TABLE 3. Nineteen Positive Microsatellite Markers From Individual Typing

Markers	Cytobands	No. of Alleles	Positive Alleles	Allele Frequencies		*P*		Odds Ratio	95% CI	Nearest Gene Name
				Case	Control	2×2	2×*m*			
HUMUT617	6q27	5	1	0.155	0.215	0.00287	0.02289	1.54	1.08 to 2.21	SMOC2
D2S0226i	2q35	16	1	0.096	0.058	0.00523	0.12651	1.73	1.18 to 2.55	XRCC5
D17S0287i	17q21.33	8	1	0.390	0.323	0.00746	0.08458	1.34	1.09 to 1.65	CROP
D17S0351i	17q24.3	10	4	0.146	0.198	0.00812	0.00229	0.69	0.53 to 0.90	KCNJ16
D19S0134i	19p13.2	28	2	0.070	0.040	0.01212	0.06104	1.80	1.14 to 2.84	ZNF358
D2S0208i	2q11.2	9	1	0.224	0.174	0.01468	0.06679	1.38	1.07 to 1.77	CHST10
D13S0183i	13q31.3	11	2	0.230	0.179	0.01545	0.13986	1.37	1.07 to 1.76	Unknown
D4S0818i	4p16.1	17	1	0.112	0.153	0.01950	0.35114	0.70	0.52 to 0.94	SORCS2
D2S0949i	2p25.1	11	2	0.105	0.070	0.02343	0.09465	1.54	1.08 to 2.21	LPIN1
D20S885	20p11.23	17	1	0.123	0.165	0.02403	0.55390	0.71	0.53 to 0.95	Unknown
D10S0517i	10q26.13	15	1	0.223	0.177	0.02542	0.10058	1.33	1.04 to 1.72	TACC2
D17S0231i	17p13.1	8	1	0.615	0.558	0.02608	0.18437	1.26	1.03 to 1.54	Unknown
D3S0865i	3p21.31	13	1	0.245	0.199	0.03162	0.27624	1.31	1.03 to 1.66	XCR1
D3S1129i	3p22.1	26	1	0.149	0.112	0.03273	0.43997	1.39	1.03 to 1.88	Unknown
D3S0046i	3p26.1	19	1	0.001	0.010	0.03862	0.59448	0.13	0.02 to 0.73	GRM7
D17S790	17q22	14	1	0.026	0.047	0.04028	0.20889	0.54	0.31 to 0.94	Unknown
D18S0390i	18q22.1	17	2	0.140	0.179	0.04227	0.11569	0.75	0.57 to 0.98	Unknown
G09023	13q33.3	8	1	0.204	0.249	0.04245	0.03990	0.78	0.61 to 0.99	Unknown
D4S0370i	4q34.4	18	1	0.052	0.078	0.04780	0.54383	0.65	0.43 to 0.98	Unknown

individual typing after all of the pooling experiments to validate the pooled frequency estimates.

Essential Hypertension Susceptibility Genes

We have identified 19 MS loci associated with essential hypertension and compared our findings with those of 6 previous large-scale genome-wide studies that are summarized in Table 4. The loci of linkage analysis in the previous 9 reports were too wide (≥5 megabases) to identify and speculate about disease susceptibility genes. Three of the 19 identified regions in our study overlapped with a region identified in other races (Table 4). The studies in Table 4, except for the admixture mapping study,[13] were linkage studies and suggest a much broader region than our results. For example, we found that the positive MS locus D2S0949i is located on cytoband 2p25.1, and this finding is in accordance with the admixture mapping results obtained by Zhu et al,[13] who found evidence for linkage with a marker on chromosome 2p25.1. This concordance between 2 different studies suggests that chromosome 2p25.1 contains an unknown candidate gene for essential hypertension. Interestingly, our MS marker is located within the LPIN1 gene sequence (NM 145693.1), and this is a candidate gene for human lipodystrophy, a disease characterized by loss of body fat, fatty liver, hypertriglyceridemia, and insulin resistance. There have been no reports to indicate that LPIN1 is a candidate gene for essential hypertension, but lipin expression is important for metabolic homeostasis.[40] In consideration that hypertension is an associated factor in the metabolic syndrome characterized by obesity, hypertriglyceridemia, and insulin resistance,[41,42] LPIN1 deserves to be studied as a new candidate gene.

TABLE 4. Summary of Genome-Wide Scan Mapping Analyses on Blood Pressure

References	Chromosome	Ethnicity
Zhu et al.[13]	2p25.1, 3q13.31-33, 6q24, 21q21	Admixture
Caulfield et al.[14]	2q24.1, 5q13.1, 6q27, 9q34.11	White
Rao et al.[15]	2p	Blacks and whites
Kardia et al.[16]	No evidence	Blacks and non-Hispanic whites
Thiel et al.[17]	1	Blacks and whites
Ranade et al.[18]	10p	Chinese and Japanese origin
Harrap et al.[19]	1p34.3-1p31, 4q21-28, 16p13.1-16p12, Xp11.4-Xq11	White
Atwood et al.[20]	2p11.2, 2q12.2, 8q24.3, 18q23, 21q22.13	Mexican Americans
Rice et al.[21]	1p22.3-p13.1, 2p11.1-q12.3, 3q13.31-q26.32, 5p15.2-p12, 7q32.1-q36.1, 8q21.11, 10p14, 12p13.33, 14q11.2-q12, 19p13.3, 22q13.1-q13.2	White

Overlapped locations mapped by this study in comparison with previous studies are underlined.

Another significant MS marker, HUMUT617 on 6q27, is in the same cytoband position reported by Caulfield et al[14] Our marker was located within the SMOC2 gene sequence (NM_022138) that codes for a modular extracellular calcium-binding protein[43] and a smooth muscle–associated protein upregulated during neointima formation.[44] There have been no previous reports suggesting any connection between SMOC2

and BP, but this gene may be involved in the progression of atherosclerosis in the aorta.[44]

Perspectives

We performed an association analysis of essential hypertension using a high-density set of polymorphic MS markers with original, multistep methodology. The outcome was a rapid and efficient path to detect genomic susceptibility loci for a highly complex disorder. MS markers basically play a role as location markers for regions containing susceptibility and protective genes. Rarely, MS markers may be the causative variance themselves. The next step is to identify susceptibility and protective genes in the 19 narrow regions by SNP, LD block, and haplotype analysis. It is also important to replicate these results in different subjects, ethnic groups, and a larger number of samples. The future successful accomplishment of such analysis will also open the door to investigating the etiology of other multifactorial disorders, including common diseases such as bronchial asthma, type 2 diabetes mellitus, obesity, arteriosclerosis, schizophrenia, and psoriasis.

BP is influenced by nongenetic factors, such as salt intake. In the present study, because we did not focus on salt-induced hypertension, the amounts of urinary excretion of sodium were not examined. It might be noteworthy to perform studies specializing in genes related to salt-induced hypertension.

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Disclosures

None.

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ORIGINAL INVESTIGATION

The *COL1A1* gene and high myopia susceptibility in Japanese

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Abstract The collagen type I alpha I (COL1A1) gene encodes the extracellular matrix component, collagen, and resides in candidate MYP5 for high myopia on the chromosome 17q22–q23.3. This locus has recently been implicated in playing an important role in the pathogenesis of experimental myopia. We investigated the association of disruptions of *COL1A1* gene with high myopia by analyzing the frequency of ten SNPs in a Japanese population of 330 subjects with high myopia of −9.25 D or less and 330 randomized controls without high myopia. Two SNPs (rs2075555 and rs2269336) were significantly associated with high myopia ($P < 0.05$, $Pc < 0.1$). Two different haplotype blocks in *COL1A1* were observed by the pair-wise linkage disequilibrium between the SNPs. The frequency of GGC/GGC diplotype constructed by the three SNPs (rs2075555, rs2269336, rs1107946) was significantly high (OR = 1.6) and associated with high myopia ($P = 0.028$, $Pc < 0.084$). Together our results provide the first evidence for *COL1A1* as a gene associated with high myopia.

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Introduction

Myopia is the most common eye disorder in the world and a significant public health problem. Myopia is far more frequent in the Asian populations than in the USA or Europe, and Japan has one of the highest incidences of myopia in the world (Saw et al. 1996). In the USA, the prevalence of myopia in high school students is approximately 25% (Burton 1989; Curtin 1970; Katz et al. 1997; Leibowitz et al. 1980; Ministry of Education 2004), compared to nearly 60% in Japan (Burton 1989). High or pathologic myopia can cause blindness, or severe visual acuity loss due to retinal detachment, submacular hemorrhage, glaucoma, or macular degeneration. High myopia is especially common in Asia, and in Japan, accounts for 6 to 18% of myopia cases and 1 to 2% of the general population (Curcio et al. 1987). Severe myopia, therefore, has been a major cause of visual affliction.

The refractive power of the eye is correlated with ocular axial length, and it is well established that myopia is caused by increased axial eye size (Tokoro and Sato 1982). The development of high myopia in humans is associated with marked thinning of the sclera, the tough outer coat of the eye that facilitates any change in eye size. Scleral thinning

is greatest at the posterior pole of the eye, the anatomical region of greatest retinal photoreceptor density and vital to detailed visual discrimination (Curtin 1985). Collagen accounts for 90% of scleral dry weight, and the majority of this collagen is collagen type I (Rada et al. 2006). Mammalian sclera also contains small amounts of other fibrillar and fibril-associated collagens (Marshall et al. 1993; Tamura et al. 1991; Wessel et al. 1997), and studies have shown that scleral fibrils are heterologous, comprising collagen types I, III, and V (Marshall et al. 1993).

Myopia is a highly prevalent, complex phenotype involving both genetic and environmental factors. Recently, myopia susceptibility genes have been identified in the 14 genomic loci (MYP1on Xq28, MYP2 on chromosome18p, MYP3 on chromosome 12q, MYP4 on chromosome 7q, MYP5 on chromosome 17q, MYP6 on chromosome 22q12, MYP7 on chromosome 11p13, MYP8 on chromosome 3q26, MYP9 on chromosome 4q12, MYP10 on chromosome 8p23, MYP11 on chromosome 4q22–q27, MYP12 on chromosome 2q37.1, MYP13 on Xq23–q25, and MYP14 on chromosome 1p36) (Hammond et al. 2004; Naiglin et al. 2002; Paluru et al. 2003; Paluru et al. 2005; Schwartz et al. 1990; Stambolian et al. 2004; Wojciechowski et al. 2006; Young et al. 1998a, b; Zhang et al. 2006).

The 18 kb *COL1A1* gene, encoded by 52 exons, is located on chromosome 17 (17q21.33) where the MYP5 (17q21-22) susceptibility locus was identified (Paluru et al. 2003). This gene encodes the major component of type I collagen. The scleral tissue contains approximately 90% collagen, consisting predominantly of type I collagen (Zorn et al. 1992). Mutations in *COL1A1* have been described in individuals with type 1 osteogenesis imperfecta, Ehlers-Danlos syndrome type VIIA and VIIB, osteoporosis, and Marfan syndrome, all systemic disorders with scleral thinning and myopia as a clinical component (Dalgleish 1997).

The association between variations in the *COL1A1* gene and high myopia is yet to be investigated. In this study, we screened the *COL1A1* gene for single-nucleotide polymorphisms (SNPs) that could potentially be associated with myopia.

Materials and methods

A total of 330 Japanese subjects with high myopia and ethnically matched unrelated control subjects ($n = 330$) attending the Ophthalmology Clinic at the Yokohama City University or the Okada eye clinic were recruited for this study. The myopia phenotypes were classified according to the mean spherical equivalent refractive error below or above the threshold level of -9.25 Dsph. The mean refractive error of myopia cases was -11.55 ± 2.17 Dsph with a mean axial length of 27.78 ± 1.30 mm. The mean age of case subjects was 37.82 ± 11.97 years, and the male–female ratio of the cases was 0.66:1.00. A group of 330 subjects of same sex and age with moderate or no myopia was used as the control group. None of the subjects involved exhibited any systemic connective tissue diseases, keratoconus, or other genetic disorders known to cause myopia. All subjects involved were Japanese from a similar social background and from the same urban area, and informed consent was obtained from all patients. The study was conducted in accordance with the Declaration of Helsinki and subsequent revisions.

SNP-specific polymerase chain reaction (PCR) primers and fluorogenic probes were designed using ABI (Applied Biosystems, Foster City, CA, USA). This technique has been utilized extensively in genotyping other candidate genes with multiple single nucleotide polymorphisms. The fluorogenic probes were labeled with a reporter dye (either FAM or VIC) and specific for one of the two possible bases in the promoter region. A MGB quencher probe was utilized on the 3′ end by a linker arm. The 2X PCR mix (TaqMan Universal PCR Master Mix , Applied Biosystems) was optimized for TaqMan reactions and contained AmpliTaq-Gold DNA polymerase, dNTPs with UTP and a passive reference dye. Primers, probes, and genomic DNA were added to final concentrations of 300 nmol/l, 100 nmol/l, and 0.5–2.5 ng/μl, respectively. Control samples (without DNA template) were run as a negative control for contaminating DNA, and a reference positive control DNA used to verify the identified polymorphisms. The amplification reactions were carried out in an ABI Prism 7700HT Sequence Detection System (Applied Biosystems GeneAmp® PCR System 9700) with an initial hold step (95°C for 10 min) followed by 40 cycles of a two-step PCR (denaturation at 92°C for 15 s, annealing at 60°C for 1 min). The fluorescence intensity of each sample was measured at each temperature change to monitor the amplification of the base pair *COL1A1* promoter region. The targeted nucleotide was determined by the fluorescence ratio of the two SNP-specific fluorogenic probes. The fluorescence signal increased when the specific probe matched the single stranded template DNA and was digested by the 5′-3′ exonuclease activity of AmpliTaq-Gold DNA polymerase (Applied Biosystems), whereupon the probe released the fluorescent reporter dye (either FAM or VIC) from the quencher dye.

We used the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) to extract the available information on the SNPs in *COL1A1* gene. Forward or reverse strand sequences of the SNP sites were selected depending on their GC content, to avoid primer dimerization and hairpin structure, and based on the absence of any other SNP site. The extension reaction was controlled by a mixture of dideoxy-terminated nucleotides, such that one single-base

extension product and one double-base extension product were created corresponding to a SNP allele. This scheme generated two peaks in the mass spectrometer separated by approximately 300 Da.

Allele frequencies of sequence alterations in patients and controls were evaluated using Chi-square tests or Fisher exact tests. Allelic frequencies of all detected SNPs were also assessed for Hardy–Weinberg equilibrium. Statistical analyses were performed on a computer using the Statview software (ver.5.0 SAS Institute Inc. USA). We corrected these *P* values (Pc) by the Bonferroni's correction where the coefficient was the total number of the contingency tables tested. *P* value < 0.05 and Pc value < 0.1 were considered statistically significant. The R package "haplo.stats" (http://www.r-project.org/) was used to evaluate haplotype structure. Based on the haplotype structures the pair-wise linkage disequilibrium between SNPs was measured with LD coefficient (Lewontin's D') obtained from the R package "genetics" in the R Project for Statistical Computing.

Results

We sequenced 10 SNPs of the *COL1A1* gene, chosen using criteria such as population-frequency validation, multiple submitters and high-profile submitters, using an electronic database 'dsSNP' (http://www.ncbi.nlm.nih.gov/SNP/) for the final selection of ten SNPs for genotyping. A total of 10 SNPs in the *COL1A1* gene were screened in all the myopia cases and control subjects (Fig. 1). Each marker was tested to see if it is in Hardy–Weinberg equilibrium, and that this hypothesis was not rejected for any of the markers. Of the SNP analyzed, one was non-synonymous, one was a synonymous substitution, three were intronic, two were untranslated, and three were unknown (Fig. 1; Table 1). Two of them, rs2586486 and rs1800211, were monomorphisms (Table 1).

Two SNPs (rs2075555 and rs2269336) were significant under a model assuming dominance of the 1st allele listed for each locus (Table 1). The G allele in intron 11 (rs2075555) was significantly more common in the patient group ($P = 0.0071$; OR = 1.36) than in the controls (Table 2). In addition, the frequencies of G allele (rs2269336) and the C allele (rs1107946) in the 5' upstream region of the *COL1A1* gene were significantly increased in the patient group ($P = 0.0140$, OR = 1.31 and $P = 0.0278$, OR = 1.28, respectively; Table 2).

The pairwise LD mapping confirmed that haplotype structure in the *COL1A1* gene was constructed by two blocks (rs748075 to rs207558 and rs207555 to rs1107946) from LD index values (Table 3). The estimated haplotype (GGC/GGC) was significantly more prevalent in the patient group than in the control group ($P = 0.0084$, Pc = 0.025, OR = 1.60 in Table 4). Frequencies of pairwise haplotypes consisting of eight polymorphic SNPs are listed in Table 5. No significant difference found between the patient and controls was detected.

Discussion

Myopia is an extremely common ocular condition that affects approximately one billion people worldwide (Marshall et al. 1993). Many studies have shown that the development of human myopia is influenced by multifactorial etiology with underlying complex genetic factors and undefined environment factors (Bear 1991; Goss et al. 1988; Goss 2000). Studies with animal models have indicated that environmental factors such as visual deprivation and the effects of a negative lens may contribute to the development of myopia. Likewise, there is a significant correlation between myopia and the quantum of near work such as reading and writing (Bear 1991, Saw et al. 2002). However, evidence for genetic pathways contributing to myopia is strengthened by the observations that myopic parents are much more likely to have myopic children (Mutti et al. 2002), and that myopia is far more frequent in the Asian populations than in the USA or Europe, even if the populations examined have performed similar quantum of near work (Feldkamper and Schaeffel 2003). Thus, in addition to environmental effects, myopia is also likely to result from alterations of multiple genetic factors.

In this study, we examined whether the *COL1A1* gene is a disease susceptibility gene for high myopia in Japan. We

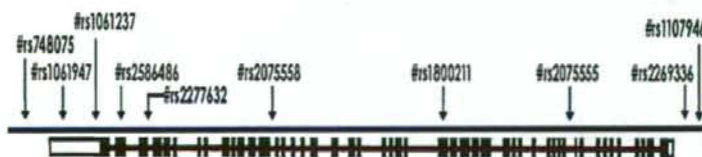

Fig. 1 Human *COL1A1* gene structure and 10 SNPs. *Vertical arrows* show 10 SNPs in this study with the #rs numbers. *Left* of the figure is 5'UTR. *White boxes* show the untranslated regions and *black boxes* show the translated regions of this gene. The size of each exon and intron is not to scale

Table 1 Association of 10 SNPs of *COL1A1* gene with high myopia

SNP rs	Public position	Function	AA change	Allele	Case	Control	χ^2	*P*	OR	95% CI
rs748075	45615428	3'Downstream		C	269 (82.8)	259 (79.7)		NS		
				G	208 (64.0)	218 (97.8)				
rs1061947	45617118	3'UTR		A	30 (9.1)	27 (8.2)		NS		
				G	328 (99.7)	328 (99.7)				
rs1061237	45617774	3'UTR		A	276 (84.1)	267 (81.4)		NS		
				G	212 (64.6)	212 (65.6)				
rs2586486	45618215	Exon	Lys→Gln	G	326 (100)	329 (100)		NS		
				T	0 (0)	0 (0)				
rs2277632	45618902	Intron		A	299 (91.4)	291 (89.8)		NS		
				G	170 (52.0)	169 (52.2)				
rs2075558	45622584	Intron		A	124 (37.8)	111 (33.9)		NS		
				C	311 (94.8)	313 (95.7)				
rs1800211	45626379	Exon		C	329 (100)	330 (100)		NS		
				T	0 (0)	0 (0)				
rs2075555	45629290	Intron		G	294 (89.6)	274 (84.0)	4.47	0.035*	1.64	1.03–2.61
				T	200 (61.0)	228 (69.9)				
rs2269336	45635355	5'Upstream		C	212 (64.4)	246 (74.5)	7.94	0.0048**	1.62	1.15–2.26
				G	280 (85.1)	270 (81.8)				
rs1107946	45635989	5'Upstream		A	191 (58.1)	214 (65.4)		NS		
				C	296 (90.0)	280 (85.6)				

SNPrs: public reference SNP number from the dbSNP database; numbers in parentheses indicate the percentage

AA change amino acid change; *95% CI* 95% confidence interval; *OR* odds ratio

NS not significant by χ^2 test 2 × 2 contingency table ($df = 1$); *P* value was calculated by χ^2 test 2 × 2 contingency table ($df = 1$)

* Pc (corrected *P* value) = 0.07

** Pc = 0.0096

Table 2 Two SNPs (rs2075555 and rs2269336) in Japanese patients with high myopia and healthy individuals

SNP rs	Genotype frequency				Allele frequency					
		Case (%)	Control (%)	*P*		Case (%)	Control (%)	*P*	OR	95%CI
rs2075555	G/G	128 (39.0)	98 (30.1)		G	422 (64.3)	372 (57.1)		1.36	1.09–1.70
	G/T	166 (50.6)	176 (54.0)	0.018*	T	234 (35.7)	280 (42.9)	0.0071***		
	T/T	34 (10.4)	52 (16.0)							
rs2269336	C/C	49 (14.9)	60 (18.2)		C	261 (39.7)	306 (46.4)			
	C/G	163 (49.5)	186 (56.4)	0.018**	G	397 (60.3)	354 (53.6)	0.014****	1.31	1.06–1.64
	G/G	117 (35.6)	84 (25.4)							

Numbers in parentheses indicate the percentage

OR odds ratio; *95% CI* 95% confidence interval

P value was calculated by χ^2 test 3 × 2 contingency table ($df = 2$), or χ^2 test 2 × 2 contingency table ($df = 1$)

* Pc (corrected *P* value) = 0.054

** Pc = 0.054

*** Pc = 0.014

**** Pc = 0.028

performed SNP analysis on 10 SNPs in 330 patients with high myopic change (refractive error greater than −9.25 Dsph) and compared the findings to those in a control group of 330 individuals with no myopia. As myopia is a globally prevalent disease, we selected universally high heterozygosity SNPs from the NCBI SNP database for the association study. However, two of these SNPs were monomorphisms in Japanese.

Table 3 Pairwise linkage disequilibrium (LD) between 8 SNPs of the *COL1A1* gene for high myopia patients and healthy control groups

		Myopia			D'				
		rs748075	rs1061947	rs1061237	rs2277632	rs2075558	rs2075555	rs2269336	rs1107946
Control	rs748075	–	**1.00**	0.78	**0.94**	0.01	0.43	0.35	0.34
	rs1061947	**1.00**	–	**1.00**	**1.00**	**1.00**	0.77	0.64	0.67
	rs1061237	**0.80**	**1.00**	–	**0.95**	0.33	0.51	0.36	0.39
D'	rs2277632	**1.00**	**1.00**	**0.96**	–	**0.80**	0.29	0.23	0.2
	rs2075558	0.2	**0.90**	0.28	**0.90**	–	0.33	0.28	0.16
	rs2075555	0.3	0.63	0.36	0.15	0.53	–	**0.90**	**0.83**
	rs2269336	0.29	0.44	0.23	0.07	0.40	**0.88**	–	**0.96**
	rs1107946	0.3	0.51	0.25	0.11	0.08	**0.81**	**0.96**	–

The degree of LD is shown as the LD index of Lewontin correlation (D'). Bold number indicates the strong LD: D' > 0.8

Table 4 Frequency analysis of the haplotype of *COL1A1* gene in Japanese high myopia patients and control groups

Haplotype	Case (%)	Control (%)	P	χ^2	OR	*
GGC/–	171(52.1)	187(57.7)	N.S.			
GGC/GGC	104(31.7)	73(22.5)	0.0084	6.94	1.60	$P = 0.028$
–/–	53(16.2)	64(19.8)	NS			$\chi^2 = 7.15$

Haplotype consists of three SNPs (rs2075555, rs2269336, rs1107946) in the *COL1A1* gene; numbers in parentheses indicate the percentage

OR odds ratio, *95% CI* 95% confidence interval

P value was calculated by 2 × 2 contingency χ^2 test, and * 3 × 2 contingency table ($df = 2$)

Table 5 Estimated haplotype frequencies of the *COL1A1* gene between control groups and Japanese high myopia patients groups

	Haplotype	748075	1061947	1061237	2277632	2075558	2075555	2269336	1107946	Frequency ($n = 310$)
Control	1	C	G	A	A	C	G	G	C	0.318
	2	G	G	G	G	C	T	C	A	0.15
	3	G	G	G	G	C	G	G	C	0.132
	4	C	G	A	A	C	T	C	A	0.081
	5	C	G	A	A	A	G	G	C	0.045
	6	G	G	G	A	A	T	C	A	0.045
	7	G	G	A	A	C	T	C	A	0.041
	8	C	A	G	A	A	T	C	A	0.035
	Haplotype	748075	1061947	1061237	2277632	2075558	2075555	2269336	1107946	Frequency ($n = 323$)
Case	1	C	G	A	A	C	G	G	C	0.342
	3	G	G	G	G	C	G	G	C	0.136
	2	G	G	G	G	C	T	C	A	0.121
	5	C	G	A	A	A	G	G	C	0.071
	4	C	G	A	A	C	T	C	A	0.06
	8	C	A	G	A	A	T	C	A	0.037
	6	G	G	G	A	A	T	C	A	0.036
	7	G	G	A	A	C	T	C	A	0.026

Two of the 10 SNPs (rs2075555 and rs2269336) showed significantly different frequencies between cases and controls ($P < 0.05$, $Pc < 0.1$). The SNPs were not missense mutations, and one of them exists in an intron while the others are upstream of the *COL1A1* gene. How these SNPs disrupt the function is unknown. Together this suggests that

the *COL1A1* gene is likely associated with high myopia, although the associated variations of this gene do not result in obvious changes in the COL1A1 protein; it is possible that these SNPs are in LD with an, as yet undiscovered, causative mutation in the COL1A1 gene. We investigated the10 SNPs genotypes and allele dominant models (Table 2) and found two SNPs to be statistically different, rs2075555(A < C) in allele2(C) dominant model($P < 0.05$), and rs2269336(C > G) in allele1(C) dominant model ($P < 0.01$).

Gentle et al. (2003) reported that collagen type I mRNA expression was reduced, and scleral collagen accumulation was decreased in the tree shrews sclera of myopic eyes, suggesting that disruption of collagen function in myopia could potentially be regulated at the transcriptional level. Together this suggests the possibility that these three SNPs could affect the promoter and/or transcriptional control of the *COL1A1* gene.

Our susceptibility gene mapping showed that the limited haplotype block (GGC/GGC) constructed by the three SNPs in 5'-genomic region of the *COL1A1* gene was significantly associated with the susceptibility to high myopia.

In conclusion, *COL1A1* may be a candidate gene for high myopia based on its mapped location within the MYP5 candidate region as well as the evidence presented in this report linking SNPs in this region to susceptibility to high myopia. The associated SNPs do not appear to be causal mutation in high myopia phenotypes in Japan. Further studies of candidate genes are needed to determine the molecular genetic factors that contribute to genetic influence of myopia.

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BRIEF COMMUNICATION

Lack of association of Toll-like receptor 9 gene polymorphism with Behçet's disease in Japanese patients

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Abstract

Toll-like receptors (TLRs) play an important role in the induction of defense mechanisms of the innate and adaptive immune responses to microbial pathogens. Genetic polymorphisms within the *TLR9* gene have been reported to be associated with a variety of inflammatory and infectious diseases. Behçet's disease (BD) is a chronic inflammatory disease, and the etiology of BD has yet to be fully elucidated. We investigated the potential association of the *TLR9* gene with susceptibility to BD by analyzing the frequency of nine single nucleotide polymorphisms (SNPs) in a population of 200 Japanese BD patients and 102 randomized controls. Our results showed that SNPs in the *TLR9* gene were not significantly associated with susceptibility to BD.

Behçet's disease (BD) is a chronic systemic inflammatory disorder characterized by four major symptoms: recurrent uveoretinitis, oral aphthosis, genital ulcers and skin lesions. BD is occasionally associated with inflammation in tissues and organs throughout the body, including the vascular system, gastrointestinal tract, central nervous system, lungs, kidneys, joints and epididymis (1). The disease generally arises in young adults, although childhood onset has also been reported (2) and usually runs a course of unpredictable exacerbations and remissions that gradually abate with time. BD patients have been diagnosed worldwide, although BD is found most commonly in Mongoloid and rarely in Caucasian populations, with a particularly high prevalence in countries along the ancient Silk Route from Japan to the Middle East and the Mediterranean basin (3).

While the immunopathogenic mechanisms of BD remain uncertain, various genetic and environmental factors, immune mechanisms, and infectious agents are implicated in being involved in the onset of BD. Our group, as well as others, has presented evidence for an association between BD and human leukocyte antigen (HLA) class I antigen, HLA-B51 (3). In addition, we suggested that the HLA-B*51 allele was a potential candidate to indicate susceptibility of developing BD (4). In addition, the streptococcal antigens (5), bacterial superantigens (6), mycobacterial antigens (7) and type 1 herpes virus (8) have been considered to function as contributory factors toward the development of BD.

Toll-like receptors (TLRs), a family of evolutionarily conserved pathogen recognition receptors, play a pivotal role in innate recognition of foreign material. TLR activation leads to induction of both the innate and the adaptive immune responses directed against invading pathogens (9–11). Notably, genetic variations in genes involved in innate immunity are associated with a range of inflammatory disorders (12).

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TLR9 is expressed in a wide variety of human cells but mainly in plasmacytoid dendritic cells (pDCs) and B cells. TLR9 recognizes unmethylated CpG motifs common in bacterial and viral DNA but rarely found in mammalian DNA (13). Activation of TLR9 in pDC induces Th1 cytokines such as interferon-α or interleukin-12 and Th1-biased immune response (14). The *TLR9* gene, located on chromosome 3p21.3, spans approximately 5 kbp and contains two exons, with the second being the major coding exon (15). Various studies have reported an association between *TLR9* polymorphisms and diseases such as asthma (16), Crohn's disease (17) and systemic lupus erythematosus (18), but no genetic study on the relationship between *TLR9* polymorphisms and BD has yet been reported. To evaluate the potential candidacy of *TLR9* as a BD susceptibility gene, we investigated the association of *TLR9* single nucleotide polymorphisms (SNPs) and BD using case–control analysis.

The total subject group consisted of 200 Japanese patients diagnosed with BD and 102 healthy Japanese controls. The BD patients were diagnosed according to the standard criteria proposed by the Japan Behçet's Disease Research Committee at the Uveitis Clinic of Yokohama City University or Hokkaido University and classified as complete-type BD or incomplete-type BD, according to these criteria (19). All the control subjects were healthy volunteers and similar in ethnic origin to the patients; control subjects were unrelated to each other and to the BD patients. The research methods were in compliance with the Declaration of Helsinki guidelines. Details of the study were explained to all patients and controls, and consent to genetic screening was obtained. Peripheral blood lymphocytes were collected, and genomic DNA was extracted from peripheral blood cells using the QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA).

Nine SNPs (rs187084, rs5743836, rs5743841, rs352139, rs5743842, rs5743843, rs352140, rs5743845 and rs5743846; named SNP1–SNP9) within the *TLR9* gene were genotyped by the TaqMan 5′ exonuclease assay using primers supplied by Applied Biosystems (Foster City, CA). Probe fluorescence signal was detected by TaqMan Assay for Real-Time PCR (7500 Real Time PCR System; Applied Biosystems) following the manufacturer's instructions.

Allelic frequencies of all detected SNPs were tested for Hardy–Weinberg equilibrium. Differences of genotype frequency between case and control were assessed by Chi-squared test and Fisher's exact test. The maximum likelihood estimates of haplotype frequencies were estimated by pairs of unphased genotypes using the expectation–maximization algorithms in the R package 'haplo.stats' (20). Statistical analyses were performed by the STATVIEW software (version 5.0; SAS Institute Inc., Cary, NC). The correction of P values (Pc) was calculated by the Bonferroni's correction where the coefficient was the total number of the contingency tables tested. P value <0.05 and Pc value <0.1 were considered as statistically significant. The strength of linkage disequilibrium (LD) between SNPs was measured with LD coefficient (Lewontin's D') (21),

Table 1 Association of nine SNPs of *TLR9* gene with Behçet's disease

Name	SNP rs	Function	AA change	Allele	Case, n (%), ($n = 200$)	Control, n (%), ($n = 102$)	χ^2	P
SNP1	rs187084	5′Untranslated		A	141 (70.5)	75 (73.5)	0.304	NS
				G	151 (75.5)	73 (71.6)	0.545	
SNP2	rs5743836	5′Untranslated		T	200 (100)	102 (100)		NS
				C	0 (0)	3 (2.9)		
SNP3	rs5743841	Exon 1	Synonymous	C	200 (100)	102 (100)		NS
				T	0 (0)	0 (0)		
SNP4	rs352139	Intron 1		T	141 (70.5)	76 (74.5)	0.537	NS
				C	150 (75.0)	72 (70.6)	0.675	
SNP5	rs5743842	Exon 2	Arg to Cys	C	200 (100)	102 (100)		NS
				T	0 (0)	0 (0)		
SNP6	rs5743843	Exon 2	Gln to His	C	200 (100)	102 (100)		NS
				A	0 (0)	0 (0)		
SNP7	rs352140	Exon 2	Synonymous	T	150 (75)	71 (69.6)	1.001	NS
				C	109 (54.5)	55 (53.9)	0.009	
SNP8	rs5743845	Exon 2	Arg to Gln	C	200 (100)	102 (100)		NS
				T	1 (0.5)	3 (2.9)		
SNP9	S5743846	Exon 2	Ala to Thr	C	200 (100)	102 (100)		NS
				T	0	0		

AA change, amino acid change; NS, not significant by chi-squared test 2 × 2 contingency table (d.f. = 1); SNP, single nucleotide polymorphism; SNP rs, public reference SNP number from the dbSNP database.

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Table 2 Pairwise LD coefficients (D' and r^2) between SNPs on *TLR9* gene[a]

D'							
		SNP	1 rs187084	2 rs5743836	4 rs352139	7 rs352140	8 rs5743845
r^2	Control	1		0.990	0.976	1.000	0.326
	Patient	rs187084		—	0.990	0.990	0.965
	Control	2	0.015		0.987	0.986	0.994
	Patient	rs5743836	—		—	—	—
	Control	4	0.606	0.010		1.000	0.932
	Patient	rs352139	0.970	—		1.000	0.965
	Control	7	0.574	0.008	0.364		0.999
	Patient	rs352140	0.970	—	1.000		0.965
	Control	8	0.019	0.085	0.100	0.100	
	Patient	rs5743845	0.003	—	0.003	0.003	

LD, linkage disequilibrium; SNP, single nucleotide polymorphism.

[a] The degree of LD is shown as the LD index of Lewontin's correlation (D') in the upper right triangle and Pearson's correlation (r^2) in the lower left triangle.

Table 3 Estimated haplotype frequencies of the *TLR9* gene between controls and patients with Behçet's disease

	SNP rs									Frequency	
	1 187084	2 5743836	3 5743841	4 352139	5 5743842	6 5743843	7 352140	8 5743845	9 5743846	Control	BD
Hp1	A	T	C	T	C	C	C	C	C	0.5098	0.4700
Hp2	G	T	C	C	C	C	T	C	C	0.4657	0.5200
Hp3	G	C	C	C	C	C	C	T	C	0.0147	0.0000
Hp4	G	T	C	T	C	C	C	C	C	0.0098	0.0050
Hp5	A	T	C	C	C	C	T	C	C	0.0000	0.0025
Hp6	A	T	C	C	C	C	C	T	C	0.0000	0.0025

SNP, single nucleotide polymorphisms.

obtained from the R package 'genetics' in the R Project for Statistical Computing (http://www.r-project.org/).

Nine SNPs in TLR9 were genotyped, four of which (SNP1, SNP4, SNP5 and SNP7) were monopolymorphic, while six (SNP3, SNP5, SNP6, SNP7, SNP8 and SNP9) are in the coding exon and SNP4 is in the intron. Allelic frequencies of nine SNPs in cases and controls are listed in Table 1. No statistically significant association (Fisher's exact test, $P < 0.05$) was observed for any of the SNPs (Table 1). The subjects used in this study were justified by the Hardy–Weinberg's exact test, and no genetic bias was observed for each SNP.

We also evaluated the LD indices for the specific LD block using five SNPs in *TLR9* in both controls and patients (Table 2). Pairwise LD mapping confirmed that all five of these alleles have a comparatively strong LD index of >0.8 for D' and >0.4 for r^2. The haplotype frequencies in BD patients were similar to those observed in controls, with no detected significant difference (Table 3).

Although the etiology of BD is still uncertain, herpes simplex virus immunopathology, autoimmunity to oral mucosa or cross-reactive microbial antigens and streptococcal infection seem to be potential candidates in inducing BD. TLR9 plays a pivotal role in the induction of first-line defense mechanisms of the innate immune system and triggers effective adaptive immune responses to different bacterial and viral pathogens. Furthermore, it has been speculated that polymorphisms in the *TLR9* gene might influence the functional capability of TLR9 to elicit effective defense mechanism against microbial pathogens, rendering a high susceptibility to microbial infections (16, 22–24). Despite the predicted candidacy of *TLR9* gene as a susceptibility marker for BD, our study clearly shows that no relationship was found.

In conclusion, our study in a group of Japanese population showed that *TLR9* gene polymorphisms were not significantly associated with the susceptibility to BD.

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Polymorphism of *IFN-γ* gene and Vogt-Koyanagi-Harada disease

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Purpose: Interferon-γ (IFN-γ) is a key cytokine in inflammatory disorders. Elevated aqueous and serum levels of IFN-γ levels have been reported to be elevated in patients with Vogt-Koyanagi-Harada (VKH) disease. The aim of this study was to determine the *IFN-γ* gene polymorphisms in VKH disease.
Methods: The study involved 136 VKH patients and 176 healthy controls, who were genotyped for functional single nucleotide polymorphism (SNP; rs2430561; A/T) and functional microsatellite (CA) repeats (rs3138557) in the first intron of the *IFN-γ* gene. Moreover, clinical manifestations of the patients were also analyzed.
Results: Diffuse choroiditis/staining of fluorescein angiography was seen in all VKH patients in this study. Sunset glow fundus and nummular chorioretinal depigmented scars were observed in 83.9%, and 36.1% of the patients, respectively. Neurological and auditory disorders were observed in 90.1% of the patients: meningismus (79.8%), tinnitus (53.0%), and cerebrospinal fluid pleocytosis (70.0%). Dermatologic manifestations were observed in 22.9% of the patients, manifesting as alopecia (6.9%), poliosis (17.6%), and vitiligo (13.0%). In addition, 22.1% of the patients were classified as having complete VKH disease, while 65.4% as having incomplete VKH disease, and 12.5% as having probable VKH disease. There were no significant differences in the allele and genotype frequencies between VKH patients and healthy controls. In addition, we found no association between each clinical manifestation and SNP (re2430561) in the healthy control subject. A strong linkage disequilibrium (LD) was found in the functional SNP T allele and functional microsatellite 12 (CA) repeats (D'=0.96-0.99).
Conclusions: The functional SNP T allele and microsatellite 12 (CA) repeats were found to have a strong LD, although a genetic susceptibility for the *IFN-γ* gene could not be demonstrated among the Japanese VKH patients.

Vogt-Koyanagi-Harada (VKH) disease is one of the most frequent forms of uveitis in Japan [1]. It is characterized as a panuveitis, and is often accompanied by headache, pleocytosis of the cerebrospinal fluid, inner ear disturbances, and skin lesions, such as vitiligo and alopecia [2,3]. VKH disease is considered an autoimmune disease against melanocytes [2-7], and is strongly associated with HLA-DRB1*0405 is strongly associated with VKH disease [8,9]. Interferon-γ (IFN-γ) is significantly elevated in the aqueous humor and sera of VKH patients [10-14]. IFN-γ may be associated with the development of predominant Th1-dominant, cell mediated immune responses which may thereby enhance the expression of HLA class II antigens [15,16].

The *IFN-γ* gene on chromosome 12q24.1 spans approximately 5.4 kb and contains four exons. Like other cytokines, the IFN-γ coding region is invariant, with no reported polymorphisms [17]. Single nucleotide polymorphism (SNP; rs2430561) and microsatellite (rs3138557) within the first intron of the *IFN-γ* gene correlate with a high amount of in vitro production of IFN-γ and are associated with disease severity or resistance to drug therapy in various autoimmune diseases [18-22]. This allele is associated with a higher or a lower risk of a variety of diseases including autoimmune and chronic inflammatory conditions [23]. The association between SNP (rs2430561) alleles T to A with a low (AA), medium (AT), and high (TT) production of cytokines has been reported in vitro [24,25].

We hypothesized that a common allelic variation in these potential functional polymorphisms may be involved in Th1-mediated autoimmune diseases, such as VKH disease. In this case-control association study of Japanese subjects, we investigated whether the aforementioned polymorphisms in the *IFN-γ* gene contribute to the development risk of VKH disease and to some of the clinical features of the disease.

METHODS

We recruited 136 VKH patients and 176 healthy controls from the Uveitis Survey Clinic of the Hokkaido University and Yokohama City University. VKH patients were approached as they visited the clinic for previously scheduled visits and were enrolled after consenting to participate in the study. The control subjects were healthy volunteers who were unrelated to each other or to the patients and had no history of any inflammatory disease. All the participants were Japanese, and they all gave their informed consent. The patients were diagnosed at the Uveitis Survey Clinic of the Hokkaido Univer-

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sity Hospital, Sapporo, Japan, according to the "Revised Diagnostic Criteria for VKH Disease" [2,3]. The procedures used in this study conformed to the tenets of the Declaration of Helsinki.

Blood samples were collected from each subject (7 ml), and genomic DNA was extracted with a QIAamp DNA blood mini kit (Qiagen, Tokyo, Japan). SNP and microsatellite repeats in the first intron of the *IFN-γ* gene were amplified using two primers: forward 5'-TGA TTC TGG CTA AGG AAT GT-3' and reverse 5'-AAT TGC AAT GTC ACA AAT GA -3'. Polymerase chain reactions (PCRs) were performed in a total volume of 10 μl containing PCR buffer, genomic DNA, 0.2 mM dinucleotide triphosphates, 0.5 μM primers, and 0.25 U Taq polymerase. The reaction mixture was denatured for 2 min at 94 °C, and then subjected to 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C, 2 min for extension at 72 °C, and 7 min for final elongation at 72 °C using a PCR thermal cycler, GeneAmp System 9700 (Applied Biosystems, Foster City, CA). The purified PCR products were used as templates for direct sequencing using BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing primer was 5'-TGA TTC TGG CTA AGG AAT GT-3', same as that used previously for amplification. The BigDye XTerminator Purification kit was used for purification of DNA sequencing reactions. Nucleotide sequences were determined by an ABI3130 Genetic Analyzer (Applied Biosystems). Microsatellite repeats were calculated by direct counting (Figure 1).

Statistical analysis: The significance of associations was tested using the chi-square test in a single-point analysis. Pairwise linkage disequilibrium (LD; D' value) between SNP (rs2430561) and microsatellite (rs3138557), and haplotypes frequency were estimated by the expectation-maximization (EM) algorithm using the SNPAlyze program (version 5.1 software; Dynacom, Yokohama, Japan). Haplotype frequencies of the patients and controls were compared using the chi-square test and by permutation p values based on 10,000 permutations.

RESULTS

The characteristics of the VKH patients are summarized in Table 1. The age of the patients ranged from 15 to 80 years (51.9±13.7, mean±SD). The study group included 65 men (47.8%) and 71 women (52.2%). Based on the diagnostic cri-

Table 1. Characteristics of the study patients

	Percentage	(n/N)
Age (years)		
Mean±SD (range)	51.9±13.7 (15-80) yo	
Sex		
Male	65 47.8	
Female	71 52.2	
Complete VKH disease	22.1	(30/136)
Incomplete VKH disease	65.4	(89/136)
Probable VKH disease	12.5	(17/136)
Bilateral ocular involvement	100	(136/136)
Diffuse choroiditis	100	(125/125)
Sunset glow fundus	83.9	(99/118)
Depigmented scars	36.1	(44/122)
Neurological auditory findings	90.1	(118/131)
Meningismus (headache, fever etc)	79.8	(103/129)
Tinnitus	53.0	(62/117)
Cerebrospinal fluid pleocytosis	70.0	(77/110)
Integumentary findings	22.9	(30/131)
Alopecia	6.9	(9/131)
Poliosis	17.6	(23/131)
Vitiligo	13.0	(17/131)

The age of the patients ranged from 15 to 80 years (51.9±13.7, mean±SD). The study group included 65 men (47.8%) and 71 women (52.2%). Based on the diagnostic criteria for VKH disease, 30 patients (22.1%) were classified as having complete VKH disease, 89 (65.4%) as having incomplete VKH disease, and 17 (12.5%) as having probable VKH disease. Diffuse choroiditis/staining of fluorescein on angiography was observed in all VKH patients. Sunset glow fundus and nummular chorioretinal depigmented scars were observed in 83.9% and 36.1% of the patients, respectively. Neurological and auditory disorders were observed in 90.1% of the patients: meningismus (e.g., headache and fever), 79.8%; tinnitus, 53.0%; and cerebrospinal fluid pleocytosis, 70.0%. Dermatologic manifestations were observed in 22.9% of the patients: alopecia (6.9%), poliosis (17.6%), and vitiligo (13.0%). n/N represents the number of patients with the characteristics/number of patients with available information. SD represents standard deviation; yo represents years old.

Figure 1. DNA sequences of rs2430561 and rs3138557 in the *IFN-γ* gene. The number of functional microsatellite (CA) repeats was calculated by direct counting. Asterisk (*) 1 indicates the position of the SNP alleles A and T. *2 and *3 indicate functional microsatellite alleles with 12 (CA) repeats and 13 (CA) repeats, respectively. Complement sequence data is available at the NCBI database.

teria for VKH disease, 30 cases (22.1%) were classified as complete VKH disease, 89 cases (65.4%) as having incomplete VKH disease, and 17 cases (12.5%) as having probable VKH disease. Both eyes were affected in all patients. Diffuse choroiditis/staining of fluorescein on angiography was observed in all patients. Sunset glow fundus and nummular chorioretinal depigmented scars were observed in 83.9% and 36.1% of the patients, respectively. Neurological and auditory disorders were observed in 90.1% of the patients: meningismus (e.g., headache and fever) in 79.8%, tinnitus in 53.0%, and cerebrospinal fluid pleocytosis in 70.0%. Dermatologic manifestations were observed in 22.9% of the patients: alopecia (6.9%), poliosis (17.6%), and vitiligo (13.0%; Table 1).

We examined the allele and genotype frequencies of the SNP and microsatellites in the VKH patients and healthy controls (Table 2). Six microsatellite alleles were observed in the study group. There was no significant difference in the allele and genotype frequencies between total the VKH patients and healthy controls.

Overall haplotype frequencies that were greater than 1% are shown in Table 3. The degree of the LD between SNP (rs2430561) and microsatellite (rs3138557) was D'=0.96 in the healthy controls and D'=0.99 in the VKH patients.

DISCUSSION

A positive association has been reported between allele 12 (CA) repeats and several autoimmune diseases, such as rheumatoid arthritis, type 1 diabetes mellitus, and aplastic anemia [19-21,26]. These reports have suggested that either the (CA) repeat sequence itself has a regulatory function or there is an allelic linkage between the CA repeat and functional polymorphism, which would account for the differences in the IFN-γ production [27]. Pravica, et al., in the United Kingdom reported that the presence of the SNP T allele was closely correlated with the 12 CA repeats [18]. In the present study, we showed that a high-IFN-γ productive T allele had strong LD with the 12 CA repeats in the Japanese population (D'=0.96-0.99). Therefore, our results regarding the correlation between with the T allele and 12 CA repeats in the Japanese patients were consistent with the study of Pravica et al. Because this SNP lies within the a binding site for the transcription factor nuclear factor-α-B (NF-α-B) it is believed that, SNP T allele causes an increased production in serum IFN-γ levels [18]. This transcription factor induces IFN-γ expression, and the T allele correlates with a high expression IFN-γ.

In the present study, no significantly different allelic or genotypic distributions of the analyzed *IFN-γ* gene polymorphisms were found between Japanese VKH patients and healthy controls. In our clinically stratified analysis, we investigated the onset of VKH disease and the presence of some of its clinical features such as diffuse choroiditis, sunset glow fundus, depigmented scars, meningismus, tinnitus, cerebrospinal fluid pleocytosis, and integumentary manifestations. None of these clinical findings were significantly associated with SNP (data not shown).

VKH disease is a multifactorial disease that results from interactions between susceptibility genes, environmental factors, and immunological responses. If IFN-γ production by the T allele is sufficient for disease onset, then VKH disease may not require the high-IFN-γ polymorphism allele. In this study, most of the patients were categorized as having incomplete VKH disease. We attributed the low number of patients with complete VKH disease to early detection. Early detection and a rapid cure can affect the prognosis of VKH disease. If the treatment of VKH is delayed, systemic complications, such as vitiligo and alopecia, will frequently arise [28]. Yang et al. recently classified 66.6% of the Chinese VKH patients in their study as having complete VKH. Most of these patients (82.2%) were referred to the uveitis center two months or later after the disease onset [29]. The clinical features are quite different between these two neighboring countries [2,29]. It is therefore still possible that the IFN-γ polymorphism is associated with disease severity and a poor prognosis. If the patients could be followed without therapy, as some of the Chinese patients were, we might have confirmed this hypothesis. Microsatellite markers are often used as a tool for sequencing disease candidate genes because they have many polymorphisms, and their LD is approximately in the 100 kb range [30]. The results of the present study indicate that the

Table 3. Haplotype distribution

	VKH patients			Healthy controls	
	SNP	MS	Percentage	Percentage	p-value
1	A	13	55.2	52.3	0.50
2	A	15	27.9	32.1	0.26
3	T	12	9.6	9.1	0.80
4	A	16	3.3	1.7	0.23
5	A	17	1.8	2.0	0.77
6	A	14	0.7	2.3	0.12

We examined the allele and genotype frequencies of the SNP and microsatellites in the VKH patients and healthy controls. Six microsatellite alleles were observed in the study group. There was no significant difference in the allele and genotype frequencies between the VKH patients and healthy controls. RR represents relative risk.

Table 2. Genotype and allele frequencies of rs2430561 (A/T) and rs3138557 functional microsatellites

	VKH patients		Healthy controls			
rs2430561 A/T	(n=136)	(%)	(n=176)	Percentage	RR	p-value
Genotype frequency	AA 111	81.6	146	83.0	0.91	0.76
	AT 24	17.6	27	15.3	1.16	0.63
	TT 1	0.7	3	1.7	0.43	0.45
T allele frequency (%)		9.6		9.3	1.03	0.94
rs3138557 CA	(2n=272)	(%)	(2n=352)	Percentage		
Allele frequency	12 30	11.0	33	9.3	1.20	0.50
	13 150	55.1	184	52.3	1.12	0.48
	14 2	0.7	8	2.3	0.32	0.13
	15 76	27.9	114	32.4	0.81	0.23
	16 9	3.3	6	1.7	1.97	0.19
	17 5	1.8	7	2.0	0.92	0.89

We examined the allele and genotype frequencies of the SNP and microsatellites in the VKH patients and healthy controls. Six microsatellite alleles were observed in the study group. There was no significant difference in the allele and genotype frequencies between the VKH patients and healthy controls. RR repesents relative risk.

IFN-γ gene and the genes surrounding it are not truly susceptible loci associated with VKH disease [31]. (4) All patients in this study had normal levels of IFN-γ. Although we did quantify IFN-g levels in the present study, some previous studies reported that serum and aqueous IFN-γ levels were elevated in VKH patients [11,14]. There was a slight possibility that only our patients had normal IFN-γ levels in the sera and aqueous humor.

In conclusion, no significant correlation was observed between the IFN-γ gene polymorphism and VKH disease onset. Because all the patients in the present study received systemic corticosteroids immediately after diagnosis, the association between polymorphisms and disease prognosis and/or complications is still unclear. Further studies are required to clarify the genetic mechanisms underlying VKH disease.

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Association of Toll-like Receptor 4 Gene Polymorphisms with Normal Tension Glaucoma

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Purpose. Toll-like receptor 4 (TLR4) is a transmembrane receptor that mediates immune responses to exogenous and endogenous ligands and interacts with heat shock proteins, which are reportedly involved in normal tension glaucoma (NTG). This study was undertaken to investigate whether TLR4 polymorphisms are associated with NTG.

Methods. Two hundred fifty Japanese patients with NTG and 318 Japanese healthy control subjects were recruited. Eight single-nucleotide polymorphisms (SNPs) in the TLR4 gene were genotyped, and allelic and phenotypic diversity was assessed between cases and control subjects.

Results. Strong linkage disequilibrium was observed among all SNPs ($D' \geq 0.85$), which were located in one haplotype block. With respect to allelic diversity, the minor allele of three SNPs (rs10759930, rs1927914, and rs7037117) carried a significantly increased risk of NTG. With regard to genotypic diversity, individuals with the minor allele of six SNPs (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, and rs7037117) had a 1.47- to 1.65-fold increased risk of NTG. rs7037117, located in the 3′-untranslated region of TLR4, was most strongly associated with NTG, and when incorporated into a haplotype with rs10759930, the strongest association was detected ($P = 0.0038$, $P_c = 0.0095$).

Conclusions. Multiple SNPs in the TLR4 gene are associated with the risk of NTG. This finding suggests that the ligands and/or cytokines involved in the TLR4 signaling network may be risk factors for the development of NTG. (*Invest Ophthalmol Vis Sci.* 2008;49:4453–4457) DOI:10.1167/iovs.07-1575

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Glaucoma is a degenerative optic neuropathy characterized by the progressive loss of retinal ganglion cells and optic nerve axons, together with visual field damage; it often occurs in relation to elevated intraocular pressure (IOP). It is the second leading cause of blindness worldwide, affecting approximately 70 million people.[1,2] Glaucoma is generally a multifactorial disorder initiated as the result of several interacting factors. Factors other than IOP are likely to play a role in the pathogenesis of glaucomatous optic neuropathy, particularly in patients with normal tension glaucoma (NTG). NTG is an important subset of primary open-angle glaucoma (POAG). Although many POAG patients have high IOP,[3] patients with NTG have statistically normal IOP.[4–6] NTG accounts for approximately 20% to 50% of all POAG cases.[7–9] In a recent study of the Japanese population, the reported prevalence of POAG was 3.9%, and 92% of patients with POAG had NTG, in which the IOP was 21 mm Hg or less.[10] Because IOP is normal, NTG is underdiagnosed and usually presents late in life, after loss of the visual field. The factors contributing to the development of NTG have not yet been determined.

Recently, it has been suggested that the immune system and heat shock proteins (HSPs) play an important role in glaucoma.[11] HSPs are highly immunogenic molecules that are widely distributed in nature; they perform important functions relating to the folding and assembly of protein complexes. Human HSPs are expressed on cell membranes in response to stress, such as physiological shock and microbial challenge. Wax et al.[12] observed that patients with NTG have increased

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