Association Between *ZIC2*, *RASGRF1*, and *SHISA6* Genes and High Myopia in Japanese Subjects

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Purpose. We investigated the association of genetic variations, which were identified recently in a large-scale genome-wide association study (GWAS) to confer risk of refractive error and common myopia in Caucasians, with high myopia in Japanese subjects.

Methods. The 5 single-nucleotide polymorphisms (SNPs) from the 5 genes *TOX*, *RDH5*, *ZIC2*, *RASGRF1*, and *SHISA6*, were genotyped in 1339 unrelated highly myopic Japanese patients and 3248 healthy Japanese participants in the Nagahama Study. In addition, genotypes were compared between high myopia patients without choroidal neovascularization (CNV) and patients with myopic CNV.

Results. Significant associations between rs8000973 near *ZIC2* ($P = 7.16 \times 10^{-7}$), rs4778879 in *RASGRF1* ($P = 3.40 \times 10^{-7}$), and rs2969180 in *SHISA6* ($P = 0.033$) and high myopia were observed. Odds ratios (95% confidence intervals) were 1.33 (1.19–1.49), 0.78 (0.71–0.86), and 1.11 (1.01–1.22) for the rs8000973 C allele, rs4778879 A allele, and rs2969180 G allele, respectively. The effect of the rs2969180 allele G contrasted with that observed in the original report, whereas the effect of the other 2 SNPs agreed. Further analysis using controls with -1.0 diopter (D) $\leq$ spherical equivalent $\leq +1.0$ D showed a significant association between *ZIC2* and *RASGRF1*, but not *SHISA6*. Among the patients with high myopia, 516 had myopic CNV in either eye, while 823 patients did not have myopic CNV in eyes. No evaluated genes showed a significant association with the development of myopic CNV.

Conclusions. *ZIC2* and *RASGRF1* are susceptibility genes, not only for common myopia, but also for high myopia.

Keywords: high myopia, ZIC2, RASGRF1, SHISA6, CNV

Myopia, or nearsightedness, is the most common ocular disorder worldwide. Recent studies reported that the prevalence of myopia is approximately 20% to 42% in the Caucasian population, and much higher (40%–70%) in East Asian populations.[1–4] High myopia is distinguished from common myopia by an excessive increase in the axial length of the eye[5,6] and is considered important because of its association with various ocular complications that lead to blindness.[7–10] For example, choroidal neovascularization (CNV) beneath the fovea is one of the most vision-threatening complications of high myopia.[11,12]

Previous studies have indicated the involvement of genetic and environmental factors in the progression of myopia.[13–16] Family-based linkage analyses and twin studies have identified MYP1-19 loci and several candidate genes,[17,18] but genetic screening studies have achieved limited success. Since 2009, several genome-wide association studies (GWAS) have reported candidate genes for myopia,[19–26] but none of the reported genes or loci, except for the 15q14 locus, showed a consistent association with either common or high myopia in later studies.[27–30] Moreover, although some loci were reported to be associated with common and high myopia,[25,27,31] it still is not clear whether common myopia and high myopia share the same genetic background.

Recently, Verhoeven et al.[32] and Kiefer et al.[33] conducted a large-scale GWAS independently, and reported multiple new susceptibility loci for refractive error and common myopia. To investigate whether these loci cause high myopia in Japanese subjects, we performed a large-scale, case-control study on high myopia. In addition, we investigated the contribution of these genetic variations to the occurrence of CNV in high myopic eyes.

Methods

All procedures used in this study adhered to the tenets of the Declaration of Helsinki. The institutional review boards and the ethics committees of each institution involved approved the protocols of this study. All patients were fully informed of the purpose and procedures of this study, and written consent was obtained from each patient.

Table 1. Characteristics of the Study Population

	Patients, High Myopia*	Controls†
Patients, *n*	1339	3248
Age in y, mean ± SD	57.13 ± 14.90	52.20 ± 14.12
Sex, *n* (%)		
Male	442 (33.0%)	1092 (33.6%)
Female	897 (67.0%)	2154 (66.4%)
Axial length, mm ± SD		
Right eyes	29.25 ± 1.87	24.11 ± 1.39
Left eyes	29.12 ± 1.83	24.07 ± 1.39
Refraction of the phakic eyes, D‡		
Right eyes	−12.39 ± 4.66	−1.73 ± 2.85
Left eyes	−12.54 ± 4.59	−1.64 ± 2.80

* Axial length of ≥26.0 mm in eyes.

† Healthy individuals recruited from Nagaha cohort study.

‡ For calculations of refraction, eyes that had undergone cataract surgery or corneal refractive surgery were excluded.

Patients and Controls

A total of 1339 unrelated highly myopic Japanese patients was recruited from Kyoto University Hospital, Tokyo Medical and Dental University Hospital, Fukushima Medical University Hospital, Kobe City Medical Center General Hospital, Ozaki Eye Hospital, and Otsu Red-Cross Hospital. All patients underwent comprehensive ophthalmic examinations, including dilated indirect and contact lens slit-lamp biomicroscopy, automatic objective refraction, and measurements of the axial length by applanation A-scan ultrasonography (UD-6000; Tomey, Nagoya, Japan) or partial coherence interferometry (IOLMaster; Carl Zeiss Meditec, Dublin, CA). Patients with an axial length of ≥26.0 mm in both eyes were placed into the high myopia group. For control subjects, we included 3248 unrelated healthy Japanese subjects (control 1) from the Nagahama Prospective Genome Cohort for the Comprehensive Human Bioscience dataset (The Nagahama Study). Automatic objective refraction and measurements of the axial length by partial coherence interferometry (IOLMaster; Carl Zeiss Meditec) were performed on all participants. For subanalysis, subjects with a spherical equivalent between −1.0 and +1.0 diopters (D) in both eyes also were included as a control group (control 2). All participants were Japanese, and subjects with any history of ocular disease were eliminated from the control group.

To evaluate the contribution of single-nucleotide polymorphisms (SNPs) to the occurrence of CNV in myopic eyes, the high myopia group was divided into 2 groups: CNV and no CNV. The inclusion criteria for the CNV group were clinical presentation and angiographic manifestations of macular CNV or Fuchs' spot in at least 1 eye.

SNP Selection

Verhoeven et al.[32] reported 26 loci (29 potential candidate genes) associated with refractive error and common myopia in a large-scale multi-ethnic GWAS. Of these loci, 8 also were reported to be associated with myopia and replicated in another recent large-scale GWAS including Caucasian participants.[33] For our analysis, we selected 8 SNPs in these 8 loci that were evaluated in the original report. Among these 8 SNPs, 3 showed extremely low minor allele frequency (MAF) in the Japanese population according to the HapMap data (rs12205363 in *LAMA2*, rs1656404 near *PRSS56*, and rs1960445 near *BMP3*; MAF 0.00, 0.01, and 0.02, respectively). In addition to these 3 SNPs, rs524952 in *GJD2* also was excluded as we had confirmed its association previously with high myopia.[27] Selected SNPs included rs7837791 near *TOX*, rs3138144 in *RDH5*, rs8000973 near *ZIC2*, and rs2969180 in *SHISA6*. Although negated by Kiefer et al.[33] at the replication stage ($P = 0.08$), rs4778879 in *RASGRF1* was included because its association with myopia still is disputed despite numerous replication studies.

Genotyping

Genomic DNAs were prepared from peripheral blood by using a DNA extraction kit (QuickGene-610L; Fujifilm, Minato, Tokyo, Japan). Genotyping of samples from 1339 high myopic patients was performed using a commercially available assay (TaqMan SNP assay with the ABI PRISM 7700 system; Applied Biosystems, Foster City, CA). For the control group, 3712 individuals from the Nagahama study were genotyped using HumanHap610K Quad Arrays, HumanOmni2.5M Arrays, and/or HumanExome Arrays (Illumina, Inc., San Diego, CA). To ensure high-quality genotype data, a series of quality control (QC) filters were applied to the data from each platform, including MAF cutoffs (MAF > 0.01), Hardy-Weinberg equilibrium (HWE; $P > 1 \times 10^{-7}$), genotypic success rate (>95%), individual call rate (>99%), and estimated relatedness (PI-HAT < 0.35). The QCs were performed using PLINK (ver.1.07; available in the public domain at http://pngu.mgh.harvard.edu/purcell/plink/). The fixed dataset consisted of 3248 individuals. Genotype data directly assessed by arrays was used for analyses. Because directly genotyped data of SNP rs4778879 in *RASGRF1* in controls was not available, we analyzed genotype counts of SNP rs6495367 whose linkage disequilibrium value (r^2) is 1.0 compared to rs4778879 (HapMap phase II + III rel 28 JPT).

Statistical Analyses

Data are presented as the mean ± SD. Deviations in the genotype distribution from the HWE were assessed for each group by using the HWE exact test. The χ^2 test for the trend or its exact counterpart was used to compare the genotype distribution of 2 groups. To adjust for age and sex, we performed multiple regression and logistic regression analyses. Two subjects in the control group were excluded from multiple regression and logistic regression analyses because of lack of information regarding age or sex. Statistical analyses were performed using SPSS software (version 21.0; SPSS Science, Chicago, IL). A P value of <0.05 was considered statistically significant. To analyze CNV, a P value of <0.01 (= 0.05/5) was considered statistically significant after Bonferroni correction. Power calculations were performed using R software, package "pwr" (v 3.0.0; R Foundation for Statistical Computing, Vienna, Austria; available in the public domain at http://www.r-project.org/).

Results

Basic information of the study population is shown in Table 1. The mean age of the 1339 high myopia cases was 57.13 ± 14.90 years and the male-to-female ratio was 33.0%:67.0%. The average axial length of cases was 29.19 ± 1.85 mm. Among the 2678 eyes included in the study, 1920 (71.7%) were phakic, and the mean refraction of the phakic eyes was −12.68 ± 4.54 D. The mean age of the 3248 control subjects was 52.20 ± 14.12 years, and the male-to-female ratio was 33.6%:66.4%. The average axial length of controls was 24.09 ± 1.39 mm, and the mean refraction of the 5572 (85.8%) phakic eyes was −1.68 ±

Table 2. Genotype Frequency, Associations, and Odds Ratios (ORs) in the High Myopia Patients and Controls (Control 1)

				Genotype Frequency								
SNP	Chr	Position	Genes	Genotype	High Myopia	Control 1	Nominal *P**	Adjusted *P*†	Adjusted OR†	95% CI†	N‡	HWE *P*§
rs7837791	8	60179086	*TOX*	GG	22.1%	21.7%	0.47	0.62	1.02	0.93–1.12	3239	0.76
				TG	50.9%	50.0%						
				TT	27.0%	28.3%						
rs3138144	12	56114769	*RDH5*	CC	19.2%	20.9%	0.41	0.28	0.95	0.85–1.05	1848	0.49
				CG	50.1%	48.7%						
				GG	30.7%	30.4%						
rs8000973	13	100691367	*ZIC2*	CC	10.2%	6.5%	8.64E-07	7.16E-07	1.33	1.19–1.49	1849	0.76
				TC	42.8%	38.6%						
				TT	47.0%	54.8%						
rs4778879	15	79372875	*RASGRF1*	AA	17.9%	(GG) 23.5%‖	1.46E-07	3.40E-07	0.78	0.71–0.86	3244‖	0.88‖
				GA	49.0%	(GA) 50.1%‖						
				GG	33.1%	(AA) 26.4%‖						
rs2969180	17	11407901	*SHISA6*	GG	24.5%	20.7%	0.023	0.033	1.11	1.01–1.22	3240	0.10
				AG	49.0%	51.2%						
				AA	26.5%	28.1%						

Chr, chromosome; CI, confidence interval.
* Differences in the observed genotypic distribution were examined by χ^2 test for trend.
† Age and sex adjustment was performed based on a logistic regression model.
‡ Number of control subjects who were genotyped directly.
§ The HWE test results for control subjects who were genotyped directly.
‖ Data of SNP rs6495367, whose linkage disequilibrium value (r^2) is 1.0 compared to SNP rs4778879.

2.82 D. Among the control group, 999 subjects had a spherical equivalent between −1.0 and +1.0 D in both eyes, and these subjects were used as control 2. Their average axial length was 23.38 ± 0.79 mm, and the mean refraction of the 1998 (100%) phakic eyes was −0.11 ± 0.53 D.

Genotype counts, associations examined using the χ^2 test for trend analysis, odds ratios for the 5 SNPs between high myopia cases and controls, number of control subjects who were genotyped directly, and the results of the HWE exact test in controls are shown in Table 2. The SNPs rs8000973 near *ZIC2*, rs4778879 in *RASGRF1*, and rs2969180 in *SHISA6* showed significant association with high myopia ($P = 7.16 \times 10^{-7}$, 3.40×10^{-7}, and 0.033, respectively). The odds ratios (95% confidence intervals) were 1.33 (1.19–1.49) for the rs8000973 C allele, 0.78 (0.71–0.86) for the rs4778879 A allele, and 1.11 (1.01–1.22) for the rs2969180 G allele. The effect of the rs2969180 allele G was contrasting to that obtained in the previous study, whereas the other 2 SNPs showed the same trend as that observed in the original report. The distributions of the genotypes for all the five SNPs were in HWE. When control group 2 was evaluated, the SNPs rs8000973 and rs4778879 showed significant association with high myopia

Table 3. Genotype Frequency, Associations, and ORs in the High Myopia Patients and Control 2

				Genotype frequency							
SNP	Chr	Position	Genes	Genotype	High Myopia	Control 2*	Nominal *P*†	Adjusted *P*‡	Adjusted OR‡	95% CI‡	N§
rs7837791	8	60179086	*TOX*	GG	22.1%	20.7%	0.20	0.24	1.07	0.95–1.21	997
				TG	50.9%	49.9%					
				TT	27.0%	29.4%					
rs3138144	12	56114769	*RDH5*	CC	19.2%	20.5%	0.26	0.21	0.91	0.79–1.05	567
				CG	50.1%	51.5%					
				GG	30.7%	28.0%					
rs8000973	13	100691367	*ZIC2*	CC	10.2%	7.0%	1.33E-05	1.29E-05	1.43	1.22–1.67	568
				TC	42.8%	34.7%					
				TT	47.0%	58.3%					
rs4778879	15	79372875	*RASGRF1*	AA	17.9%	(GG) 26.4%‖	1.28E-07	1.01E-07	0.72	0.64–0.82	998‖
				GA	49.0%	(GA) 47.8%‖					
				GG	33.1%	(AA) 25.8%‖					
rs2969180	17	11407901	*SHISA6*	GG	24.5%	21.2%	0.043	0.076	1.11	0.99–1.25	996
				AG	49.0%	49.5%					
				AA	26.5%	29.3%					

* Healthy individuals with spherical equivalent between −1.00 and +1.00 in eyes.
† Differences in the observed genotypic distribution were examined by χ^2 test for trend.
‡ Age and sex adjustment was performed based on a logistic regression model.
§ Number of control subjects who were genotyped directly.
‖ Data of SNP rs6495367 whose linkage disequilibrium value (r^2) is 1.0 compared to SNP rs4778879.

Table 4. Characteristics of the High Myopic Patients With CNV and With No CNV

	CNV	No CNV
Patients, *n*	516	823
Age in y, mean ± SD	60.99 ± 13.28	54.56 ± 15.56
Sex, *n* (%)		
Male	112 (21.7%)	330 (40.1%)
Female	404 (78.3%)	493 (59.9%)
Axial length, mm ± SD		
Right eyes	29.29 ± 1.71	29.22 ± 1.96
Left eyes	29.10 ± 1.69	29.13 ± 1.91

(Table 3, $P = 1.29 \times 10^{-5}$ and 1.01×10^{-7}, respectively). In contrast, rs2969180 in *SHISA6* showed a marginal association with high myopia (nominal $P = 0.043$ and adjusted $P = 0.076$). The SNPs in *RDH5* (rs3138144) and near *TOX* (rs7837791) showed no association with high myopia for all settings examined in this study.

Among the 1339 high myopic patients, 516 had CNV, while 823 did not. The demographics of the CNV group and the no CNV group are shown in Table 4. There was no difference in the axial lengths in each group ($P > 0.05$), whereas the age and female ratios were significantly higher in the CNV group ($P < 0.05$), as was reported previously.[12,34] The results of the association between the genetic variants and myopic CNV in this study are shown in Table 5. None of these 5 SNPs showed significant associations with CNV occurrence in the high myopia patients after Bonferroni correction.

Discussion

In the present study, we showed that SNPs rs8000973 near *ZIC2* and rs4778879 in *RASGRF1*, which were reported recently as susceptibility loci for common myopia, were significantly associated with high myopia in Japanese subjects. Our study also suggested that rs2969180 in *SHISA6* is associated with high myopia. Although it is unclear whether common and high myopia share the same genetic background, our results indicated the existence of some overlap.

The association between the 15q25 locus/*RASGRF1* region and myopia still is controversial; however, our findings strongly suggested the contribution of the 15q25 locus/*RASGRF1* region to high myopia. The 15q25 locus/*RASGRF1* region was reported initially by Hysi et al.[21] to be associated with refractive error and common myopia in a large-scale GWAS by using Caucasian cohorts. However, later studies could not replicate its association with common myopia,[28–30] and its association with high myopia remains controversial. We showed that this locus had a weak association ($P = 0.031$ for rs8027411 and $P = 0.047$ for rs17175798) with high myopia in Japanese subjects,[27] but a Chinese study showed no association of 15q25 with moderate or high myopia. In contrast with these 2 reports on high myopia, our study used a larger number of cases and a larger control group, which differed from that used in our previous study, and the examined SNP also was different from those in previous reports. Because rs4778879 showed weak linkage disequilibrium with previously investigated SNPs, the number of samples would lead to the contradictory results obtained for high myopia between the present and previous studies. Further study on common myopia by using a relatively larger number of samples may confirm the association between the 15q25 locus/*RASGRF1* region and common myopia.

The risk allele in rs8000973 near *ZIC2* and rs4778879 in *RASGRF1* was the same as that observed in the previous study, but the effect of rs2969180 in *SHISA6* differed from that observed in the previous study. Of the SNPs examined in this study, the MAFs in the control group and those obtained from the HapMap data were fairly consistent. The significance of the association of *SHISA6* was weaker than that of *ZIC2* and *RASGRF1* when compared with the population controls (control 1), and it was marginal when compared with the subjects with emmetropic refractive error (−1.0 to +1.0 D) in eyes (control 2). In control 1, the average axial length and mean refraction of the phakic eyes were slightly shifted to a myopic range (24.09 ± 1.39 mm, and −1.68 ± 2.82 D, respectively) as a logical outcome of the high prevalence of myopia (40%–70%) in the Japanese population. Because control 1 included high myopia participants, as the Japanese general population includes 1% to 5% high myopia, analysis of control 1 may have less power to detect the genetic association with high myopia. Although using emmetropic subjects as

Table 5. Genotype Frequency, Associations, and ORs in the High Myopia Patients With CNV and With No CNV

					Genotype Frequency					
SNP	Chr	Position	Genes	Genotype	CNV, %	No CNV, %	Nominal *P**	Adjusted *P*†	Adjusted OR†	95% CI†
rs7837791	8	60179086	*TOX*	GG	22.9	21.6	0.50	0.33	0.92	0.78–1.09
				TG	47.5	52.9				
				TT	29.5	25.5				
rs3138144	12	56114769	*RDH5*	CC	19.7	18.9	0.94	0.80	0.98	0.83–1.16
				CG	49.3	50.6				
				GG	31.1	30.6				
rs8000973	13	100691367	*ZIC2*	CC	11.2	9.7	0.11	0.14	1.14	0.96–1.37
				TC	44.7	41.6				
				TT	44.1	48.7				
rs4778879	15	79372875	*RASGRF1*	AA	17.	18.1	0.60	0.65	0.96	0.81–1.14
				GA	48.5	49.2				
				GG	34.0	32.6				
rs2969180	17	11407901	*SHISA6*	GG	23.2	25.3	0.12	0.04	0.84	0.71–0.99
				AG	47.6	49.8				
				AA	29.2	24.9				

* Differences in the observed genotypic distribution were examined by χ^2 test for trend.
† Age and sex adjustment was performed based on a logistic regression model.

controls by excluding high myopia will improve the power for detecting a genetic association with high myopia, analysis with control 2 further decreased the significance of the association, partly because of the cohort size. Taken together with its contrasting results relative to those from the original report, we must interpret the association of SNP rs2969180 in the present study with caution.

Genetic factors influencing the risk of developing CNV in myopic eyes have been evaluated in many studies because myopic CNV is the most prominent complication leading to severe visual function loss.[35–39] Genetic variants strongly related to age-related macular degeneration (AMD), another degenerative retinal disease characterized by neovascularization in the macula, have been examined to explain the development of CNV in highly myopic eyes. However, several studies showed that susceptibility genes for AMD did not affect the occurrence of myopic CNV.[35–38] In addition, axial elongation of highly myopic eyes results in the thinning of the retina and choroid, patchy chorioretinal atrophy, and lacquer cracks, all of which are important predisposing conditions for the development of CNV.[12,40,41] Therefore, as another approach, we hypothesized that CNV could occur when the eye is affected strongly by susceptibility genes for myopia. We evaluated the genetic difference between high myopia patients with CNV and those without CNV; however, we found that genotype distribution of the SNPs evaluated did not differ significantly. Among the 5 SNPs, rs2969180 in the *SHISA6* gene showed a *P* value of 0.040, but it was not statistically significant after Bonferroni correction. Because the genetic variants contributing to high myopia and to CNV in high myopic eyes may differ, further analyses are required to assess myopic CNV independent of the susceptibility genes for myopia.

In the current study, we used genotype data in controls that were directly genotyped by arrays to eliminate a possibility of imputation error, which may affect the results. Because two SNPs, rs3138144 in *RDH5* and rs8000973 near *ZIC2*, were not genotyped directly by HumanHap610K Quad Arrays, the number of directly-genotyped control subjects in these two SNPs was smaller than that in the other 3 SNPs.

One of the possible limitations is that the current study may be that it was underpowered for detecting associations with SNPs in *RDH5* (rs3138144) and near *TOX* (rs7837791). A power calculation indicated that to obtain 80% power, we would require odds ratios of >1.22 for SNP rs3138144 and odds ratios of >1.20 for SNP rs7837791 by using the sample size used in the present study. Although we cannot estimate the odds ratios in the case-control study for high myopia, the original report showed that SNPs rs3138144 and rs7837791 had a larger effect on common myopia compared to the other 3 SNPs examined in this study,[32] thereby suggesting that these 2 SNPs required a smaller sample size for their association study. The nonsignificant associations in this study may be caused by other factors, such as heterogeneity across the populations or the discrepancy of responsible genes between common myopia and high myopia. Because the associations between these 2 SNPs and common myopia were replicated successfully in the East Asian population in the original study, these 2 SNPs may explain the difference between the mechanisms involved in the development of common myopia and high myopia. In addition, we examined only the top SNP in each susceptibility locus; therefore, our results do not necessarily negate the associations of the *RDH5* and *TOX* locus to high myopia. To investigate the contribution of these loci to myopia, more detailed, confirmatory studies with larger sample sizes are required.

In conclusion, we showed that genetic variants of SNP rs8000973 near the *ZIC2* gene and rs4778879 in the *RASGRF1* gene are associated with high myopia in Japanese subjects. This result, together with previous GWAS, implied that these SNPs may be the susceptibility loci for myopia and high myopia. However, we were not able to identify genetic factors influencing CNV risk in high myopic patients among these 5 SNPs.

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APPENDIX

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