>G mutations in both alleles.

TABLE 4. Summarization of the Data Obtained by Haplotype Analysis of the 13 LCD4 Patients and the 91 Normal Volunteers

Haplotype	Normal Volunteers Healthy Allele	LCD4 Patients	
		Healthy Allele	Disease-Carrying Allele
C-A-A-C-G-C	37	3	0
C-G-A-C-G-C	23	3	0
T-G-T-C-A-C	57	4	0
T-G-T-T-A-T	65	2	14

dominant in the current Japanese population. Among the 96 normal samples, 91 produced data with sufficient quality for the subsequent analysis, but data from the other 5 samples were omitted due to insufficient quality. In the 91 normal samples, four haplotypes were found, which were identical with those found in the healthy alleles of the LCD4 patients. Of those, the mutation-related haplotype (T-G-T-T-A-T) appeared the most abundant (65/182; 35.7%) in the current Japanese population (Table 4). Statistical significance was found (P = 0.00003, χ^2 test, or P = 0.00002, Fisher's exact test) in difference between the haplotype distribution in the disease-carrying and the healthy alleles. The statistical power for our enrolled samples was 1 at the 0.05 level of significance.

DISCUSSION

Allelic homogeneity is a prominent feature of the TGFBI-related corneal dystrophies.[22,23] This fact can be well explained by two different mechanisms: the first is the presence of mutation hot spots,[24] and the second is a founder mutation. The mutation hot spots are mainly located at cytosine or guanine within CpG dinucleotides. Cytosine within the CpG dinucleotide is frequently modified by methylation (5-methylated cytosine; 5meC) in mammalian cells, mainly for epigenetic regulation.[25] The 5meC can be spontaneously deaminated, thus producing thymine which results in C>T or G>A conversion when the deamination occurs in a sense or antisense strand, respectively.[26] Although a DNA mismatch-repair mechanism normally recognizes and repairs such heteroduplex sites,[27] the conversions are occasionally passed over unnoticed, possibly due to the insufficient stringency of the repair mechanism. Eventually, when such a conversion occurs in a germ-line cell, the C>T or G>A conversions can be inherited over generations.

Within the spectrum of *TGFBI*-related corneal dystrophies, LCD1 and granular corneal dystrophy type 1 (GCD1) are caused by the C>T conversion, granular corneal dystrophy type 2 (GCD2, alternatively designated as Avellino corneal dystrophy) and Thiel-Behnke corneal dystrophy (TBCD)[28] are caused by the G>A conversion, with all these conversions occurring at CpG sites (Table 5). Therefore, it is strongly supposed that these four dominant *TGFBI*-related corneal dystrophies are caused by that mutation mechanism. In actuality, these dystrophies occur at a relatively high frequency in many countries, thus implicating the existence of multiple, independently occurring founders, in different areas of the world.

Apart from the mutation mechanism, other types of mutations, including non-C>T or non-G>A mutations or C>T or G>A mutations occurring at non-CpG sites, sometimes occur. Such types of mutations seem to be mainly caused by the accidentally occurring replication error during cell division that may escape the proofreading function of DNA polymerase, as well as the DNA mismatch-repair process, with a roughly estimated frequency of 1 in 10^9 to 10^{10} base pairs per cell division.[27] Hence, these mutations are predisposed to be much

TABLE 5. Suspected Mutation Mechanism for the *TGFBI*-Related Corneal Dystrophies

Corneal Dystrophy	Nucleotide Change	Protein Change	Mutated at CpG or Non-CpG Site	Mutation Mechanism	Reported Countries
LCD1	c.370C>T	p.Arg124Cys	CpG	Deamination	Brazil, Bulgaria, Chile, China, Czech Republic, France, Germany, Hungary, India, Japan, South Korea, Spain, Switzerland, Thailand, United Kingdom, Ukraine, United States, Vietnam
GCD1	c.1663C>T	p.Arg555Trp	CpG	Deamination	China, Czech Republic, France, Germany, Hungary, India, Japan, Mexico, New Zealand, Poland, Spain, Switzerland, Taiwan, Turkey, United Kingdom, Ukraine, United States, Vietnam
GCD2	c.371G>A	p.Arg124His	CpG	Deamination	China, France, Germany, Hungary, India, Iran, Japan, South Korea, Spain, Switzerland, United Kingdom, United States, Vietnam
TBCD	c.1664G>A	p.Arg555Gln	CpG	Deamination	Brazil, China, Czech Republic, France, Hungary, Japan, New Zealand, Switzerland, United Kingdom, United States
RBCD	c.371G>T	p.Arg124Leu	CpG	Replication error	Brazil, China, Czech Republic, Denmark, France, India, Japan, Switzerland
LCD3A	c.1501C>A	p.Pro501Thr	non-CpG	Replication error	Japan
LCD4	c.1580T>G	p.Leu527Arg	non-CpG	Replication error	Japan
Variant LCD	c.1640T>C	p.Phe547Ser	non-CpG	Replication error	Hungary
Variant LCD	c.1874T>A	p.Val625Asp	non-CpG	Replication error	China

LCD1, lattice corneal dystrophy type 1; GCD1, granular corneal dystrophy type 1; GCD2, granular corneal dystrophy type 2; TBCD, Thiel-Behnke corneal dystrophy; RBCD, Reis-Bückler's corneal dystrophy; LCD3A, lattice corneal dystrophy type IIIA; LCD4, lattice corneal dystrophy type IV.

less frequent and tend to become a founder mutation. Other than the four dominant *TGFBI*-related corneal dystrophies described earlier, those including LCD4 clearly meet the criteria for this mutation mechanism (Table 5). In fact, most of these corneal dystrophies occur infrequently and are reported in only one, or at most, only a few countries. For example, in the Japanese population, founder mutations have been reported in corneal dystrophies of LCD type IIIA[23] (currently designated as variant LCD in the IC3D classification), GCD2,[22] and GDLD with a p.Gln118X mutation.[7] Our current haplotype analysis of the 13 LCD4 patients revealed that all their disease-carrying alleles share an identical SNP-haplotype. Since the residences of the 13 patients were not restricted to a small geographic area, but in fact, extended across six different prefectures in Japan (Fig. 1), such biased data seem to be ascribed, not to the preference of a certain SNP-haplotype that was due to a bias toward their place of residence, but rather to the occurrence of a founder mutation in a Japanese ancestor. We imagine that almost all infrequently occurring corneal dystrophies may have been related to the occurrence of founder mutations.

One exception to the hypothesis for the occurrence of a mutation among the *TGFBI*-related corneal dystrophies appears to be Reis-Bücklers corneal dystrophy (RBCD). The mutation of RBCD is located on the same CpG site as that of LCD1 and GCD2 but its substitution is G>T, not C>T or G>A. Therefore, the cause of mutation in RBCD seems to be a simple replication error not a deamination. However, RBCD has been reported in many countries, similar to the other four dominant *TGFBI*-related corneal dystrophies. Therefore, the exact mechanism for the occurrence of the RBCD mutation cannot be

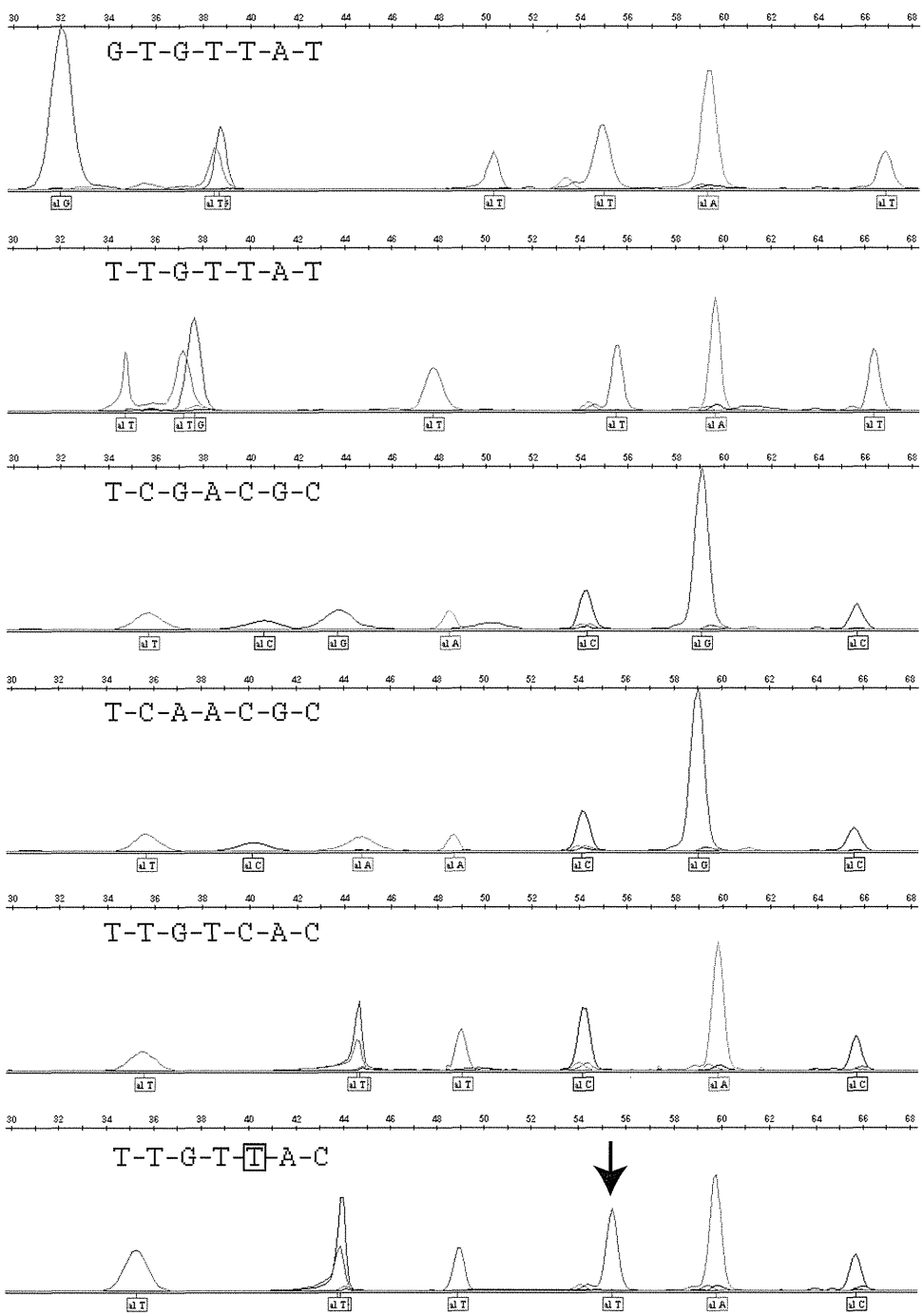

FIGURE 4. Results of the 1-base primer extension assay. Shown are representative chromatogram data for all the observed haplotypes, as indicated at the *top left* corners. The first base (G or T) is of the site of the mutation (c.1580), not of the SNP. The T-T-G-T-T-A-C haplotype is an artificial product by the recombination of the T-T-G-T-C-A-C and the T-T-G-T-T-A-T haplotypes at the indicated site (*square*).

explained by our hypothesis, but it is hoped that it will be elucidated in the future.

We performed a 1-base primer extension assay for the identification of the SNP-haplotype. The reason we chose that method rather than the standard sequencing analysis is that the former method can be easily multiplexed and hence largely save time and costs compared with the latter method. In addition, a commercially available phi29 polymerase-based, isothermal plasmid amplification procedure has been developed.[29] This procedure is much more time-efficient than the standard plasmid DNA extraction procedure and has been subjected to various applications.[30–33] Thus, we examined whether the phi29 polymerase-amplified plasmid DNA can also be used as the template for the 1-base primer extension assay. As is shown in Figure 4, the chromatogram data of this combined assay was of sufficient quality for the identification of each of the SNPs examined in this study. We think that this combined procedure is quite useful for experimentally determining haplotypes.

We initially expected that data from the 16 plasmids investigated for each sample could be easily divided into one or two haplotype groups without any confusion. However, the actual data were sometimes quite complicated, possibly due to the artificial recombination during the consecutive processes of PCR and bacterial transformation, as reported previously.[34] In that study, a method of eliminating such an artificial recombination was also reported. However, although we performed an additional three-cycle PCR amplification against a 10-times diluted initial PCR product, according to this reconditioning PCR method, the artificial recombination was still observed, possibly due to the insufficiency of the reconditioning PCR. For example, a mutation/SNP-combination T-T-G-T-T-A-C was observed in a normal volunteer who was found to have two haplotypes (T-T-G-T-C-A-C and T-T-G-T-T-A-T; Fig. 4). Therefore, we created a Perl-based software program, to identify the most probable haplotype pair by calculating a score for each of the possible haplotypes estimated from the genotype data of each sample. We carefully reviewed the processed data and found that the software worked properly. We think that this software may be useful for studies conducted in the future that are similar to the present study.

In summary, our findings indicate that LCD4 was caused by a founder mutation that occurred in a single Japanese ancestor. In addition, we have established a time- and cost-efficient new procedure through the combination of an isothermal amplification of plasmid DNA and a 1-base primer extension assay. We created a Perl-based software program that helps estimate the most probable haplotype pair from blended data hampered by randomly occurring artificial recombinations. We hope that the results of this study, as well as the newly developed procedures, will contribute to the further understanding of the etiology, populational genetics, and pathogenesis of inherited dystrophies of the cornea and of other organs.

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References

1. Nishida K, Adachi W, Shimizu-Matsumoto A, et al. A gene expression profile of human corneal epithelium and the isolation of human keratin 12 cDNA. *Invest Ophthalmol Vis Sci.* 1996;37:1800–1809.
2. Kinoshita S, Adachi W, Sotozono C, et al. Characteristics of the human ocular surface epithelium. *Prog Retin Eye Res.* 2001;20:639–673.
3. Munier FL, Korvatska E, Djemai A, et al. Kerato-epithelin mutations in four 5q31-linked corneal dystrophies. *Nat Genet.* 1997;15:247–251.
4. Irvine AD, Corden LD, Swensson O, et al. Mutations in cornea-specific keratin K3 or K12 genes cause Meesmann's corneal dystrophy. *Nat Genet.* 1997;16:184–187.
5. Nishida K, Honma Y, Dota A, et al. Isolation and chromosomal localization of a cornea-specific human keratin 12 gene and detection of four mutations in Meesmann corneal epithelial dystrophy. *Am J Hum Genet.* 1997;61:1268–1275.
6. Tsujikawa M, Tsujikawa K, Maeda N, et al. Rapid detection of M1S1 mutations by the protein truncation test. *Invest Ophthalmol Vis Sci.* 2000;41:2466–2468.
7. Tsujikawa M, Kurahashi H, Tanaka T, et al. Identification of the gene responsible for gelatinous drop-like corneal dystrophy. *Nat Genet.* 1999;21:420–423.
8. Mannis MJ, De Sousa LB, Gross RH. The stromal dystrophies. In: Krachmer JH, Mannis MJ, Holland EJ, eds. *Cornea.* St. Louis, MO: Mosby; 1997:1048–1051.
9. Weiss JS, Moller HU, Lisch W, et al. The IC3D classification of the corneal dystrophies. *Cornea.* 2008;27(suppl 2):S1–S83.
10. Fujiki K, Hotta Y, Nakayasu K, et al. A new L527R mutation of the betaIGH3 gene in patients with lattice corneal dystrophy with deep stromal opacities. *Hum Genet.* 1998;103:286–289.
11. Hotta Y, Fujiki K, Ono K, et al. Arg124Cys mutation of the betaig-h3 bene in a Japanese family with lattice corneal dystrophy type I. *Jpn J Ophthalmol.* 1998;42:450–455.
12. Fujiki K, Hotta Y, Nakayasu K, et al. Six different mutations of TGFBI (betaig-h3, keratoepithelin) gene found in Japanese corneal dystrophies. *Cornea.* 2000;19:842–845.
13. Fujiki K, Nakayasu K, Kanai A. Corneal dystrophies in Japan. *J Hum Genet.* 2001;46:431–435.
14. Funayama T, Mashima Y, Kawashima M, Yamada M. Lattice corneal dystrophy type III in patients with a homozygous L527R mutation in the TGFBI gene. *Jpn J Ophthalmol.* 2006;50:62–64.
15. Hirano K, Hotta Y, Nakamura M, Fujiki K, Kanai A, Yamamoto N. Late-onset form of lattice corneal dystrophy caused by leu527Arg mutation of the TGFBI gene. *Cornea.* 2001;20:525–529.
16. Hirano K, Nakamura M, Yamamoto N, Hotta Y. Geographical feature of lattice corneal dystrophy patients in Aichi Prefecture: an analysis of the TGFBI gene (in Japanese). *Nippon Ganka Gakkai Zasshi.* 2002;106:352–359.
17. Kawashima M, Yamada M, Funayama T, Mashima Y. Six cases of late-onset lattice corneal dystrophy associated with gene mutations induced by the transforming growth factor-beta (in Japanese). *Nippon Ganka Gakkai Zasshi.* 2005;109:93–100.
18. Nakagawa E, Sakimoto T, Inada N, et al. Histopathological study of lattice corneal dystrophy with L 527 R mutation of transforming growth factor-beta induced gene (in Japanese). *Nippon Ganka Gakkai Zasshi.* 2004;108:118–123.
19. Yamada N, Chikama TI, Morishige N, et al. Homozygous mutation (L527R) of TGFBI in an individual with lattice corneal dystrophy. *Br J Ophthalmol.* 2005;89:771–773.
20. Mashima Y, Yamamoto S, Inoue Y, et al. Association of autosomal dominantly inherited corneal dystrophies with BIGH3 gene mutations in Japan. *Am J Ophthalmol.* 2000;130:516–517.
21. Nakagawa Asahina S, Fujiki K, Enomoto Y, Murakami A, Kanai A. Case of late onset and isolated lattice corneal dystrophy with Asn544Ser (N544S) mutation of transforming growth factor beta-induced (TGFBI, BIGH3) gene (in Japanese). *Nippon Ganka Gakkai Zasshi.* 2004;108:618–620.
22. Tsujikawa K, Tsujikawa M, Watanabe H, et al. Allelic homogeneity in Avellino corneal dystrophy due to a founder effect. *J Hum Genet.* 2007;52:92–97.
23. Tsujikawa K, Tsujikawa M, Yamamoto S, Fujikado T, Tano Y. Allelic homogeneity due to a founder mutation in Japanese patients with lattice corneal dystrophy type IIIA. *Am J Med Genet.* 2002;113:20–22.
24. Korvatska E, Munier FL, Djemai A, et al. Mutation hot spots in 5q31-linked corneal dystrophies. *Am J Hum Genet.* 1998;62:320–324.
25. Holliday R. DNA methylation and epigenetic inheritance. *Philos Trans R Soc Lond B Biol Sci.* 1990;326:329–338.
26. Cooper DN, Youssoufian H. The CpG dinucleotide and human genetic disease. *Hum Genet.* 1988;78:151–155.
27. Hsieh P, Yamane K. DNA mismatch repair: molecular mechanism, cancer, and ageing. *Mech Ageing Dev.* 2008;129:391–407.
28. Ridgway AE, Akhtar S, Munier FL, et al. Ultrastructural and molecular analysis of Bowman's layer corneal dystrophies: an epithelial origin? *Invest Ophthalmol Vis Sci.* 2000;41:3286–3292.

29. Nelson JR, Cai YC, Giesler TL, et al. TempliPhi, phi29 DNA polymerase based rolling circle amplification of templates for DNA sequencing. *BioTechniques.* 2002;Jun(suppl):44–47.
30. Li J, Zhong W. Typing of multiple single-nucleotide polymorphisms by a microsphere-based rolling circle amplification assay. *Anal Chem.* 2007;79:9030–9038.
31. Hutchison CA, 3rd, Smith HO, Pfannkoch C, Venter JC. Cell-free cloning using phi29 DNA polymerase. *Proc Natl Acad Sci U S A.* 2005;102:17332–17336.
32. Reddy PS, Mahanty S, Kaul T, Nair S, Sopory SK, Reddy MK. A high-throughput genome-walking method and its use for cloning unknown flanking sequences. *Anal Biochem.* 2008;381:248–253.
33. Li N, Jablonowski C, Jin H, Zhong W. Stand-alone rolling circle amplification combined with capillary electrophoresis for specific detection of small RNA. *Anal Chem.* 2009;81:4906–4913.
34. Thompson JR, Marcelino LA, Polz MF. Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. *Nucleic Acids Res.* 2002;30:2083–2088.

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ORIGINAL ARTICLE

Metabolic syndrome as a risk factor for high-ocular tension

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Objective: To investigate the relationship between the metabolic syndrome and intraocular pressure (IOP).
Methods: An observational study was conducted in a medical health checkup program at a general hospital. This study involved 14 003 apparently healthy Japanese men and women, 18–83 years of age, with a mean IOP of 14.8 (3.0) mm Hg. IOP was examined by noncontact tonometer. High-ocular tension was defined as IOP >21 mm Hg without optic-disc abnormalities or history of receiving any anti-glaucoma therapy. Modified criteria of the revised National Cholesterol Education Program Adult Treatment Panel III (rATPIII), the new International Diabetes Federation definition, and the Japan Society for The Study of Obesity definition were used to characterize the metabolic syndrome. Air temperature was assessed from the Gifu Meteorological Observatory, Gifu, Japan.
Results: In the male and female subjects, mean IOP and the prevalence of high-ocular tension became high in direct correlation with the increased number of metabolic syndrome components. To analyze by logistic regression, the metabolic syndrome defined by rATPIII was positively and maximum temperature was negatively correlated with high-ocular tension in males (adjusted odds ratio: 2.0 [95% confidence interval, CI, 1.43–2.78] and 0.63 [95% CI, 0.54–0.73], respectively) and in females (adjusted odds ratio: 7.09 [95% CI, 3.74–13.43] and 0.67 [95% CI, 0.53–0.87], respectively). Three of five metabolic syndrome components (fasting plasma glucose, blood pressure, and triglycerides) were related to high-ocular tension.
Conclusion: The metabolic syndrome is a risk factor for high-ocular tension.
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Keywords: intraocular pressure; ocular hypertention; metabolic syndrome; fasting plasma glucose; blood pressure; triglycerides

Introduction

The metabolic syndrome is a collection of risk factors that increase a person's chance of developing heart disease, stroke, and diabetes. The original description of the metabolic syndrome by Reaven consisted of factors including obesity, insulin resistance, hypertension, impaired glucose tolerance or diabetes, hyperinsulinemia, and dyslipidemia characterized by elevated triglyceride, and low high-density lipoprotein (HDL) concentrations.[1] It affects a great number of people worldwide, and the prevalence of this syndrome increases with age. Some studies estimate the current prevalence in the United States to be up to 25% of the population,[2] and among native Japanese to be 41% (range: 19–60%) in men and 51% (range: 31–89%) in women.[3] As our understanding of the action of insulin evolves to comprehensively include the recent insulin-related discoveries,[4] it becomes easier to see that insulin resistance is the basis of most, if not all, of the features of the metabolic syndrome. The original conceptualization of the metabolic syndrome was on the basis of resistance to the metabolic actions of insulin.[5]

Ocular hypertension (OH) is one of the major risk factors of primary open angle glaucoma.[6,7] OH is the state in which

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intraocular pressure (IOP) is >21 mm Hg, the anterior chamber angle is open, and both optic-disc abnormality and visual-field defect are not detectable. Meanwhile, glaucoma is a type of optic neuropathy, and glaucoma patients suffer from a decrease in the quality of vision, which causes a progressive visual-field defect.

An earlier study has shown that the mean IOP value tends to increase linearly according to the number of risk factors for the metabolic syndrome.[8] However, the relationship between the metabolic syndrome and OH is unknown. We designed a cross-sectional study in a healthy Japanese population to examine the possibility of an association between high-ocular tension, which represents OH in this study, and the metabolic syndrome.

Materials and methods

Study design

We performed a cross-sectional study involving participants of a medical health checkup program, including abdominal ultrasonography and IOP measurement. The study was approved by the ethics committee of Murakami Memorial Hospital, Gifu, Japan. The program was conducted in the Medical Health Checkup Center at Murakami Memorial Hospital. The purpose of the medical health checkup program is to promote public health through early detection of chronic diseases and the evaluation of their underlying risk factors. Known as a 'human dock,' medical services of this kind are very popular in Japan.

Study population

All of the subjects participating in such health checkup programs at Murakami Memorial Hospital between January 2004 and December 2008 were invited to join this study. This study was approved by the ethics committee of Murakami Memorial Hospital. Participants who reported earlier ophthalmic anti-glaucoma therapy or surgery, or the use of steroid drugs were excluded from this study.

In addition, participants who reported the use of any drugs, which could influence the metabolic syndrome such as anti-diabetic drugs, anti-hypertensive drugs, anti-dyslipidemic drugs, anti-goat drugs, and/or anti-obesity drugs, were also excluded from the main analysis, but were used in a subgroup analysis. In the main analysis, we assessed the direct effects of the metabolic syndrome and its components on IOP. In the subgroup analysis, we assessed whether the participants who had well-controlled abnormal metabolic profiles because of medication had a decreased risk of high-ocular tension. In this study, all participants who reported the use of any drugs were categorized as the 'medication group.' Those participants discontinued their use of the medication on the day when they received the health check up.

Data collection

The health checkup programs that were used for the collection of data in this study included the following tests: eye examinations, urinalysis, blood-cell counts, blood chemistry, electrocardiography, chest radiography, barium examination of the upper gastrointestinal tract, and abdominal ultrasonography. Trained technicians, hepatologists, and radiologists conducted the abdominal ultrasonography, and hepatologists or radiologists diagnosed the ultrasonographic findings in the Japanese 'human dock.' Ophthalmologic specialists performed the eye examinations. High-ocular tension was defined as right-eye IOP of >21 mm Hg without optic-disc abnormalities or history of receiving any anti-glaucoma therapy. The medical history and lifestyle factors of all participants, including physical activity and habits pertaining to smoking and alcohol consumption, were surveyed by use of a self-administered questionnaire. When the participants had difficulty completing the questionnaire, trained nurses provided assistance. We undertook blood and urine examinations with MODULAR ANALYTICS (Hitachi High-Technologies Corp., Ltd, Tokyo, Japan). Body mass index was calculated as body weight in kilograms divided by the square of the participant's height in meters. The eye examinations consisted of visual acuity, IOP measurement between 9- and 10-o'clock AM by noncontact tonometry (TOPCON CT-90A; TOPCON Corp., Tokyo, Japan) and fundus photography (TOPCON TRC-NW200; TOPCON Corp.) with no mydriasis. Three successive IOP measurements per eye were averaged and the values of right-eye IOP were adopted. Weather parameters were obtained from the Gifu Meteorological Observatory, Gifu, Japan.

Metabolic syndrome

There are several differing criteria for the metabolic syndrome worldwide.[9–14] In this study, we used the following three definitions for metabolic syndrome: (1) the revised National Cholesterol Education Program Adult Treatment Panel III (rATPIII) definition,[13] (2) the new International Diabetes Federation (IDF) definition[11] (the IDF consensus worldwide definition of the metabolic syndrome [article online]: available from http://www.idf.org/webdata/docs/IDF_Meta_def_final.pdf/), and (3) the Japan Society for The Study of Obesity definition.[14]

According to the rATPIII definition,[9] subjects who had three or more of the following criteria were identified as having the metabolic syndrome: (1) triglycerides $\geqslant 1.69\,mmol\,l^{-1}$ ($\geqslant$150 mg per 100 ml), (2) HDL cholesterol $<1.03\,mmol\,l^{-1}$ (<40 mg per 100 ml) for men and $<1.29\,mmol\,l^{-1}$ (<50 mg per 100 ml) for women, (3) blood pressure $\geqslant$130/85 mm Hg, (4) fasting glucose $\geqslant 5.56\,mmol\,l^{-1}$ ($\geqslant$100 mg per 100 ml)[13] instead of $\geqslant 6.11\,mmol\,l^{-1}$ ($\geqslant$110 mg per 100 ml), or (5) abdominal obesity—modified waist circumference cutoffs ($\geqslant$90 cm for men and $\geqslant$80 cm for women) were used[13,15] instead of the waist circumference cutoffs ($\geqslant$102 cm for men and $\geqslant$88 cm for women) proposed in the existing definition.

According to the new IDF definition, Japanese people were defined as having the metabolic syndrome if the subjects had abdominal obesity (waist circumference cutoffs $\geqslant$90 cm for men and $\geqslant$80 cm for women) plus two or more of the following risk factors: (1) elevated triglyceride level $\geqslant$1.69 mmol l^{-1} ($\geqslant$150 mg per 100 ml) or on treatment, (2) low HDL cholesterol $<$1.03 mmol l^{-1} ($<$40 mg per 100 ml) for men and $<$1.29 mmol l^{-1} ($<$50 mg per 100 ml) for women or on treatment, (3) high blood pressure $\geqslant$130/85 mm Hg, or (4) high fasting glucose $\geqslant$5.56 mmol l^{-1} ($\geqslant$100 mg per 100 ml).

The Japan Society for The Study of Obesity, as well as study conducted by Matuzawa, another Japanese researcher, recommended the following four abnormalities to define the metabolic syndrome in Japan: (1) abdominal obesity (abdominal circumference $\geqslant$85 cm for men and $\geqslant$90 cm for women); (2) elevated serum triglyceride level ($\geqslant$150 mg per 100 ml) and/or decreased HDL cholesterol level ($<$40 mg per 100 ml); (3) elevated blood pressure (systolic blood pressure $\geqslant$130 mm Hg and/or diastolic blood pressure $\geqslant$85 mm Hg); and (4) an elevated fasting glucose level ($\geqslant$110 mg per 100 ml). Of the four abnormalities listed above, subjects with (1) plus two or more of (2) through (4) were considered to have the metabolic syndrome.[14]

Sample size

As the prevalence of high-ocular tension in persons with the metabolic syndrome was unknown, a formal sample size estimate was not made a priority in this study.

Statistical methods

The *R* version 2.4.1 (available from http://www.r-project.org/) was used for statistical analyses. Data was expressed as mean (s.d.). Two groups of subjects were compared by using the unpaired *t*-test and the χ^2 test, and a *P*-value of $<$0.05 was accepted as a significant level.

The difference of continuous variable between two groups among multiple groups was analyzed by one-way analysis of variance followed by the Tukey's Honestly Significantly Different test. We analyzed the association of the number of components of the metabolic syndrome with IOP by using a linear regression model. Logistic regression was used to analyze associations between the incidence of high-ocular tension and the metabolic syndrome while controlling for other parameters such as age, duration of sunshine, and maximum temperature. The adjusted odds ratio and 95% confidence intervals (CIs) were calculated.

Results

Between January 2004 and December 2008, we invited 20 012 participants in the health checkup program to enroll in the study. Of those, a total of 16 929 Japanese participants (10 124 men and 6805 women) were enrolled after giving informed consent to be included in the study. We excluded 841 participants (527 men and 314 women) who reported earlier ophthalmic anti-glaucoma therapy or surgery, or usage of steroid drugs. Moreover, 2085 participants (1566 men and 519 women) who reported the use of any drugs, which could influence the metabolic syndrome such as anti-diabetic drugs, anti-hypertensive drugs, anti-dyslipidemic drugs, anti-goat drugs, and/or anti-obesity drugs, were used for a subgroup analysis (medication group). As a result, this study ultimately consisted of 14 003 apparently healthy participants (8031 men and 5972 women). The mean age was 46.0 years (s.d.: 9.2) (range: 18–83 years) for men and 44.1 years (s.d.: 9.1) (range: 18–78 years) for women, respectively. The mean body mass index was 23.2 kg m^{-2} (s.d.: 3.0) (range: 14.3–41.8 kg m^{-2}) in men and 21.1 kg m^{-2} (s.d.: 3.0) (range: 14.0–58.3 kg m^{-2}) in women, respectively. The mean abdominal circumference was 81.4 cm (s.d.: 8.0) (range: 48.5–132.0 cm) in men and 71.4 cm (s.d.: 8.3) (range: 49.0–145.0 cm) in women, respectively. Among the medication group, the mean age, body mass index, and abdominal circumference were significantly higher than those among the main group of analyzed subjects group (Table 1).

High-ocular tension was detected in 2.4% of the apparently healthy males (95% CI, 2.0–2.7%) (n=190 of 8031 subjects) and in 1.1% of the apparently healthy females (95% CI, 0.8–1.3%) (n=65 of 5972 subjects). The prevalence of high-ocular tension was higher in the males than in the females ($P<0.001$).

The metabolic syndrome defined by rATPIII was detected in 15.3% of the apparently healthy males (95% CI, 14.5–16.1%) (n=1226 of 8031 subjects) and in 5.1% of the apparently healthy females (95% CI, 4.6–5.7%) (n=306 of 5972 subjects) (Table 1). High-ocular tension was detected in 2.1% of the males without the metabolic syndrome defined by rATPIII (95% CI, 1.7–2.4%) (n=140 of 6805 subjects) and in 0.9% of the females without it (95% CI, 6.7–11.8%) (n=51 of 5666 subjects). High-ocular tension was detected in 4.1% of the apparently healthy males with the metabolic syndrome defined by rATPIII (95% CI, 3.0–5.3%) (n=50 of 1226 subjects) and in 4.6% of the apparently healthy females with it (95% CI, 2.5–7.6%) (n=14 of 306 subjects). The prevalence of high-ocular tension was higher in males and in females with the metabolic syndrome than in those without it ($P<0.001$). The metabolic syndrome defined by IDF was detected in 8.2% of the apparently healthy males (95% CI, 7.6–8.8%) (n=655 of 8031 subjects) and in 4.3% of the apparently healthy females (95% CI, 3.8–4.8%) (n=256 of 5972 subjects) (Table 1).

As the definitions of metabolic syndrome used in this study require information about drug medication, we compared the IOP between metabolic syndrome participants who were free from medication and those who were receiving medication. By using three definitions of the metabolic syndrome, we separated 9597 males and 6491 females into four groups according to the absence or presence of

Table 1 The difference of basic characteristics between study population who were free from medication and the subgroup who were receiving medication

	Men			*Women*		
	Apparently healthy group	*Medication group*	P	*Apparently healthy group*	*Medication group*	P
N	8031	1566		5972	519	
Age	46.0 ± 9.2	54.2 ± 8.4	<0.001	44.1 ± 9.1	55.6 ± 7.3	<0.001
BMI	23.2 ± 3.0	24.5 ± 3.2	<0.001	21.1 ± 3	23.6 ± 3.7	<0.001
Abdominal circumference (cm)	81.4 ± 8.0	85.3 ± 8.4	<0.001	71.4 ± 8.3	78.6 ± 9.7	<0.001
Systolic blood pressure (mm Hg)	119.6 ± 15.0	131.7 ± 16.5	<0.001	109.6 ± 15	129.5 ± 18.6	<0.001
Diastolic blood pressure (mm Hg)	75.8 ± 10.0	83.5 ± 10.2	<0.001	68.2 ± 9.8	79.8 ± 11	<0.001
Fasting glucose level (mg per 100 ml)	98.7 ± 15.1	113.4 ± 30.4	<0.001	90.4 ± 9.2	103.3 ± 28.8	<0.001
Triglyceride level (mg per 100 ml)	110.8 ± 87.0	130.4 ± 100.8	<0.001	62.6 ± 42.3	91.4 ± 56.3	<0.001
HDL cholesterol level (mg per 100 ml)	48.3 ± 12.5	46.4 ± 12.2	<0.001	60.6 ± 13.5	57.4 ± 14.6	<0.001
Intraocular pressure (mm Hg)	15.0 ± 3.0	15.5 ± 3.2	<0.001	14.5 ± 2.9	15.1 ± 3	<0.001
rATPIII-defined MS	15.3% (1226)	50.0% (783)	<0.001	5.1% (306)	50.1% (260)	<0.001
IDF-defined MS	8.2% (655)	23.1% (33)	<0.001	4.3% (256)	34.3% (178)	<0.001
JASSO-defined MS	9.4% (757)	33.5% (524)	<0.001	0.6% (35)	7.1% (37)	<0.001
High-ocular tension	2.4% (190)	3.8% (60)	0.002	1.1% (65)	2.1% (11)	<0.001

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; IDF, the new International Diabetes Federation definition; JASSO, the Japan Society for the Study of Obesity; MS, the metabolic syndrome; rATPIII, the revised National Cholesterol Education Program Adult Treatment Panel III definition.

Table 2 The rate of high-ocular tension and the averaged intraocular pressure in each MS criteria

	Men				*Women*			
	Total number	*High-ocular tension*	*Intraocular pressure (mm Hg)*	P	*Total number*	*High-ocular tension*	*Intraocular pressure (mm Hg)*	P
rATPIII-defined MS								
Participants free from MS and medication	6805	2.1% (140)	14.8 ± 3	b,c	5666	0.9% (51)	14.4 ± 2.8	b
Participants who met the criteria of MS, but received no medication	1226	4.1% (50)	15.9 ± 3.1	a	306	4.6% (14)	15.8 ± 3	a
Participants who received medication, but did not satisfy the criteria of MS	783	2.8% (22)	15.2 ± 3.2	a,b	259	1.5% (4)	14.5 ± 2.9	b
Participants who met the criteria of MS and received medication	783	4.9% (38)	15.7 ± 3.3	a,c	260	2.7% (7)	15.7 ± 3	a,c
IDF-defined MS								
Participants free from MS and medication	7376	2.3% (171)	14.9 ± 3	b,c	5716	0.9% (53)	14.4 ± 2.8	b
Participants who met the criteria of MS, but received no medication	655	2.9% (19)	15.9 ± 3.1	a	256	4.7% (12)	15.7 ± 3.1	a
Participants who received medication, but did not satisfy the criteria of MS	1204	3.8% (46)	15.3 ± 3.2	a,b	341	2.3% (8)	14.7 ± 2.9	b
Participants who met the criteria of MS and received medication	362	3.9% (14)	15.9 ± 3.3	a,c	178	1.7% (3)	15.8 ± 14.4	a,c
JASSO-defined MS								
Participants free from MS and medication	7274	2.2% (161)	14.8 ± 3	b,c	5937	1% (61)	14.5 ± 2.9	b
Participants who met the criteria of MS, but received no medication	757	3.8% (29)	16.1 ± 3	a	35	11.4% (4)	16.8 ± 3.3	a
Participants who received medication, but did not satisfy the criteria of MS	1042	3.3% (34)	15.2 ± 3.2	a,b	482	2.1% (10)	15 ± 3	a,b
Participants who met the criteria of MS and received medication	524	5% (26)	16 ± 3.2	a,c	37	2.7% (1)	16.6 ± 2.5	a,c

Abbreviations: IDF, the new International Diabetes Federation definition; JASSO, the Japan Society for the Study of Obesity; MS, the metabolic syndrome; rATPIII, the revised National Cholesterol Education Program Adult Treatment Panel III definition. [a]Indicates $P<0.05$ when compared with participants who were free from MS and medication. [b]Indicates $P<0.05$ when compared with participants who met the criteria of MS, but received no medication. [c]Indicates $P<0.05$ when compared with participants who received medication, but did not satisfy the criteria of MS.

metabolic syndrome and medication. When participants satisfied the criteria for metabolic syndrome, the prevalence of high-ocular tension was up to 2.9–4.1% of the apparently healthy males, and up to 4.6–11.4%, the apparently healthy females (Table 2). On the other hand, when participants did not satisfy the criteria for metabolic syndrome, the prevalence of high-ocular tension was down to 2.1–2.3% of the apparently healthy males, and down to 0.9–1.0% of the apparently healthy females. High-ocular tension was detected in 3.9–5.0% of the males and in 1.7–2.7% of the females who were diagnosed as metabolic syndrome and received medication. The mean IOP was at the same level, with and without medication, when participants satisfied the criteria of metabolic syndrome. The mean IOP was higher in participants who were diagnosed as metabolic syndrome than in those who were not diagnosed as metabolic syndrome, despite the use of medication. When participants received medication, but were not metabolic

Table 3 The effect of medication for the rate of high-ocular tension and the averaged intraocular pressure

	Men				*Women*			
	Total number	*High-ocular tension*	*Intraocular pressure (mm Hg)*	P	*Total number*	*High-ocular tension*	*Intraocular pressure (mm Hg)*	P
Elevated fasting glucose level								
Participants under criterion and free from medication	5687	1.8% (105)	14.7 ± 3		5593	0.8% (47)	14.4 ± 2.8	
Participants over criterion and free from medication	3549	3.6% (126)	15.4 ± 3.1	[a]	827	3.1% (26)	15.4 ± 3.1	[a]
Participants under criterion and controlled by medication	16	0% (0)	16.9 ± 2.5	[a]	3	33.3% (1)	16.7 ± 5.5	
Participants over criterion despite of medication	345	5.5% (19)	15.8 ± 3.3	[a]	68	2.9% (2)	16.6 ± 2.5	[a,b]
Elevated blood pressure								
Participants under criterion and free from medication	6449	2% (130)	14.7 ± 3		5526	0.9% (47)	14.3 ± 2.8	
Participants over criterion and free from medication	2217	3.8% (85)	15.8 ± 3.1	[a,c]	634	3.2% (20)	15.7 ± 3	[a]
Participants under criterion and controlled by medication	250	2% (5)	14.6 ± 3.2	[b]	108	1.9% (2)	14.8 ± 3.2	[b]
Participants over criterion despite of medication	681	4.4% (30)	15.8 ± 3.2	[a,c]	223	3.1% (7)	15.6 ± 2.9	[a,c]
Decreased HDL cholesterol level								
Participants under criterion and free from medication	6713	2.4% (159)	15 ± 3		4873	1.1% (52)	14.5 ± 2.9	
Participants over criterion and free from medication	2485	3% (75)	15.1 ± 3.2		1405	1.6% (22)	14.7 ± 2.9	
Participants under criterion and controlled by medication	274	4% (11)	15.3 ± 3.1		143	0.7% (1)	14.6 ± 2.8	
Participants over criterion despite of medication	125	4% (5)	15.4 ± 3.1		70	1.4% (1)	15 ± 2.8	
Elevated triglyceride level								
Participants under criterion and free from medication	7328	2.1% (153)	14.9 ± 3		6045	1% (63)	14.5 ± 2.9	
Participants over criterion and free from medication	1870	4.3% (81)	15.7 ± 3.2	[a,c]	233	4.7% (11)	15.3 ± 3.2	[a]
Participants under criterion and controlled by medication	256	3.9% (10)	15.1 ± 3.1	[b]	185	1.1% (2)	14.7 ± 2.8	
Participants over criterion despite of medication	143	4.2% (6)	15.8 ± 3.1	[a]	28	0% (0)	14.8 ± 2.7	

Abbreviation: HDL, high-density lipoprotein. In this assessment, we used the revised national cholesterol education program adult treatment Panel III definition. [a]Indicates $P<0.05$ when compared with participants who were under the criterion and were free from medication. [b]Indicates $P<0.05$ when compared with participants who were over the criterion and were free from medication. [c]Indicates $P<0.05$ when compared with participants who were under the criterion and controlled by medication.

syndrome, the mean IOP was lower than in those metabolic syndrome participants who were free from medication.

Next, we separated participants into four groups as follows to assess the effect of medication and controlling states: (1) participants who were under the criterion and were free from medication, (2) participants who were over the criterion and were free from medication, (3) participants who were under the criterion controlled by medication, and (4) participants who were over criterion despite of medication (Table 3). IOP was higher in participants whose blood sugar level was over the criterion. The medication and controlling states for blood sugar was found to have no effect on IOP. When we turned our attention toward blood pressure and triglycerides, we discovered that IOP was lower in participants whose levels were controlled within the criteria by medication than in those who had not received medication. On the other hand, HDL criterion was found to be not associated with IOP, regardless of whether the participants received medication or not, or whether the participants satisfied the criterion or not.

As for the main analysis, we analyzed 14 003 apparently healthy participants (8031 men and 5972 women) to assess the direct effects of the metabolic syndrome and its components on IOP. Linear regression analysis indicated an association between the average IOP and the number of components of the metabolic syndrome, both in males ($\beta=0.17$, $P<0.001$) and females ($\beta=0.13$, $P<0.001$). We calculated the odds ratio of the metabolic syndrome, age, smoking habits, and temperature for high-ocular tension using a logistic model (Table 4). The rATPIII-defined metabolic syndrome was a statistically significant explanatory variable both in males (odds ratio: 2.02, 95% CI, 1.46–2.81) and females (odds ratio: 5.28, 95% CI, 2.89–9.64). The Japan Society for The Study of Obesity-defined metabolic syndrome was also a statistically significant explanatory variable both in males (odds ratio: 2.02, 95% CI, 1.46–2.81) and females (odds ratio: 5.28, 95% CI, 2.89–9.64). However, the IDF-defined metabolic syndrome was not a statistically significant explanatory variable in males. One of the reasons for that was the fact that increased waist circumference was not a statistically significant explanatory variable in males. Temperature was a negative explanatory variable.

From the univariate logistic model, we selected age, maximum temperature, and the existence of the rATPIII-defined metabolic syndrome, or the number of its components, or each criterion as an explanatory variable in the multivariate logistic analyses. We applied three models of multivariate logistic analyses to investigate the association of high-ocular tension with the metabolic syndrome (Table 5). In Model 1, each component of the metabolic syndrome, including increased abdominal circumference, elevated fasting glucose level, elevated blood pressure, decreased HDL

Table 4 The unadjusted odds ratio of the metabolic syndrome for high-ocular tension

	Men (n = *8031*)		*Women* (n = *5972*)	
rATPIII-defined MS	2.02 (1.46–2.81)	<0.001	5.28 (2.89–9.64)	<0.001
IDF-defined MS	1.26 (0.78–2.04)	0.35	5.25 (2.77–9.96)	<0.001
JASSO-defined MS	1.76 (1.18–2.63)	0.006	12.43 (4.26–36.28)	<0.001
Number of components of rATPIII-defined MS	1.37 (1.24–1.53)	<0.001	1.70 (1.42–2.04)	<0.001
rATPIII and IDF criteria				
Increased waist circumference (⩾90 cm for men and ⩾80 cm for women)	1.29 (0.88–1.9)	0.19	2.23 (1.29–3.87)	<0.001
Elevated fasting glucose level (⩾100 mg per 100 ml)	2.04 (1.53–2.72)	<0.001	3.65 (2.16–6.17)	<0.001
Elevated blood pressure (⩾130/85 mmHg)	1.79 (1.33–2.42)	<0.001	4.09 (2.4–6.98)	<0.001
Decreased HDL cholesterol level (<40 mg per 100 ml for men and <50 mg per 100 ml for women)	1.3 (0.96–1.78)	0.094	1.28 (0.73–2.23)	0.39
Elevated triglyceride level (⩾150 mg per 100 ml)	2.04 (1.5–2.77)	<0.001	4.68 (2.28–9.6)	<0.001
JASSO criteria				
Increased waist circumference (⩾85 cm for men and ⩾90 cm for women)	1.19 (0.88–1.61)	0.26	3.29 (1.30–8.31)	0.012
Elevated fasting glucose level (⩾110 mg per 100 ml)	2.34 (1.64–3.33)	<0.001	6.34 (3.08–13.06)	<0.001
Elevated blood pressure (⩾130/85 mmHg)	1.79 (1.33–2.42)	<0.001	4.09 (2.4–6.98)	<0.001
Abnormal cholesterol level (HDL cholesterol <40 mg per 100 ml or triglyceride level⩾150 mg per 100 ml)	1.5 (1.12–2.01)	0.006	2.63 (1.33–5.2)	0.005
Current smoker	1.11 (0.83–1.49)	0.47	0.71 (0.22–2.28)	0.57
Age (s.d.)	0.97 (0.84–1.12)	0.69	0.81 (0.63–1.03)	0.09
Maximum temperature (s.d.)	0.63 (0.54–0.73)	<0.001	0.69 (0.54–0.89)	<0.001
Minimum temperature (s.d.)	0.63 (0.54–0.73)	<0.001	0.71 (0.55–0.9)	0.006

Abbreviations: HDL, high-density lipoprotein; IDF, the new International Diabetes Federation definition; JASSO, the Japan Society for the Study of Obesity; MS, the metabolic syndrome; rATPIII, the revised National Cholesterol Education Program Adult Treatment Panel III definition. Univariate logistic analysis was applied for high-ocular tension in 8031 males and 5972 females. Data was expressed as the odds ratio (95% confidence interval). The odds of the number of components mean the odds ratio of one component. For age and temperatures, the odds of 1 s.d. for high-ocular tension were expressed.

Table 5 The adjusted odds ratio of the metabolic syndrome for high-ocular tension

Multivariate analysis	*Men* (n = *8031*)		*Women* (n = *5972*)	
Model 1				
Age (s.d.)	0.91 (0.78–1.06)	0.21	0.54 (0.41–0.72)	<0.001
Maximum temperature (s.d.)	0.64 (0.56–0.75)	<0.001	0.7 (0.54–0.9)	0.005
Increased abdominal circumference	0.9 (0.6–1.36)	0.63	1.37 (0.72–2.59)	0.34
Elevated fasting glucose level	1.75 (1.29–2.37)	<0.001	2.94 (1.6–5.42)	0.001
Elevated blood pressure	1.52 (1.11–2.09)	0.009	3.84 (2.04–7.23)	<0.001
Decreased HDL cholesterol level	1.06 (0.76–1.47)	0.74	0.78 (0.42–1.46)	0.45
Elevated triglyceride level	1.7 (1.22–2.38)	0.002	3.07 (1.33–7.13)	0.009
Model 2				
Age (s.d.)	0.94 (0.81–1.09)	0.41	0.62 (0.48–0.81)	<0.001
Maximum temperature (s.d.)	0.64 (0.55–0.74)	<0.001	0.67 (0.52–0.86)	0.002
Number of components of rATPIII-defined MS	1.37 (1.23–1.52)	<0.001	1.92 (1.58–2.33)	<0.001
Model 3				
Age (s.d.)	0.97 (0.84–1.12)	0.69	0.67 (0.52–0.87)	0.003
Maximum temperature (s.d.)	0.63 (0.54–0.73)	<0.001	0.67 (0.53–0.87)	0.002
rATPIII-defined MS	2 (1.43–2.78)	<0.001	7.09 (3.74–13.43)	<0.001

Abbreviations: HDL, high-density lipoprotein; MS, the metabolic syndrome; rATPIII, the revised National Cholesterol Education Program Adult Treatment Panel III definition. Multivariate logistic analysis was applied for high-ocular tension in 8031males and 5972 females. Data was expressed as the odds ratio (95% confidence interval). The odds of the number of components mean the odds ratio of one component. For age and temperatures, the odds of 1 s.d. for high-ocular tension were expressed.

cholesterol level, and elevated triglyceride level, was listed as independent variables. Model 2 replaced the diagnosis of the metabolic syndrome in Model 1 with the number of metabolic syndrome components. In Model 3, each component of the metabolic syndrome was switched to the diagnosis of metabolic syndrome.

In both males and females, high-ocular tension was correlated positively with the metabolic syndrome and

negatively with maximum temperature. There was also a significantly positive relationship between the number of metabolic syndrome components and high-ocular tension.

When each component of the metabolic syndrome was analyzed, elevated fasting glucose level, elevated blood pressure, elevated triglyceride level, and maximum temperature were found to be significantly associated with high-ocular tension both in males and females.

To clarify the effect of the other two components, increased abdominal circumference and decreased HDL cholesterol level, for high-ocular tension, we categorized the subjects into one of the two groups. The first group included subjects who satisfied none of the three criteria such as elevated fasting glucose level, elevated blood pressure, and elevated triglyceride level. The second group included subjects who satisfied at least one of these three criteria.

The prevalence of high-ocular tension was not increased in subjects who had increased abdominal circumference and/or a decreased HDL cholesterol level among each subgroup (Figure 1)

Discussion

Principal findings

In this study, we showed the association between rATPIII-defined metabolic syndrome and both IOP and high-ocular tension. It has been reported that subjects with more metabolic syndrome components had higher IOP.[8] However, we found that IDF-defined metabolic syndrome was not linked to high-ocular tension in males. IDF is different from rATPIII in that increased waist circumference is not one of the components, but is needed to conduct a proper

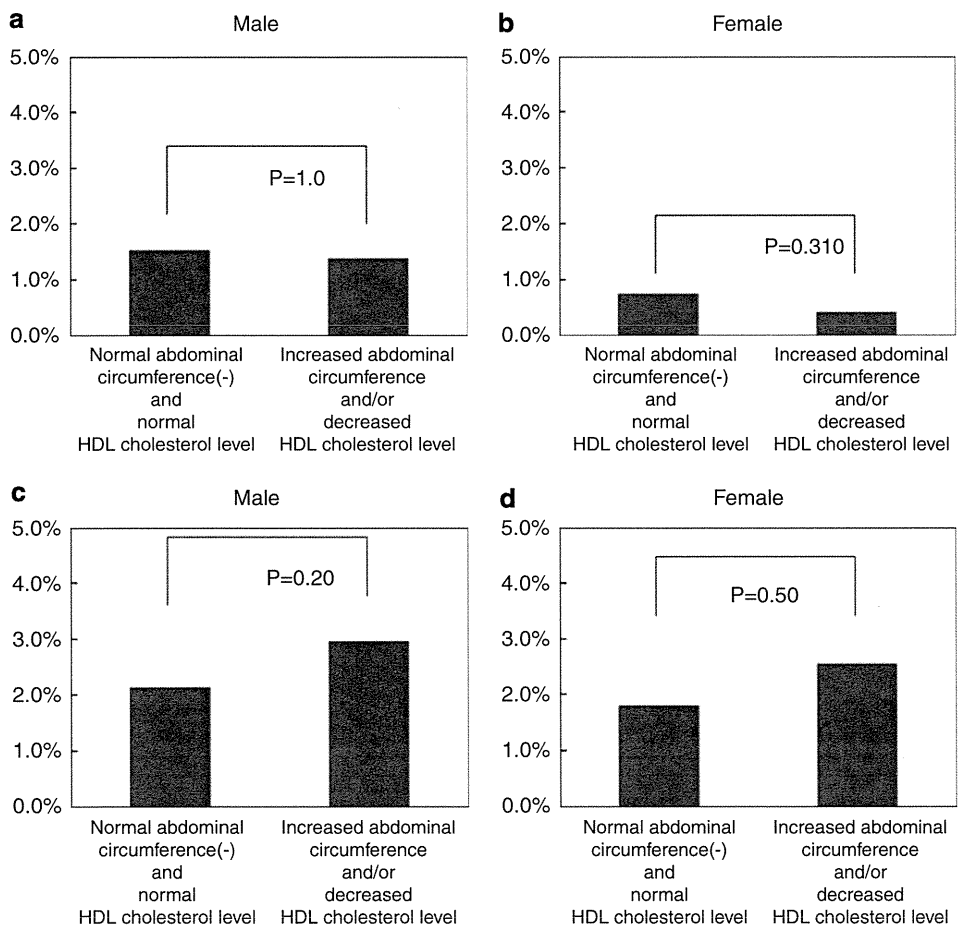

Figure 1 When each component of the metabolic syndrome was analyzed, three of the five metabolic syndrome components (fasting plasma glucose, blood pressure, and triglycerides) were found to be related to high-ocular tension. The study subjects were then categorized into one of the two groups. The first group (**a**, **b**) included subjects who satisfied none of the three criteria such as elevated fasting glucose level, elevated blood pressure, and elevated triglyceride level. The second group (**c**, **d**) included subjects who satisfied at least one of these three criteria. Figures indicate the prevalence of subjects with high-ocular tension among subjects with increased abdominal circumference and/or decreased HDL cholesterol level, and those with high-ocular tension among subjects with normal abdominal circumference and normal HDL cholesterol level. Two groups of subjects were compared by using the χ^2 test, and $P<0.05$ was accepted as the significant level.

diagnosis. Therefore, we also evaluated which components of the metabolic syndrome are related to high-ocular tension. Interestingly, this study showed that three of the five components (fasting plasma glucose, blood pressure, and triglycerides) were associated with high-ocular tension and that the other two were not.

We also assessed the participants who were receiving medication as a subgroup analysis. The subgroup analysis indicated that IOP was higher in participants who were diagnosed as having metabolic syndrome, whether they were receiving medication or not. When we assessed the effect of medication and the controlling states for each component of the metabolic syndrome, the control for blood pressure or triglycerides level by medication decreased the IOP. However, the medication for plasma glucose did not present this phenomenon.

Limitations of the study

High-ocular tension is a concept that is included in health checkup programs, yet it is not necessarily equivalent to OH. To diagnose OH, additional evaluations including slit-lamp examination and a visual-field test are required, yet they were not performed because of economical and temporal reasons. IOP was measured by noncontact tonometer. When comparing the noncontact tonometer with the Goldmann applanation tonometer, the noncontact tonometer showed a slightly higher IOP reading, but still close to those obtained with the Goldmann applanation tonometer.[16] Other limitations may include complications arising from un-measured factors such as central corneal thickness (CCT) or other known risk factors for higher IOP.

It should be noted that the results of the subgroup analysis that showed that the medication for blood pressure or triglycerides could decrease IOP still requires validation by a future longitudinal study.

Interpretation

Several earlier studies showed that the rise of IOP is associated with diabetes mellitus and systemic hypertension,[17,18] whereas other studies have reported no association.[19] The multivariate analysis in this study indicates the correlation between high-ocular tension and both hyperglycemia and systemic hypertension in both males and females. Although the mechanism behind how hyperglycemia affects IOP is unclear, it has been reported that there is possibly more than one effect and that some effects may operate in opposite directions.[20] It has been suggested that the cause of the rise in IOP in systemic hypertension is the excessive production of aqueous humor or the increase of episcleral vein pressure.[21]

The positive relationship between IOP and total cholesterol has been reported,[17] whereas to the best of our knowledge, the association between IOP and the other lipid metabolisms have not been well discussed. In our multivariate data, triglycerides were correlated with high-ocular tension. One possible reason for this is that hypertriglyceridemia is commonly associated with diabetes and obesity. Higher IOP has also been found to be related to obesity,[17,22] probably because of excess intraorbital fat tissue and an increase in viscosity of the blood, which consequently decreases outflow facility.[23]

Individually, three components of the metabolic syndrome, fasting plasma glucose, blood pressure, and triglycerides, do not affect IOP elevation to a large degree, but when taken into consideration as a whole, they do affect the development of high-ocular tension. Therefore, we suspect that a common background exists between high-ocular tension and the metabolic syndrome. As mentioned above, the increase in episcleral venous pressure, which is caused by excess orbital fat, may occur with the decrease of outflow facility. Episcleral venous pressure also increases the insulin resistance mechanism, which has been found in an earlier study to induce the retension of sodium when extracellular fluid volume is expanded.[24]

Recent studies have suggested that higher glucose[25] and the number of metabolic syndrome components[26] were associated with thicker CCT. The mean CCT of the participants with metabolic syndrome who met the three components was 6 μm thicker than that of paticipants without metabolic syndrome.[26] Murase *et al.*[27] reported that a 10 μm increase in CCT led to an IOP 0.29 mm Hg higher when measured with a noncontact tonometer. Accordingly, the influence of CCT to IOP in the metabolic syndrome participants who met the three components was calculated at almost 0.17 mm Hg, whereas the difference of mean IOP between participants with no component and participants with three components was 1.4 mm Hg in this study. We consider CCT to be merely one of the factors that elucidate high IOP in the metabolic syndrome.

Numerous studies have reported that there are seasonal variations of IOP and that in winter, IOP rises more than in summer.[17,28] Our results also indicate that maximal temperature is negatively related to the occurrence of high-ocular tension. These phenomena show that IOP is affected by the endogenous rhythm.[28]

Conclusion

The prevalence of metabolic syndrome was associated with high-ocular tension, which represents OH in this study. The high IOP related to the metabolic syndrome is not desirable in patients with not only OH, but also normal tension glaucoma. It has been reported that primary open angle glaucoma develops from 1 to 2% of OH cases every year[7] and that the higher IOP the patients have, the greater possibility they have of developing primary open angle glaucoma. However, an earlier report has shown that normal tension glaucoma makes up 90% of Japanese primary open angle glaucoma cases.[29]Even when IOP is in the normal range, the

risk of the progression of visual-field defect in normal tension glaucoma increases 10% with each 1 mm Hg rise in IOP.[30] Therefore, it is important to keep the metabolic syndrome in mind as a consideration when managing IOP. To improve glaucoma treatment in patients with the metabolic syndrome, those patients should be provided with instruction on how to lessen their metabolic syndrome component levels. The medication for blood pressure or triglycerides could decrease high IOP, which often accompanies the metabolic syndrome. However, this possibility requires validation by a future longitudinal study.

Conflict of interest

The authors declare no conflict of interest.

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References

1 Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes* 1988; **37**: 1595–1607.
2 Ford ES, Giles WH, Dietz WH. Prevalence of the metabolic syndrome among US adults: findings from the third national health and nutrition examination survey. *JAMA* 2002; **287**: 356–359.
3 Oda E, Abe M, Veeraveedu PT, Watanabe K. Considerable disagreement among definitions of metabolic syndrome for Japanese. *Circ J* 2007; **71**: 1239–1243.
4 Dandona P, Aljada A, Mohanty P. The anti-inflammatory and potential anti-atherogenic effect of insulin: a new paradigm. *Diabetologia* 2002; **45**: 924–930.
5 Dandona P, Aljada A, Chaudhuri A, Mohanty P, Garg R. Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. *Circulation* 2005; **111**: 1448–1454.
6 Sommer A, Tielsch JM, Katz J, Quigley HA, Gottsch JD, Javitt J *et al.* Relationship between intraocular pressure and primary open angle glaucoma among white and black Americans. The baltimore eye survey. *Arch Ophthalmol* 1991; **109**: 1090–1095.
7 Kass MA, Heuer DK, Higginbotham EJ, Johnson CA, Keltner JL, Miller JP *et al.* The Ocular Hypertension Treatment Study: a randomized trial determines that topical ocular hypotensive medication delays or prevents the onset of primary open-angle glaucoma. *Arch Ophthalmol* 2002; **120**: 701–713 discussion 829-730.
8 Oh SW, Lee S, Park C, Kim DJ. Elevated intraocular pressure is associated with insulin resistance and metabolic syndrome. *Diabetes Metab Res Rev* 2005; **21**: 434–440.
9 Executive summary of the third report of the national cholesterol education program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). *JAMA* 2001; **285**: 2486–2497.
10 Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA *et al.* Diagnosis and management of the metabolic syndrome: an American heart association/national heart, lung, and blood institute scientific statement. *Circulation* 2005; **112**: 2735–2752.
11 Alberti KG, Zimmet P, Shaw J. Metabolic syndrome—a new world-wide definition. A consensus statement from the international diabetes federation. *Diabet Med* 2006; **23**: 469–480.
12 Definition and the diagnostic standard for metabolic syndrome—committee to evaluate diagnostic standards for metabolic syndrome. *Nippon Naika Gakkai Zasshi* 2005; **94**: 794–809.
13 Stone NJ, Bilek S, Rosenbaum S. Recent national cholesterol education program adult treatment panel III update: adjustments and options. *Am J Cardiol* 2005; **96**: 53E–59E.
14 Matuzawa Y. Metabolic syndrome-definition and diagnostic criteria in Japan. *J Jpn Soc Int Med* 2005; **94**: 188–203.
15 World Health Organization Western Pacific Region/International Association for the Study of Obesity/International Obesity Task Force. The Asia-Pacific perspective: redefining obesity and its treatment. *Health Communications Australia* 2000. Melbourne (Australia).
16 Quigley HA, Langham ME. Comparative intraocular pressure measurements with the pneumatonograph and Goldmann tonometer. *Am J Ophthalmol* 1975; **80**: 266–273.
17 Klein BE, Klein R, Linton KL. Intraocular pressure in an American community. The beaver dam eye study. *Invest Ophthalmol Vis Sci* 1992; **33**: 2224–2228.
18 Hennis A, Wu SY, Nemesure B, Leske MC. Hypertension, diabetes, and longitudinal changes in intraocular pressure. *Ophthalmology* 2003; **110**: 908–914.
19 Uhm KB, Shin DH. Glaucoma risk factors in primary open-angle glaucoma patients compared to ocular hypertensives and control subjects. *Korean J Ophthalmol* 1992; **6**: 91–99.
20 Armaly MF, Baloglou PJ. Diabetes mellitus and the eye. 11. Intraocular pressure and aqueous outflow facility. *Arch Ophthalmol* 1967; **77**: 493–502.
21 Bulpitt CJ, Hodes C, Everitt MG. Intraocular pressure and systemic blood pressure in the elderly. *Br J Ophthalmol* 1975; **59**: 717–720.
22 Mori K, Ando F, Nomura H, Sato Y, Shimokata H. Relationship between intraocular pressure and obesity in Japan. *Int J Epidemiol* 2000; **29**: 661–666.
23 Shiose Y, Kawase Y. A new approach to stratified normal intraocular pressure in a general population. *Am J Ophthalmol* 1986; **101**: 714–721.
24 Sechi LA, Bartoli E. Molecular mechanisms of insulin resistance in arterial hypertension. *Blood Press* 1996; **5** (Suppl 1): 47–54.
25 Su DH, Wong TY, Wong WL, Saw SM, Tan DT, Shen SY *et al.* Diabetes, hyperglycemia, and central corneal thickness: the Singapore Malay Eye Study. *Ophthalmology* 2008; **115**: 964–968 e961.
26 Su DH, Wong TY, Foster PJ, Tay WT, Saw SM, Aung T. Central corneal thickness and its associations with ocular and systemic factors: the Singapore Malay Eye Study. *Am J Ophthalmol* 2009; **147**: 709–716 e701.
27 Murase H, Sawada A, Mochizuki K, Yamamoto T. Effects of corneal thickness on intraocular pressure measured with three different tonometers. *Jpn J Ophthalmol* 2009; **53**: 1–6.
28 Blumenthal M, Blumenthal R, Peritz E, Best M. Seasonal variation in intraocular pressure. *Am J Ophthalmol* 1970; **69**: 608–610.
29 Iwase A, Suzuki Y, Araie M, Yamamoto T, Abe H, Shirato S *et al.* The prevalence of primary open-angle glaucoma in Japanese: the Tajimi Study. *Ophthalmology* 2004; **111**: 1641–1648.
30 Nemesure B, Honkanen R, Hennis A, Wu SY, Leske MC. Incident open-angle glaucoma and intraocular pressure. *Ophthalmology* 2007; **114**: 1810–1815.

A significant association of viral loads with corneal endothelial cell damage in cytomegalovirus anterior uveitis

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A significant association of viral loads with corneal endothelial cell damage in cytomegalovirus anterior uveitis

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ABSTRACT

Aim The aim of the study was to investigate the correlation between the clinical manifestation and the cytomegalovirus (CMV) viral load in the aqueous humour of patients with CMV anterior uveitis.

Methods Seven patients with CMV-associated iridocyclitis and four patients with CMV-associated corneal endotheliitis were enrolled. Presence of CMV, but not other human herpes viruses, was confirmed by multiplex polymerase chain reaction (PCR). Viral load was measured using real-time PCR. Clinical manifestations were examined using a slit-lamp microscope and ophthalmoscope, applanation tonometer and specular microscope.

Results All 11 patients had unilateral recurrent anterior uveitis with high intraocular pressure and mutton fat keratic precipitates with pigmentation. Stromal oedema of the cornea was found in CMV-associated endotheliitis, but not in CMV-associated iridocyclitis patients. A significant corneal endothelium cell loss was recorded in all 11 patients with CMV-associated endotheliitis and iridocyclitis patients. High viral loads of CMV were detected in the aqueous humour of all 11 patients. A significant association was found between the corneal endothelial cell loss intensity and CMV viral load in the aqueous humour.

Conclusion There is a significant correlation between the CMV viral load and corneal endothelial cell loss in both CMV-associated iridocyclitis and corneal endotheliitis.

INTRODUCTION

Cytomegalovirus (CMV) is a member of the human herpes virus family and is found in latent infections in the majority of the adult population. In immunocompromised hosts, the virus causes necrotising retinitis,[1] but has been thought not to cause any diseases in immunocompetent hosts. However, a previous study showed local production of anti-CMV antibodies in the aqueous humour of an immunocompetent patient with iridocyclitis with elevated intraocular pressure (IOP).[2] In addition, recent studies using qualitative PCR have demonstrated that genomic CMV DNA is present in the aqueous humour of immunocompetent patients with unilateral iridocyclitis[3–6] as follows. Markomichelakis *et al*[3] reported two cases of iridocyclitis with sectoral iris atrophy in which CMV was detected by PCR, and de Schryver *et al*[4] also reported five similar cases. In the recent report by Chee *et al*,[5] they studied if there was a relationship between the CMV viral load in the aqueous and clinical manifestation of the diseases such as either acute or chronic iridocyclitis, eg Posner–Schlossman syndrome and Fuchs heterochromic iridocyclitis. CMV genomic DNA was also detected in the aqueous humour of immunocompetent patients with another inflammatory condition of the eye, ie corneal endotheliitis, in three previous reports.[7–9] Corneal endotheliitis is an inflammatory condition at the corneal endothelium in which keratic precipitates (KPs) develop together with severe stromal oedema in the cornea, whereas iridocyclitis has cells and flare in the anterior chamber with or without KPs but no stromal oedema in the cornea.

The real-time PCR made it possible to measure the viral load quantitatively. Thus, the use of this assay makes it possible to determine the clinical significance of the viral infection in the pathogenesis of human diseases. Our previous report showed a high CMV genomic DNA load in the aqueous humour in an immunocompetent patient with unilateral iridocyclitis with high IOP.[6] However, the correlation between the viral load in the aqueous humour and the clinical manifestation of the disease (iridocyclitis versus corneal endotheliitis) was not investigated. Therefore, we examined if there was any correlation between the CMV viral load in the aqueous humour and the clinical manifestation of anterior inflammatory diseases associated with CMV. We showed a significant correlation between the CMV viral load in the aqueous humour and the endothelial cell damage of the cornea in patients with iridocyclitis and corneal endotheliitis associated with CMV.

MATERIALS AND METHODS

Subjects

Between 2006 and 2008, 11 patients with CMV-associated inflammation in the anterior segment of the eye, ie seven patients with CMV-associated iridocyclitis and four patients with CMV-associated corneal endotheliitis, were enrolled. These patients were from Tokyo Medical and Dental University Hospital (Tokyo, Japan), Miyata Eye Hospital (Miyakonojo, Miyazaki, Japan) and Kyoto Prefectural University Hospital (Kyoto, Japan). Diagnosis was made based on clinical manifestations and the qualitative detection of the CMV genomic DNA in the aqueous humour by the multiplex PCR. The viral load in the aqueous humour was further measured quantitatively by the real-time PCR.

An aliquot of 0.1 ml of the aqueous humour was aspirated with a 30G needle after disinfection and

processed for PCR. Anti-viral therapy was not given before the PCR assay, but topical corticosteroids were given by local ophthalmologists to treat intense anterior uveitis. The interval between the disease onset and the aqueous humour sampling varied among the patients.

Polymerase chain reaction

The aqueous humour samples were centrifuged at 1000 *g* for 5 min and used for multiplex PCR and real-time PCR.[10 11] Multiplex PCR was designed to qualitatively measure the genomic DNA of eight human herpes viruses: herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella zoster virus (VZV), Epstein–Barr virus (EBV), CMV, and human herpes virus type 6 (HHV-6), type 7 (HHV-7) and type 8 (HHV-8). DNA was extracted from the aqueous humour samples using a DNA minikit (Qiagen, Valencia, California, USA). Multiplex PCR was performed using LightCycler (Roche, Basle, Switzerland). The primers of the glycoprotein gene sequences for CMV were TACCCCTATCGCGTG TGTTC (forward) and ATAG-GAGGCGCCACGTATTC (reverse). The probes used included 3′-fluorescein isothiocyanate: TCGTCGTAGCTACGCTTACAT and LcRed705-5′: ACACCACTTATCTGCTGGGCAGC. Specific primers for the virus were used in conjunction with Accuprim Taq (Invitrogen, Carlsbad, California, USA). PCR amplification conditions used in the current study have been reported previously.[12]

Real-time PCR was only performed for the HHV, with multiplex PCR used to detect the genomic DNA. Amplitaq Gold, with a Real-Time PCR 7300 system (ABI, Foster City, California, USA), was used to perform the procedure. The forward and reverse primers of immediate early (IE)-1 were CATGAAGGT-CTTTGCCCAGTAC and GGCCAAAGTGTAGGCTACAATAG, respectively. FAM-TGGCCCGTAGGTCATCCACACTAGG-TAMRA was used as the probe. The PCR amplification conditions used in the current study were previously reported by Sugita *et al*.[11] When more than 50 copies per tube (5×10^3/ml) were observed, the value of the sample's viral copy number was considered to be significant.

Clinical evaluation

Clinical manifestations of the eye were determined by a slit-lamp microscopic and ophthalmoscopic examination. Each patient underwent best corrected visual acuity (BCVA) measurement using a Japanese standard decimal visual acuity chart (Landolt ring chart) after treatment. Anterior chamber flare was measured by a laser flare photometer (FC-1000; Kowa Electronics, Nagoya, Japan). A photograph of the central cornea using a specular microscope (NONCON ROBO FA-3509; Konan Medical, Nishinomiya, Japan) was used for evaluation of the corneal endothelial cells. In cases of corneal endotheliitis, intense corneal oedema disturbed the measurements of the corneal endothelium, and we measured corneal endothelial cell counts after the inflammation was reduced by the treatment.

Evaluation of corneal endothelial cell loss

The relationship between the CMV viral load in the aqueous humour and the intensity of the corneal endothelial cell loss was assessed. The corneal endothelial cell loss was determined according to the following formula:

$$\text{Corneal endothelial cell loss}(\%) = 100 - (\text{endothelial cell counts in affected eye})/(\text{endothelial cell counts in the fellow eye})\times 100$$

Statistical analysis

Statistical analysis was performed using the Mann–Whitney U test. Statistical significance was set at $p<0.05$. Linear regression analysis was performed using the Spearman's correlation coefficient by rank test.

RESULTS

Clinical manifestations

Nine men and two women ranging in age from 23 to 71 years (mean age 60.6 years) were enrolled in the study. No abnormalities were found in the systemic investigations and laboratory tests. Serology examinations for human immunodeficiency virus were all negative. None of the patients had any history of eye surgery prior to the onset of uveitis. Clinical findings of the CMV-associated iridocyclitis patients (n=7) and corneal endotheliitis patients (n=4) are shown in table 1. A unilateral mild anterior uveitis with high IOP was noted in all 11 patients. There were no significant differences between the iridocyclitis and corneal endotheliitis groups in the cells and flare values in the anterior chamber, nor were there any differences noted for the elevated levels of IOP, KPs, gonioscopic findings and iris atrophy. Stromal oedema of the cornea was seen in all corneal endotheliitis but not in iridocyclitis patients. While the stromal oedema was diffuse in three out of the four patients, it was localised at upper cornea in one of the corneal endotheliitis patients. Representative cases for iridocyclitis and corneal endotheliitis are shown in figures 1 and 2, respectively. As for the IOP elevation, all 11 eyes required anti-glaucoma medications, with two eyes (cases 1 and 2) requiring trabeculectomy. With regard to the iris atrophy, no sectorial iris atrophy was seen in all 11 eyes, although four eyes (two each in the iridocyclitis and the corneal endotheliitis groups, respectively) presented diffuse iris atrophy.

Systemic valganciclovir therapy (1800 mg/day for longer than 3 weeks) in conjunction with topical corticosteroids and

Table 1 Clinical findings in patients with CMV anterior uveitis

Case	Age (years)	Sex	Eye	Diagnosis	Corneal oedema	KPs	Cells in AC	Flare in AC	IOP (mmHg)	Pigmentation in the AC angle	Iris atrophy
1	66	M	R	Iridocyclitis	-	Mutton-fat	1+	17	38	Depigmentation	None
2	62	M	R	Iridocyclitis	-	Mutton-fat	1+	26	40	PAS and pigment	Diffuse
3	56	M	L	Iridocyclitis	-	Mutton-fat	1+	13	44	Depigmentation	Diffuse
4	53	F	R	Iridocyclitis	-	Mutton-fat	1+	13	36	Depigmentation	None
5	71	M	L	Iridocyclitis	-	Mutton-fat	2+	28	25	PAS	None
6	63	M	R	Iridocyclitis	-	Fine	1+	Nt	50	Depigmentation	None
7	23	M	R	Iridocyclitis	-	Fine	1+	Nt	25	Depigmentation	None
8	71	M	R	Endotheliitis	+ (diffuse)	Mutton-fat	2+	151	37	PAS	None
9	67	M	R	Endotheliitis	+ (diffuse)	Fine	1+	14	25	Depigmentation	Diffuse
10	64	F	L	Endotheliitis	+ (superior)	Fine	1+	21	28	Depigmentation	None
11	71	M	R	Endotheliitis	+ (diffuse)	Mutton-fat	1+	12	43	PAS	Diffuse

Information from 11 patients with CMV anterior uveitis were reviewed. Data collected included intraocular pressure and clinical manifestation of the anterior segments in the affected eye.
AC, anterior chamber; F, female; KP, keratic precipitate; M, male; Nt, not tested; PAS, peripheral anterior synechia.