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Tyrosinase gene family and Vogt-Koyanagi-Harada disease in Japanese patients

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Purpose: The aim of the present study was to examine the genetic background of Vogt-Koyanagi-Harada (VKH) disease in a Japanese population by analyzing the tyrosinase gene family (TYR, TYRP1, and dopachrome tautomerase (DCT)).
Methods: 87 VKH patients and 122 healthy controls were genotyped using seven microsatellite markers on the candidate loci. We analyzed microsatellite (MS) polymorphisms at regions within tyrosinase gene family loci. In addition, the haplotype frequencies were also estimated and statistical analysis was performed. HLA-DRB1 genotyping was performed by the PCR-restriction fragment length polymorphism (RFLP) method.
Results: No significant evidence for an association was found. HLA-DRB1*0405 showed a highly significant association with VKH disease compared with the healthy controls (Pc=0.000000079), as expected.
Conclusions: We concluded that there is no genetic susceptibility or increased risk attributed to the tyrosinase gene family. Our results suggest the need for further genetic study and encourage a search for novel genetic loci and predisposing genes in order to elucidate the genetic mechanisms underlying VKH disease.

Vogt-Koyanagi-Harada (VKH) disease is one of the most frequent forms of uveitis in Japan, and is characterized as a panuveitis accompanied by neurological lesions such as headache and pleocytosis of the cerebrospinal fluid, skin lesions such as vitiligo, alopecia, and inner ear disturbances. This disease is considered to be an autoimmune disease against melanocytes [1]. Numerous studies have shown that about 90% of VKH patients have human leukocyte antigen (HLA) DRB1*0405 [2-4]. However, the true pathogenic gene related to VKH remains unclear. Recently, the genetic contribution of single nucleotide polymorphisms (SNPs) to autoimmune disease has been documented and shown to be consistently associated with numerous diseases, including Graves' disease, type 1 diabetes, and rheumatoid arthritis [5-7].

In earlier studies, melanocyte-specific proteins, tyrosinase-related protein (TRP) 1 and TRP2, induced an experimental autoimmune disease in Lewis rats that resembled human VKH disease [8]. Inflammation induced by TRP1 in Akita dogs also resembled human VKH disease [9]. Lymphocytes extracted from VKH disease patients were reactive to peptides derived from tyrosinase family proteins [10]. These studies suggest that tyrosinase family proteins may be responsible for human VKH disease.

As for the investigation of disease susceptibility genes, association studies are now primarily conducted with single nucleotide polymorphisms or microsatellites because they are ubiquitous in the genome. Microsatellite (MS) polymorphisms show a greater diversity than SNPs and have been widely used in both linkage and association studies of disease. Microsatellite linkage disequilibrium (LD) length is in the approximately 100 kb range [11] when compared with the shorter range for SNPs. Therefore, the advantage of microsatellite analysis is that a collection of relatively small numbers of polymorphic markers can make association analyses an immediate reality [12]. To investigate whether the tyrosinase gene family is responsible for VKH disease or not, we analyzed polymorphisms in MSs among tyrosinase gene family loci.

METHODS

We recruited 87 Japanese VKH patients and 122 healthy Japanese controls for this study. Patients were diagnosed according to the criteria of the American Uveitis Society [13] (at the uveitis clinic of Hokkaido University). All control subjects were healthy volunteers unrelated to each other or to the patients. Informed consent was obtained from all patients and controls, and the procedures used conformed to the tenets of the Declaration of Helsinki.

Genomic DNA was extracted by the QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan) or guanidine method. The tyrosinase gene family was selected as target regions: TYR (11q14-q21), TYRP1 (9q23), and DCT (13q32) [14]. Seven informative microsatellite markers in these regions which had polymorphisms [11] (D9S0128i, D9S0926i, D9S267, D11S0260i, D11S0529i, D13S0450i, and D13S0504i,) were

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selected to perform linkage analysis. These makers were distributed at the following distances from the tyrosinase gene family: TRY gene: D11S0529i, located between exon 2 and exon 3, D11S0260i, between exon 4, and exon 5; TYRP1 gene: D9S0128i, 70 kb telomeric, D9S0926i, 4 kb telomeric, D9S267, 200 kb centromeric; and DCT: D13S0450i, 120 kb centromeric, and D13S0504i, 30 kb telomeric.

Oligonucleotide primers for microsatellites were synthesized according to the previously described method [11]. PCR reactions were performed in a total volume of 12.5 µl containing PCR buffer, genomic DNA, 0.2 mM dinucleotide triphosphates (dNTPs), 0.5 µM primers, and 0.35 U Taq polymerase. The reaction mixture was subjected to 5 min at 94 °C, then 35 cycles of 1 min for denaturing at 94 °C, 1 min for annealing, 2 min for extension at 72 °C, and 10 min for final elongation at 72 °C using a PCR thermal cycler, GeneAmp System 9700 (Applied Biosystems, Foster City, CA). PCR annealing temperatures, depending on the primers used, are indicated in Table 1. Each forward primer was labeled at the 5' end with 6-FAM (Sigma, Japan), NED, or VIC (Applied Biosystems). To determine the number of microsatellite repeats, PCR-amplified products were denatured for 2 min at 97 °C mixed with formamide, and electorphoresed using an ABI3130 Genetic Analyzer (Applied Biosystems). Fragment length analysis was performed by an ABI3130 automatic sequencer (Applied Biosystems) with GeneScan software (Applied Biosystems). The number of microsatellite repeats was estimated with GeneMapper v3.5 software (Applied Biosystems) using GS500(-250)Liz (Applied Biosystems) as a size marker.

HLA-DRB1 genotyping by the PCR-RELP method: Genomic amplification of the HLA-DRB1 gene was performed using local primers, as previously described [15]. After PCR amplification, 7 micro liter of PCR products were digested with 2-5 units of allele-specific restriction endonucleases at an appropriate temperature for 3 h in an incubator. These digested products were subjected to electrophoresis in 12% polyacrylamide gels, and HLA genotypes were determined on the basis of the obtained specific band pattern (PCR-RFLP method) [15].

Statistical analysis: Allele frequencies were calculated by direct counting. The significance of association was tested using Fisher's exact method. The probability of an association was corrected with the Bonferroni inequality method, i.e., by multiplying the p-values obtained by the number of alleles compared. The haplotypes frequencies were estimated using the SNPAlyze program version 5.1 software (Dynacom, Yokohama, Japan), which uses the Expectation-Maximization (EM) algorithm. Where there were incomplete genotype data, potential haplotype reconstructions were inferred, given the genotype data observed at the other loci. The Chi-square test was used to detect the difference between VKH patients and healthy controls in haplotype frequencies. Permutation tests were used to assess the significance of VKH patient and healthy control haplotype frequency differences [16].

TABLE 1.

Marker	GenBank accession number	Repeat unit	Primers	Sequences(5' - 3')	Tm(°C)
D9S0128i	AB133511	TG	5-primer	AGTTAGACAGAACTGATATGGCAC	59
			3-primer	GACTGCTTCCAGACTAAACTGTAG	
D9S0926i	AB133446	TG	5-primer	AGTATTGACCAAATGTCAGTTGTC	58
			3-primer	CAAGCAACTTGTATACGTTTAGAAG	
D9S267	Z23807	TG	5-primer	TTACTGATTGAGGCAACCA	58
			3-primer	GCAATGTTTTGGGCTCT	
D11S0529i	AB143188	AC	5-primer	AACGTGATCTAATTGGCTATTAC	57
			3-primer	TATAAGCCATGAAATAACGAGT	
D11S0260i	AB139945	AC	5-primer	TTCTTAGGTTGCTCAGTATGAT	56
			3-primer	CATTAGAGAACAGCATTTCTAAAT	
D13S0450i	AB146899	CA	5-primer	CTCACTTTACCAGTGAGAACAC	59
			3-primer	GATCCAAGGAGTCTGATTCTAC	
D13S0504i	AB147548	GGAA	5-primer	AAGCCAAAGCATGCTAGTA	60
			3-primer	TTAGTTGCCAGTTGCTGAC	

RESULTS

Table 2 shows the allele frequencies in VKH patients and healthy controls at seven microsatellites. All alleles in each microsatellite marker were named on the basis of the amplified fragment length. Among the seven microsatellite markers, the frequency of allele 162 of D9S267 (55.1% in VKH patients and 65.2% in healthy controls) was shown significant association (p=0.039). However, this increase did not reach significance when the p-value was corrected via multiplication by the number of alleles (pc=0.39). Table 3 displays the results of the estimated haplotype frequency analyses for microsatellite markers at TYR loci. The estimated haplotype frequencies for VKH patients and healthy controls are shown for configurations as well as chi-square, p-value and permutation tests. There was no significant difference between VKH patients and healthy controls in the TYR loci, nor in haplotype frequency.

The phenotype frequency of HLA-DRB1*0405 was 61 (70.1%) in VKH patients, which was remarkably higher than that of healthy controls (35 (28.6%), pc=0.000000079; Table 4).

DISCUSSION

The tyrosinase gene family encodes the enzymes involved in melanin formation and is expressed specifically in melanocytes [17,18]. Yamaki et al. suggested that VKH disease may be induced by tyrosinase family proteins [10]. We speculated that polymorphisms within the tyrosinase gene family may be related to VKH disease. As previously described, the candidate tyrosinase gene family, which codes for melanosome proteins [14], is a class of genes that have been associated with depigmentation and ocular developmental defects. In previous studies, mutations of TYR and TYRP1 caused oculocutaneous albinism (OCA) 1, OCA3, and microphthalmia [19-22]. Recently, it has been reported that mutation of DCT may cause microcoria [23]. These mutations include missense, nonsense, frameshift, and splice site mutations, and deletion of the entire coding sequence [24,25]. At present, six polymorphisms in TYR and seven polymorphisms in TYRP1 have been identified (Albinism Database).

In the present study, we examined 87 VKH patients and 122 healthy controls for tyrosinase gene family loci. None of these regions showed evidence for a significant association with VKH disease. D9S267 showed a marginally significant p-value ($p<0.05$). However, this did not reach significance when the P-value was corrected (pc=0.39); furthermore, the

Table 2. Genotype frequency of VKH patients and healthy controls at tyrosinase gene family loci

			VKH patients		Healthy controls				
Chromosome	Marker	Allele size	(2n=174)	(%)	(2n=244)	(%)	R.R.	P	Pc
Chr 9									
	D9S0128i	257	2	(1.1)	0	(0.0)	0.00	0.09	0.65
		259	117	(67.2)	178	(73.0)	0.76	0.21	1.45
		261	1	(0.6)	3	(1.2)	0.46	0.50	3.49
		263	0	(0.0)	1	(0.4)	0.00	0.40	2.79
		265	9	(5.1)	5	(2.0)	2.61	0.08	0.56
		267	33	(19.0)	40	(16.4)	1.19	0.50	3.46
		269	12	(6.9)	17	(7.0)	0.99	0.98	6.84
	D9S0926i	297	0	(0.0)	1	(0.4)	0.00	0.40	4.77
		301	15	(8.6)	13	(5.3)	1.68	0.18	2.21
		303	2	(1.1)	0	(0.0)	0.00	0.09	1.12
		305	0	(0.0)	1	(0.4)	0.00	0.40	4.77
		307	4	(2.3)	8	(3.3)	0.40	0.13	1.55
		309	10	(5.7)	9	(3.7)	0.91	0.85	10.21
		311	46	(26.4)	60	(24.6)	1.10	0.67	8.03
		313	86	(49.4)	140	(57.4)	0.73	0.11	1.29
		315	7	(4.0)	7	(2.9)	1.42	0.52	6.22
		317	3	(1.7)	4	(1.6)	1.05	0.95	11.36
		319	0	(0.0)	1	(0.4)	0.00	0.40	4.77
		321	1	(0.6)	0	(0.0)	0.00	0.24	2.83
	D9S267	158	0	(0.0)	1	(0.4)	0.00	0.40	3.98
		160	14	(8.0)	14	(5.7)	1.44	0.35	3.52
		162	96	(55.1)	159	(65.2)	0.66	0.04	0.39
		164	6	(3.4)	11	(4.5)	0.76	0.59	5.89
		166	3	(1.7)	5	(2.0)	0.84	0.81	8.11
		168	9	(5.2)	4	(1.6)	3.27	0.04	0.40
		170	41	(23.6)	45	(18.4)	1.38	0.19	1.87
		172	4	(2.3)	3	(1.2)	1.89	0.40	4.01
		174	1	(0.6)	0	(0.0)	0.00	0.24	2.36
		178	0	(0.0)	2	(0.8)	0.00	0.23	2.31
Chr 11									
	D11S0529i	404	1	(0.6)	0	(0.0)	0.00	0.24	2.36
		408	1	(0.6)	2	(0.8)	0.70	0.77	7.70
		410	1	(0.6)	3	(1.2)	0.46	0.50	4.98
		412	18	(10.3)	20	(8.2)	1.29	0.45	4.51
		414	50	(28.7)	60	(24.6)	1.24	0.34	3.43
		416	56	(32.2)	83	(34.0)	0.92	0.70	6.95
		418	16	(9.2)	32	(13.1)	0.67	0.22	2.15
		420	28	(16.1)	40	(16.4)	0.98	0.93	9.34
		422	3	(1.7)	3	(1.2)	1.41	0.68	6.75
		424	0	(0.0)	1	(0.4)	0.00	0.40	3.98
	D11S0260i	232	25	(14.4)	32	(13.1)	1.11	0.71	4.28
		234	20	(11.5)	38	(15.6)	0.70	0.23	1.41
		236	56	(32.2)	59	(24.1)	1.49	0.07	0.43
		238	70	(40.2)	108	(44.3)	0.85	0.41	2.47
		240	2	(1.1)	4	(1.6)	0.70	0.68	4.07
		242	1	(0.6)	3	(1.2)	0.46	0.50	2.99
Chr 13									
	D13S0450	383	0	(0.0)	1	(0.4)	0.00	0.40	3.98
		387	32	(18.4)	51	(20.9)	0.85	0.53	5.26
		389	1	(0.6)	6	(2.5)	0.23	0.14	1.39
		391	2	(1.1)	0	(0.0)	0.00	0.09	0.93
		393	41	(23.6)	43	(17.6)	1.44	0.14	1.35
		395	67	(38.5)	107	(43.9)	0.80	0.27	2.74
		397	15	(8.6)	21	(8.6)	1.00	1.00	9.96
		399	15	(8.6)	10	(4.1)	2.21	0.06	0.55
		401	1	(0.6)	4	(1.6)	0.35	0.32	3.24
		403	0	(0.0)	1	(0.4)	0.00	0.40	3.98
	D13S0504	426	6	(3.4)	11	(4.5)	0.76	0.59	4.12
		430	31	(17.8)	53	(21.7)	0.78	0.33	2.28
		434	85	(48.9)	110	(45.1)	1.16	0.45	3.13
		438	31	(17.8)	38	(15.6)	1.18	0.54	3.80
		442	12	(6.9)	27	(11.1)	0.60	0.15	1.04
		446	7	(4.0)	5	(2.0)	2.00	0.23	1.64
		450	2	(1.1)	0	(0.0)	0.00	0.09	0.65

Chr represents Chromosome. R.R. represents relative risk. Pc represents corrected P-values by Bonferroni's correction.

remaining MS marker (D9S0128i and D9S0926i) at this locus did not yield increased association. To exclude an association with VKH at TYR loci, we also estimated and analyzed haplotype frequencies. This haplotype analysis did not show an association at the level of TYR either (the haplotype 259-313-162; p=0.058). The allele 162 of D9S267 was also included in the haplotype which was the lowest p-value. Considering about the over collection of the p-value, the allele 162 of D9S267 may be associated with VKH disease. According to previous reports, T-cells established from VKH patients responded to peptides derived from tyrosinase family proteins, implying a role in VKH disease [8-10]. Our results did not contradict these reports because VKH disease may be triggered by the breakdown of self-tolerance, in a similar way to other autoimmune diseases [26,27].

We also performed HLA-DRB1 genotyping, and DRB1*0405 was strongly associated with VKH (pc=0.000000079). This result was almost the same as that previously reported [2-4]. We could not identify any association of sites around the tyrosinase gene family loci between VKH patients and healthy controls.

In conclusion, we speculated that polymorphisms in MSs among the tyrosinase gene family loci of VKH patients were different from those of healthy controls, but no significant difference was noted in both single microsatellite marker analysis and haplotype analysis. HLA-DRB1*0405 showed a highly significant association (pc=0.000000079), as expected. The mutational characterization of genes involved in VKH disease will provide additional insight into the molecular mechanisms underlying this common uveitis in the Japanese population.

Table 3. Estimated haplotype frequencies for TYR loci

1 - 2 - 3	All cases (%)	VKH patients (n=87; %)	Healthy controls (n=122; %)	Chi-square	p-value	permutation p-value
259-313-162	32.25	27.43	37.12	4.30	0.038	0.058
259-311-162	7.43	8.25	6.62	0.40	0.528	0.643
259-313-170	7.18	7.02	7.21	0.01	0.941	0.958
267-311-162	4.20	2.18	6.25	3.87	0.049	0.174
259-313-160	3.25	4.97	1.25	5.17	0.023	0.085
267-311-170	3.20	5.52	1.31	6.06	0.014	0.094
269-301-162	2.54	3.19	2.08	0.51	0.477	0.535
259-311-178	2.37	3.02	2.25	0.24	0.624	0.777
265-311-162	2.04	3.30	0.46	5.39	0.020	0.118
267-313-162	2.02	1.92	1.86	0.00	0.964	0.985
259-311-160	1.76	1.65	2.12	0.12	0.730	0.786
259-313-164	1.67	0.34	2.69	4.76	0.029	0.159
267-309-162	1.60	2.08	0.93	0.98	0.323	0.438
259-315-170	1.53	1.51	1.56	0.00	0.967	0.973
259-309-170	1.35	2.52	0.44	3.41	0.065	0.144
259-313-172	1.15	1.95	0.45	3.15	0.143	0.273
259-313-168	1.14	1.38	1.02	0.06	0.804	0.928
267-301-170	1.13	1.66	0.33	2.04	0.153	0.374
259-307-162	1.12	0.57	1.54	0.84	0.359	0.509
259-317-162	1.10	1.15	0.90	0.06	0.800	0.778

Association analysis of haplotype in each candidate loci. All haplotypes with a frequency >1% are displayed. Numbers in the heading are as follows: 1 represents D9S0128i, 2 represents D9S0926i, 3 represents D9S267. p-value based on chi-square distribution. Permutation p-values based on 10,000 permutations.

Table 4. HLA-DR4 phenotype frequency in Japanese VKH patients and healthy controls

DR serotype	DRB1 genotype	VKH patients (n=87)	(%)	Healthy controls (n=122)	(%)	R.R.	P	Pc
DR4		71	(81.6)	35	(28.7)	11.03	4.60E-14	1.10E-12
	DRB1*0401	0	(0.0)	4	(3.3)	0	0.09	2.20
	DRB1*0403	3	(3.4)	8	(6.6)	0.51	0.32	8.03
	DRB1*0405	61	(70.1)	35	(28.7)	5.83	3.16E-09	7.90E-08
	DRB1*0406	3	(3.3)	4	(3.3)	0.69	0.68	16.98
	DRB1*0407	0	(0.0)	3	(2.5)	0	0.14	3.52
	DRB1*0408	0	(0.0)	2	(1.6)	0	0.23	5.75
	DRB1*0410	10	(11.5)	2	(1.6)	6.9	0.01	0.13
not DR4		28	(32.2)	115	(94.3)	0.01	1.80E-21	4.40E-20

Not DR4 means the number of the people who have the HLA-DR alleles other than DR4. R.R. represents relative risk. Pc represents corrected P-value by Bonferroni's correction.

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Genetic Polymorphisms in the Promoter of the Interferon Gamma Receptor 1 Gene Are Associated with Atopic Cataracts

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Purpose. Previous reports have shown genetic predisposition for atopic dermatitis (AD). Some of the severe complications of AD manifest in the eye, such as cataract, retinal detachment, and keratoconjunctivitis. This study was conducted to examine the genetic association between the atopy-related genes and patients with ocular complications (ocular AD).

Methods. Seventy-eighty patients with ocular AD and 282 healthy control subjects were enrolled in an investigation of the association between the atopy-related genes (*FCERB*, *IL13*, and *IFNGR1*) and ocular AD. Genetic association studies and functional analysis of single nucleotide polymorphisms (SNPs) were performed.

Results. The −56TT genotype in the *IFNGR1* promoter region was significantly associated with an increased risk of ocular AD under recessive models (χ^2 test, raw $P = 0.0004$, odds ratio 2.57). The −56TT genotype was more common in atopic cataracts. A reporter gene assay showed that, after stimulation with IFN-γ, the *IFNGR1* gene promoter construct that contained the −56T allele, a common allele in ocular AD patients, manifested higher transcriptional activity in lens epithelial cells (LECs) than did the construct with the −56C allele. Real-time PCR analysis demonstrated higher *IFNGR1* mRNA expression in the LECs in atopic than in senile cataracts. iNOS expression by *IFNGR1*-overexpressing LECs was enhanced on stimulation with IFN-γ and LPS.

Conclusions. The −56T allele in the IFNGR1 promoter results in higher IFNGR1 transcriptional activity and represents a genetic risk factor for atopic cataracts. (*Invest Ophthalmol Vis Sci.* 2007;48:583–589) DOI:10.1167/iovs.06-0991

Atopic dermatitis (AD) is a chronic inflammatory skin disease. In the acute stage, there is local infiltration by T-helper type 2 (Th2) cells; the subsequent infiltration by T-helper type 1 (Th1) cells produces chronic AD lesions.[1] Genetic epidemiologic studies on monozygotic twins[2] and genetic association studies[3,4] suggested a genetic susceptibility for AD. Because the severe complications of AD manifest in the eye as keratoconjunctivitis,[5] retinal detachment,[6] cataract,[7] and keratoconus, it is important to identify the genetic risk factors for ocular AD. Our group previously reported several atopy-related genes including high-affinity IgE receptor beta (FCERB),[8] interleukin 13 (IL-13),[9] and interferon gamma receptor (IFNGR),[10] and elucidated their functional roles. In the present study, we genotyped these candidate genes and compared the results in patients with AD with and without ocular AD and normal control subjects. As we found a strong association between the −56C/T single nucleotide polymorphism (SNP) in the promoter region of IFNGR1 and ocular AD, we further investigated the role of this SNP. IFNGR comprises the two transmembrane subunits IFNGR1 and IFNGR2. IFNGR1 is encoded by a 30-kb gene (chromosome 6) consisting of 7 exons,[11] and its expression is essential for ligand binding and signaling through Jak1 and STAT1; IFNGR2 transduces IFN-γ (IFNG) signals through Jak2.[11,12] Reduced IFNGR1 expression results in diminished JAK1/JAK2/STAT1 signaling,[13] and the expression of IFNGR1 is downregulated by *Mycobacterium* infections[14] and the TLR2 ligand[15]—factors known to counteract atopic diseases.[15,16] There is growing evidence of a role for IFNG in the effector phase of chronic AD[1] and allergic conjunctivitis.[17] Furthermore, overexpression of the IFNG gene in mouse epidermis produces eczema-like phenotypes,[18] and its overexpression in the lens induces cataracts in transgenic mice.[19] We found that among patients with ocular AD, in those with atopic cataracts, there was a strong genetic association with the IFNGR1 −56C/T SNP. Because the SNPs that placed individuals at high risk for ocular AD manifested higher IFNGR1 promoter activity in lens epithelial cells (LECs), we investigated the IFNGR1 mRNA levels in LECs obtained at cataract surgery.

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TABLE 1. Clinical Characters of the Ocular AD Patients

	Ocular AD	Control
Total subjects	78	282
AD + cataract*	48	—
AD + retinal detachment (RD)†	15	—
AKC‡	35	—
Mean age	27.18 (6–48 y)	38.21 (19–68 y)
Male:Female ratio	1.2:1.0	1.5:1.0

* Cataract only, 30 cases; caratact + RD, 10 cases; cataract + AKC, 6 cases; catract + RD + AKC, 2 cases.
† RD only, 3 cases; cataract + RD, 10 cases; cataract + RD + AKC, 2 cases.
‡ AKC only, 27 cases; cataract + AKC, 6 cases; cataract + RD + AKC, 2 cases.

MATERIALS AND METHODS

Antibodies and Cell Lines

We purchased anti-human major histocompatibility complex (MHC) class II antibody from Dako Japan (Kyoto, Japan) and Alexa-488 goat anti-mouse IgG antibody from Invitrogen Japan (Tokyo, Japan). Human immortalized LECs (SRA01/04), obtained from RIKEN cell bank (Tsukuba, Japan),[20] were maintained with 10% fetal bovine serum (FBS) in minimum essential medium (MEM, Invitrogen).

Subjects

In all patients with AD, the disease was diagnosed according to the criteria of Hanifin and Rajka.[21] Peripheral blood was obtained from 78 patients with (Table 1) and 186 without ocular AD.[4] The patients were recruited at Juntendo University Hospital, Yamaguchi University Hospital, Takao Hospital, Kyoto Prefectural University Hospital, Japan Red Cross Society Nagoya 2nd Hospital, Hokkaido University Hospital, and Yokohama City University Hospital. Atopic keratoconjunctivitis (AKC) was diagnosed according to the criteria of the Japanese Ophthalmological Society, and atopic cataracts were detected by slit lamp examination. The control subjects were 282 randomly selected, population-based individuals 19 to 68 years of age (mean, 38.21) with no atopy-related diseases. All study subjects were ethnic Japanese. According to the rules of the process committee at SNP Research Center of RIKEN, written informed consent was obtained from all participants; parental consent was obtained for individuals younger than 16 years. The study was conducted in accordance with the tenets of the Declaration of Helsinki.

Screening for Genetic Polymorphisms

Genetic polymorphisms screening was carried out essentially as previously described.[3,4] The IFNGR1 genomic region targeted for SNP discovery included a 2.5-kb continuous region 5′ to exon 1 (promoter region) and 11 exons, each with a minimum of 200 bases of flanking intronic sequences. We designed primer sets on the IFNGR1 genomic sequence (GenBank: AL050337; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). Each polymerase chain reaction (PCR) was performed with 5 ng of genomic DNA from 24 individuals (12 patients with AD and 12 control subjects). Sequence reactions were performed (Big Dye Terminator ver. 3.1 using a 3700 DNA analyzer; Applied Biosystems [ABI], Foster City, CA).

Genotyping

Initial screening genotyping of SNPs in the *FCER1B*, *IL13*, and *IFNGR1* gene regions has been described.[9,10,22] We further genotyped the *IFNGR1* gene by allele frequency (minor allele frequency [MAF] >10%) and based on intragenic linkage disequilibrium (LD) information. We genotyped the SNPs either with the invader assay,[23] by PCR-RFLP, direct sequencing, or with a genotyping assay (Taqman; ABI). An invader assay was performed with multiplex PCR products as the template. Genotyping was performed on a sequence-detection system (Prism 7700; ABI), according to the manufacturer's protocol.

Statistical Analysis

Statistical analysis was carried out essentially as previously described.[4] Allele frequencies in patients with AD and the control subjects were compared by the contingency χ^2 test. $P < 0.01$ (also in the case of multiple comparisons after Bonferroni adjustment) was considered to be statistically significant. Odds ratio (OR) and 95% confidence interval (CI) were also calculated.

Reporter Gene Assay. Reporter gene assay was carried out essentially as previously described.[4] A pair of 753-base IFNGR1 promoter sequences was subcloned continuous to exon 1 into pGL3 basic vector (Promega Corp., Madison, WI). Two clones were made; the first was −611G, −56C, and the second was −611G, −56T. After all subcloned plasmids were verified by direct sequencing, pGL3-IFNGR1 promoter plasmids and pRL-TK (as an internal control for transfection efficiency) were transfected into immortalized human LECs (Lipofectamine 2000; Invitrogen). The medium was changed 24 hours later, and the LECs were stimulated with lipopolysaccharide (LPS, 1 μg/mL, L4391; Sigma-Aldrich) or recombinant human IFN-γ (1000 U/mL, R&D Systems). Luciferase activity was measured with a dual luciferase reporter assay kit (Promega) at 36 hours after transfection.

TABLE 2. Genotyping of Candidate Genes for Ocular AD

6886G/A (E237G)			329G/A (R110Q)			−56C/T		
FCERBI	Normal	Ocular AD	*IL13*	Normal	Ocular AD	*IFNGR1*	Normal	Ocular AD
GG	7	2	GG	130	28	CC	69	14
GA	72	24	GA	120	37	CT	153	32
AA	200	51	AA	28	12	TT	60	32
NS*			NS			$P = 0.0004$†		

* NS, no significant associations.
† P values for comparisons of genotype −56TT versus −56CT + −56CC between cases and controls.

TABLE 3. List of *IFNGR1* SNPs Identified in a Japanese Population

SNP			Position	Amino Acid	MAF (%)
1	5′-Promoter	A/T	−766	—	2
2	5′-Promoter	C/T	−731	—	2
3	5′-Promoter	A/G	−611	—	12
4	5′-Promoter	C/T	−255	—	2
5	5′-Promoter	C/T	−56	—	46
6	Exon 1	G/A	40	Val/Met	2
7	Exon 1	G/A	48	Arg/Arg	2
8	Intron 1	C/T	95	—	46
9	Intron 1	A/G	130	—	48
10	Exon 2	C/T	12300	Ile/Ile	4
10	Intron 6	G/A	18693	—	2
12	Intron 6	C/T	20488	—	2
13	Intron 6	A/G	20685	—	35
14	Exon 7	T/G	20877	Ser/Ser	2
15	Exon 7	T/C	21227	Pro/Leu	2
16	Exon 7	A/G	21499	3′-UTR	2
17	Exon 7	A/G	21503	3′-UTR	2
18	Exon 7	G/A	21514	3′-UTR	2
19	Exon 7	A/C	21663	3′-UTR	4

MAF, minor allele frequency.

IFNGR1 Overexpression in LECs

Full length human *IFNGR1* cDNA was generated by PCR and then subcloned into pcDNA-V5-His vectors (Invitrogen), and the sequence was verified by direct sequences. LECs in six-well culture plates were transfected with 500 ng of the plasmid/well, using 1 μL of transfection reagent (Lipofectamine 2000; Invitrogen) according to the manufacturer's protocol. Twenty-four hours later, the culture medium was changed; the cells were stimulated with 1 μg/mL LPS and 1000 U/mL human IFN-γ. Twelve hours after stimulation, they were washed extensively with phosphate-buffered saline (PBS). Total RNA was extracted with an RNA isolation kit (NucleoSpin II; Macherey-Nagel, Duren, Germany), and cDNAs were prepared using random primers and the reverse transcriptase (Revertra Acc; both from Toyobo, Osaka, Japan) according to the manufacturer's protocol.

Reverse Transcription and Real-Time PCR Analysis

Reverse-transcription (RT) and real-time PCR analysis was carried out essentially as previously described.[24] Anterior capsules, obtained at cataract surgery with written informed consent, were immediately stored in stabilizer (RNAlater reagent; Ambion, Austin, TX) to protect the RNA. The procedure was approved by the ethics committees of Kyoto Prefectural University of Medicine. Total RNA was isolated with the (Micro RNA extraction kit; Qiagen Japan, Tokyo) from the anterior capsules or LECs, and then cDNA was prepared as described earlier. We used real-time PCR probes and primers specific for human IFNGR1, inducible nitric oxide synthase (iNOS), and GAPDH (Assay-on-Demand gene expression products; ABI). Real-time PCR analysis was performed on a sequence-detection system (Prism 7300; ABI). The relative expression of IFNGR1 in LECs was quantified by the standard curve method using GAPDH expression in the same cDNA as the control.

Immunohistochemistry

Lens capsules obtained at cataract surgery were frozen in OCT compound, cryostat sections were cut, mounted on slides, and fixed in 4% paraformaldehyde in PBS. Nonspecific staining was blocked (30 minutes) with blocking buffer (10% normal goat serum, and 1% bovine serum albumin [BSA] in PBS). Anti-MHC class II monoclonal antibody (1:200 dilution) was then applied and incubated overnight at 4°C. After they were washed with PBS, the slides were incubated for 30 minutes with Alexa 488-conjugated anti-mouse IgG. The slides were inspected under a confocal microscope (Leica, Tokyo, Japan).

RESULTS

Genotyping of the Candidate Genes

First, we screened for SNPs in the *FCER1B*, *IL13*, and *IFNGR1* gene regions. Although we observed no associations between the SNPs in the *FCER1B* and *IL13* regions (Table 2), there was a statistically significant association between the −56C/T SNP in the *IFNGR1* region and ocular AD ($P = 0.0004$).

SNP Discovery and Case–Control Association Study in the *IFNGR1* Region

Our genotyping of the candidate genes prompted the additional screening for other SNPs in the *IFNGR1* region. As shown in Table 3, we detected 19 SNPs. Their position is numbered relative to their position in the published *IFNGR1* gene sequence (GenBank: AL050337). Position 1 is the adenine

TABLE 4. Pair-Wise LD Calculated for Common SNPs and Tag SNP Typings (MAF > 10%)

SNPs	r^2
3–5	0.1074
3–8	0.1074
3–9	0.0988
3–13	0.0499
5–8	1
5–9	0.9197
5–13	0.464
8–9	0.9197
8–13	0.464
9–13	0.5045

−611G/A (SNP 3)			206856A/G (SNP 13)		
IFNGR1	Normal	Ocular AD	*IFNGR1*	Normal	Ocular AD
GG	257	59	AA	139	49
GA	23	13	AG	123	24
AA	2	0	GG	20	5
$P = 0.02$*			NS		

* Comparisons of genotype −611GG versus −611GA + −611AA between cases and controls.

Table 5. Genotype Frequencies and Case Control Analysis of *IFNGR1* −56 C/T SNPs in Ocular AD

				Genotype TT versus CT + TT between Cases and Controls		
	−56CC	−56CT	−56TT	OR (95% CI)	χ^2	*P*
Healthy controls (n = 282)	69 (25%)	153 (54%)	60 (21%)	—	—	—
Atopic dermatitis (n = 192)	48 (25%)	102 (53%)	42 (22%)	1.04 (0.60–1.62)	0.024	0.88
Ocular AD (n = 78)	14 (21%)	32 (37%)	32 (42%)	2.57 (1.51–4.39)	12.53	0.0004
Atopic cataract (n = 48)	5 (10%)	19 (40%)	24 (50%)	3.70 (1.96–6.97)	17.83	0.000024
AKC (n = 35)	8 (21%)	14 (36%)	13 (43%)	2.19 (1.04–4.59)	4.4	0.035

of the first methionine. Among the 19 SNPs, there were five common SNPs with MAF greater than 10%. We selected SNP 3 (−611G/A) and SNP 5 (−56C/T) in the promoter region and SNP 13 (20685 A/G) in intron 6, as tag SNPs because of intragenic pair-wise LD expressed as r^2 (Table 4). SNP 206856A/C (No. 13) did not show an association with ocular AD, SNP −611G/A (No. 3) exhibited marginal association not stronger than SNP -56C/T (No. 5). Therefore, we focused on SNP −56C/T. There was a significant association between the −56C/T SNP and ocular AD (raw P = 0.0004, OR = 2.57, 95% CI = 1.51–4.39), the association became stronger for the atopic cataracts (raw P = 0.000024, OR = 3.70, 95% CI = 1.96 to 6.97; Table 5). All the genotype frequencies of the SNPs were concordant with Hardy-Weinberg equilibrium.

Haplotype Analysis of *IFNGR1* (−611/−56) SNPs

We also tested the distribution of two-locus haplotypes in AD and control samples (Table 6). Among the two-locus haplotypes of SNPs in the promoter region (−611G/A and −56T/C), the −611G/−56C haplotype showed decreased risk for ocular AD (G-C versus others; P = 0.00,003, OR = 2.26). The IFNGR1 haplotype −611G/−56C showed decreased risk for atopic cataracts (G-C versus others; P = 0.000003, OR = 3.16), to a degree that was greater than that of single SNP genotype association (−56TT versus others, P = 0.00002).

Reporter Gene Analysis

Using pGL3-basic vector, we prepared a construct for −611G/−56C, the major haplotype, and for −611G/−56T, the common haplotype among patients with AD. The primers used for subcloning were 5′-aggtgagatcattagacatt-3′ (forward) and 5′-gctgctaccgacggtcgctggct-3′ (reverse). All assays were performed in triplicate. In Figure 1, a representative result of three independent experiments is shown as the mean ± SD. In the absence of stimulation, luciferase activity was not significantly different between −56C/T SNPs containing constructs in the LECs. The genotype −56T containing construct induced stronger IFNGR1 promoter activity than the −56C construct when stimulated for 12 hours with IFNG or IFNG+LPS (P = 0.01 and 0.02, respectively, by Student's t-test).

IFNGR1 Overexpression Experiment in LECs

LECs transfected with an IFNGR1 expression plasmid showed approximately a fivefold increase in IFNGR1 expression (Fig. 2, left). iNOS mRNA expression was only observed in cells stimulated with IFNG+LPS. iNOS gene expression was upregulated approximately threefold in IFNGR1-overexpressing LECs compared with mock-transfected LECs (Fig. 2, right).

Real-Time PCR Analysis

cDNAs were synthesized from total RNA isolated from the anterior lens capsules of patients undergoing surgery for atopic (n = 5) and senile cataracts (n = 5). The expression of IFNGR1 mRNA was significantly higher in the atopic than the senile cataracts (Fig. 3, P = 0.00005 by Mann-Whitney's U-test).

Anti-MHC Class II Immunostaining of Lens Epithelium

Anti-MHC class II immunohistochemistry was performed on senile and atopic cataract lens capsules. LECs in atopic but not in senile cataracts were positive for MHC class II immunostaining (Fig. 4).

Discussion

Although Nishimura et al.[25] reported genetic linkage in allergic conjunctivitis, ours is the first genetic association study of ocular AD, which tends to be more severe and longer lasting than allergic conjunctivitis without AD. Initial genotyping screening showed that atopy- or AD-related genes did not necessarily show an association with ocular AD. Among our candidate SNPs, we found a strong genetic association between the IFNGR1 −56C/T SNP and ocular AD. We previously reported an association between IFNGR1 SNPs and the serum IgE concentration in patients with atopic asthma.[10] Herein, we

Table 6. Structure and Frequencies of Two-Locus Haplotype in *IFNGR1*

	Frequency				
Haplotype	Control	Ocular AD	Atopic Cataract	*P**	*P*†
−611G/−56C	0.52	0.32	0.25	0.000030	0.0000030
−611G/−56T	0.44	0.56	0.64	0.014	0.00032
−611A/−56T	0.048	0.058	0.054	0.850	1.590
−611A/−56C	0	0.064	0.05	NA	NA

NA, not applicable.
* Ocular AD versus control.
† Atopic cataract versus control.

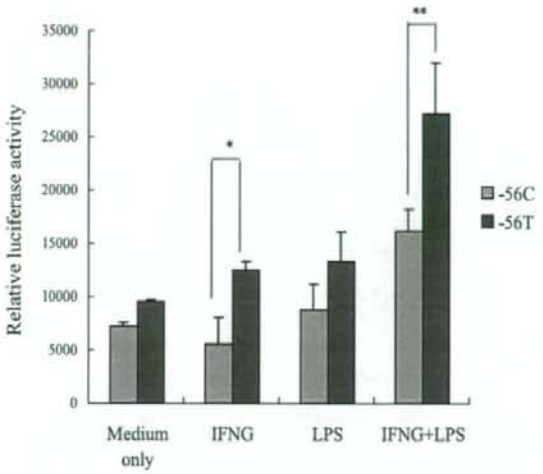

FIGURE 1. Reporter gene assay of IFNGR1 promoter region. IFNGR1 promoter-pGL3 vector consisting of −611G/−56C or -611G/−56T SNPs were transfected into human lens epithelial cells. Twenty-four hours after transfection, the cells were stimulated with human recombinant IFNG and/or LPS. Relative luciferase activity was measured 12 hours after stimulation. $^{*}P = 0.01$, $^{**}P = 0.02$; Student's *t*-test.

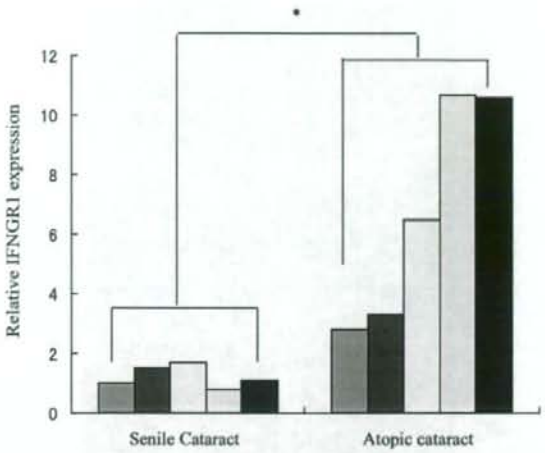

FIGURE 3. Real-time PCR analysis of IFNGR1 mRNA expression in human lens anterior capsules. Total RNA was extracted from the anterior lens capsule of atopic/senile cataracts. cDNA was synthesized from the total RNA. Real-time PCR analysis was performed with expression assay probes. The amount of relative expression was normalized to that of GAPDH ($^{*}P = 0.00003$; Mann-Whitney test.)

document that the association between the SNPs and ocular AD was stronger than in the previous study. As surprisingly, there was no association between SNP and AD without ocular complications (Table 4), we postulated that in addition to its effect on serum IgE, the IFNGR1 gene plays some role as an organ-specific susceptibility gene for ocular AD. In African populations, there is a genetic associations between the IFNGR1 −56C/T SNP and *Helicobacter pylori* infection,[26] and cerebral malaria[27]; the −56T genotype was associated with higher serum *H. pylori* antibody concentrations, and −56C/T heterozygosity was protective against cerebral malaria infection. These results suggest that the −56C/T SNP plays some functional role(s) not only in the Japanese, but also in the African population.

We examined the association between atopic cataracts and the IFNGR1 SNP, because cataract formation was observed in IFNG transgenic mice,[19] and IFNG treatment of LECs induced apoptosis,[28] one of the pathologic features of atopic cataracts.[29] Using a reporter gene assay, we first examined the effect of the −56C/T SNP in human immortalized LECs.[20] We made 753-bp IFNGR1 promoter region constructs to analyze the −611G/−56C and −611G/−56T haplotypes, because these two major haplotypes make up more than 90% of all haplotypes (Table 5). After LEC stimulation with IFNG and LPS, we found significantly higher transcriptional activity in the presence of the −56T allele, the risk allele for atopic cataracts, than the −56C allele (Fig. 1). This result is consistent with the findings of Juliger et al.,[30] whose reporter gene assay showed a higher level of IFNGR1 transcriptional activity with the −56T allele, and is well matched to our haplotype association study which showed that −611G/−56C is a protective and −611G/−56T is a risk haplotype for ocular AD induction (Table 5). In our experiments, we used LPS/IFNG stimulation because LPS/

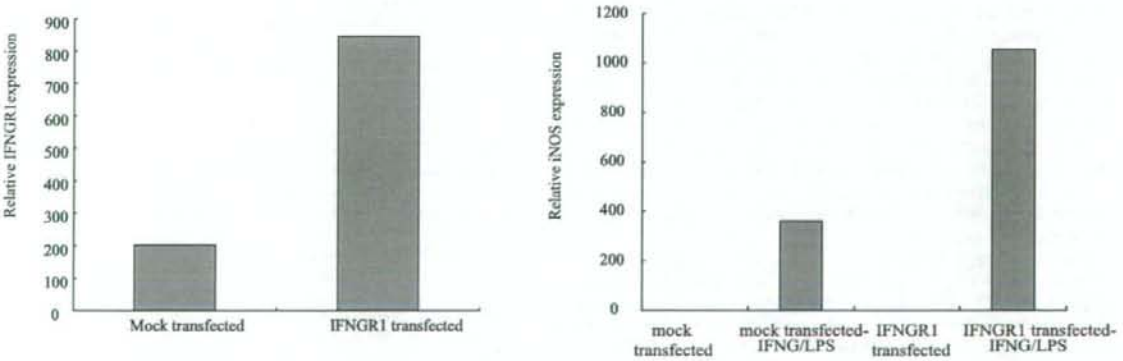

FIGURE 2. The effect of IFNGR1 overexpression for iNOS expression in LECs. *Left*: real-time PCR analysis of IFNGR1 expression of IFNGR1-overexpressing LECs. An approximately five-fold overexpression of IFNGR1 mRNA was detected. *Right*: real-time PCR analysis of iNOS mRNA expression. IFNGR1-transfected LECs showed higher iNOS expression than that of mock-transfected LECs when stimulated with IFN-γ+LPS. No iNOS expression was observed without IFN-γ+LPS stimulation.

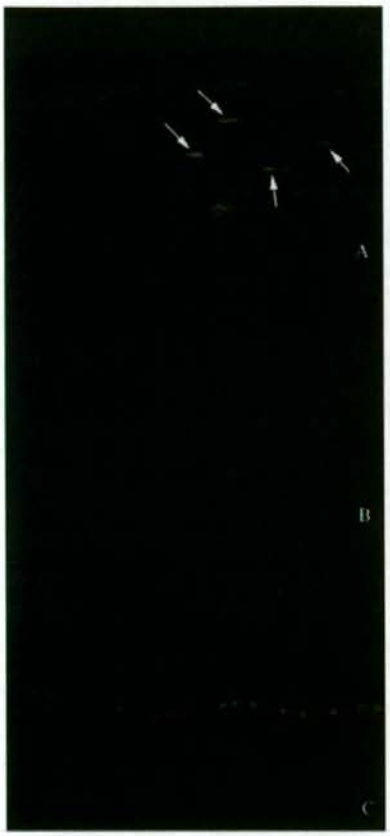

FIGURE 4. Immunohistochemical staining of human anterior lens capsules with MHC class II antibody. Cryosections of anterior lens capsules were immunostained with anti-MHC class II antibody. (**A**) In an anterior lens capsule of an atopic cataract, positive immunostaining was observed in some of the lens epithelial cells (*arrows*). (**B**, **C**) Anterior lens capsule of a senile cataract; no MHC class II staining was observed. The existence of lens epithelial cells was verified with nuclear DAPI staining. Original magnification, ×400.

IFNG treatment of LECs induced iNOS expression,[31] a known cataract-inducing factor.[32] As a downstream signal of IFNG, iNOS has been intensively studied in macrophages,[33] and in airway[34] and lens epithelium.[31] To clarify the role of IFNGR1 in the induction of iNOS in LECs, we transfected LECs with IFNGR1 and stimulated them with IFNG+LPS. Cells that overexpressed IFNGR1 generated higher amounts of iNOS mRNA (Fig. 2), a finding consistent with that reported by Li et al.[31] As the NOS inhibitor could prevent the development of cataracts in selenite-treated rats,[32] iNOS expression may play a role in the genesis of cataracts.

Furthermore, LECs from atopic cataracts manifested higher IFNGR1 mRNA expression than did LECs from senile cataracts (Fig. 3), and LECs from atopic cataracts were positive for MHC class II staining (Fig. 4). Our results are consistent with those of Egwuagu et al.,[35] who showed that ectopic MHC class II expression due to IFNG overexpression resulted in ocular disease including cataract formation. Based on these considerations, we postulate that IFNG-IFNGR signals are active in the development of atopic cataracts and that higher IFNGR1 expression may be a predisposing factor for atopic cataracts. We are in the process of measuring IFNG concentration in aqueous humor samples from patients with atopic and senile cataracts.

Although topical steroids are frequently used to treat ocular atopic conditions, they are causative reagents for cataracts.[36] Therefore, treatment of ocular AD with inhibitors of T-cell activation (e.g., cyclosporine and tacrolimus) or with NOS inhibitors may be more successful in preventing IFNG-mediated atopic cataract formation. Our findings identified a genetic risk factor for ocular complications in patients with AD. We are planning additional genotyping and functional studies on other candidate genes and are investigating antiapoptotic molecules Bcl-2[29] and major basic protein.[37] The roles of glutathione should also be investigated because of a possible relationship with subcapsular cataracts.[38]

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LABORATORY INVESTIGATION

Exclusion of Transforming Growth Factor-β1 as a Candidate Gene for Myopia in the Japanese

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Abstract

Purpose: To determine whether single-nucleotide polymorphisms (SNPs) within the transforming growth factor-β1 (*TGF-βI*) gene are associated with high myopia in Japanese. Previous studies have indicated that the gene expression products, regulators of the *TGF-βI* gene, are involved in high myopia.

Methods: Genomic DNA samples were obtained from 330 Japanese patients with high myopia and 330 Japanese controls without high myopia who were chosen at random. SNPs were genotyped by the TaqMan system, using primer extension and polymerase chain reaction amplification.

Results: Ten SNPs were identified in the high-myopia patients and controls, with four of the ten SNPs having nonsynonymous changes. However, no statistical differences in the SNPs were detected between the high-myopia cases and the controls.

Conclusions: Sequence variants of the *TGF-βI* gene were not associated significantly with high myopia, and further studies are needed to identify which genes are responsible for high myopia. **Jpn J Ophthalmol** 2007;51:96–99 © Japanese Ophthalmological Society 2007

Key Words: Japanese population, myopia, single-nucleotide polymorphism, transforming growth factor-β1

Introduction

Myopia affects approximately 25% of the adult population of the United States, and is a significant public health problem in Asian populations such as the Chinese and Indian populations, as it is associated with an increased risk of visual loss.[1–6] Japan has one of the highest rates of myopia, with 59.6% of Japanese high school students and approximately 3% of the general population affected and at risk of developing irreversible, sight-threatening pathology of the retina and choroid.[1]

Excessive enlargement of the eye, predominantly in the axial dimension, results in the development of high myopia, both in human and in animal models.[7,8] In 1996, Honda et al.[9] used reverse transcriptase-polymerase chain reaction, immunoblotting, and immunostaining and showed that the expression of the *TGF-β* gene decreased in a chicken experimental myopic model.[9] Subsequently, *TGF-βI* expression was found to be reduced in an isoform- and time-specific manner in the sclera[10] and retina/choroid[11] of chickens during myopia development, whereas the TGF-β2 content increased in both the retina/choroid and sclera of the chickens.[10,11] In addition, cultured human retinal pigment epithelial cells expressed *TGF-βI* and *-β2* genes, but gene expression of *TGF-β3* was not confirmed.[10] Taken together,

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the results of these and other studies[12–15] suggest that *TGF-βI* plays an important role in the development of myopia.

Recently, human population studies on gene variants of the TGF-β-induced factor (*TGIF*) gene, the product of which can be induced by the activation of *TGF-β*, have suggested that *TGIF* is a possible candidate gene for the development of high myopia. For example, single-nucleotide polymorphisms (SNPs) of the *TGIF* gene were found to be associated with high myopia in some Chinese.[16] Recently, family linkage studies of autosomal dominant high myopia have associated at least ten candidate myopia-susceptible genomic loci (MYP1–MYP10) with the occurrence of myopia. The *TGF-β* gene is located on chromosome 19 (19q13.1), and the *TGIF* gene is located on chromosome 18 (18p11.31), where an MYP2-susceptible locus was identified. Since the *TGF-β* gene is regulated by *TGIF*, the association of *TGF-β* gene variants with high myopia also warrants investigation even though they have not been studied previously and even though they do not belong to genes associated with autosomal dominant high myopia. As of September 2005, when our gene analysis was completed, this is the first report to have investigated SNPs in the *TGF-βI* gene. Studies on other ethnic groups have not been performed.

The purpose of the present study was to determine whether SNPs within the *TGF-βI* gene are associated with high myopia in Japanese.

Methods

We recruited 660 Japanese subjects, 330 with high myopia of −9.25 D or greater and 330 unrelated control subjects at the Yokohama City University or the Okada eye clinic. The mean (± SD) age of the patients was 37.82 ±1 1.97, and the male:female ratio was 0.66 (131/199). The mean refraction was −11.54 ± 2.17, and the mean axial length was 27.78 ± 1.30 mm. The control subjects had no myopia or mild myopia with refractive errors of −2.00 D or smaller. The mean age and the male:female ratio of the controls were unknown. All patients and controls were Japanese from similar social backgrounds and the same urban area. This study was performed after careful examination by the Ethics Committee of the Yokohama City University School of Medicine. All subjects involved in this study provided informed consent orally or in writing. This study is considered to have been performed based on Institutional Review Board (IRB) approval as the Ethics Committee in the Yokohama City University School of Medicine belongs to the IRB, Yokohama City University School of Medicine.

For study and sequencing, we selected ten of the SNPs known to be in the *TGF-βI* gene, on the basis of criteria that included population-frequency validation, multiple gene submitters, high-profile submitters, and the absence of repeat masking in the sequence, in order to determine their association with high myopia. In the Japanese population, there are ten SNP locations available in Internet databases. Six SNP locations were selected from the HapMap database (http://www.hapmap.org/index.html), and four SNP locations were selected from the Japanese Single Nucleotide Polymorphisms (JSNP) database (http://snp.ims.u-tokyo.ac.jp/index.html).

Genotyping of candidate SNPs was performed with the TaqMan system (Applied Biosystems, Foster City, CA, USA). SNP-specific polymerase chain reaction (PCR) primers and fluorogenic probes were modified using TaqMan Search (Applied Biosystems). This technique has been utilized extensively in genotyping other candidate genes with multiple SNPs. The fluorogenic probes were labeled with a reporter dye (either FAM or VIC) and were specific for one of the two possible bases in the promoter region. An MGB quencher probe was utilized on the 3′ end by a linker arm. A TaqMan Universal PCR Master Mix (Applied Biosystems) was used to prepare the PCR. The 2× mix was optimized for TaqMan reactions and contained AmpliTaq-Gold DNA polymerase, dNTPs with UTP, and a passive reference. Primers, probes, and genomic DNA were added to final concentrations of 300 nM, 100 nM, and 0.5–2.5 ng/μl, respectively. Controls (no DNA template) were run to ensure that there was no amplification of contaminating DNA. The amplification reactions were carried out in an ABI Prism 7700 sequence detection system (Applied Biosystems) with two initial hold steps, 95°C for 10 min and 40 cycles of a two-step PCR (92°C for 15 s, 60°C for 1 min). The fluorescence intensity of each sample was measured at each temperature change to monitor amplifica-

Table 1. Ten SNPs of *TGF-βI* in patients with high myopia and controls

SNPs	Myopia	Group	Control	Group	*P* value
#rs1800820	G330 (100%)	T0 (0%)	G330 (100%)	T0 (0%)	NS
#rs1054797	C330 (100%)	T0 (0%)	C330 (100%)	T0 (0%)	NS
#rs1800468	C330 (100%)	T0 (0%)	C330 (100%)	T0 (0%)	NS
#rs1800469	T93 (49%)	C97 (51%)	T87 (46%)	C101 (54%)	0.609
#rs2241715	T93 (49%)	G97 (51%)	T87 (46%)	G101 (54%)	0.603
#rs11466324	A0 (0%)	C330 (100%)	A0 (0%)	C330 (100%)	NS
#rs2241717	T93 (49%)	C97 (51%)	T93 (49%)	C95 (51%)	0.919
#rs11672143	G330 (100%)	T0 (0%)	G330 (100%)	T0 (0%)	NS
#rs11466334	A0 (0%)	G330 (100%)	A0 (0%)	G330 (100%)	NS
#rs2278422	C182 (96%)	G8 (4%)	C175 (94%)	G11 (6%)	0.45

SNP, single-nucleotide polymorphism; TGF, transforming growth factor; NS, not significant.

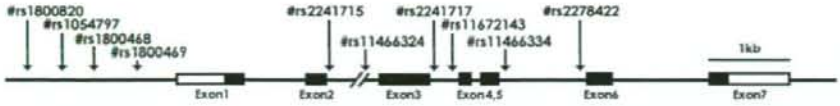

Figure 1. Map of the positions of ten single-nucleotide polymorphisms within the transforming growth factor-β1 gene.

tion of the *TGF-β1* promoter region. The target nucleotide was determined by the fluorescence ratio of the two SNP-specific fluorogenic probes. The fluorescence signal increased when the probe with the exact sequence match had bound to the single-stranded template DNA and was digested by the 5′-3′ exonuclease activity of AmpliTaq-Gold DNA polymerase (Applied Biosystems). Digestion of the probe released the fluorescent reporter dye (either FAM or VIC) from the quencher dye.

Results

Some of the genotyping data of the candidate SNPs performed by TaqMan for 95 of the 330 patients are shown in Fig. 1. First, we selected only six SNPs from within the *TGF-β1* gene using the HapMap database. The six SNPs selected were, in the promoter region, #rs1800820 (G → T), #rs1054797 (C → T), and #rs1800468 (C → T), and #rs11466324 (A → C) in intron 2, #rs11672143 (G → T) in intron 3, and #rs11466334 (A → G) in intron 5 (Fig. 1). However, none of these six SNPs was significantly different between the patients with high myopia and the controls in this study (Table 1).

Second, four SNPs previously detected within the *TGF-β1* gene were extracted from the JSNP database for analysis in this study. Only four of the nine possible SNP regions were selected because none of the other SNPs had nonsynonymous changes, or they were not present in the Japanese population. The four SNPs selected were #rs1800469 (T → C) in the promoter region, #rs2241715 (T → G) in intron 2, #rs2241717 (T → C) in intron 3, and #rs2278422 (C → G) in intron 5 (Fig. 1). However, none of these four SNPs was significantly different between the patients with high myopia and the controls (Table 1).

Discussion

Japan is one of the world's most myopia-prevalent countries, where severe myopia has long been a leading cause of adventitious blindness.[17] Myopia is a multifactorial disorder and is thought to become manifest because of the interaction between genetic and environmental factors,[18] as previously noted in experimental models.[19,20] The main purpose of this study was to determine whether the *TGF-β* gene is a disease-susceptible gene for high-grade myopia, that is, a mean refractive error of –9.25 D or greater, by screening for four SNPs in the high-grade myopia cases and controls. Statistical analysis of the allele frequencies and genotypes revealed that there was no significant difference between the myopia cases and the controls.

Only four of the possible ten SNPs within the *TGF-β* gene were analyzed in the present study for three reasons. First, nonsynonymous rather than synonymous SNPs can be more readily related to disease and located more efficiently by analyzing regions directly related to gene function than adjacent noncoded or nonregulated SNPs. Second, the JSNP/NCBI/EMBL SNP databases are global front-runners in cataloging SNPs, and in providing easy access to cDNA information. Third, some of the *TGF-β* SNPs reported in the SNP databases are not found in Japanese and therefore were not relevant to this study.

This is the first reported study on the association between high myopia and the *TGF-β* polymorphisms. Our findings suggest that *TGF-β* cannot be considered a disease-susceptible gene on the basis of SNP differences and variations.

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High-Resolution Mapping for Essential Hypertension Using Microsatellite Markers

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Abstract—During the past decade, considerable efforts and resources have been devoted to elucidating the multiple genetic and environmental determinants responsible for hypertension and its associated cardiovascular diseases. The success of positional cloning, fine mapping, and linkage analysis based on whole-genome screening, however, has been limited in identifying multiple genetic determinants affecting diseases, suggesting that new research strategies for genome-wide typing may be helpful. Disease association (case–control) studies using microsatellite markers, distributed every 150 kb across the human genome, may have some advantages over linkage, candidate, and single nucleotide polymorphism typing methods in terms of statistical power and linkage disequilibrium for finding genomic regions harboring candidate disease genes, although it is not proven. We have carried out genome-wide mapping using 18 977 microsatellite markers in a Japanese population composed of 385 hypertensive patients and 385 normotensive control subjects. Pooled sample analysis was conducted in a 3-stage genomic screen of 3 independent case–control populations, and 54 markers were extracted from the original 18 977 microsatellite markers. As a final step, each single positive marker was confirmed by individual typing, and only 19 markers passed this test. We identified 19 allelic loci that were significantly different between the cases of essential hypertension and the controls. (***Hypertension*. 2007;49:446-452.)**

Key Words: essential hypertension ■ genome-wide ■ association study ■ Japanese ■ new candidate regions

Hypertension is a leading risk factor for cerebrovascular disease, coronary heart disease, and renal failure.[1] It is the major cause of morbidity and mortality and also the third highest risk factor for lifetime burden worldwide.[2,3] Kearney et al[4] reported that there were 972 million hypertension patients in the world, accounting for 26.4% of the adult population in 2000, and predicted that this figure will increase to 1.56 billion patients (29.2%) by 2025. The present pandemic of cardiovascular diseases has been attributed largely to the high prevalence of hypertension, suggesting that more emphasis should be placed on the prevention, detection, and treatment of hypertension.

Elucidation of the genetic etiology of hypertension has been increasingly emphasized as important for a better understanding of the pathogenesis of this disease and for ultimately improving the prevention strategies, diagnostic tools, and therapy in the new millennium.[5] Hypertension is one of the risk factors for coronary heart disease, which is a common complex human genetic disease, and its genetic variance accounts for 30% to 70% of the trait variance.[6,7] The sibling recurrent risk ratio of hypertension is reportedly to be 2 to 3.[8] Each of the hypertension-causing gene recurrent risk ratios is less than the aggregate sibling recurrent risk ratio. There are now many reports describing the results of genome-wide screens for genes controlling blood pressure (BP). The National Heart, Lung, and Blood Institute Genelink project website (https://genelink.nhlbi.nih.gov) lists the National Heart, Lung, and Blood Institute–supported genome scans for BP. The majority of these reports have described numerous chromosomal regions with suggestive evidence of linkage.[9] However, the application of linkage analysis to hypertension, with the exception of obvious Mendelian inheritance, has achieved only limited suc-

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cess thus far.[10,11] Since 2000, ≥6 large genome scans have been reported,[12] namely, an admixture mapping study,[13] a Medical Research Council Program-funded British Genetics of Hypertension Study,[14] the US National Institutes of Health-funded Family Blood Pressure Program studies,[15–18] the Victorian Family Heart Study,[19] the San Antonio Heart Study,[20] and the Quebec Family Study.[21] Except for the admixture mapping study, all of these studies were based on linkage analysis.

In many cases, complex diseases are complicated by genetic heterogeneity and small effects of each gene. In 1996, Risch and Merikangas reported[22] that numerous genetic effects in complex diseases were too weak to be identified by linkage analysis and could be better detected by genomic association studies. Thus, the new challenges to identify disease-predisposing variants in human genome research have resulted in approaches, such as the Hapmap project,[23] high-density single nucleotide polymorphism (SNP) analysis,[24] and microsatellite (MS) association analysis.[25] Disease association studies using MS markers distributed across the human genome every 100 to 150 kb have distinct advantages over linkage analysis, the candidate approach, and SNP typing in terms of linkage disequilibrium (LD).[25] The MS markers are highly polymorphic, showing a high degree of heterozygosity (on average, ≈70%) and LD lengths in the 100- to 200-kb range.[26–31] As compared with MS markers, SNPs have a low degree of genetic polymorphism (biallelic) and have a shorter, by ≈30 kb, LD range, probably because of their older age. Varilo et al[32] reported that highly polymorphic MS markers can provide much greater power for detection of intermarker LD than can either single SNPs or SNP haplotypes on chromosomes 1q and 5q. Therefore, it is possible to carry out substantial whole genome association analysis using a smaller number of MS markers than SNPs (eg, tens of thousands of MS markers versus hundreds of thousands or millions of SNPs). Recently, the usefulness of the haplotype approach and haplotype-tagging SNPs from the HapMap project has been questioned.[33]

Methods

Subjects for MS Typing

A total of 425 (stage 1: 95; stage 2: 131; stage 3: 199) patients with essential hypertension and 467 (stage 1: 103; stage 2: 132; stage 3: 232) normotensive healthy individuals participated in this study. The number of subjects for pooled DNA typing was 95 versus 95 for stage 1, 120 versus 120 for stage 2, and 170 versus 170 for stage 3. After pooled DNA typing, individual typing for the same samples was performed. The difference in number in each stage was derived from the time of sample collection. It was aimed to collect 100 volunteers for each stage each in case and control subjects, but many more subjects were collected beyond our expectations. So, we made the most of all of the subjects to increase the statistical power. The subjects were of Japanese origin from Hokkaido, Tokyo, Kanagawa, Shiga, Osaka, Kyoto, and Ehime. The subjects for the stage 1 and stage 2 screens were recruited from the Millennium Genome Project, and the subjects for the stage 3 screen were recruited from Yokohama City University School of Medicine. The diagnosis of essential hypertension was made according to the guidelines of the Japanese Society of Hypertension (declared in 2000), which include a sitting systolic BP of >140 mm Hg and/or diastolic BP of >90 mm Hg on ≥2 occasions after the first medical examination. Furthermore, subjects in this study were selected as follows, as shown in Table 1. Our criteria were classification as moderate or severe hypertension.

TABLE 1. Characteristics of Subjects

Character	Hypertensive Patients	Normotensive Control Subjects
Gender	Male	Male
Age of onset	30 to 59	≥50
Family history	Within parents and siblings	None
Body mass index, kg/m^2	≤25	≤25
BP, mm Hg	Systolic BP≥160 and/or diastolic BP≥100	Systolic BP≤120 and diastolic BP≤80 and no antihypertensive treatment

We obtained informed consent from all of the patients and healthy individuals whose DNA samples were used in the analyses. Our experimental procedures were approved by the relevant ethical committee in each participating university and center. All of the personal identities associated with medical information and blood samples were carefully eliminated and replaced with anonymous identities in each recruiting institution.

Pooled DNA and Genotyping

Ninety-five subjects in stage 1, 120 in stage 2, and 170 in stage 3 were selected from each group (case and control subjects) based on the DNA quality and quantity for DNA pooling analysis. The DNA pooling method was adopted to bring down the cost and the technical burden linked to genotyping thousands of MSs without losing any significant amount of data.

The DNA pooling method for MS typing was carried out by making slight modifications[34] of the protocol of Collins et al.[35] The key factor in this methodology is the absolute equality of individual DNA quantities, so we used a highly accurate quantitative procedure to construct a pooled DNA template for PCR amplification.[35] This pool was composed of strictly measured DNA concentrations, extracted from 95 stage 1, 120 stage 2, and 170 stage 3 Japanese individuals. We checked each DNA concentration ≥3 times and equalized each DNA concentration by dilution. Multiple peak patterns in the pooled DNA showed the distribution of allele frequencies in the subjects.[35] The DNA pooling method enabled us to obtain the allele frequencies of MSs in pooled Japanese individuals by measuring the heights of multiple peaks and to apply this approach to an association study. The quality of the pooled DNA was confirmed by comparing the allelic distributions between individual and pooled typing results using 23 MS markers, unless there was the absence of any significant difference ($P \leq 0.05$) in allele frequencies between pooled DNA typing and individual. This comparison of allele frequencies for the same allele was performed by Fisher's exact test.

DNA was extracted using a QIAamp DNA blood kit (Qiagen) under standardized conditions to prevent variation in DNA quality. This was followed by 0.8% agarose gel electrophoresis to check for DNA degradation and RNA contamination. After measurement of the optical density to check for protein contamination, the DNA concentration was determined through 3 successive measurements using the PicoGreen fluorescence assay (Molecular Probes). Standardized pipetting and aliquoting of the DNA samples were robotically performed using a Biomek 2000 and Multimek 96 (Beckman). The pooled DNA template for typing with 2×18 977 MS markers (first set: case subjects; second set: control subjects) was prepared immediately after DNA quantification. After the initial tests, the 18 977 PCR reaction mixtures containing all of the components except primers were prepared and then aliquoted into 96-well reaction plates and stored until use. The MS pooled typing and individual genotyping procedures after the PCR reaction were carried out according to standard protocols using ABI3700 and 3730 DNA analyzers (Applied Biosystems). The standardized preparations allowed the reproducibility and accuracy to be maintained for the pooled DNA typing throughout the experiment. Various kinds of

information, such as the peak positions and heights, were manually extracted by the PickPeak and MultiPeaks programs, developed by Applied Biosystems Japan, from the multipeak pattern in the chromatogram ABI fsa files.

In the first stage, 95 case and 95 control subjects were subjected to association analysis using all of the 18 977 markers. Among them, markers showing statistical significance of $P<0.05$ were subjected to the second stage with another 120 case subjects and 120 control subjects. The markers showing statistical significance of $P<0.05$ in the second screening were subjected to a third stage with another 170 case subjects and 170 control subjects. All of the positive markers that remained statistically significant ($P<0.05$) in the stage 3 screening were confirmed by individual genotyping using the same set of 385 case subjects and 385 control subjects as the final step.

Marker Information

MS sequences were computationally detected from all of the chromosomes except for the Y chromosome (in 4 versions of the human genome draft sequence: Golden Path Jun 2004 to the National Center for Biotechnology Information build 35). At present, our laboratory has built 27 037 markers as a full set.[25]

In this study, we used 18 977 markers with an average spacing of 145.9 kb (Tables I and II, available online at http://hyper.ahajournals.org.) from 19 654 markers in the first built set. The other 678 markers were excluded because of problems with the PCR reaction and marker quality.

The MS markers were investigated for repeat polymorphisms in 200 healthy Japanese by using the DNA pooling method. Our criteria for selection of MS markers for the hypertension association study were dinucleotide repeats with >10 repeats; tri-, tetra-, and pentanucleotide repeats with >5 repeats; and polymorphic MS markers with heterozygosity of >30% but not those with heterozygosity of >85% to eliminate any unstable and highly mutated MS markers. We chose PCR primers that contained no SNPs in the sequences to prevent differential amplification. Seven PCR primer pairs of 54 MS markers for individual typing were redesigned to improve the efficiency of PCR (Table III). Detailed information on the 27 039 MS markers is available on the Japan Biological Information Research Center homepage (http://www.jbirc.aist.go.jp/gdbs/).

Statistical Analysis

The measurements of the heights of multiple peaks in the pooled DNA were applied to association analysis. To calculate P values, we used 2 types of Fisher's exact test for 2×2 contingency tables for each individual allele and $2\times m$ contingency tables for each locus, where m refers to the number of marker alleles observed in a population. The Markov chain/Monte Carlo simulation method was used to execute Fisher's exact test for the $2\times m$ contingency table. The simple "allelic" but not "genotype" association was presented for the 2×2 contingency tables for MSs. These analyses were executed using the software package, AStat. The method of Pritchard and Rosenberg[36] was used for the detection of stratification in case and control populations using 23 MS markers. The Hardy–Weinberg test for allele frequency distributions at the MS loci was performed by P test for differentiation, as determined by GenePop 3.4. Other basic analyses were carried out using Microsoft Excel.

The authors had full access to the data and take responsibility for its integrity.

Results

Three-Stage Screening: Pooled DNA Typing

Before the 3-stage screenings, we verified spurious associations through the method of Pritchard and Rosenberg[36] using 23 randomly selected MS markers from each of chromosomes 1 to 22 and X, with an absence of any significant stratification in either the case or control populations (data not shown). This test is important to prevent spurious associations by population stratifications, especially for late-onset diseases, such as essential hypertension.

We initially identified 54 markers as potential hypertension susceptibility loci by 3-stage screening of 3 independent case–control populations (stage 1: 95; stage 2: 120; stage 3: 170 patients with essential hypertension and normotensive healthy individuals; Table 2). Three-stage screenings were intended to sequentially replicate the results in the 3 independent sample populations and eliminate pseudopositive markers resulting from type I errors.[37,38]

The number of markers decreased from 18 977 to 1160 markers in the first screening, then to 284 markers in the second screening, and finally to 54 markers in the third screening. The significance ($P<0.05$) of the association of positive markers was assessed by the Fisher's exact test, using either 2×2 or $2\times m$ (m=number of alleles) contingency tables. Both 2×2 and $2\times m$ analysis were performed together at each marker. If either of the 2 had $P<0.05$, the marker was judged as a positive marker. Finally, 54 markers significant in all of the screening sets were significant in the 2×2 test, and some of the markers were significant in the $2\times m$ test. The concordance between the 2×2 and $2\times m$ tests was relatively low (stage 1: 60%; stage 2: 64%; stage 3: 81%). All of the positive markers were checked by the Hardy–Weinberg test for allele frequency distributions at the MS loci, and then significant markers ($P\leq0.05$) in the Hardy–Weinberg test were excluded. The positive rates in the second and third screenings were higher than that in the first. This might be partially because of experimental artifacts of the pooled DNA method as reported by Sham et al[39] and Shaw et al.[40]

Individual Typing

The results of pooled typing were presumptive, so we genotyped a total of 770 individuals (385 case subjects versus 385 control subjects) and reanalyzed the 54 markers in the 3-stage screening procedure. These individuals are the same individuals as used in pooled typing and were not from a new cohort. Ultimately, we reduced the number of positive markers from 54 to 19 loci by using individual genotyping in the genome-wide association study for hypertension (Table 2). All 19 of the markers were significant ($P<0.05$) by 2×2 analysis, but only 3 markers were also significant ($P<0.05$) by $2\times m$ analysis. In addition, the odds ratios ranged from 0.13 to 1.8 (Table 2).

The 19 genomic loci were observed on chromosome 2, 3, 4, 6, 10, 13, 17, 18, 19, and 20 (Table 3). The observed and expected frequencies of each genotype for the 19 markers in the case and control subjects were in Hardy–Weinberg equilibrium (data not shown). In considering the LD range, the susceptibility genes for hypertension were estimated to reside in a 100- to 150-kb region from each marker. We have also provided a list of the genes that are known to be positioned closest to the centromeric and telomeric side of each marker (Table 3) to highlight the locations of the 19 positive markers. The chromosomal location of the 19 markers in our study (Table 3) was compared with those identified in previous studies (Table 4). Essentially, 3 chromosomal locations were found to overlap in comparison with other studies: chromosome locations 2p11.1-q12.3, 2p25.1, and 6q27.

TABLE 2. Summary of the Phased Genome Screen by the DNA Method and Individual Typing

	First Screening			Second Screening		Third Screening		Individual Typing	
Chromosome	No. of MS	No. of Positives	Positive Rate, %	No. of Positives	Positive Rate, %	No. of Positives	Positive Rate, %	No. of Positives	Positive Rate, %
1	1516	146	10	31	21	7	23	0	0
2	1847	112	6	29	26	5	17	3	60
3	1422	66	5	15	23	3	20	3	100
4	1157	69	6	13	19	7	54	2	29
5	1173	71	6	14	20	0	0	0	0
6	1204	89	7	22	25	7	32	1	14
7	1266	66	5	18	27	2	11	0	0
8	830	58	7	12	21	0	0	0	0
9	838	32	4	6	19	3	50	0	0
10	907	45	5	11	24	2	18	1	50
11	916	40	4	10	25	0	0	0	0
12	684	25	4	6	24	0	0	0	0
13	609	32	5	11	34	2	18	2	100
14	571	29	5	6	21	1	17	0	0
15	458	34	7	9	26	2	22	0	0
16	508	25	5	9	36	0	0	0	0
17	495	42	8	17	40	5	29	4	80
18	539	27	5	8	30	2	25	1	50
19	358	33	9	8	24	3	38	1	33
20	430	23	5	7	30	3	43	1	33
21	260	15	6	2	13	0	0	0	0
22	228	16	7	5	31	0	0	0	0
X	756	64	8	15	23	0	0	0	0
Y	5	1	20	0	0	0	0	0	0
Total	18,977	1160	6	284	24	54	19	19	35

P value<0.05 was set at statistical significance by Fisher's test for the 2×2 or 2×*m* contingency table. The positive rates represented the rate of the markers, which were positive in the 2×2 or the 2×*m* analysis, to analyzed 18 977 (first), 1160 (second), and 284 markers (third).

Most of the 35 markers that were eliminated by individual typing after the 3-stage screening procedure may have been experimental artifacts or pseudopositive markers because of the DNA pooling method, PCR assay conditions, faulty peak heights during electrophoresis, PCR ghost peaks because of dissociation of labeled fluorescent reagents from a primer oligonucleotide, complications resulting from stutter, and additional nucleotide bands inherent to a particular MS.

Discussion

High-Density MS Markers

We conducted a 3-stage genome-wide scan of 3 independent case–control populations by an association test using 18 977 MS markers to identify susceptible genes for essential hypertension. In this study, we used 18 977 markers with an average spacing of 145.9 kb as the first built set (Tables I and II). Based on recent knowledge, the average length of LD between the disease-susceptible SNPs and the nearby MS alleles is ≥100 kb.[26–31] In other words, if the disease-susceptible SNPs are harbored between 2 neighboring MS markers at an interval ≤200 kb, LD between the disease-susceptible SNPs and either of the nearby MS will be proved. The use of average spacing of genetic markers across 100 to 200 kb of the entire genome is the best practical solution in genome-wide association analysis before availability of a genome-wide LD map, because the LD pattern varies between different regions of the human genome depending on several factors, such as allele frequency, mutation, and recombination.[33] Therefore, our first step for genome-wide analysis was to collect enough MS markers (>18 000 MS, 1 MS at every 150 kb) to cover the euchromatic area (≈90%) of the human genome (3 giga base; 3×10^9 kb ×0.9/150 kb=18 000). The remaining part of the genome was mostly heterochromatin restricted mainly to centromeres and telomeres, rich in repetitive sequences and believed to lack expressed genes. This 150-kb spacing of MS markers would enable us to assure an average 75-kb LD interval, which was presumed to detect the presence of disease-susceptible loci flanked by 2 neighboring MS markers across the whole genome.

Although 54 MS markers were found significant in all 3 stages of the pooling experiments, only 19 (35%) of them were confirmed to be significant when individual typing was performed. This indicates the importance of performing