Table 1 Observed frequency of 39 SNPs in Japanese patients with high myopia and healthy individuals

SNP No.	SNP rs	Genes		Observed Allele (1/2)	Frequency (Allele 1)		Genotype distribution						P-value		exact2x2		OR	95% CI
		Name	Portion				Allele 1/1		Allele 1/2		Allele 2/2		Allele	Genotype	(+/*\-/-)			
					Case	Control	Case	Control	Case	Control	Case	Control			Allele	P		
A1	rs17114134	ZNF295	Intergenic/unknown	A/G	0.602	0.603	200	192	221	232	95	87						
A2	rs2839430	ZNF295	3′UTR	T/A	0.943	0.912	457	426	59	82	0	4	0.008	0.008	2(A)	0.015	0.640	0.448–0.912
A3	rs3746912	ZNF295	3′UTR	G/A	0.619	0.609	203	197	232	231	80	85						
A4	rs915837	ZNF295	Silent mutation	T/C	0.515	0.556	128	171	275	228	113	114						
A5	rs987523	ZNF295	Intron	A/C	0.870	0.841	386	360	129	143	3	10						
A6	rs1628526	ZNF295	Intron	G/A	0.864	0.832	381	352	130	153	5	10	0.043					
A7	rs2839438	C21orf121, ZNF295	Intergenic/unknown	G/A	0.979	0.983	496	498	22	18	0	0						
A8	rs220219	C21orf121	Acceptor splice site	C/G	0.671	0.699	240	251	210	212	64	48						
A9	rs2839442	C21orf121	Intergenic/unknown	C/G	0.695	0.667	244	233	231	218	42	62			1(C)	0.039	1.555	1.029–2.348
A10	rs220238	C21orf121	Intergenic/unknown	A/G	0.621	0.630	192	207	259	235	67	73						
A11	rs2839448		Intergenic/unknown	C/A	0.628	0.624	196	195	260	254	63	67						
A12	rs150796		Intergenic/unknown	A/G	0.583	0.599	168	193	251	221	84	93						
A13	rs2298688		Intergenic/unknown	C/G	0.834	0.853	357	373	148	127	12	12						
A14	rs462098	UMODL1	Intergenic/unknown	G/C	0.654	0.684	213	246	248	211	54	57			2(C)	0.039	1.302	1.017–1.665
A15	rs220262	UMODL1	Intergenic/unknown	T/A	0.744	0.766	283	308	208	175	29	33						
A16	rs220263	UMODL1	Intergenic/unknown	A/G	0.526	0.556	135	158	274	254	108	101						
A17	rs220271	UMODL1	Intron	C/T	0.547	0.497	145	126	274	261	97	129	0.028	0.045	1(C)	0.020	1.440	1.069–1.939
A18	rs220282	UMODL1	Intron	G/A	0.576	0.611	161	194	274	241	82	80			2(A)	0.031	1.336	1.033–1.729
A19	rs749020	UMODL1	Intron	A/G	0.524	0.544	137	154	269	250	112	109						
A20	rs2839466	C21orf128, UMODL1	Intron	G/A	0.677	0.683	234	238	233	226	51	50						
A21	rs220308	C21orf128, UMODL1	Intron	T/G	0.590	0.608	184	191	243	245	91	80						
A22	rs220110	C21orf128, UMODL1	Intron, 3′UTR	C/A	0.566	0.552	165	157	256	256	97	103						
A23	rs220120	C21orf128, UMODL1	Intron	C/G	0.743	0.775	287	309	199	182	34	25						
A24	rs220137	C21orf128, UMODL1	Intron	G/A	0.819	0.842	347	365	156	139	16	12						
A25	rs220143	C21orf128, UMODL1	Intron	G/A	0.712	0.755	256	297	227	184	36	34	0.029	0.023	2(A)	0.0074	1.400	1.095–1.789
A26	rs220148	UMODL1	Intron	A/C	0.705	0.747	248	290	230	188	37	36	0.034	0.024	2(C)	0.0088	1.394	1.090–1.782
A27	rs220149	UMODL1	Intron	T/G	0.498	0.540	134	152	250	254	136	111						
A28	rs220151	UMODL1	Intron	G/C	0.592	0.563	182	162	250	258	87	97						
A29	rs220154	UMODL1	Intron	C/T	0.627	0.655	198	224	256	228	66	64						
A30	rs220159	UMODL1	Missense mutation	G/A	0.638	0.644	216	211	232	243	72	62						
A31	rs220163	UMODL1	Intron	A/G	0.870	0.894	394	409	117	103	9	3						
A32	rs2839471	UMODL1	Intron	C/T	0.490	0.554	114	166	282	241	124	110	0.004	0.001	2(T)	0.00027*	1.684	1.276–2.224

Table 1 (Continued)

SNP No.	SNP rs	Genes		Observed Allele (1/2)	Frequency (Allele 1)		Genotype distribution				P-value	exact2x2		OR	95% CI
		Name	Portion		Case	Control	Allele 1/1 Case	Allele 1/1 Control	Allele 1/2 Case	Allele 1/2 Control	Allele Genotype	Allele (+/*1-/-)	P		
A33	rs3819141	UMODL1	Silent mutation	T/A	0.543	0.538	159	140	247	273	114	101			
A34	rs915840	UMODL1	Intron	T/C	0.973	0.976	492	492	28	25	0	0			
A35	rs9976212	UMODL1	Intergenic/unknown	G/A	0.788	0.790	327	324	162	166	29	25			
A36	rs2839474	UMODL1	Intergenic/unknown	T/C	0.579	0.573	180	165	241	260	98	90			
A37	rs220186		Intergenic/unknown	C/G	0.607	0.591	196	182	239	247	85	88			
A38	rs220321		Intergenic/unknown	A/G	0.582	0.578	179	174	244	246	94	94			
A39	rs220324		Intergenic/unknown	T/C	0.582	0.580	181	176	243	245	96	94			

CI = confidence interval; OR = odds ratio SNP rs = public reference SNP number from the dbSNP database.
Only statistically significant *P*-values ($P<0.05$) are noted in table.
*P_c (corrected *P*-value) = 0.01.

Schiavi *et al*[39] demonstrated through *in situ* hybridization that mouse *UMODL1* was preferentially expressed in the olfactory and vomeronasal sensory neurons, starting at embryonic day 16.5, during embryonic development. Structure of *UMODL1* gene in mouse and human is very similar to each other in their sequence and domain organization. The olfactory system provides an excellent model for the development neurobiology of cell migration, axonal projections, and synaptic connections. Thus, we speculate that aberrations of *UMODL1* in cases of high myopia might be implicated in scleral thinning and neuronal disorder in postnatal eye development.

Eight SNPs were significantly associated with high myopia ($P<0.05$). Four statistically significant SNPs were involved in 10 haplotype blocks identified by LD analysis. However, four other SNPs (rs2839430, rs220271, rs220282, and rs2839471; SNP 2, 25, 26, and 32) were not in the haplotype blocks. Of these results, allele T-positivity of rs283971 (SNP No.32) indicated a strong association ($P=0.00027$; OR = 1.684) even after correction ($P_c=0.01$) for multiple testing. This result suggests that rs283971 is located in the frequent recombinant region on the *UMODL1*, and that this region might play a critical role in disease susceptibility to high myopia.

Our susceptibility gene mapping study in a Japanese population used SNP genotyping to identify the novel high-myopia candidate gene *UMODL1* around *D21S0083i*. However, further investigations (eg, replication studies) are required to confirm whether *UMODL1* is a high-myopia susceptibility gene, and functional investigations are also required to demonstrate the mechanisms by which aberrations in the *UMODL1* gene are related to and contribute to susceptibility for disease.

Electronic database information

The following URLs were used in this study for the analysis of data.

dbSNP: http://www.ncbi.nlm.nih.gov/SNP/index.html

A database of Japanese single nucleotide polymorphisms (JSNP): http://snp.ims.u-tokyo.ac.jp/index.html

UCSC Genome Browser: http://genome.ucsc.edu/cgi-bin/hgGateway

Genome diversity database system (GDBS) Gene diversity DB: http://www.jbirc.aist.go.jp/gdbs/index.html

Genepop on the web: http://wbiomed.curtin.edu.au/genepop/index.html (statistical analysis)

JavaStat—2-way contingency table analysis: http://statpages.org/ctab2x2.html (OR calculation)

Table 2 Estimated haplotype frequencies in Japanese high-myopia patients and healthy individuals

Locus	*Haplotype block*			*Haplotype Frequency*			P-value	OR (95% CI)
	Name	*SNP No.*	*Haplotype*	*All*	*Case*	*Control*		
21q22.3	Block 1	A3-A5-A6	GAG	0.462	0.483	0.440	0.0493*	1.189 (1.000–1.414)
			AAG	0.386	0.381	0.391	0.6193	
			GCA	0.145	0.130	0.160	0.0569	
	Block 2	A9-A10	CG	0.374	0.379	0.370	0.6448	
			GA	0.319	0.305	0.334	0.1478	
			CA	0.306	0.316	0.296	0.3271	
	Block 3	A13-A14-A15	CGT	0.66	0.642	0.678	0.0913	
			GCA	0.156	0.164	0.147	0.2925	
			CCT	0.094	0.100	0.088	0.3708	
			CCA	0.081	0.081	0.081	0.9859	
	Block 4	A19-A20	AG	0.527	0.520	0.535	0.4803	
			GA	0.313	0.317	0.308	0.6577	
			GG	0.152	0.157	0.148	0.5558	
	Block 5	A22-A23	AC	0.441	0.434	0.448	0.5341	
			CC	0.318	0.309	0.327	0.3688	
			CG	0.241	0.257	0.225	0.0890	
	Block 6	A25-A26-A27	GAT	0.519	0.498	0.540	0.0583	
			ACG	0.265	0.285	0.245	0.0394*	1.228 (1.010–1.494)
			GAG	0.206	0.204	0.208	0.8164	
	Block 7	A28-A29	CC	0.419	0.404	0.434	0.1549	
			GT	0.356	0.370	0.342	0.1897	
			GC	0.221	0.222	0.221	0.9460	
	Block 8	A30-A31	GA	0.637	0.634	0.640	0.7617	
			AA	0.245	0.237	0.254	0.3689	
			AG	0.113	0.124	0.102	0.1082	
	Block 9	A33-A35	TG	0.536	0.537	0.534	0.8798	
			AG	0.253	0.250	0.256	0.7553	
			AA	0.206	0.206	0.205	0.9668	
	Block 10	A37-A38-A39	CAT	0.571	0.572	0.569	0.8904	
			GGC	0.392	0.383	0.400	0.4188	
			CGC	0.024	0.029	0.020	0.1576	

CI = confidence interval; OR = odds ratio. *$P < 0.05$.

LocusView 2.0: http://www.broad.mit.edu/mpg/locusview/ (generating images)

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Investigation of the association between the GLC3A locus and normal tension glaucoma in Japanese patients by microsatellite analysis

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Purpose: To investigate whether the GLC3A locus harboring the CYP1B1 gene is associated with normal tension glaucoma (NTG) in Japanese patients.

Materials and Methods: One hundred forty-two Japanese patients with NTG and 101 Japanese healthy controls were recruited. Patients exhibiting a comparatively early onset were selected as this suggests that genetic factors may show stronger involvement. Genotyping and assessment of allelic diversity was performed on 13 highly polymorphic microsatellite markers in and around the GLC3A locus.

Results: There were decreased frequencies of the 444 allele of D2S0416i and the 258 allele of D2S0425i in cases compared to controls ($P = 0.022$ and $P = 0.034$, respectively). However, this statistical significance disappeared when corrected ($Pc > 0.05$). We did not find any significant association between the remaining 11 microsatellite markers, including D2S177, which may be associated with CYP1B1, and NTG ($P > 0.05$).

Conclusions: Our study showed no association between the GLCA3 locus and NTG, suggesting that the CYP1B1 gene, which is reportedly involved in a range of glaucoma phenotypes, may not be an associated factor in the pathogenesis of NTG.

Keywords: GLCA3, microsatellite, normal tension glaucoma, polymorphism

Introduction

Glaucoma is a progressive optic neuropathy leading to permanent visual loss that is often associated with elevated intraocular pressure (IOP). Primary open-angle glaucoma (POAG) is the most common type of glaucoma. Normal tension glaucoma (NTG) is an important subset of POAG; while many POAG patients have high IOP,[1] patients with NTG have statistically normal IOP.[2–4] The prevalence of NTG is higher among the Japanese population than among Caucasians, and recent studies reported that 92% of POAG patients in Japan had NTG.[5–8] The diagnosis of glaucoma is based on a combination of factors including optic nerve damage and specific field defects for which IOP is the only treatable risk factor. NTG, however, tends to be underdiagnosed because of an accompanying high myopia in many cases and the lack of elevated IOP. Although relatively higher IOP, myopia, and older age are known to be factors associated with the development of NTG,[9] they are not pathognomonic and there remains a growing interest in the identification of pathogenetic factors associated with NTG.

Glaucoma is genetically heterogeneous and the detection of susceptibility genes could provide useful information for early diagnosis of glaucoma. To date, over 30 genetic loci for glaucoma have been identified by linkage analysis in multiple pedigrees;[10–12] 14 loci of POAG, 3 loci of primary congenital glaucoma (PCG), and 1 locus of pigment dispersion syndrome have been designated GLC1A-GLC1N, GLC3A-GLC3C, and GPDS1 (with approval from the HUGO Genome Nomenclature Committee), respectively. Among them, GLC3A harbors cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1).[13] CYP1B1 sapans 8.5 kb on chromosome 2p21 with three exons, and mutation of this gene is a major cause of PCG.[10,13] Most CYP1B1 mutations are genetic insertions, deletions,

or missense mutations, indicating that CYP1B1 is relatively susceptible to recombination events.[10] CYP1B1 has also been found to be involved in the development of juvenile-onset glaucoma (juvenile open angle glaucoma [JOAG]).[14–16] In addition, recent studies have shown that CYP1B1 mutations are related to POAG in several ethnic groups,[10] and there have been reports of delayed expression of a CYP1B1 mutation and coexistence of PCG and POAG in the same pedigree.[17,18] These recent studies suggest that CYP1B1 mutations in the GLC3A locus may contribute to a broader range of glaucoma phenotypes than the PCG phenotype alone, including NTG.

In this study, with prospective SNP analysis of the CYP1B1 gene in NTG patients in mind, we performed comprehensive microsatellite mapping in and around the GLC3A locus and investigated the disease-susceptibility of this locus in NTG patients.

Materials and methods

Subjects

We recruited 243 Japanese subjects from the Yokohama City University, Yamanashi University, Gifu University, Kobe University, Yamaguchi University, Kumamoto University, Hokkaido University, Tokyo University, Niigata University, Kanazawa University, Hiroshima University, Tajimi Municipal Hospital, and Tokai University in Japan. Of these subjects, 142 were diagnosed with NTG, and 101 were control subjects. The control subjects were of the same age and sex as the NTG patients, and they were not affected by glaucoma or any ophthalmological or systemic diseases that could result in optic nerve or visual field changes. Furthermore, the control cases either had no myopia or had mild myopia with refractive errors of –3.00 D or less. All patients and control subjects were of Japanese ethnicity, with similar social backgrounds and residing in the same urban area. Informed consent was obtained from all patients, and the study was conducted in accordance with the Declaration of Helsinki and subsequent revisions thereof.

NTG patients enrolled in this study were diagnosed as such if the patient had the following conditions: glaucomatous changes in the optic nerve head with or without retinal nerve fiber layer defect and corresponding glaucomatous visual field defects; normal open angle with angle width greater than Shaffer grade 2; absence of intraocular pressure greater than 21 mmHg on repeated examination by Goldmann applanation tonometry without medication; neurologic, rhinologic, and general medical examinations including magnetic resonance imaging that failed to disclose any pathology responsible for optic nerve change. Glaucomatous optic nerve abnormality was diagnosed when the vertical cup/disc ratio of the optic nerve head was more than or equal to 0.7, or the rim width at the superior portion (11–1 hour) or inferior portion (5–7 hour) was less than or equal to 0.1 of disc diameter, or the difference of the vertical cup/disc ratio was greater than or equal to 0.2 between both eyes, or a nerve fiber layer defect was found. Glaucomatous visual field defect was defined on a hemifield basis using a reliable field data examined by the Humphrey® static visual field analyser (Carl Zeiss Meditec, Oberkochen, Germany,) C-30-2 program according to the Anderson and Patella's criteria; [19] the hemifield was judged abnormal when the pattern deviation probability plot showed a cluster of three or more nonedge-contiguous points having sensitivity with a probability of less than 5% in the upper or lower hemifield, and in one of these with a probability of less than 1%.

The following inclusion and exclusion criteria were used to stringently categorize the patient groups in this study. We excluded individuals who were diagnosed under 20 or over 60 years of age and who had –8.0 D or higher myopic refractive error of spherical equivalence. The selection criteria of HFA mean deviation were stratified depending on the subjects' age in order to minimize the effect of aging on retinal ganglion cell loss and subsequent visual field defect(s) (i) no limitation if the patient was diagnosed under 50 years of age (ii) –10.00 dB or worse in at least one eye if the patient was diagnosed between 50 and 55 years of age (iii) –15.00 dB or worse in at least one eye if the patient was diagnosed above 55 years of age. In this study, the cases exhibiting a comparatively early onset were selected as they suggest that genetic factors may show stronger involvement.

During diagnosis, patients whose refraction values had changed due to cataract surgery, refractive surgery, or other intervention were excluded from the study. In cases where a glaucomatous visual field defect was present only in one eye, the refraction value and glaucomatous visual field defect of the affected eye were adopted. In cases where a glaucomatous visual field defect was present in both eyes, the refraction value and glaucomatous visual field defect of the more severely affected eye were adopted.

Analysis of repeat polymorphisms for 13 microsatellite markers

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) or the guanidine method. In this association study, we selected 13 highly polymorphic microsatellite markers that are located in and around the GLC3A locus as shown in Figure 1. The markers were determined based on the National Center for Biotechnology Information for fine mapping. Polymerase chain

reaction (PCR) was performed in a reaction mixture with 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 PCR conditions were as follows: 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 56 °C for 30 sec, extension at 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. The reaction was carried out in a PCR thermal cycler (GeneAmp System 9700, Applied Biosystems, Foster City, CA, USA). The forward primer was labelled at the 5′ end with 6-FAM, VIC, PET, or NED (Sigma-Aldrich, St. Louis, MO, USA) (Table 1). To determine the number of microsatellite repeats, the PCR products were denatured at 97 °C for 2 min, mixed with formamide, and electrophoresed using an ABI3130 Genetic Analyser (Applied Biosystems). The number of microsatellite repeats was estimated automatically using the GeneScan 672 software (Applied Biosystems) by the local Southern method with a size marker of GS500 TAMRA (Applied Biosystems).

Statistical analysis

Allelic frequencies were estimated by direct counting. The significance of allelic frequencies between the patient and control groups was evaluated by Fisher's exact test. The probability of association was corrected by the Bonferroni inequality method, ie, by multiplying the obtained P values with the number of alleles compared. A corrected P (Pc) value of <0.05 was considered statistically significant. Statistical analyses were performed on a computer using the SPSS software (version 10.1; SPSS Inc., Chicago, IL, USA). Haplotype frequencies and linkage disequilibrium (LD) in the multi-locus analyses were calculated using PyPop.[20] Haplotype frequencies were estimated using the iterative Expectation-Maximization algorithm. LD was measured using Hedrick's multiallelic D' statistic.[21]

Results

Patient age range was 21–58 years (mean 49.1 ± 9.7); 47.2% were male and 52.8% were female. The mean refraction value was −3.74 ± 3.02 diopters (D), and the mean deviation observed in the Humphrey® static visual field determination (Carl Zeiss Meditec, Oberkochen, Germany) was −10.14 ± 8.04 dB.

We genotyped 13 polymorphic microsatellite markers in and around the GLC3A locus in 142 patients and 101 controls (Figure 1). The observed and expected frequencies of each genotype for the 13 markers in the case and control subjects were in Hardy–Weinberg equilibrium (data not shown). Only two adjacent markers, D2S0416i and D2S0425i, were significantly positive, as shown in Table 2, and the frequency of the 444 allele of D2S0416i and the 258 allele of D2S0425i were decreased in cases compared to controls (P = 0.022, OR = 0.59 and P = 0.034, OR = 0.42, respectively). However, this statistical significance disappeared (Pc > 0.05) when evaluated by Bonferroni correction. The magnitude of LD between these two markers was low, with pair-wise D' = 0.25, and the comparison of haplotype consisting of two alleles (D2S0416i_444 and D2S0425i_258) rendered no significant difference between cases and controls (cases vs controls = 3.5% vs 7.3%, P = 0.055) (data not shown).

Discussion

The purpose of this study was to investigate whether the GLC3A locus is associated with NTG in Japanese subjects, based on results from recent studies reporting that the CYP1B1 gene, located at the GLC3A locus on chromosome 2p21, could be a causative gene in POAG as well as PCG. To this end, we genotyped 13 microsatellite markers in and around the GLC3A locus. Here we report a lack of association between the GLC3A locus and NTG in Japanese patients, suggesting that genetic variation at this locus may not play an important role in the development of NTG.

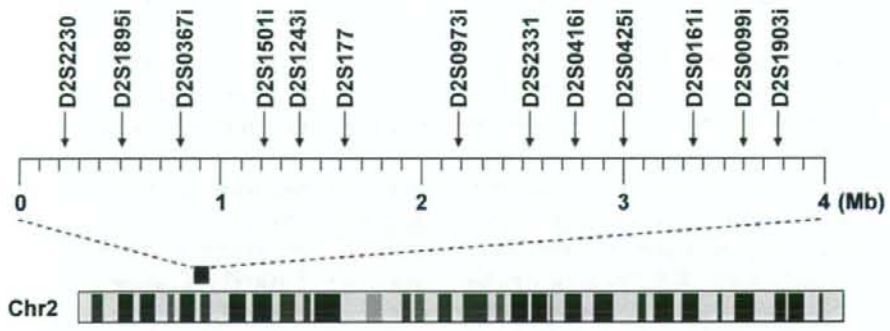

Figure 1 Location of 13 microsatellite markers used in this study. The heavy bar above the chromosome represents the 2p22.1-p22.3 region including the GLC3A locus.

Table 1 Primer sequences of 13 microsatellite markers used in this study

Locus	Dye label		Primer sequences (5'-to-3')	Allele size range
D2S2230	VIC	F	CTTAGAAACGAACCACTGGA	224–232
		R	GTGGAAATAAGTGCAACCAT	
D2S1895i	VIC	F	AAGGATAACCACTCTATCTGCTAC	285–295
		R	GAGCTCTGATTCTATGTATCAAAG	
D2S0367i	6-FAM	F	CTATCAATTCCTGCATCTAACTAT	297–335
		R	TAGTTACTGGATGGCTGTGTAC	
D2S1501i	PET	F	AGTCAATCTGGACTGCTGG	124–142
		R	AGCCCTCAGTACGCAGTAG	
D2S1243i	NED	F	TCCTCAGATTCACTAAACTCATAC	284–298
		R	TGTCAAGTGATGTTAAGTGCTA	
D2S177	PET	F	AGCTCAGAGACACCTCTCCA	287–311
		R	CTGTATTAGGATACTTGGCTATTGA	
D2S0973i	6-FAM	F	AGTTGTAAAGTATCAAGCCATAAA	243–269
		R	GCAGAATGGAACTTAGTGTCTA	
D2S2331	NED	F	ATTAGCACTTACCTGGCACA	126–140
		R	AGTTTATGCTGTGATTAATACCTGG	
D2S0416i	6-FAM	F	CACTCAGTGGCCATTAGAG	440–454
		R	GTAGATTCAGAATTTATGAACCAC	
D2S0425i	PET	F	CTTAAGTACCTTCATGAAGTTCAG	254–260
		R	CAGGAGTTCAAGGTTAAAGTAAG	
D2S0161i	NED	F	GTCTAAGTCAGTAGCATAGCCAAG	292–322
		R	ACGGCTTGAAGTTATAGGAGAC	
D2S0099i	PET	F	GCTAACTTTAACTGATTAAGCAAA	351–363
		R	CATTACCAGTGAGCCTCAC	
D2S1903i	NED	F	TTACCAGTATGCCACTAAACTTAC	242–268
		R	CAGCTTTCTTAATACAGCAAAG	

Sarfarazi and colleagues mapped GLC3A as a putative PCG locus to 2p21 in 11 Turkish families, and the D2S177 microsatellite, located 270 kb telomeric to the CYP1B1 gene, showed a maximal LOD score of 9.40.[22] Bejjani and colleagues also reported that the maximal LOD score was 15.76 with D2S177 in 25 Saudi Arabian PCG families.[23] Recently, it has been reported that the D2S177 allele was associated with CYP1B1 mutations related to PCG.[24] Therefore, D2S177 may be potentially useful as a marker for genetic events associated with glaucoma, such as CYP1B1 mutations. In the present study, we did not find any significant association between D2S177 alleles and NTG, suggesting that CYP1B1 mutations may not be a risk factor for NTG. It has been hypothesized that mutations of CYP1B1 cause developmental abnormality in the structure and function of the anterior ocular segment.[25] However, NTG patients have statistically normal IOP with no abnormality in the structure of anterior ocular segment, suggesting that CYP1B1 is a potential disease susceptibility gene of PCG, POAG, and JOAG, but not NTG. On the other hand, we found a weak association between two adjacent markers, D2S0416i and D2S0425i, and NTG, although this link did not reach statistical significance when corrected. These two markers were located ~860 kb centromeric to the CYP1B1 gene. The nearest gene of D2S0416i is the SOS1 (son of sevenless homolog 1 [Drosophila]) gene, which is about 40 kb from the marker. SOS1 encodes a protein that is a guanine nucleotide exchange factor for RAS proteins,[26] and mutations in this gene are associated with gingival fibromatosis and Noonan syndrome.[27–29] Another marker, D2S0425i, is within the CDKL4 (cyclin-dependent kinase-like 4) gene, a member of the Ser/Thr protein kinase family. Currently, there are no reports suggesting any connection between these two genes and NTG.

Previous studies reported that mutations in genes such as optic atrophy 1 (OPA1),[30–32] apolipoprotein E (APOE),[33,34] and optineurin (OPTN)[35–37] are implicated in NTG. Recently, WD repeat-domain 36 (WDR36),[38] endothelin receptor

Table 2 Frequencies of 13 microsatellite markers in NTG cases and controls

Marker	No. of alleles	Allele*	Frequency, n (%)				P	Pc	Odds ratio (95% CI)
			Cases (2n = 284)		Controls (2n = 202)				
D2S2230	5	228	135	(47.5)	102	(50.5)	0.67		
D2S1895i	6	285	19	(6.7)	15	(7.4)	0.75		
D2S0367i	14	309	33	(11.6)	36	(17.8)	0.054		
D2S1501i	12	136	18	(6.3)	15	(7.4)	0.64		
D2S1243i	8	294	13	(4.6)	16	(7.9)	0.13		
D2S177	13	297	52	(18.3)	48	(23.8)	0.14		
D2S0973i	8	253	226	(79.6)	164	(81.2)	0.66		
D2S2331	8	132	250	(88.0)	170	(84.2)	0.22		
D2S0416i	5	444	44	(15.5)	48	(23.8)	0.022	0.11	0.59 (0.37–0.93)
D2S0425i	4	258	10	(3.5)	16	(7.9)	0.034	0.13	0.42 (0.19–0.96)
D2S0161i	7	297	14	(4.9)	16	(7.9)	0.18		
D2S0099i	7	353	7	(2.5)	11	(5.4)	0.086		
D2S1903i	12	262	12	(4.2)	5	(2.5)	0.30		

Notes: *Alleles with >1% of frequency and found to have the most difference in allele frequency between cases and controls in each marker are shown. Each allele was named by the size of its amplification.

type A (EDNRA)[39,40] methylenetetrahydrofolate reductase (MTHFR),[41] and β-1-adrenergic receptor (ADRB1)[42] have also been reported as risk factors for NTG. However, associations between these genes and NTG were not strong or not often replicated in other populations,[43–47] suggesting that there may be other unknown genetic factors having more powerful effects on the development of NTG than those identified so far.

In conclusion, we performed an association analysis of the GLC3A locus including the CYP1B1 gene using microsatellite markers in NTG patients, but the marker involved in NTG was not detected in this locus. In NTG patients, diagnosis is usually made after a visual field defect has occurred, due to few subjective symptoms and normal IOP. Therefore it is necessary to identify a disease susceptibility gene and elucidate pathogenic mechanisms of NTG for early diagnosis, prevention, and therapeutic development.

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Association of microsatellite polymorphisms of the GPDS1 locus with normal tension glaucoma in the Japanese population

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Background: To investigate whether the GPDS1 locus, a potential causative locus of pigment-dispersion syndrome, is associated with normal-tension glaucoma (NTG) in Japanese patients.

Materials and methods: We used polymerase chain reaction amplification with sequence-specific primers to analyze 20 polymorphic microsatellite markers in and around the GPDS1 locus with an automated DNA analyzer and automated fragment detection by fluorescent-based technology. The DNA samples used for these analyses were obtained from ethnicity- and gender-matched patients, including 141 Japanese patients with NTG and 101 healthy controls. Patients exhibiting a comparatively early onset were selected as this suggests that genetic factors may show stronger involvement.

Results: One allele of D7S2462 exhibited a frequency that was significantly decreased in NTG cases compared to controls ($P = 0.0013$, $Pc = 0.019$, $OR = 0.48$, 95% CI = 0.30–0.75). Alleles at another six microsatellite loci were positively or negatively associated with NTG, but these associations did not retain statistical significance after Bonferroni correction ($P < 0.05$, $Pc > 0.05$).

Conclusion: Our study showed a significant association between the GPDS1 locus and NTG, suggesting that there may be some genetic risk factor(s) in the development of NTG.

Keywords: microsatellite, normal tension glaucoma, glaucoma-related pigment dispersion syndrome, GPDS1, DPP6

Background

Glaucoma is the second most common cause of blindness worldwide.[1] This condition has a high socioeconomic impact, affecting over 67 million people in the western world.[2] The disease is characterized by a loss of visual field corresponding to the excavation of the optic disc. It is considered to be a multifactorial disorder that occurs because of the interaction of genetic and environmental factors. In addition to an association with intraocular pressure (IOP), the onset and progression of glaucoma is involved with several other risk factors, including ischemia, refraction, systemic illness, and genetic predisposition.

Primary open-angle glaucoma (POAG) is usually associated with elevated IOP. The pathogenesis of glaucomatous optic neuropathy is not completely understood, although elevated IOP is a generally accepted major risk factor for this condition. Normal-tension glaucoma (NTG) is an important subtype of POAG and accounts for approximately one-third of all POAG cases in Caucasians.[3] Notably, the incidence of NTG is higher in Japan than in the western world, and it occurs in approximately 92% of all Japanese POAG patients, as reported in the Tajimi Study.[4] The IOP of NTG patients consistently lies within the statistically normal range; thus, although IOP is suggested to play a role in the development of NTG, its involvement may be less in NTG than that in high-tension glaucoma (HTG). Other pathogenic factors such as vasospasm,

ischemia, and vascular insufficiencies are indicated to be associated with the development of NTG.[5–8]

Pigment dispersion syndrome (PDS) and pigmentary glaucoma (PG) are characterized by a disruption of iris pigment epithelium and deposition of the dispersed pigment granules throughout the anterior segment. The classic diagnostic triad consists of corneal pigmentation (Krukenberg spindle); slit-like, radial, or mid-peripheral iris transillumination defects; and dense trabecular pigmentation.[9] PDS/PG usually affects individuals under the age of 30 years. In the early stages of the disease, affected individuals may exhibit clinical evidence of dispersed pigment without an associated elevation of IOP or optic nerve degeneration. However, as the disease progresses, some patients develop increased IOP and degeneration of the optic nerve, causing permanent vision loss. Recent linkage studies have identified several genes or chromosomal loci in some types of glaucoma.[10–12] In PDS-affected families, significant linkage has been observed between the disease phenotype and markers located on several chromosomal loci. GPDS1 (OMIM ID 600510) was the first genetic locus on chromosome 7q35–q36 found to be associated with PDS.[13]

Although the GPDS1 locus may include a gene associated with NTG, an association between the GPDS1 locus and NTG had not been previously investigated. The purpose of this study is to determine whether the GPDS1 locus is one of the candidate loci for developing NTG. We explored this potential in an association study using 20 microsatellite markers around the GPDS1 locus.

Materials and methods

Participants

We recruited 242 Japanese participants from the Yokohama City University, Yamanashi University, Gifu University, Kobe University, Yamaguchi University, Kumamoto University, Hokkaido University, Tokyo University, Niigata University, Kanazawa University, Hiroshima University, Tajimi Municipal Hospital, and Tokai University in Japan. Of these participants, 141 were diagnosed with NTG, and 101 served as controls. The criteria applied for the diagnosis of NTG were those proposed in our previous study.[14] The mean patient age was 49.2 ± 9.6 years, and the male:female ratio was 0.87. The mean refraction value was −3.75 ± 3.02 diopters (D), and the mean deviation observed in the Humphrey® static visual field determination (Carl Zeiss Meditec, Oberkochen, Germany) was −10.13 ± 8.04 dB. The control participants were of the same age and sex as the NTG patients, and they were not affected by glaucoma or any local or systemic illnesses that could result in optic disc or visual field changes. Furthermore, the control cases either had no myopia or had mild myopia with refractive errors of −3.00 D or less. All patients and control participants were of Japanese ethnicity, with similar social backgrounds and residing in the same urban area. Informed consent was obtained from all participants, and the study was conducted in accordance with the Declaration of Helsinki and its subsequent revisions.

Analysis of repeat polymorphisms for 12 microsatellite loci

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) or the guanidine method. In this association study, we selected 20 microsatellite loci that are located around the GPDS1 region on chromosome 7q35–36.3 and that have been established as informative polymorphic genetic markers (Figure 1). The markers were determined based on the National Center for Biotechnology Information guidelines for fine mapping. A polymerase chain reaction (PCR) was performed in a reaction mixture with 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 PCR conditions were as follows: 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 56 °C for 30 sec, extension at 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. The reaction was carried out in a PCR thermal cycler (GeneAmp System 9700, Applied Biosystems, Foster City, CA, USA). The forward primer was labeled with 6-FAM (Sigma-Aldrich, St. Louis, USA) at the 5′ end (Table 1). To determine the number of microsatellite repeats, the PCR products were denatured at 97 °C for 2 min, mixed with formamide, and electrophoresed in an ABI3130 Genetic Analyzer (Applied Biosystems). The number of microsatellite repeats was estimated automatically using GeneScan 672 software (Applied Biosystems) and the local Southern method with a size marker of GS500 TAMRA (Applied Biosystems).

Statistical analysis

Allele frequencies were estimated by direct counting. The significance of allele frequencies between the patient and control groups was evaluated by the Fisher's exact test. The probability of association was corrected by the Bonferroni inequality method, ie, by multiplying the obtained P values with the number of alleles compared. A Pc value of <0.05 was considered statistically significant. Statistical analyses were performed on a computer using SPSS software (version 10.1; SPSS Inc., Chicago, IL, USA).

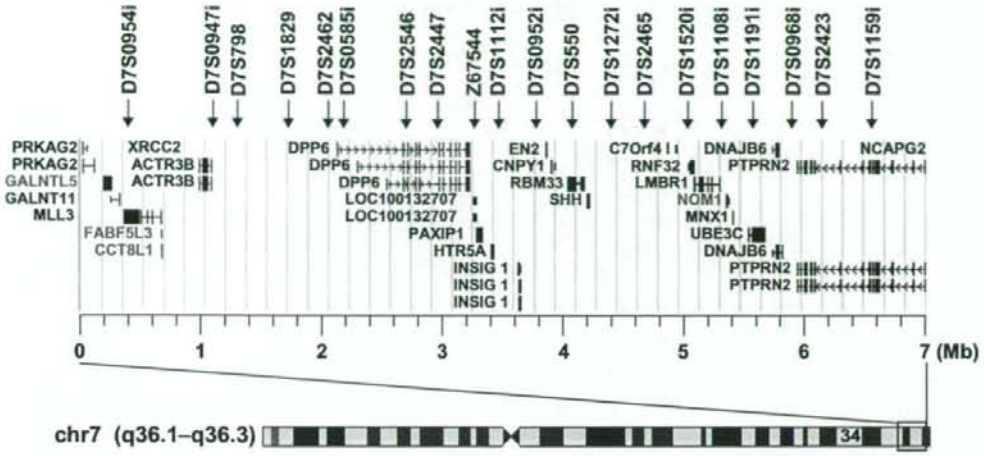

Figure 1 Location of 20 microsatellite markers used in this study and the genes around the GPDS1 locus. Display of genes and chromosome seven results in the UCSC genome browser on Human Mar. 2006 Assembly at chr7: 151,120,000–158,120,000.

Results

We analyzed 20 microsatellite markers localized around GPDS1 for all patients and control participants (Figure 1). All allele frequencies were found to be in Hardy–Weinberg equilibrium proportion. Table 2 lists the allele frequencies of microsatellite markers in patients and control participants, with each allele designated by the size of its amplification. The 132 allele of D7S2462 was significantly associated with NTG, and the allele frequency was decreased in NTG cases as compared to controls (14.5% vs 26.2%, $P = 0.0013$, $Pc = 0.019$, OR = 0.48, 95% CI = 0.30–0.75). Another two markers, D7S2465 and D7S2423, were also negatively associated with the risk of NTG. On the other hand, four markers – D7S2546, Z67544, D7S1272i, and D7S1108i – were positively associated with the risk of NTG. However, these two negative associations and four positive associations did not retain statistical significance after Bonferroni correction ($P < 0.05$, $Pc > 0.05$).

Discussion

Criteria for PDS were two of the following: midperipheral, radial iris transillumination defects; Krukenberg spindle; heavy trabecular meshwork pigmentation; and wide-open angle.[9] Criteria for PG were PDS and two or more of three findings: IOP greater than 21 mmHg, optic nerve damage, or visual field loss. The probability of converting to pigmentary glaucoma was 10% at 5 years and 15% at 15 years. Young, myopic men were most likely to have pigmentary glaucoma. The most significant risk factor for conversion to pigmentary glaucoma was an IOP greater than 21 mmHg at initial examination, whereas age, refractive error, and family history of glaucoma were not correlated with conversion.[15]

NTG is a critical and major subtype of POAG and shares many of the same pathologic characteristics with HTG except for elevated IOP. Notably, many other diseases cause atrophy of the optic nerve similar to that caused by NTG. Since the coexistence of these different diseases decreases statistical validation in the case-control analysis, we carefully selected the NTG patients using highly stringent criteria to select and distinguish the NTG phenotype from the other diseases that cause optic nerve atrophy.

The GPDS1 locus is responsible for PDS.[13] An association between the locus and NTG, however, had not been previously investigated. Therefore, in this study, we sequenced the repeat polymorphisms of 20 microsatellite loci within and around the GPDS1 locus to determine whether these repeat polymorphisms are associated with NTG in a Japanese population.

The 20 microsatellite markers selected for this study were located almost 100–700 kb apart from each other on chromosome 7q35–36.3; they were chosen to achieve dense coverage of the regions located around GPDS1 and to detect susceptible loci related to NTG. As a result, a relatively increased allele frequency was observed in the patient group for the four microsatellite markers D7S2546, Z67544, D7S1272i, and D7S1108i. Another three markers – D7S2462, D7S2465, and D7S2423 – were associated with lower risk of NTG. Of these markers, only D7S2462 remained

Table 1 Primers used in polymerase chain reaction for amplification of microsatellite markers in this study

Location	Orientation*	Sequence
D7S0954i	F (NED)	5'TTACATAAGGTACTTTCTGGTCAT3'
	R	5'TGGTCTCCAGCTACATCAG3'
D7S0947i	F (NED)	5'CTACTTTCCTGGTGTCTAGCTT3'
	R	5'GATAACAGCTGATAGGTAGATCAG3'
D7S798	F (VIC)	5'AGCTGCAAAATAGTGGAAGTAG3'
	R	5'CATCAATTCACATAATGACCG3'
D7S1829	F (VIC)	5'GGGGGTTTCAGAAGAGATGT3'
	R	5'CCTCCAAAATTTCTGAGCAA3'
D7S2462	F (FAM)	5'GCCAAGATTGCACCACTAC3'
	R	5'TGTTAAAAGTTCCCCAAGC3'
D7S0585i	F (NED)	5'AATGAGCATTCTCAGTTTGAATAC3'
	R	5'ATCTGGTGAACGAGTAAATAACAC3'
D7S2546	F (FAM)	5'GGAGGTTGAACAACTCTGAATAC3'
	R	5'CACGCCAGGGTCTATCTT3'
D7S2447	F (NED)	5'GTCAGATGTAGGAACCAAGC3'
	R	5'TCTCACTGTACTCAGCCTGT3'
Z67544	F (VIC)	5'CAACTTAAAATACGCTGTTACC3'
	R	5'GCTTTTTCAGACCAAATAACTTAC3'
D7S1112i	F (NED)	5'GAGAATCTAATGAAAGCTGTGG3'
	R	5'TTCAGGGCATCCAAGAAG3'
D7S0952i	F (NED)	5'AGATATTTGGCTAAACATGGTC3'
	R	5'TCTTCAGAGATACAGAAGCAATAG3'
D7S550	F (FAM)	5'TCTCATCTGTGAATGCACTATC3'
	R	5'GCAGTTGGGTTATTTCAAGTC3'
D7S1272i	F (PET)	5'AATGTCCTGGAGGCTGAG3'
	R	5'AAAGACTTGGTGAGACCTTCTC3'
D7S2465	F (PET)	5'ACCTGGGCAACAGAGTGAG3'
	R	5'CTTCAAAGAGTTTATGCTTATGTGG3'
D7S1520i	F (PET)	5'GCTGTTAGCATCTGGTTAATTTAC3'
	R	5'AAACTGAAGTCTGCCATCTATTAG3'
D7S1108i	F (FAM)	5'ACTTACTTATCCTACTGATCCGTG3'
	R	5'TGAACCATAAATTACCTCCATTC3'
D7S1191i	F (VIC)	5'CACTCTATTTGCATGGTGAAC3'
	R	5'TCCAGTAGAGGGAGCTCAG3'
D7S0968i	F (NED)	5'CACATCCTTTATACCTACATTCAG3'
	R	5'TAATTTATCTAGAAGGTCAGCAAA3'
D7S2423	F (NED)	5'CTTCAGACCTTCAGTTGATGAT3'
	R	5'GCTCTCAGACACATTTTCCA3'
D7S1159i	F (VIC)	5'CTTGGAGTCAAAGAGGCC3'
	R	5'CTTTCCTGACTCCTTGATTAGAG3'

Note: *Dye label.

significantly associated with NTG after Bonferroni correction ($Pc < 0.05$). Therefore, there is one statistically appreciable difference between the NTG group and the control group in these 20 microsatellite markers located over a 6-Mb genomic region around GPDS1.

The dipeptidyl-peptidase 6 (DPP6) gene is the nearest gene to the D7S2462 marker. DPP6 is a type II membrane protein that binds voltage-gated neuronal potassium channels (A-type) and alters their expression and biophysical properties.[16] Recently, genetic variants in DPP6 have been

Table 2 Frequencies of 20 microsatellite markers in normal-tension glaucoma (NTG) patients and healthy participants

Marker	Occurrence of allele	Allele*	Frequencies (%)		P	Pc	Odds ratio (95% CI)
			NTG patients (2n = 282)	Healthy participants (2n = 202)			
D7S0954i	9	310	3.5	2.0	0.31		
D7S0947i	7	151	3.2	2.5	0.64		
D7S798	8	211	7.8	4.0	0.08		
D7S1829	13	391	6.0	4.0	0.31		
D7S2462	15	132	14.5	26.2	0.0013	0.019	0.48
D7S0585i	9	324	4.3	3.0	0.46		
D7S2546	8	239	13.8	6.4	0.0096	0.077	2.33
D7S2447	4	175	53.5	52.5	0.82		
Z67544	6	151	6.4	1.5	0.0091	0.055	3.99
D7S1112i	6	121	44.7	42.1	0.57		
D7S0952i	3	450	1.2	0.5	0.21		
D7S550	13	192	1.2	0.5	0.21		
D7S1272i	6	156	20.2	13.4	0.0499	0.30	1.64
D7S2465	14	175	22.7	32.2	0.020	0.28	0.62
D7S1520i	8	300	3.2	3.0	0.58		
D7S1108i	14	188	3.2	0.5	0.040	0.56	6.63
D7S1191i	6	181	0.7	0.5	0.77		
D7S0968i	6	357	50.0	44.6	0.24		
D7S2423	5	250	19.9	29.2	0.017	0.086	0.60
D7S1159i	8	115	13.8	9.4	0.14		

Note: *Alleles found to exhibit the most difference in frequencies between patients and healthy participants for each marker are shown. Each allele was designated by the size of its amplification.

reported to show a significant association with human neural diseases such as amyotrophic lateral sclerosis and autism.[17–21] Since potassium channels are considered to play an important role in protecting against ischemia and the development of apoptosis,[22] DPP6 variants may act as risk factors in the development of NTG.

In conclusion, we performed an association analysis of the GPDS1 locus using microsatellite markers in NTG patients and found that there was an NTG-associated region in the GPDS1 locus. Therefore, further association studies using single nucleotide polymorphisms in the resistant region might help to identify the pathogenic gene(s) related to NTG.

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