

Comprehensive Replication of the Relationship Between Myopia-Related Genes and Refractive Errors in a Large Japanese Cohort

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PURPOSE. We investigated the association between refractive error in a Japanese population and myopia-related genes identified in two recent large-scale genome-wide association studies.

METHODS. Single-nucleotide polymorphisms (SNPs) in 51 genes that were reported by the Consortium for Refractive Error and Myopia and/or the 23andMe database were genotyped in 3712 healthy Japanese volunteers from the Nagahama Study using HumanHap610K Quad, HumanOmni2.5M, and/or HumanExome Arrays. To evaluate the association between refractive error and recently identified myopia-related genes, we used three approaches to perform quantitative trait locus analyses of mean refractive error in both eyes of the participants: per-SNP, gene-based top-SNP, and gene-based all-SNP analyses. Association plots of successfully replicated genes also were investigated.

RESULTS. In our per-SNP analysis, eight myopia gene associations were replicated successfully: *GJD2*, *RASGRF1*, *BICC1*, *KCNQ5*, *CD55*, *CYP26A1*, *LRR4C*, and *B4GALNT2*. Seven additional gene associations were replicated in our gene-based analyses: *GRIA4*, *BMP2*, *QKI*, *BMP4*, *SFRP1*, *SH3GL2*, and *EHHF1L1*. The signal strength of the reported SNPs and their tagging SNPs increased after considering different linkage disequilibrium patterns across ethnicities. Although two previous studies suggested strong associations between *PRSS56*, *LAMA2*, *TOX*, and *RDH5* and myopia, we could not replicate these results.

CONCLUSIONS. Our results confirmed the significance of the myopia-related genes reported previously and suggested that gene-based replication analyses are more effective than per-SNP analyses. Our comparison with two previous studies suggested that BMP3 SNPs cause myopia primarily in Caucasian populations, while they may exhibit protective effects in Asian populations.

Keywords: refractive error, myopia, genome-wide association study, Japanese, gene-based replication

Myopia is one of the most common ocular disorders worldwide. Recent studies reported that the prevalence of myopia is much higher in East Asian populations (40%–70%) than in Caucasian populations (20%–42%).^{1–3} Additionally, the prevalence of high myopia, which could give rise to various ocular complications and lead to blindness, also is much higher in East Asian populations.^{4–8} However, the regional and/or ethnic differences in the genetic background of myopia between Asians and Caucasians have not been fully investigated.

Previously, several candidate loci have been identified using family-based linkage analyses or twin studies; however, the mechanisms underlying myopia development have not been fully elucidated through these findings.⁹ Research on myopia-related genetic regions has progressed greatly after genome-wide association studies (GWASs) have been performed for myopia.¹⁰ To date, more than 10 GWASs have identified several

genes associated with myopia or related phenotypes; two of these were *15q14* and *15q25*, which showed potent and consistent associations beyond regional and racial variations.^{11–20} Among them, the two largest GWASs, published in 2013 by the Consortium for Refractive Error and Myopia (CREAM) and the 23andMe database, identified as many as 51 genes that account for most of the myopia-related genes that have ever been reported by GWASs.^{19,20}

In the CREAM GWAS discovery stage, 21 single-nucleotide polymorphisms (SNPs) from the Caucasian dataset and eight SNPs from the combined datasets of Caucasians and Asians showed significant associations with refractive error. Although 23andMe performed GWASs for the age of myopia onset using only Caucasians, these two studies showed remarkable overlaps in the associated SNPs and even in their effect sizes.²¹ Further replication studies in different populations would narrow down

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Method	Target	Comment	Range of interest	Significant P-value
Per-SNP replication	Purple circle in B (SNP)	Reported SNP	Single SNP	0.05
Gene-based top-SNP analysis	Purple circle in C (SNP)	Top SNP within the gene	Gene ±10kb	0.05/Number of tag-SNPs
Gene-based all-SNP analysis	All SNPs within the gene	Using VEGAS software	Gene ±50kb	0.05

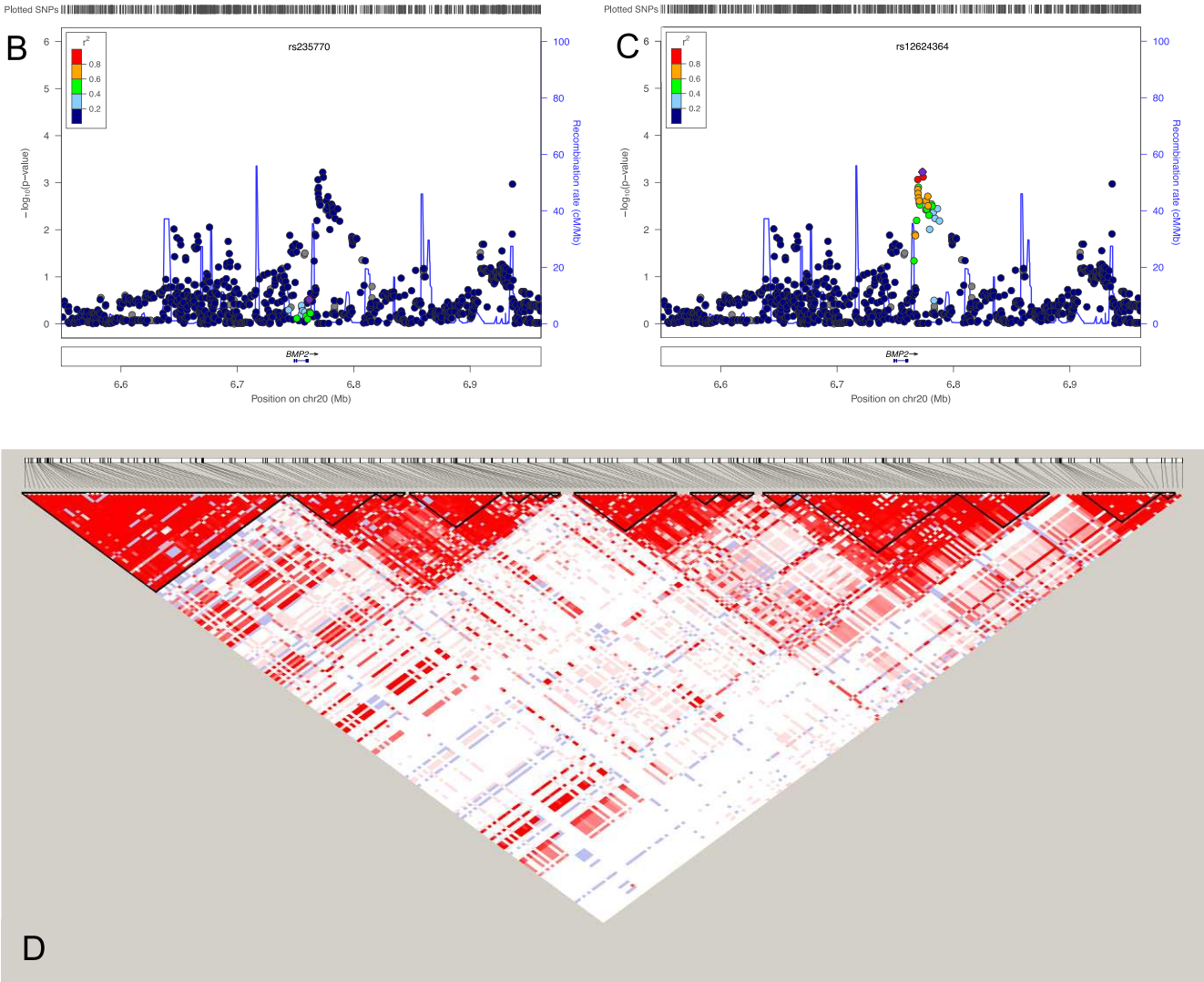


FIGURE 1. Description and illustration of three replication methods used in our analysis, using *BMP2* as an example. (A) Definitions of the three methods are summarized. (B, C) Association plots of the SNPs near *BMP2* in our dataset, showing the target SNPs of per-SNP analysis (B) and gene-based top-SNP analysis (C). Genetic regions ± 200 kb are shown in each plot. (D) An LD plot within the genetic region ± 50 kb of *BMP2*, comprised of 240 SNPs in our dataset. Totals of 17 haplotype blocks and 19 SNPs were not included in any of the blocks. Thus, the number of tag-SNPs was counted to be 36 ($= 17 + 19$). The top SNP of *BMP2* that is shown in (C) should be corrected by the number of tag-SNPs and a *P* value of < 0.0014 ($= 0.05/36$) would be significant for gene-based top-SNP analysis.

the target genes and help elucidate variable genetic backgrounds in myopia across ethnicities.

In this study, we analyzed myopia-related genes that were reported by these two GWASs as disease-susceptible polymorphisms related to refractive error in a relatively large Japanese cohort. We analyzed 51 genes, even ones with marginal significance or without successful replication in their dataset, so that the genes that contribute dominantly to Asian myopia would not be eliminated. In addition, three replication methods including gene-based approaches were performed to avoid excluding the genes by the heterogeneous distribution of

SNP associations or different linkage disequilibrium (LD) patterns across ethnicities.

METHODS

All procedures used in this study adhered to the tenets of the Declaration of Helsinki. The institutional review board and ethics committee of Kyoto University Graduate School and the Faculty of Medicine Ethics Committee, the Ad Hoc Review Board of the Nagahama Cohort Project, and the Nagahama Municipal Review Board of Personal Information Protection approved the protocols

TABLE 1. Characteristics of the Study Population According to Age

	30–39 y	40–49 y	50–59 y	60–69 y	70–75 y	Total
Patients, <i>n</i>	1047	367	518	874	276	3082
Age,* y	34.60 ± 2.76	44.24 ± 2.87	55.17 ± 2.88	64.4 ± 2.93	72.2 ± 1.57	51.02 ± 14.09 (30–5)
Sex, <i>n</i> (%)						
Male	294 (28.1)	124 (33.8)	152 (29.3)	338 (38.7)	121 (43.8)	1029 (33.4)
Female	753 (71.9)	243 (66.2)	518 (70.7)	536 (61.3)	155 (56.2)	2053 (66.6)
MSE,* D (range)	−2.63 ± 2.53 (−13.25–7.44)	−2.75 ± 2.82 (−15.38–2.19)	−1.84 ± 2.81 (−15.69–6.69)	−0.75 ± 2.52 (−14.81–4.38)	0.20 ± 2.28 (−13.31–4.63)	−1.72 ± 2.78 (−15.69–7.44)
Right eyes	−2.66 ± 2.58	−2.82 ± 2.90	−1.90 ± 2.89	−0.78 ± 2.60	0.18 ± 2.44	−1.76 ± 2.85
Left eyes	−2.59 ± 2.52	−2.68 ± 2.79	−1.78 ± 2.84	−0.71 ± 2.56	0.22 ± 2.40	−1.68 ± 2.80
AL,* mm (range)	24.51 ± 1.30 (20.47–28.92)	24.54 ± 1.40 (21.92–28.99)	23.98 ± 1.37 (21.03–29.80)	23.70 ± 1.19 (21.07–28.37)	23.41 ± 1.11 (21.29–28.83)	24.10 ± 1.34 (20.47–29.80)
Right eyes	24.53 ± 1.32	24.57 ± 1.44	24.00 ± 1.38	23.72 ± 1.21	23.42 ± 1.14	24.12 ± 1.36
Left eyes	24.48 ± 1.30	24.51 ± 1.37	23.96 ± 1.40	23.69 ± 1.20	23.39 ± 1.11	24.08 ± 1.35

* Age, MSE, and AL are shown in mean ± SD.

of this study. All participants were fully informed of the purpose of and procedures involved in this study, and written informed consent was obtained from each participant.

Study Populations

The individuals studied were healthy Japanese volunteers enrolled in the Nagahama Prospective Genome Cohort for the Comprehensive Human Bioscience dataset (The Nagahama Study, *n* = 9809). This community-based prospective multi-omics cohort study has been described in detail previously.^{22,23} This cohort was recruited from the general population living in Nagahama City, a large rural city of 125,000 inhabitants in the Shiga Prefecture, located in the center of Japan. All participants voluntarily joined the study, which resulted in the difference in the number of participants of each sex. All eligible participants were included in this study and underwent ophthalmic evaluations: automatic objective refractometry and corneal curvature calculation (Autorefractor ARK-530; Nidek, Tokyo, Japan), axial length (AL) measurement (IOL Master; Carl Zeiss, Jena, Germany), and fundus photography using a digital retinal camera (CR-DG10; Canon, Tokyo, Japan) in a darkened room.²⁴ History of cataract surgery, ocular surgery other than cataract surgery, and ocular laser treatment including photocoagulation were obtained through a questionnaire. Anthropometric parameters and genomic information also were available. We excluded participants with history of any intraocular procedures that could distort the mean spherical equivalent (MSE). Only participants with analyzable spherical equivalent refraction in both eyes were included in this study.

Genotyping and Imputation

The DNA samples were prepared and genotyped as described previously.²⁵ Briefly, 3712 samples were genotyped using at least one of the three genotyping platforms, HumanHap610K Quad Arrays, HumanOmni2.5M Arrays, or HumanExome Arrays (Illumina, Inc., San Diego, CA, USA). To ensure high-quality genotype data, a series of quality control (QC) filters were applied to the data in each platform: sample success rate (>90% for HumanHap610K Arrays, >95% for HumanOmni2.5M Arrays, and >99% for HumanExome Arrays), individual call rate (>99%), minor allele frequency (MAF) cutoffs (>0.01), *P* value for the Hardy-Weinberg test of equilibrium (>1 × 10^{−6}), and estimated relatedness (PI-HAT < 0.35). After these preliminary QC procedures were performed using PLINK (ver. 1.07; available in the public domain at <http://pngu.mgh.harvard.edu/~purcell/plink/>), SNP genotype imputation was conducted for these samples using the MaCH program (version 1.0.10; available in the public domain at <http://www.sph.umich.edu/csg/abecasis/MACH/>) with 500 Markov sampler rounds and 200 haplotype states.²⁶ Genotypes of East Asian samples in the 1000 Genomes Project (release3) were set as reference sequences and standard QC was applied again to the postimputed dataset (sample success rate [>90%], individual call rate [>90%], MAF cutoffs [>0.01], and HWE *P* value [>1 × 10^{−7}]). The SNPs with low imputation quality (*r*² < 0.5) were excluded from the following association analysis.

Myopia-Related Genes and SNPs and the Methods Used for Replication

From the previously reported results for myopia in the two largest GWASs, we included 51 genes that showed associations in at least one GWAS, even without successful replication in their dataset. These genes included 61 SNPs (henceforth referred to as “myopia-related genes and SNPs”). To replicate these myopia-related genes and SNPs, we conducted GWAS for

TABLE 2. Characteristics of the Study Population According to Sex

	Male	Female	<i>P</i> †
Patients, <i>n</i>	1029	2053	
Age,* y	53.02 ± 14.24	50.02 ± 13.91	<0.001
MSE,* D (range)	−1.56 ± 2.68 (−14.75–7.44)	−1.80 ± 2.82 (−15.69–6.69)	0.023
Right eyes	−1.59 ± 2.75	−1.85 ± 2.90	0.020
Left eyes	−1.53 ± 2.71	−1.75 ± 2.83	0.039
AL,* mm (range)	24.37 ± 1.32 (21.06–9.80)	23.96 ± 1.33 (20.47–8.99)	<0.001
Right eyes	24.39 ± 1.35	23.98 ± 1.35	<0.001
Left eyes	24.34 ± 1.31	23.94 ± 1.34	<0.001

* Age, MSE, and AL are shown in mean ± SD.

† Student's *t*-test.

MSE refraction of both eyes using our dataset. These association results were adapted to the replication analysis in three different approaches: one was SNP-based replication and the others involved gene-based replications. Each method is illustrated in Figure 1. For the per-SNP replication method, we directly examined myopia-related SNPs or SNPs with complete LD ($r^2 = 1$) in our dataset. The LD between associated SNPs and SNPs from 1000 Genomes Pilot 1 of CHB/JPT was calculated using the SNAP software (available in the public domain at <http://www.broadinstitute.org/mpg/snap/ldsearch.php>). A *P* value of <0.05 was considered statistically significant. The SNPs were excluded from this analysis if neither the original SNP nor the SNP with complete LD was included in our dataset. For gene-based replications, we conducted two methods: one was gene-based top-SNP replication and the other was gene-based all-SNP replication reflecting association signals of all SNPs. For gene-based top-SNP replication, we selected SNPs that showed the strongest association for MSE within each genetic region ±50 kb of myopia-related genes. The *P* value was multiplied by the number of tagging SNPs and a corrected *P* value of <0.05 was considered statistically significant. All of the imputed SNP genotypes in our dataset were imported into Haploview 4.2 to obtain the r^2 - and D' -based LD plots for each genetic region. Haplotype blocks were defined by the confidential blocks and the number of tagging SNPs was manually counted from these LD plots.²⁷ For gene-based all-SNP replication, we used the VEGAS software (available in the public domain at <http://gump.qimr.edu.au/VEGAS/>) that incorporated information from all SNPs within each genetic region ±50 kb.²⁸ Gene associations with MSE were calculated from the list of SNPs and their *P* values in our dataset. This software provides powerful information on whether multiple risk variants exist within a gene.^{29,30}

Statistical Analysis

The associations between MSE and SNP genotypes were analyzed as a quantitative trait using linear regression analysis in PLINK, assuming additive regression models with adjustment for age, sex, and principal components. Statistical significance of each replication method was assessed as stated above. Deviations from the Hardy-Weinberg equilibrium (HWE) in genotype distributions were assessed using the HWE exact test. We further highlighted regional association signals near the replicated genes to visualize the effect of different LD across ethnicities using LocusZoom.³¹

RESULTS

A total of 3655 individuals passed a series of QC filters after genotyping, and 3082 individuals were analyzed, excluding

those with conditions as stated above. The evaluated genomic variances were 6,746,251 SNPs after imputation and QC. The demographics of the study population are shown in Table 1. The age of the subjects ranged from 30 to 75 years, with spherical equivalent refraction ranging from −15.38 to +7.44 diopters (D) with an MSE of -1.69 ± 2.78 D. Subgroup analysis of MSE by age suggested that the refractive status could be shifted to a hyperopic state in older populations. In addition, female subgroups had significantly ($P = 0.023$) higher myopic refraction compared to male subgroups (Table 2), suggesting that the analysis should be performed with adjustment for age and sex in the linear regression analysis. The genomic inflation factor (λ) in our cohort was 1.055 after including the first two principal components as covariates, suggesting proper adjustment for population stratification.

In the per-SNP replication of the 61 myopia-related SNPs, 16 SNPs were not available in our dataset. Of those, 13 (81%) showed extremely low MAF (≤ 0.0056) in JPT samples of the 1000 Genomes database build 37 (Supplementary Table S1). We analyzed 45 originally reported myopia-related SNPs and one SNP (rs4458448 in the BMP3 region) that showed complete LD ($r^2 = 1$) to the original SNP (rs1960445 in the BMP3 region), and found that 11 SNPs in nine genetic regions showed $P < 0.05$ for the association with MSE (Table 3 and Supplementary Table S2). In the BMP3 region, rs4458448 did not show significant association with MSE, while rs5022942 had a significant *P* value of 0.0496. However, the association direction of rs5022942 was opposite to the original SNP results and we did not regard BMP3 as significantly replicated (Supplementary Table S3). In the gene-based top-SNP replication, 12 genetic regions showed $P < 0.05$ after Bonferroni correction by the number of tagging SNPs (Table 4). In the gene-based all-SNP replication study performed using VEGAS software, eight genes showed $P < 0.05$ (Table 5). A total of 15 genetic regions showed $P < 0.05$ in at least one of the three analyses and were considered to be myopia-associated genes in the Japanese (Table 6). Among these, genetic regions near *KCNQ5*, *GJD2*, *RASGRF1*, *BICC*, and *CD55* showed $P < 0.05$ in all analyses, and regions near *BMP4*, *SH3GL2*, and *B4GALNT2* showed $P < 0.05$ in two of the three analyses. Our findings were compared to the results of two previous GWASs in Supplementary Table S4. Association plots of the eight genes that were replicated by per-SNP replication are shown in Figure 2. Three of them showed peak association signals with high LD in the originally reported SNPs (Fig. 2A), while the other five genes did not (Fig. 2B). Figure 3 shows association plots of seven genetic regions that were only replicated by gene-based analyses and failed to be replicated by per-SNP analysis. Peak association signals and the originally reported SNPs had separated chromosomal positions in our dataset. We further evaluated the effect of different LD structures on the

TABLE 3. Genome-Wide Association Results of the Nagahama Study for Myopia-Related SNPs by the per-SNP Replication Method

Gene Symbol	SNP*	CHR	BP†	MAF	A1/A2‡	β ‡	SE	P
<i>GPR25</i>	rs6702767	1	200844547	0.26	G/A	−0.05	0.08	0.54
<i>CD55</i>	rs1652333	1	207470460	0.44	G/A	−0.14	0.07	0.043
<i>PABPCP2</i>	rs17412774	2	146773948	0.36	A/C	−0.08	0.07	0.23
<i>DLX1</i>	rs17428076	2	172851936	0.03	G/C	0.21	0.21	0.31
<i>PRSS56</i>	rs1656404	2	233379941	0.02	A/G	0.05	0.19	0.78
<i>PRSS56</i>	rs1550094	2	233385396	0.09	G/A	−0.05	0.11	0.67
<i>CHRNA3</i>	rs1881492	2	233406998	0.16	T/G	−0.05	0.10	0.62
<i>SETMAR</i>	rs1843303	3	4185124	0.46	T/C	−0.01	0.07	0.94
<i>LOC100506035</i>	rs9307551	4	80530671	0.34	A/C	0.06	0.07	0.36
<i>BMP3</i>	rs1960445 (rs4458448)	4	81927206	0.03	T/C	0.24	0.18	0.20
<i>BMP3</i>	rs5022942	4	81959966	0.34	A/G	0.14	0.07	0.0496
<i>KCNQ5</i>	rs7744813	6	73643289	0.21	C/A	0.23	0.08	0.0026
<i>QKI</i>	rs9365619	6	164251746	0.34	A/C	−0.01	0.07	0.87
<i>ZMAT4</i>	rs7829127	8	40726394	0.07	G/A	0.17	0.13	0.20
<i>SFRP1</i>	rs2137277	8	40734662	0.04	G/A	0.14	0.16	0.39
<i>TOX</i>	rs7837791	8	60179086	0.46	G/T	0.02	0.07	0.80
<i>TOX</i>	rs72621438	8	60178580	0.47	G/C	0.03	0.07	0.65
<i>CHD7</i>	rs4237036	8	61701057	0.20	C/T	0.04	0.08	0.62
<i>SH3GL2/ADAMTSL1</i>	rs10963578	9	18338649	0.33	A/G	0.09	0.07	0.20
<i>RORB</i>	rs7042950	9	77149837	0.31	A/G	0.04	0.07	0.54
<i>BICC1</i>	rs7084402	10	60265404	0.49	A/G	0.17	0.06	0.010
<i>BICC1</i>	rs4245599	10	60365755	0.46	G/A	0.20	0.06	0.0019
<i>KCNMA1</i>	rs6480859	10	79081948	0.17	T/C	−0.07	0.09	0.44
<i>RGR</i>	rs745480	10	85986554	0.33	C/G	0.03	0.07	0.65
<i>CYP26A1</i>	rs10882165	10	94924324	0.04	T/A	−0.40	0.18	0.023
<i>LRRC4C</i>	rs1381566	11	40149607	0.22	G/T	−0.16	0.08	0.040
<i>DLG2</i>	rs2155413	11	84634790	0.21	C/A	0.06	0.08	0.46
<i>GRIA4</i>	rs11601239	11	105556598	0.34	G/C	0.06	0.07	0.36
<i>PZP</i>	rs6487748	12	9435768	0.34	G/A	−0.13	0.07	0.069
<i>RDH5</i>	rs3138142	12	56115585	0.02	T/C	0.30	0.21	0.16
<i>PTPRR</i>	rs12229663	12	71249996	0.38	G/A	0.10	0.07	0.14
<i>ZIC2</i>	rs8000973	13	100691367	0.25	C/T	−0.11	0.08	0.14
<i>ZIC2</i>	rs4291789	13	100672921	0.27	G/A	−0.11	0.08	0.14
<i>PCCA</i>	rs2184971	13	100818092	0.29	A/G	0.02	0.07	0.83
<i>BMP4</i>	rs66913363	14	54413001	0.22	C/G	0.08	0.08	0.33
<i>66</i>	rs1254319	14	60903757	0.38	G/A	0.05	0.07	0.44
<i>GJD2</i>	rs524952	15	35005886	0.48	A/T	−0.30	0.07	3.7E-06
<i>RASGRF1</i>	rs4778879	15	79372875	0.49	A/G	0.22	0.07	0.00094
<i>RASGRF1</i>	rs28412916	15	79378167	0.48	A/C	0.21	0.07	0.0014
<i>RBFOX1</i>	rs17648524	16	7459683	0.05	C/G	−0.19	0.15	0.19
<i>SHISA6</i>	rs2969180	17	11407901	0.46	G/A	0.11	0.07	0.084
<i>SHISA6</i>	rs2908972	17	11407259	0.45	C/A	0.10	0.07	0.12
<i>B4GALNT2</i>	rs9902755	17	47220726	0.16	C/T	0.19	0.09	0.039
<i>KCNJ2</i>	rs4793501	17	68718734	0.44	T/C	−0.01	0.07	0.83
<i>CNDP2</i>	rs12971120	18	72174023	0.32	G/A	0.09	0.07	0.20
<i>BMP2</i>	rs235770	20	6761765	0.31	T/C	−0.07	0.07	0.32

CHR, chromosome; BP, base pair; A1/A2, reference/variant allele.

* SNPs that were reported by the CREAM and/or 23andME. Rs1960445 was not included in our dataset and we replicated rs4458448 instead, which showed complete LD ($r^2 = 1$) in the Hapmap release 22 by SNAP software.

† Positions and alleles are given relative to the positive strand of NCBI build 37 of the human genome.

‡ Effect size on spherical equivalent in diopters based on allele A1.

association signals of the reported SNPs and their tagging SNPs. We plotted six SNPs of seven genes in Figure 3 (excluding *EHBP1L1*) using two LD patterns in the 1000 Genomes datasets of EUR and ASN, released in March 2012 (hg19), and found that the tagging SNPs of rs66913363 (*BMP4*) and rs235770 (*BMP2*) showed increased associations with MSE using LD patterns of Caucasians (Supplementary Table S1). Tagging-SNPs of the other four SNPs did not show remarkable changes regardless of the applied LD structures (data not shown).

DISCUSSION

In the present study, we evaluated the associations between refractive error and myopia-related genes reported previously in two large GWASs for myopia: survival analysis for the onset age of myopia in Caucasians by 23andME, and quantitative trait loci analysis for spherical error using Caucasian and Asian populations by the CREAM. Our per-SNP analysis successfully replicated the associations of eight genes related to myopia, while our gene-based top-SNP and

TABLE 4. Genome-Wide Association Results of the Nagahama Study for Myopia-Related Genes by Gene-Based Top-SNP Replication Methods With Bonferroni Corrections by the Number of Each Tagging SNPs

Gene Symbol	SNP*	CHR	BP†	MAF	A1/A2‡	β‡	P	Number of Tagging SNPs§	P _{corrected}
<i>GPR25</i>	rs91564	1	200893050	0.05	T/C	0.27	0.0044	21	0.093
<i>CD55</i>	rs12116783	1	207556770	0.08	A/G	0.22	0.0045	7	0.031
<i>PABPCP2</i>	rs10202376	2	147315208	0.77	T/C	0.22	0.14	6	0.85
<i>DLX1</i>	rs79886888	2	173004317	0.17	T/C	0.28	0.10	34	1
<i>PDE11A</i>	rs13006877	2	178984328	0.32	T/A	−0.20	0.0043	32	0.14
<i>PRSS56</i>	rs115279622	2	233375977	0.37	T/C	−0.65	0.0065	40	0.26
<i>CHRNA2</i>	rs12617942	2	233416068	0.02	T/C	−0.73	0.017	37	0.63
<i>SETMAR</i>	rs79901438	3	4391460	0.15	G/T	0.20	0.015	23	0.34
<i>CACNA1D</i>	rs73841203	3	53875801	0.27	G/A	0.39	0.0020	122	0.24
<i>ZBTB38</i>	rs1993904	3	141003354	0.02	T/C	0.32	0.0016	88	0.14
<i>LOC100506035</i>	rs9684343	4	80546040	0.10	G/C	0.21	0.051	10	1
<i>ANTXR2</i>	rs11099009	4	80988658	0.08	A/G	−0.24	0.023	35	0.80
<i>BMP3</i>	rs7659948	4	81979993	0.31	C/T	0.17	0.039	19	0.74
<i>KCNQ5</i>	rs6929988	6	73914319	0.44	A/G	0.28	4.7E-05	102	0.0048
<i>LAMA2</i>	rs10080659	6	129817349	0.03	T/C	0.23	0.0016	82	0.13
<i>QKI</i>	rs9346961	6	163905968	0.10	T/C	−0.89	5.2E-05	32	0.0017
<i>ZMAT4</i>	rs7816960	8	40354396	0.18	A/C	−0.29	0.0020	55	0.11
<i>SFRP1</i>	rs148016338	8	41103891	0.04	A/G	1.07	0.00074	19	0.014
<i>TOX</i>	rs139199809	8	59755748	0.02	C/T	0.89	0.0031	72	0.22
<i>CHD7</i>	rs6984384	8	61809929	0.21	C/T	−0.31	0.0068	40	0.27
<i>SH3GL2/ (ADAMTSL1)</i>	rs10963177	9	17639458	0.50	C/T	0.24	0.00042	106	0.044
<i>(SH3GL2) /ADAMTSL1</i>	rs16937047	9	18770943	0.36	T/C	−0.26	0.00067	216	0.14
<i>TJP2</i>	rs4515614	9	71742683	0.02	T/C	−0.86	0.0091	44	0.40
<i>RORB</i>	rs11144053	9	77284559	0.27	G/A	−0.25	0.02886	45	1
<i>BICC1</i>	rs893369	10	60360901	0.01	T/A	0.23	0.00052	34	0.018
<i>KCNMA1</i>	rs11001900	10	78606671	0.22	A/G	0.22	0.00086	256	0.22
<i>RGR</i>	rs11817115	10	86018811	0.02	G/A	−0.31	0.0032	16	0.051
<i>CYP26A1</i>	rs117520829	10	94791300	0.05	G/C	−0.51	0.0034	19	0.065
<i>TCF7L2</i>	rs12573128	10	114730797	0.27	A/C	0.16	0.030	120	1
<i>LRRC4C</i>	rs58287560	11	40810557	0.38	C/A	0.25	0.00060	168	0.10
<i>EHBP1L1</i>	rs931127	11	65405300	0.12	A/G	0.21	0.0013	19	0.025
<i>DLG2</i>	rs145062356	11	83631501	0.03	A/G	−1.00	0.00080	359	0.29
<i>GRIA4</i>	rs78925386	11	105753469	0.05	A/C	−0.96	0.0018	27	0.049
<i>PZP</i>	rs717180	12	9395807	0.05	A/G	0.20	0.011	17	0.19
<i>RDH5</i>	rs11171667	12	56131052	0.13	A/C	−0.20	0.054	23	1
<i>PTPRR</i>	rs151294916	12	71325795	0.04	G/A	−0.75	0.0062	51	0.32
<i>ZIC2</i>	rs35140645	13	100649321	0.39	G/A	−0.18	0.014	23	0.32
<i>PCCA</i>	rs9513744	13	100935665	0.01	T/A	−0.80	0.0018	44	0.081
<i>LRFN5</i>	rs79467137	14	42096662	0.03	A/T	−0.54	0.0068	35	0.24
<i>BMP4</i>	rs7149027	14	54473305	0.50	A/G	0.36	0.00079	18	0.014
<i>66</i>	rs1015119	14	61027510	0.60	C/T	−0.19	0.040	2	0.080
<i>GJD2</i>	rs589135	15	35001442	0.27	C/G	−0.31	1.8E-06	45	0.000082
<i>RASGRF1</i>	rs57488047	15	79403002	0.51	C/T	0.25	0.00031	81	0.025
<i>RBFOX1</i>	rs79266634	16	7309047	0.54	A/G	0.40	0.00074	649	0.48
<i>SHISA6</i>	rs11651793	17	11267101	0.15	G/A	0.30	0.0083	105	0.88
<i>MYO1D</i>	rs117769171	17	30852727	0.45	C/T	−0.84	0.0049	71	0.35
<i>B4GALNT2</i>	rs4438351	17	47240493	0.20	C/T	0.21	0.0025	31	0.079
<i>KCNJ2</i>	rs11077480	17	68214161	0.12	A/G	0.45	0.012	15	0.18
<i>NPLC4</i>	rs76645549	17	79645253	0.12	G/A	0.20	0.0096	42	0.40
<i>CNDP2</i>	rs78754702	18	72155813	0.32	G/A	−0.79	0.0054	49	0.27
<i>BMP2</i>	rs12624364	20	6773370	0.49	A/G	−0.23	0.00059	36	0.021

* Top SNPs within each myopia-related genomic regions $\pm$ 50 kb were selected from our dataset.

† Positions and alleles are given relative to the positive strand of NCBI build 37 of the human genome.

‡ Effect size on spherical equivalent in diopters based on allele A1.

§ The number of the tagging SNPs is manually counted from LD plots using Haploview 4.2.

|| Each SNP is tested by Bonferroni correction using the number of tagging SNPs within high LD in each LD plot.

all-SNP analyses further revealed seven genes that were significantly associated with refractive error in the Japanese population. Simpson et al.³² reported the limit of the per-SNP replication method and showed the efficacy of region-based analysis for myopia. While they evaluated only two

widely known myopia-susceptible genes in Caucasians, we clearly demonstrated the usefulness of gene-based testing in that the associations of seven genes could be replicated with the gene-based approach out of 15 successfully replicated genes in our study. Considering the heterogeneous traits of

TABLE 5. Gene-Based Association Analysis Incorporating all SNPs Within Each Myopia-Related Genetic Region Using VEGAS Software

Gene Symbol*	CHR	Position NCBI37/hg19		nSNPs*	P
<i>GPR25</i>	1	200842083	200843306	80	0.59
<i>CD55</i>	1	207494817	207534311	88	0.04995
<i>PABPCP2</i>	2	147344625	147348558	NA	NA
<i>DLX1</i>	2	172950208	172954401	58	0.45
<i>PDE11A</i>	2	178487977	178973066	614	0.15
<i>PRSS56</i>	2	233385173	233390425	NA	NA
<i>CHRNA1D</i>	2	233404437	233411038	174	0.13
<i>SETMAR</i>	3	4344988	4358949	134	0.16
<i>CACNA1D</i>	3	53529076	53846492	399	0.19
<i>ZBTB38</i>	3	141043055	141168632	136	0.47
<i>LOC100506035</i>	4	80413747	80497614	NA	NA
<i>ANTXR2</i>	4	80822771	80994626	142	0.25
<i>BMP3</i>	4	81952119	81978685	105	0.18
<i>KCNQ5</i>	6	73331571	73908573	650	0.0015
<i>LAMA2</i>	6	129204286	129837710	701	0.37
<i>QKI</i>	6	163835675	163999628	172	0.073
<i>ZMAT4</i>	8	40388111	40755343	435	0.31
<i>SFRP1</i>	8	41119476	41166990	105	0.52
<i>TOX</i>	8	59717977	60031767	502	0.93
<i>CHD7</i>	8	61591324	61780586	240	0.51
<i>SH3GL2/(ADAMTSL1)</i>	9	17578953	17797122	460	0.047
<i>(SH3GL2)/ADAMTSL1</i>	9	18474079	18910947	825	0.12
<i>TJP2</i>	9	71736180	71870124	176	0.72
<i>RORB</i>	9	77112252	77302117	241	0.77
<i>BICC1</i>	10	60272904	60588845	303	0.0060
<i>KCNMA1</i>	10	78629359	79397577	1035	0.074
<i>RGR</i>	10	86004809	86018944	176	0.71
<i>CYP26A1</i>	10	94833232	94837641	55	0.070
<i>TCF7L2</i>	10	114710009	114927436	170	0.95
<i>LRRC4C</i>	11	40135751	41481186	319	0.14
<i>EHBP1L1</i>	11	65343509	65360116	58	0.088
<i>DLG2</i>	11	83166056	85338314	1377	0.32
<i>GRIA4</i>	11	105480800	105852819	433	0.35
<i>PZP</i>	12	9301436	9360966	185	0.76
<i>RDH5</i>	12	56114151	56118526	42	0.27
<i>PTPRR</i>	12	71031853	71314584	384	0.67
<i>ZIC2</i>	13	100634026	100639019	45	0.30
<i>PCCA</i>	13	100741269	101182691	294	0.75
<i>LRFN5</i>	14	42076764	42373752	316	0.59
<i>BMP4</i>	14	54416455	54423554	96	0.013
<i>66</i>	14	60975938	60978525	102	0.11
<i>GJD2</i>	15	35044642	35046782	142	0.00084
<i>RASGRF1</i>	15	79252289	79383215	185	0.014
<i>RBFOX1</i>	16	6069132	7763340	3526	0.30
<i>SHISA6</i>	17	11144740	11467380	NA	NA
<i>MYO1D</i>	17	30819628	31203902	266	0.93
<i>B4GALNT2</i>	17	47209822	47247351	94	0.031
<i>KCNJ2</i>	17	68165676	68176183	108	0.56
<i>NPLOC4</i>	17	79523909	79596831	102	0.29
<i>CNDP2</i>	18	72163500	72190689	147	0.30
<i>BMP2</i>	20	6748745	6760910	110	0.052

HapMap 2 CHB+JPT was used as the reference.

* SNPs within these genetic regions $\pm$ 50 kb were extracted and set for the gene-based test.

refractive error and the different patterns of LD across ethnicities, gene-based analysis would be a useful approach for the present study.

Of the eight genes that showed significant association with myopia in our per-SNP analysis, six genes had been evaluated in CREAM Asian cohorts and five of the six genes had shown significant association with MSE. Our per-SNP analysis found only one newly replicated gene, *CYP26A1*, in Asian populations. In the genes reported in the 23andME study that used

Caucasian subjects, our per-SNP analysis could replicate only two genes, *LRRC4C* and *B4GALNT2*.

In contrast to per-SNP analysis, gene-based analysis would be a more powerful tool in replication studies for myopia across ethnicities. Our gene-based analysis found seven newly replicated genes: *GRIA4*, *BMP2*, *QKI*, *BMP4*, *SFRP1*, *SH3GL2*, and *EHBP1L1*. In the GWAS reported by the CREAM, the per-SNP analysis in the Asian cohort showed nonsignificant *P* values for *BMP2*, which may be due to the difference in

TABLE 6. Summary of the Three Replication Analyses for the Japanese Cohort That Showed $P < 0.05$ in at Least One Analysis

				Gene-Based		
Gene Symbol	CHR	Position NCBI37/hg19		SNP-Based	Bonferroni	VEGAS
CD55	1	207494817	207534311	0.043	0.031	0.04995
KCNQ5	6	73331571	73908573	0.0026	0.0048	0.0015
QKI	6	163835675	163999628	0.87	0.0017	0.073
SFRP1	8	41119476	41166990	0.39	0.014	0.52
SH3GL2/(ADAMTSL1)	9	17578953	17797122	0.20	0.044	0.047
BICC1	10	60272904	60588845	0.0019	0.018	0.0060
CYP26A1	10	94833232	94837641	0.023	0.065	0.070
LRRC4C	11	40135751	41481186	0.040	0.10	0.14
EHBP1L1	11	65343509	65360116	NA	0.025	0.088
GRIA4	11	105480800	105852819	0.36	0.049	0.35
BMP4	14	54416455	54423554	0.33	0.014	0.013
GJD2	15	35044642	35046782	3.7E-06	0.000082	0.00084
RASGRF1	15	79252289	79383215	0.00094	0.025	0.014
B4GALNT2	17	47209822	47247351	0.039	0.079	0.031
BMP2	20	6748745	6760910	0.32	0.021	0.052

ethnicity between their Caucasian discovery and Asian replication. Gene-based analysis in their Asian cohort might have been able to show significant P values for this gene. In addition, our gene-based studies confirmed the association of

BMP4, *SFRP1*, *SH3GL2*, and *EHBP1L1* with myopia that failed to be replicated by the 23andMe study. These four genes of newly replicated Asian samples would be susceptibility genes for myopia across ethnicities.

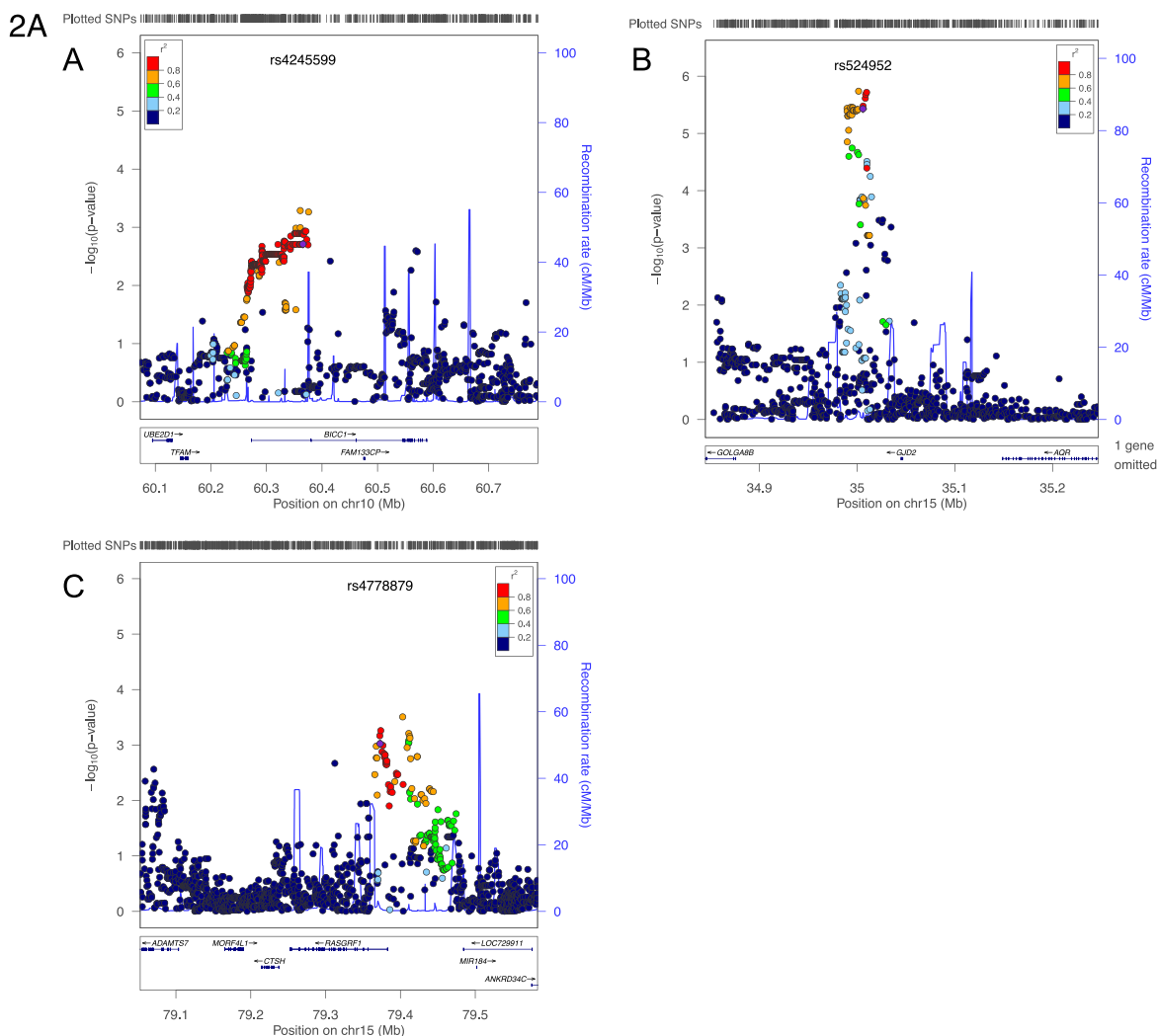


FIGURE 2.

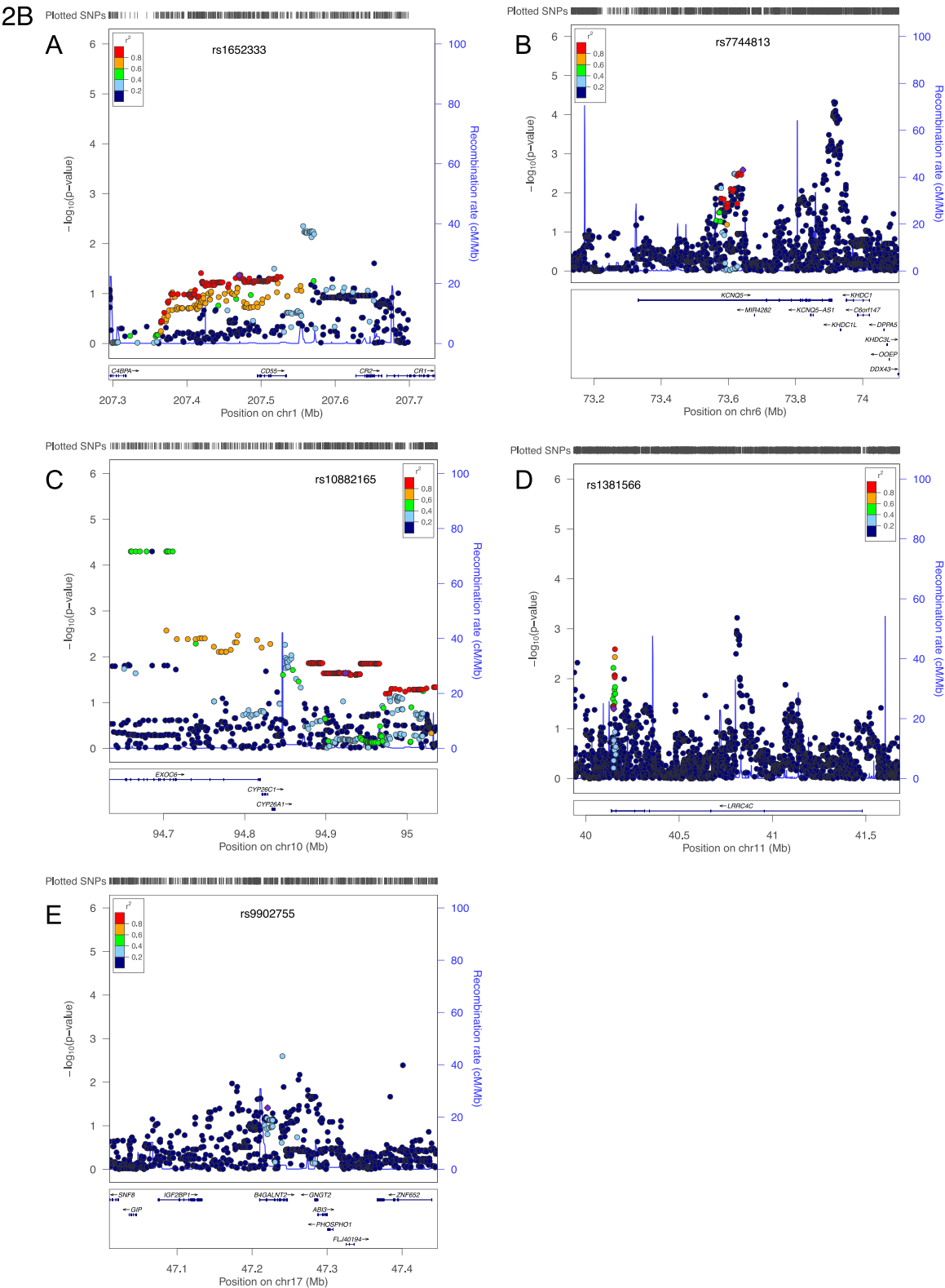


FIGURE 2. Continued Association plots of the eight genes that were significantly replicated in our per-SNP analysis. Reported SNPs near *BICC1*, *GJD2*, and *RASGRF1* showed strong associations with MSE and composed one of the peak signals in our dataset (A, A-C). In contrast, association signals of the reported SNPs of *CD55*, *KCNQ5*, *CYP26A1*, *LRRK4C*, and *B4GALNT2* did not show the highest associations within each genetic region in our dataset (B, A-E). All plots are shown in chromosomal order.

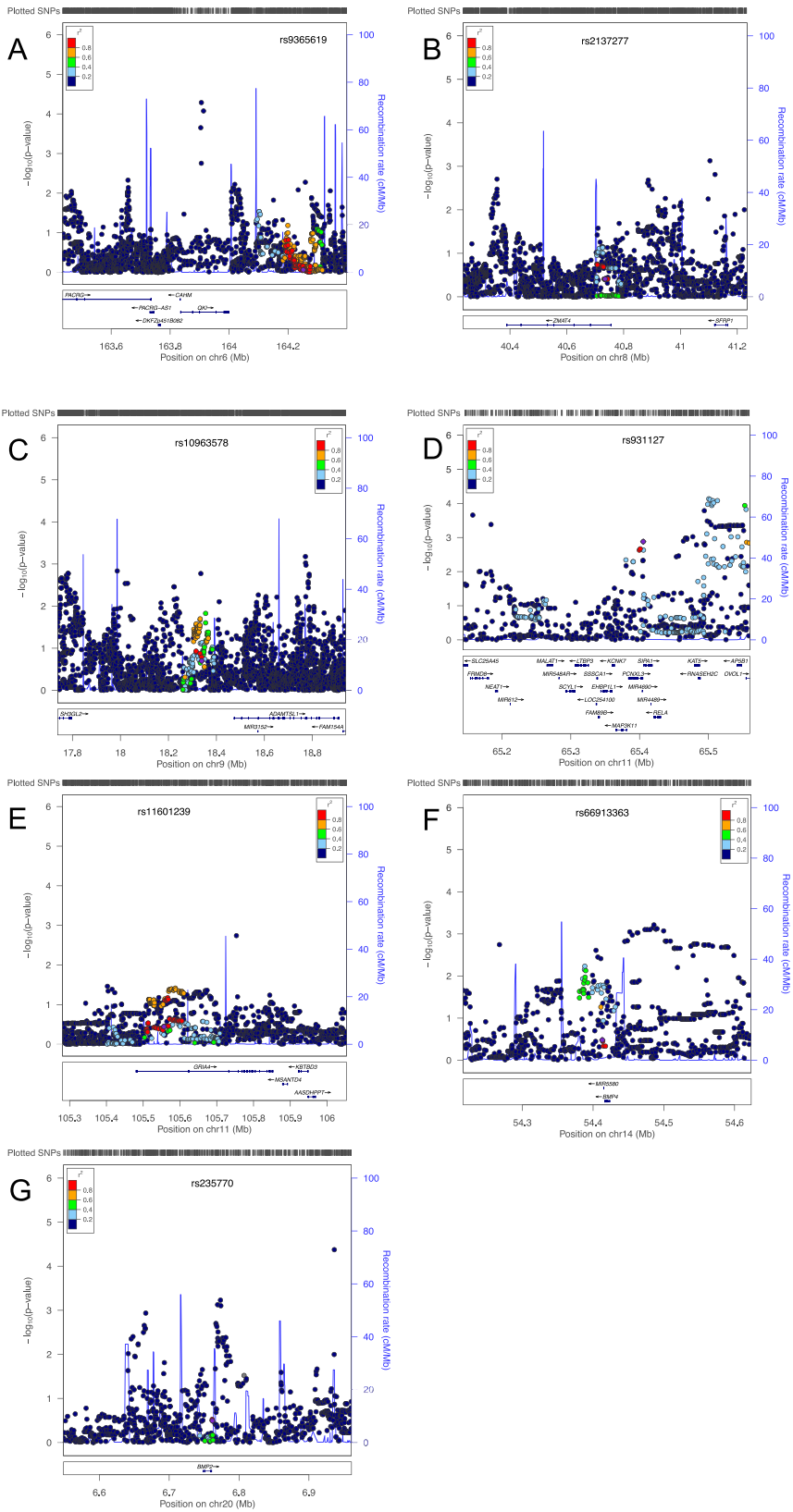


FIGURE 3. Association plots of the SNPs within seven genetic regions near *QKI*, *SFRP1*, *SH3GL2*, *EHBPI1*, *GRIA4*, *BMP4*, and *BMP2* that were replicated in our gene-based analyses but failed to be replicated in our per-SNP analysis. Reported SNPs are highlighted in purple and SNPs within high LD to the reported SNPs are colored according to the strength of LD. Reported SNP of *EHBPI1* was not available in our dataset and the top-SNP was shown instead (D). These LD were calculated using the 1000 Genomes dataset of ASN, reported in March 2012 (hg19) using the LocusZoom software. Association signals of the reported SNPs were relatively low and genetic positions of the original SNPs were apart from the peak signals in each association plot (A–C, E–F).

The advantage of gene-based analysis against per-SNP analysis can be explained in three ways. First, per-SNP analysis is affected by allele frequency. As we have shown in Supplementary Table S1, as many as 13 of 61 reported SNPs showed extremely low MAF in the Japanese population, which consequently would lead to replication failure by per-SNP approach. One example is rs72939141 near *EHBPI1* that showed marginally significant association with myopia in the 23andMe GWAS. We successfully replicated *EHBPI1* by gene-based analysis despite low allele frequencies across ethnicities (MAF was 0 in CEU and JPT populations in the 1000 Genomes dataset released in March 2014) that could have prevented us from examining the true association of the gene by the per-SNP method. The second problem in per-SNP analysis is the narrow genetic regions that could be tested for the associations with phenotype. In our association plots of the eight genes replicated by per-SNP analysis, three genes clearly showed peak association signals with high LD in the reported SNPs (Fig. 2A). However, the other five genes did not show close relationships between peak association signals and the reported SNPs (Fig. 2B). Even though the latter five SNPs also were replicated by per-SNP analysis, investigating wider genetic regions (e.g., region-based analysis shown by Simpson et al.³²) would make the associations still more significant. The association strength of a single SNP only reflects signals including nearby SNPs with moderate LD, and is far from reflecting genetic influences of the gene itself. The last problem in per-SNP analysis is the heterogeneity of LD patterns across ethnicities. Figure 3 shows different association signals of *GRIA4*, *BMP2*, *QKI*, *BMP4*, *SFRP1*, *SH3GL2*, and *EHBPI1* between Caucasians and Asians. Reported SNPs of these genes could not be replicated by per-SNP methods, probably due to the different LD patterns. This issue was further evaluated in Supplementary Figure S1 in that more intense association signals of the reported SNPs would be illustrated when considering the variability of LD patterns between Asians and Caucasians. Our successful replication of these genes by gene-based approaches shows the limitations of per-SNP replication for ethnicities with different LD patterns.

Although LD patterns are different across ethnicities, our findings suggested a similar effect direction of most myopia-related genes across ethnicities. When our per-SNP analysis was compared to the CREAM GWAS results, the evaluated SNPs showed consistent effect direction among Japanese, other Asians, and Caucasians. Supplementary Table S3 shows a comparison of effect size and direction for 24 SNPs that were reported by the CREAM study, which also were included in our dataset. Of the 24 SNPs, 19 (79.2%) have the same effect direction for myopia. However, it was interesting that *BMP3* showed the opposite effect for myopia between Caucasians and Japanese, as well as between Caucasians and Asians. Rs1960445/rs4458448 of *BMP3* was considered to be nonsignificant for myopia in the CREAM Asian samples. However, the consistent effect direction with our Japanese dataset suggested a different effect of *BMP3* on Caucasian and Asian myopia. The minor allele of rs1960445/rs4458448 would have risk effects for myopia in Caucasians, while it has protective effects in Japanese and other Asians.

For further replication, the following two sets of genes should be considered. First, we successfully replicated *CYP26A1* among 11 genes that did not show associations in the CREAM Asian samples. In our previous study, we also showed that *ZIC2* was significantly associated with high myopia in Japanese.²⁵ Further replication study with larger Asian cohorts may reveal associations of *ZIC2* with myopia. For the remaining nine genes that showed consistently negative results in our cohorts and the CREAM Asian samples, further replications of these genes are necessary using more Asian

samples. Second, among the 22 genes that showed associations only in the 23andMe dataset and are yet to be examined in Asian samples, seven genes, *LRR4C*, *QKI*, *BMP4*, *SFRP1*, *SH3GL2*, *B4GALNT2*, and *EHBPI1* were replicated in our samples. For the remaining 15 genes, further replications are necessary using Asian samples.

There were three limitations in this study. First, in our dataset, some SNPs were not genotyped directly but had imputed genotypes. Additionally, we could not find all of the reported SNPs in the first analysis; 16 of 61 reported SNPs were not available in our imputed dataset. After screening other SNPs with complete LD to original ones, only rs1960445 became analyzable through rs4458448 (Supplementary Table S2). However, this issue was resolved by gene-based analysis of replicating association signals by using multiple SNPs within the gene. Second, we could not replicate *ZIC2* in this study that is incompatible with our previous report.²⁵ We have shown that *ZIC2* is significantly associated with high myopia (AL ≥ 26.0 mm) in Japanese, which might be a result of the different genetic contributions to various myopic ocular traits. Thus, further investigation should be carried out to clarify these genetic variations. Third, we confirmed strong associations of four genes, *GJD2*, *RASGRF1*, *KCNQ5*, and *BICC1*, in the Japanese population, consistent with the previous reports on Asians and Caucasians. However, we could not replicate four genes, *PRSS56*, *LAMA2*, *TOX*, and *RDH5*, which consistently showed significant associations throughout the two previous GWASs. These genes are highly likely to be strongly associated with myopia in Caucasians and Asians and, thus, these replication failures would be caused by our sample size and/or ethnic differences between Japanese and other Asian ethnicities.

In conclusion, we selected myopia-related SNPs that had been reported by GWASs and thoroughly replicated these SNPs in a relatively large Japanese cohort. Our results suggested the efficacy of combining gene-based analysis with per-SNP analysis to replicate association signals across ethnicities. We replicated 15 genes and confirmed strong associations of *GJD2*, *RASGRF1*, *KCNQ5*, and *BICC1* with myopia across Caucasian, Asian, and Japanese populations, whereas *BMP3* might have ethnic specificity to Caucasians for associations with myopia. These analyses would support further replications and investigations regarding the contributions of these genes to myopia across ethnicities.

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APPENDIX

The Nagahama Study Group

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