and sleep duration. However, very few studies investigated the correlation between dietary behaviors and GERD with sleep duration, and the relationships among these three factors have never been evaluated comprehensively in a cohort study.[16–18]

Given this background, we analyzed the cross-sectional interrelationships among sleep duration, GERD symptoms, and dietary behaviors simultaneously in a large-scale sample of the general population.

METHODS

Study Participants

Included in the current analysis were participants of the Nagahama Prospective Genome Cohort for Comprehensive Human Bioscience (The Nagahama Study). The Nagahama Study is a longitudinal genetic epidemiological study aimed at clarifying unidentified factors and pathways relating genetic variants and disease phenotypes of common diseases and disorders, such as cardiovascular, endocrine, metabolic, and immunological diseases via the comprehensive analysis of omics data. The Nagahama Study cohort was recruited from the general population living in Nagahama City, a largely rural city of 125,000 inhabitants in Shiga Prefecture, located in the center of Japan. Among the total of 9,804 study participants recruited from 2008 to 2010, persons who had a history of malignant diseases of the upper alimentary tract (n = 79), who were pregnant (n = 43) or who did not complete the questionnaires (n = 39) were excluded from the analysis. All study procedures were approved by the ethics committee of Kyoto University Graduate School of Medicine.

Basic Clinical Parameters

Basic clinical parameters, including age, body mass index (BMI), and clinical history were obtained from the personal health records collected at the baseline examination for the Nagahama Study. Smoking history and drinking habits were obtained using a structured questionnaire. An individual who consumed alcohol more than 4 days/w was defined as a frequent drinker.

Assessment of Sleep Habits

Hours of sleeping were assessed by the following question: "On average, how many hours do you sleep per day?" Subjects were categorized into five groups according to sleep duration: less than 5 h, 5 to less than 6 h, 6 to less than 7 h, 7 to less than 8 h, and 8 or more h per day. Short sleep duration was defined as less than 6 h of sleep per day according to previous studies.[19,20] The regularity of the sleep schedule was also investigated by the following "yes-no" question: "Are your waking time and bedtime regular?"

Assessment of GERD Symptoms

The GERD symptoms were evaluated using the Frequency Scale for the Symptoms of GERD (FSSG),[21] a well-validated and widely used questionnaire for the diagnosis of GERD and also for evaluating the effectiveness of the treatment of GERD.[22,23] The 12 questions of the FSSG cover various symptoms related to the upper alimentary tract. A higher score indicates more severe GERD symptoms and 8 points are frequently used as a cutoff point for the diagnosis of GERD. All the participants were asked to respond to the FSSG scale questionnaire and participants with an FSSG score of 8 or higher or who were undergoing treatment of GERD were defined as having GERD.

Assessment of Dietary Behaviors

Unfavorable dietary behaviors that were expected to be closely correlated with both sleep duration and GERD symptoms were assessed by the following four "yes-no" questions that are used in the standard health checkup program performed by the Japanese government: 1. Do you have dinner within 2 h before going to bed more than 3 days a week? 2. Do you snack after dinner more than 3 days a week? 3 Do you have a habit of eating rapidly? 4. Do you skip breakfast more than 3 days a week? A score of one was assigned to each "yes" response.

Statistical Analysis

Differences in numeric variables among subgroups were determined by an analysis of variance for continuous variables and a chi-square test for categorical variables. Trend testing was performed by the Cochrane-Armitage trend test (categorical variables) or the Jonckheere trend test (numeric variables). In comparison of FSSG score among groups categorized by sleep duration and regularity of the sleep schedule, Dunnet test was performed using the group with 7 to less than 8 h/day sleep duration as the reference. We performed multivariate logistic regression analysis to specify the factors independently associated with short sleep duration. Two-tailed $P < 0.05$ were considered statistically significant. All statistical analyses were performed using JMP 7.0.2 statistical software (SAS Institute Inc., Cary, NC, USA) and R software (http://www.r-project.org/).

RESULTS

Basic clinical characteristics of study participants are summarized in Table 1. Of the total of 9,643 participants, the diagnosis of GERD was made in 2,210 (22.9%), and the prevalence of GERD as well as the mean FSSG score did not differ between men and women. In contrast, unfavorable dietary behaviors except for snacking after dinner were more frequent in men than in women. Frequency of an irregular sleep schedule was also higher in male than in female participants.

Table 2 shows the differences in clinical characteristics of subjects according to sleep duration. In the trend analysis, factors positively associated with short sleep duration were female sex, body mass index, irregular sleep schedule, and consumption of hypnotic or analgesic drugs, whereas frequent drinking and current smoking showed opposite associations. The frequency of GERD as well as the number of unfavorable dietary behaviors were also increased with decreasing sleep duration.

Because the frequency of an irregular sleep schedule was approximately three times higher in the highest group than in the lowest group, we conducted a separate analysis of the regularity of the sleep schedule. Results of trend analysis showed that an inverse association between sleep duration and the FSSG score was only seen in participants having a regular sleep schedule. Even though the relationship between FSSG score and sleep duration in participants with regular sleep schedule seemed to be inverse J-shaped curvilinear, a significant difference in FSSG score was not observed between groups with 7 to less

Table 1—Clinical characteristics of study participants.

	All (n = 9,643)	Men (n = 3,164)	Women (n = 6,479)	P
Age, y	54 ± 13	56 ± 14	53 ± 13	< 0.01
Body mass index, kg/m^2	22.3 ± 3.3	23.4 ± 3.1	21.8 ± 3.2	< 0.01
Current smoker, %	14.6	31.0	6.5	< 0.01
Frequent drinker, %	22.7	49.5	9.6	< 0.01
Irregular sleep schedule, %	10.7	13.7	9.2	< 0.01
Unfavorable dietary behavior, %				
Dinner within 2 h of bedtime	18.5	28.8	13.5	< 0.01
Snacking after dinner	20.9	19.4	21.6	0.01
Rapid eating	35	41.7	31.7	< 0.01
Skipping breakfast	9.2	12.4	7.6	< 0.01
Medication, %				
Hypnotic drugs	5.4	4.9	5.6	0.17
Steroids	0.7	0.6	0.7	0.33
Analgesic drugs	3.3	1.7	4.1	< 0.01
GERD treatment, %	1.1	0.9	1.2	0.16
FSSG score	4.7 ± 5.0	4.6 ± 4.9	4.7 ± 5.0	0.14
GERD, %	22.9	22.4	23.2	0.43

Values are expressed as mean ± standard deviation or percentage. Gastroesophageal reflux disease (GERD) was defined by a score of eight points or more on the Frequency Scale for the Symptoms of GERD (FSSG) or taking medication for GERD. An individual who consumed alcohol more than 4 days/w was defined as a frequent drinker.

Table 2—Differences in clinical characteristics according to sleep duration.

	less than 5 h (n = 595)	5 to less than 6 (n = 2,246)	6 to less than 7 (n = 3,732)	7 to less than 8 (n = 2,316)	8 or more h (n = 754)	P ANOVA or chi-square	P Trend
Women, %	69.6	71.6	69.0	62.9	56.6	< 0.01	< 0.01
Age, y	54 ± 13	54 ± 12	53 ± 13	54 ± 14	53 ± 15	0.72	0.34
Body mass index, kg/m^2	22.8 ± 3.6	22.4 ± 3.3	22.3 ± 3.2	22.1 ± 3.2	22.2 ± 3.5	< 0.01	< 0.01
Current smoker, %	14.0	13.4	13.7	15.8	19.0	< 0.01	< 0.01
Frequent drinker, %	17.3	21.3	21.2	25.0	31.4	< 0.01	< 0.01
Irregular sleep schedule, %	25.7	14.8	8.4	6.9	9.3	< 0.01	< 0.01
Medication, %							
Hypnotic drugs	9.6	6.9	4.2	4.7	5.6	< 0.01	< 0.01
Steroids	0.7	0.7	0.7	0.7	0.8	0.99	0.87
Analgesic drugs	4.4	3.8	3.4	2.6	2.5	0.06	< 0.01
No. unfavorable dietary behaviors	1.0 ± 1.0	0.9 ± 0.9	0.8 ± 0.9	0.8 ± 0.8	0.8 ± 0.9	< 0.01	< 0.01
FSSG score	5.6 ± 5.7	4.9 ± 5.0	4.7 ± 4.9	4.3 ± 4.8	4.5 ± 5.2	< 0.01	< 0.01
GERD, %	30.3	25.1	22.8	19.8	20.6	< 0.01	< 0.01

Values are expressed as mean ± standard deviation or percentage. Differences in numeric variables among subgroups were determined by an analysis of variance for continuous variables and a chi-square test for categorical variables. Trend testing was also performed by the Cochrane-Armitage trend test (categorical variables) or the Jonckheere trend test (numeric variables). P values for both ANOVA and trend tests are shown. ANOVA, analysis of variance; FSSG, Frequency Scale for the Symptoms of GERD; GERD, gastroesophageal reflux disease.

than 8 h and 8 h or longer per day sleep duration. However, there were significant differences between the group with 7 to less than 8 h/day sleep duration and each group with less than 5 h, 5 to less than 6 h, and 6 to less than 7 h per day sleep duration. (Figure 1)

The association between each dietary habit and the FSSG scores are summarized in Table 3. All of the investigated dietary behaviors were associated with a significantly higher FSSG score. Further, the accumulation of unfavorable dietary behaviors showed a stepwise association with the FSSG score (Figure 2).

To further identify factors independently associated with short sleep duration, multiple logistic regression analysis was performed with adjustments for possible covariates (Table 4,

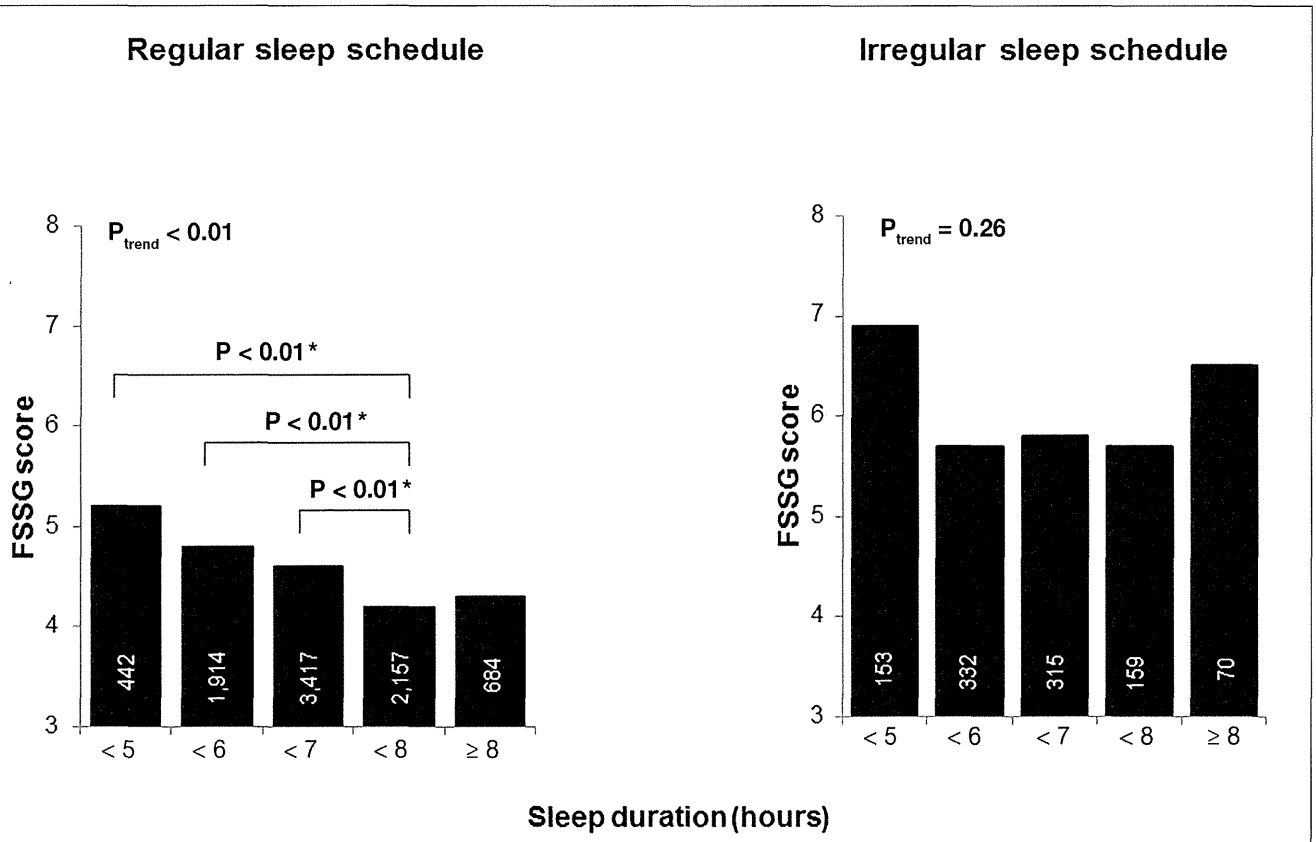

Figure 1—FSSG score for participants by sleep duration and with or without a regular sleep schedule. The bars represent the mean FSSG scores in each group. The numeral in each bar represents the number of participants in each group. The asterisk is explained as follows: The comparisons of FSSG score among groups categorized by sleep duration were performed with Dunnet test using the group with 7 h to less than 8 h per day sleep duration as the reference. In participants with regular sleep schedule, there were significant differences between the reference group and each of group with less than 5 h, 5 to less than 6 h, and 6 to less than 7 h per day sleep duration, whereas there was no significant differences with the group with 8 or more h per day sleep duration. However, in participants with an irregular sleep schedule, the significant difference was not found between the reference and any of the other groups. FSSG, Frequency Scale for the Symptoms of GERD. GERD, Gastroesophageal reflux disease.

Table 3—Frequency Scale for the Symptoms of Gastroesophageal Reflux Disease scores in subjects with or without each examined dietary behavior.

		n	FSSG score	P
Dinner within 2 h of sleep	+	1,785	5.4 ± 5.5	< 0.01
	−	7,858	4.5 ± 4.8	
Snacking after dinner	+	2,013	5.4 ± 5.1	< 0.01
	−	7,630	4.5 ± 4.9	
Rapid eating	+	3,374	5.0 ± 5.2	< 0.01
	−	6,269	4.5 ± 4.8	
Skipping breakfast	+	887	6.0 ± 5.7	< 0.01
	−	8,756	4.6 ± 4.9	

FSSG score values are expressed as mean ± standard deviation. Statistical significance was assessed by analysis of variance.

Model 1). Results showed that both GERD and the number of unfavorable dietary behaviors were independently associated with short sleep duration, even in the analysis that did not include participants having an irregular sleep schedule. (Table 4, Model 2) No interaction was observed between GERD and the number of unfavorable dietary habits (P = 0.82).

DISCUSSION

The current result showed that the frequency of GERD as well as the number of unfavorable dietary behaviors were also increased with decreasing sleep duration, and that both GERD symptoms and unfavorable dietary behaviors were associated with short sleep duration independently of other clinical variables in a large sample from the general population. To the best of our knowledge, this is the first study that showed the prevalence of GERD in the general population according to their sleep duration, and evaluated GERD symptoms and dietary behaviors comprehensively to determine if they were significant correlates of short sleep duration in a large sample from the general population.

Several previous studies have investigated the associations between only two of these three factors, i.e., sleep duration, GERD symptoms, and dietary behaviors. Matsuki et al. showed in their hospital-based study that subjects with GERD symptoms were more likely to report short sleep duration than those without such symptoms[14] and suggested that the relationship between sleep duration and GERD was bidirectional based on

other previous studies.[24–26] With regard to the correlation between dietary behaviors and sleep duration, Kim et al. found in their epidemiologic study that female subjects with short sleep duration tended to eat meals during unconventional hours.[17] Persons with short sleep duration may tend to go to bed later and thereby have more opportunities to eat at later hours. Change in the physiological regulation of metabolic hormones that influence diet and eating patterns is another possible explanation.[27] In addition, a positive association between GERD symptoms and unfavorable dietary behaviors also was reported.[16,28] However, no previous study has investigated whether GERD and dietary behaviors are independently associated with sleep duration. This is the first study to clarify that both GERD symptoms and unfavorable dietary behaviors were correlated with short sleep duration independently of each other.

We evaluated sleep duration with a questionnaire. Although sleep duration examined by an objective measurement such as actigraphy may be desirable, self-reported sleep duration assessment was reported to be as valid as objective measurements.[29] Because individuals with a short sleep duration were more likely to have an irregular sleep schedule, there might be a misperception of sleep duration in this group.[30] However, in our analysis GERD and dietary behaviors remained significant determinants of sleep duration, except for in participants with an irregular sleep schedule. This finding emphasizes the result that GERD symptoms and dietary behaviors were associated with sleep duration independently of each other. In the current study, we did not obtain data about the specific types of sleep problems, such as difficulty getting to sleep and early morning awakening. Investigations of sleep problems specifically correlated with GERD symptoms and dietary behavior are warranted.

We also evaluated the severity of GERD symptoms with the questionnaire. Several diseases, such as functional dyspepsia and nonerosive reflux disease, can cause GERD symptoms; the sensitivity of FSSG scale for detecting the patients with abnormal endoscopic findings of GERD has been reported to be 60%.[21] Further, the severity of GERD symptoms is not always proportional to that of findings in endoscopy and pH monitoring.[31,32] Therefore, further studies may be needed to examine whether the objectively measured GERD findings are a stronger explanation of short sleep duration than self-reported GERD symptoms.

In the current study, female sex, older age, and having a higher BMI were also positively associated with short sleep duration. Whereas many preceding studies reported a positive association between short sleep duration and obesity,[1–3] the relationship between sleep duration and sex or age was inconsistent in previous studies.[19,33–35] Although this finding may be caused by different ethnic and cultural influences or lifestyles, these previous studies did not take into account GERD symptoms and dietary behaviors as the determinants of sleep duration, which might explain these conflicting results. By taking

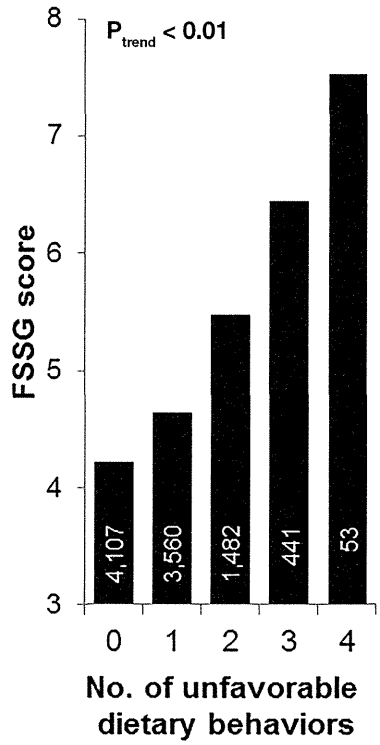

Figure 2—Association between FSSG score and the number of unfavorable dietary behaviors. The bars represent the mean FSSG score in each group. The numeral in each bar represents the number of participants in each group. FSSG, Frequency Scale for the Symptoms of GERD. GERD, Gastroesophageal reflux disease.

Table 4—Multivariate logistic regression analysis to determine the factors identifying participants with short sleep duration.

	Model 1 (n = 9,643)		Model 2 (n = 8,614)	
	Odds ratio (95%CI)	P	Odds ratio (95%CI)	P
Female	1.43 (1.27-1.60)	< 0.01	1.45 (1.28-1.65)	< 0.01
Age	1.00 (1.00-1.01)	0.02	1.00 (0.99-1.01)	0.09
Body mass index	1.02 (1.01-1.04)	< 0.01	1.02 (1.01-1.04)	0.01
Current smoker	0.92 (0.80-1.05)	0.22	0.90 (0.77-1.05)	0.17
Frequent alcohol drinker	0.94 (0.83-1.06)	0.33	0.99 (0.86-1.13)	0.83
Irregular sleep schedule	2.26 (1.97-2.58)	< 0.01	–	–
Taking hypnotic drugs	1.59 (1.32-1.92)	< 0.01	1.69 (1.38-2.07)	< 0.01
Taking analgesic drugs	1.15 (0.91-1.47)	0.24	1.11 (0.85-1.43)	0.44
Gastroesophageal reflux disease	1.19 (1.07-1.32)	< 0.01	1.19 (1.06-1.33)	0.03
No. unfavorable dietary behaviors	1.19 (1.13-1.26)	< 0.01	1.20 (1.13-1.27)	< 0.01

these factors into account, the current study led us to a more sophisticated evaluation of the relationship between sleep duration and age or sex compared with previous studies.

We recognize the limitations of this study. First, the questionnaire that we adopted could not evaluate the sleep quality of each participant in detail. Second, we did not assess details of participants' socioeconomic background such as income, education level, and marital status, factors that were also reported to be associated with sleep duration.[34,36] However, because Jansson et al. reported that GERD symptoms were associated with sleep problems independently of socioeconomic status, these factors might not have materially affected the current results.[12] Third, because this study was based on cross-sectional observations, we could not show a causal relationship between sleep duration and GERD symptoms or dietary behaviors. To clarify the causal relationship among them, further studies investigating whether clinical interventions for GERD and dietary behaviors improve sleep shortage are warranted.

In conclusion, GERD symptoms and unfavorable dietary behaviors were significantly associated with short sleep duration in the general population independently from each other. Further studies are warranted to investigate whether interventions for GERD and dietary behaviors lead to improvement of sleep shortage.

DISCLOSURE STATEMENT

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

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See the appendix for the members of the Nagahama Study Group.

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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*, *LRRC4C*, and *B4GALNT2*.Seven additional gene associations were replicated in our gene-based analyses: *GRIA4*, *BMP2*, *QKI*, *BMP4*, *SFRP1*, *SH3GL2*, and *EHBP1L1*. 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

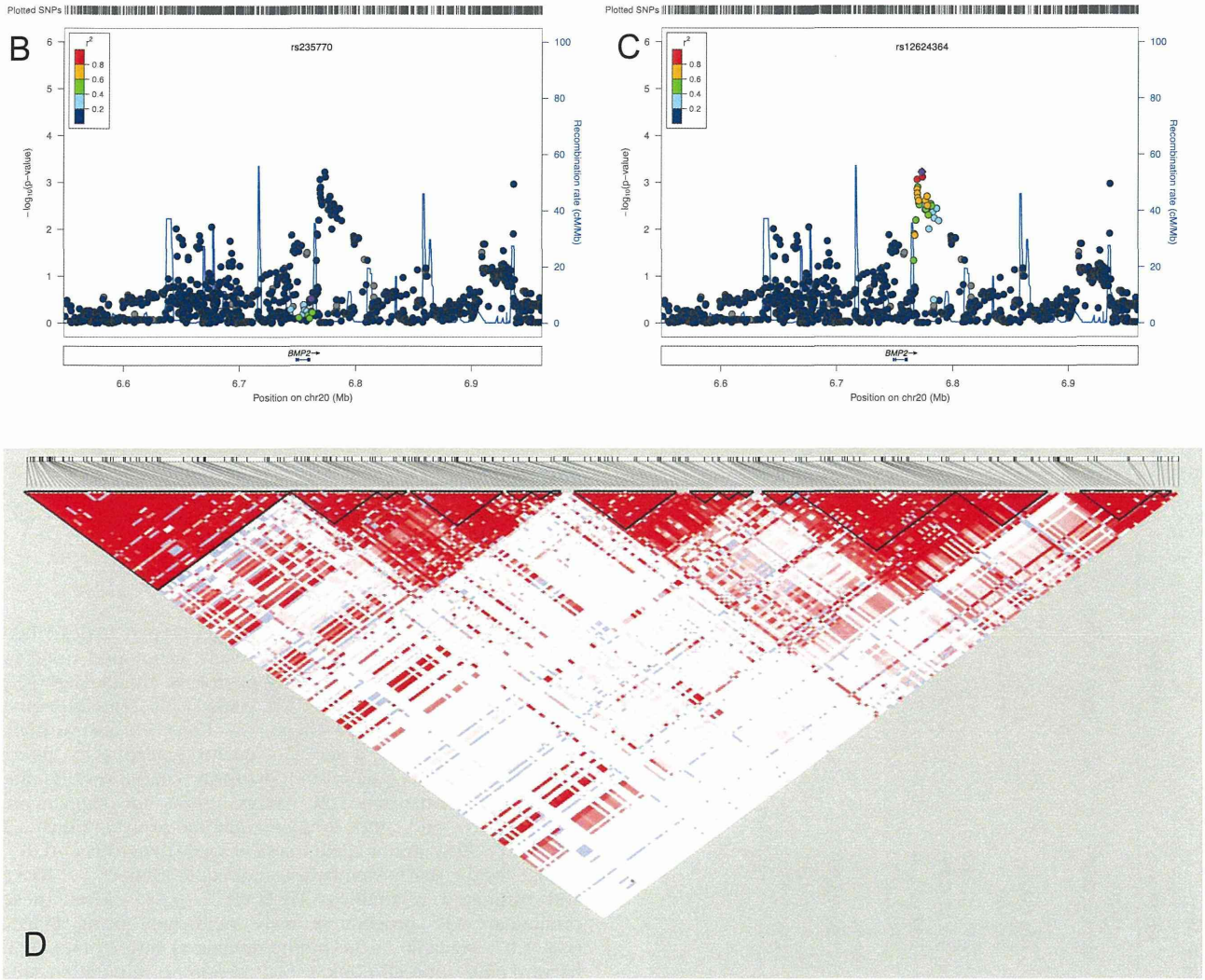

Figure 1. Description and illustration of three replication methods used in our analysis, using *BMP2* as an example. (**A**) Definitions of the three methods are summarized. (**B**, **C**) Association plots of the SNPs near *BMP2* in our dataset, showing the target SNPs of per-SNP analysis (**B**) and 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, *n*	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, *n* (%)						
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 ($>1 \times 10^{-6}$), 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 [$>1 \times 10^{-7}$]). 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	P†
Patients, *n*	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
GPR25	rs6702767	1	200844547	0.26	G/A	−0.05	0.08	0.54
CD55	rs1652333	1	207470460	0.44	G/A	−0.14	0.07	0.043
PABPCP2	rs17412774	2	146773948	0.36	A/C	−0.08	0.07	0.23
DLX1	rs17428076	2	172851936	0.03	G/C	0.21	0.21	0.31
PRSS56	rs1656404	2	233379941	0.02	A/G	0.05	0.19	0.78
PRSS56	rs1550094	2	233385396	0.09	G/A	−0.05	0.11	0.67
CHRNG	rs1881492	2	233406998	0.16	T/G	−0.05	0.10	0.62
SETMAR	rs1843303	3	4185124	0.46	T/C	−0.01	0.07	0.94
LOC100506035	rs9307551	4	80530671	0.34	A/C	0.06	0.07	0.36
BMP3	rs1960445 (rs4458448)	4	81927206	0.03	T/C	0.24	0.18	0.20
BMP3	rs5022942	4	81959966	0.34	A/G	0.14	0.07	0.0496
KCNQ5	rs7744813	6	73643289	0.21	C/A	0.23	0.08	0.0026
QKI	rs9365619	6	164251746	0.34	A/C	−0.01	0.07	0.87
ZMAT4	rs7829127	8	40726394	0.07	G/A	0.17	0.13	0.20
SFRP1	rs2137277	8	40734662	0.04	G/A	0.14	0.16	0.39
TOX	rs7837791	8	60179086	0.46	G/T	0.02	0.07	0.80
TOX	rs72621438	8	60178580	0.47	G/C	0.03	0.07	0.65
CHD7	rs4237036	8	61701057	0.20	C/T	0.04	0.08	0.62
SH3GL2/ ADAMTSL1	rs10963578	9	18338649	0.33	A/G	0.09	0.07	0.20
RORB	rs7042950	9	77149837	0.31	A/G	0.04	0.07	0.54
BICC1	rs7084402	10	60265404	0.49	A/G	0.17	0.06	0.010
BICC1	rs4245599	10	60365755	0.46	G/A	0.20	0.06	0.0019
KCNMA1	rs6480859	10	79081948	0.17	T/C	−0.07	0.09	0.44
RGR	rs745480	10	85986554	0.33	C/G	0.03	0.07	0.65
CYP26A1	rs10882165	10	94924324	0.04	T/A	−0.40	0.18	0.023
LRRC4C	rs1381566	11	40149607	0.22	G/T	−0.16	0.08	0.040
DLG2	rs2155413	11	84634790	0.21	C/A	0.06	0.08	0.46
GRIA4	rs11601239	11	105556598	0.34	G/C	0.06	0.07	0.36
PZP	rs6487748	12	9435768	0.34	G/A	−0.13	0.07	0.069
RDH5	rs3138142	12	56115585	0.02	T/C	0.30	0.21	0.16
PTPRR	rs12229663	12	71249996	0.38	G/A	0.10	0.07	0.14
ZIC2	rs8000973	13	100691367	0.25	C/T	−0.11	0.08	0.14
ZIC2	rs4291789	13	100672921	0.27	G/A	−0.11	0.08	0.14
PCCA	rs2184971	13	100818092	0.29	A/G	0.02	0.07	0.83
BMP4	rs66913363	14	54413001	0.22	C/G	0.08	0.08	0.33
66	rs1254319	14	60903757	0.38	G/A	0.05	0.07	0.44
GJD2	rs524952	15	35005886	0.48	A/T	−0.30	0.07	3.7E-06
RASGRF1	rs4778879	15	79372875	0.49	A/G	0.22	0.07	0.00094
RASGRF1	rs28412916	15	79378167	0.48	A/C	0.21	0.07	0.0014
RBFOX1	rs17648524	16	7459683	0.05	C/G	−0.19	0.15	0.19
SHISA6	rs2969180	17	11407901	0.46	G/A	0.11	0.07	0.084
SHISA6	rs2908972	17	11407259	0.45	C/A	0.10	0.07	0.12
B4GALNT2	rs9902755	17	47220726	0.16	C/T	0.19	0.09	0.039
KCNJ2	rs4793501	17	68718734	0.44	T/C	−0.01	0.07	0.83
CNDP2	rs12971120	18	72174023	0.32	G/A	0.09	0.07	0.20
BMP2	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§	$P_{corrected}$‖
GPR25	rs91564	1	200893050	0.05	T/C	0.27	0.0044	21	0.093
CD55	rs12116783	1	207556770	0.08	A/G	0.22	0.0045	7	0.031
PABPCP2	rs10202376	2	147315208	0.77	T/C	0.22	0.14	6	0.85
DLX1	rs79886888	2	173004317	0.17	T/C	0.28	0.10	34	1
PDE11A	rs13006877	2	178984328	0.32	T/A	−0.20	0.0043	32	0.14
PRSS56	rs115279622	2	233375977	0.37	T/C	−0.65	0.0065	40	0.26
CHRNG	rs12617942	2	233416068	0.02	T/C	−0.73	0.017	37	0.63
SETMAR	rs79901438	3	4391460	0.15	G/T	0.20	0.015	23	0.34
CACNA1D	rs73841203	3	53875801	0.27	G/A	0.39	0.0020	122	0.24
ZBTB38	rs1993904	3	141003354	0.02	T/C	0.32	0.0016	88	0.14
LOC100506035	rs9684343	4	80546040	0.10	G/C	0.21	0.051	10	1
ANTXR2	rs11099009	4	80988658	0.08	A/G	−0.24	0.023	35	0.80
BMP3	rs7659948	4	81979993	0.31	C/T	0.17	0.039	19	0.74
KCNQ5	rs6929988	6	73914319	0.44	A/G	0.28	4.7E-05	102	0.0048
LAMA2	rs10080659	6	129817349	0.03	T/C	0.23	0.0016	82	0.13
QKI	rs9346961	6	163905968	0.10	T/C	−0.89	5.2E-05	32	0.0017
ZMAT4	rs7816960	8	40354396	0.18	A/C	−0.29	0.0020	55	0.11
SFRP1	rs148016338	8	41103891	0.04	A/G	1.07	0.00074	19	0.014
TOX	rs139199809	8	59755748	0.02	C/T	0.89	0.0031	72	0.22
CHD7	rs6984384	8	61809929	0.21	C/T	−0.31	0.0068	40	0.27
SH3GL2/ (ADAMTSL1)	rs10963177	9	17639458	0.50	C/T	0.24	0.00042	106	0.044
(SH3GL2) /ADAMTSL1	rs16937047	9	18770943	0.36	T/C	−0.26	0.00067	216	0.14
TJP2	rs4515614	9	71742683	0.02	T/C	−0.86	0.0091	44	0.40
RORB	rs11144053	9	77284559	0.27	G/A	−0.25	0.02886	45	1
BICC1	rs893369	10	60360901	0.01	T/A	0.23	0.00052	34	0.018
KCNMA1	rs11001900	10	78606671	0.22	A/G	0.22	0.00086	256	0.22
RGR	rs11817115	10	86018811	0.02	G/A	−0.31	0.0032	16	0.051
CYP26A1	rs117520829	10	94791300	0.05	G/C	−0.51	0.0034	19	0.065
TCF7L2	rs12573128	10	114730797	0.27	A/C	0.16	0.030	120	1
LRRC4C	rs58287560	11	40810557	0.38	C/A	0.25	0.00060	168	0.10
EHBP1L1	rs931127	11	65405300	0.12	A/G	0.21	0.0013	19	0.025
DLG2	rs145062356	11	83631501	0.03	A/G	−1.00	0.00080	359	0.29
GRIA4	rs78925386	11	105753469	0.05	A/C	−0.96	0.0018	27	0.049
PZP	rs717180	12	9395807	0.05	A/G	0.20	0.011	17	0.19
RDH5	rs11171667	12	56131052	0.13	A/C	−0.20	0.054	23	1
PTPRR	rs151294916	12	71325795	0.04	G/A	−0.75	0.0062	51	0.32
ZIC2	rs35140645	13	100649321	0.39	G/A	−0.18	0.014	23	0.32
PCCA	rs9513744	13	100935665	0.01	T/A	−0.80	0.0018	44	0.081
LRFN5	rs79467137	14	42096662	0.03	A/T	−0.54	0.0068	35	0.24
BMP4	rs7149027	14	54473305	0.50	A/G	0.36	0.00079	18	0.014
66	rs1015119	14	61027510	0.60	C/T	−0.19	0.040	2	0.080
GJD2	rs589135	15	35001442	0.27	C/G	−0.31	1.8E-06	45	0.000082
RASGRF1	rs57488047	15	79403002	0.51	C/T	0.25	0.00031	81	0.025
RBFOX1	rs79266634	16	7309047	0.54	A/G	0.40	0.00074	649	0.48
SHISA6	rs11651793	17	11267101	0.15	G/A	0.30	0.0083	105	0.88
MYO1D	rs117769171	17	30852727	0.45	C/T	−0.84	0.0049	71	0.35
B4GALNT2	rs4438351	17	47240493	0.20	C/T	0.21	0.0025	31	0.079
KCNJ2	rs11077480	17	68214161	0.12	A/G	0.45	0.012	15	0.18
NPLOC4	rs76645549	17	79645253	0.12	G/A	0.20	0.0096	42	0.40
CNDP2	rs78754702	18	72155813	0.32	G/A	−0.79	0.0054	49	0.27
BMP2	rs12624364	20	6773370	0.49	A/G	−0.23	0.00059	36	0.021

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

HapMap 2 CHB+JPT was used as the reference.

* SNPs within these genetic regions ± 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 Symbol	CHR	Position NCBI37/hg19		SNP-Based	Gene-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.

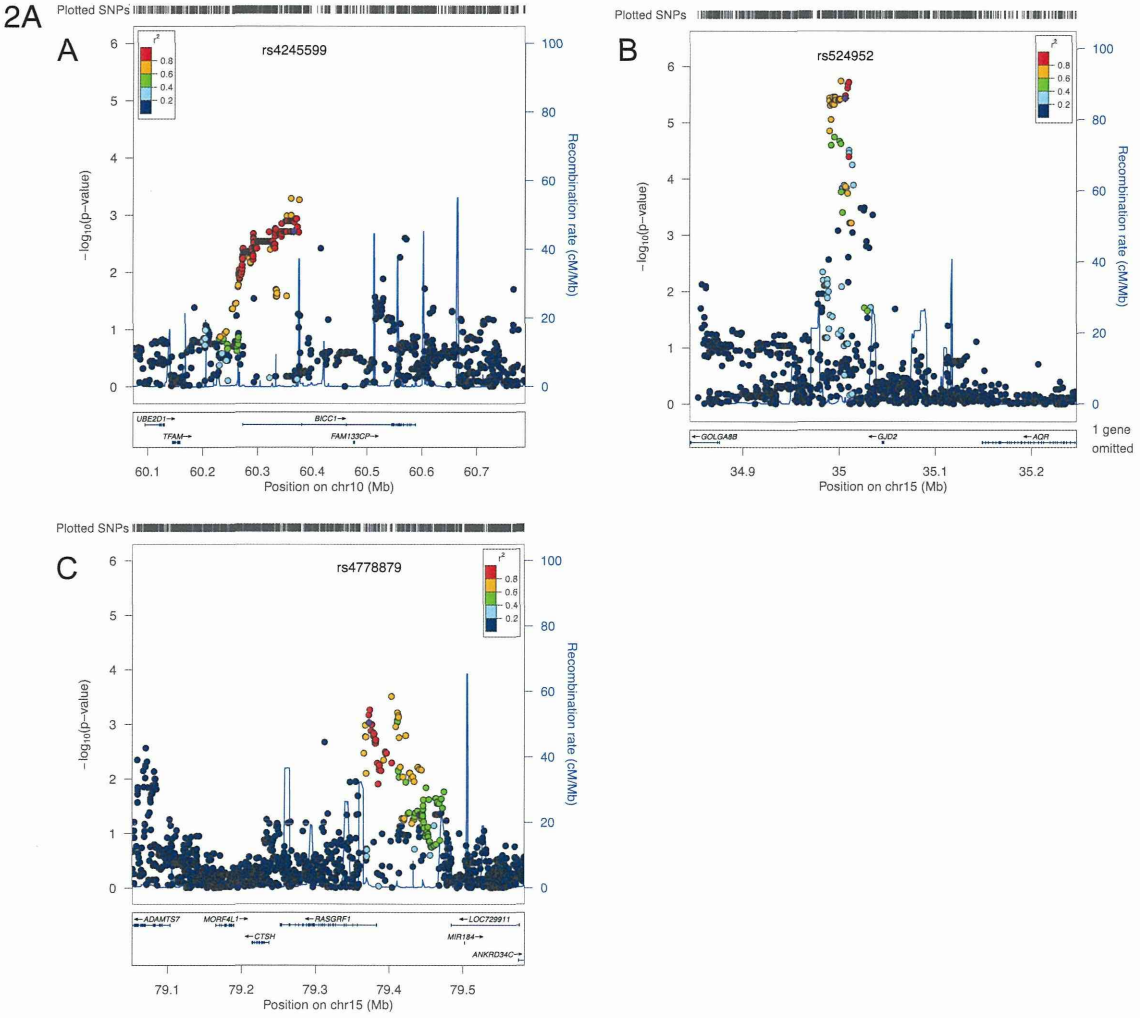

Figure 2.

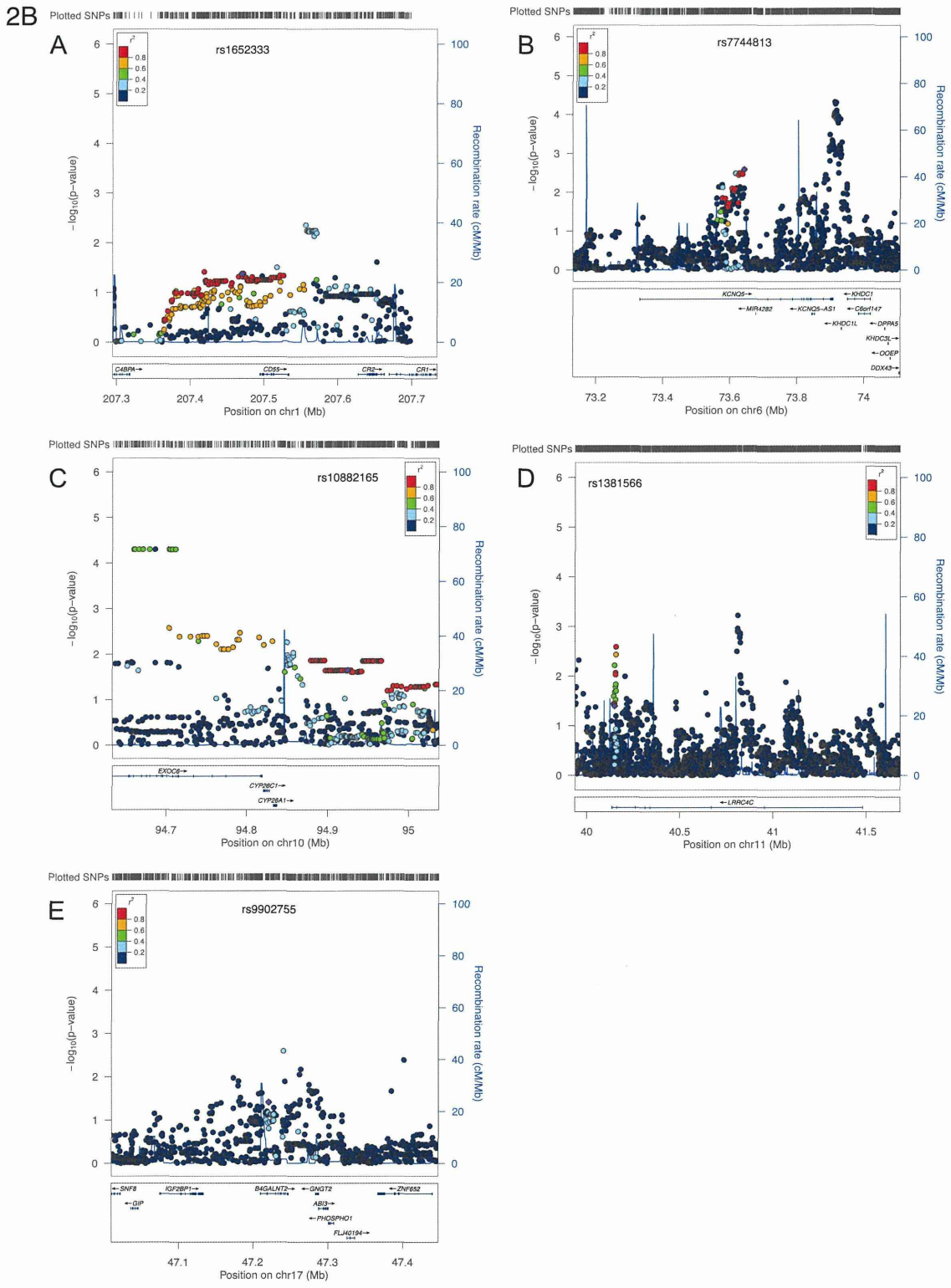

Figure 2. Continued Association plots of the eight genes that were significantly replicated in our per-SNP analysis. Reported SNPs near *BICC1*, *GJD2*, and *RASGRF1* showed strong associations with MSE and composed one of the peak signals in our dataset (**A**, *A–C*). In contrast, association signals of the reported SNPs of *CD55*, *KCNQ5*, *CYP26A1*, *LRRC4C*, 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.

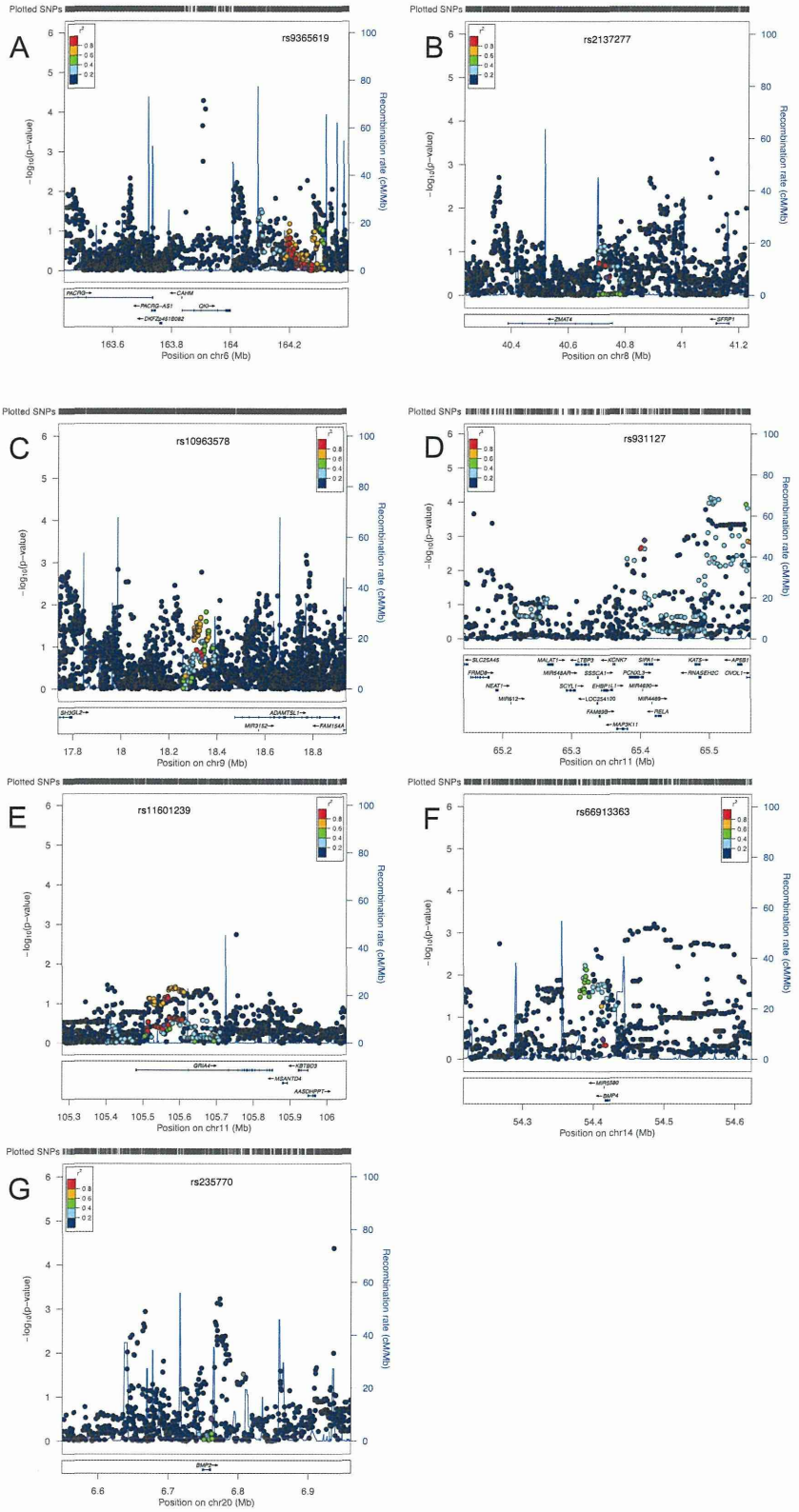

Figure 3. Association plots of the SNPs within seven genetic regions near *QKI*, *SFRP1*, *SH3GL2*, *EHBP1L1*, *GRIA4*, *BMP4*, and *BMP2* that were replicated in our gene-based analyses but failed to be replicated in our per-SNP analysis. Reported SNPs are highlighted in *purple* and SNPs within high LD to the reported SNPs are colored according to the strength of LD. Reported SNP of *EHBP1L1* 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 *EHBP1L1* that showed marginally significant association with myopia in the 23andMe GWAS. We successfully replicated *EHBP1L1* 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 *EHBP1L1* 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, *LRRC4C, QKI, BMP4, SFRP1, SH3GL2, B4GALNT2,* and *EHBP1L1* 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 $\geq$ 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

The following investigators were core members of the Nagahama Cohort Research Group: Takeo Nakayama (Department of Health Informatics, Kyoto University School of Public Health, Kyoto, Japan), Akihiro Sekine (Center for Genomic Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan), Shinji Kosugi (Department of Medical Ethics, Kyoto University School of Public Health, Kyoto, Japan), Takahisa Kawaguchi (Center for Genomic Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan), Ryo Yamada (Center for Genomic Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan), Yasuharu Tabara (Center for Genomic Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan), and Fumihiko Matsuda (Center for Genomic Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan).

Association of Serum–Free Fatty Acid Level With Reduced Reflection Pressure Wave Magnitude and Central Blood Pressure

The Nagahama Study

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Abstract—Central blood pressure (BP) has been suggested to be a better predictor of cardiovascular disease risk than brachial BP. Given that central BP and arterial waveform are both influenced by insulin resistance, major initiators of insulin resistance, such as serum–free fatty acid (FFA), are suspected of potentially being involved in central hemodynamics. To confirm that insulin signaling is an important modulator of central hemodynamics, we investigated this hypothesis in a large-scale general population. Brachial BP and radial arterial waveform were measured simultaneously in 9393 middle-aged to elderly individuals. The augmentation index was calculated from the radial waveform as the ratio of the height of the late systolic peak to that of the first peak. Central systolic BP was defined as the absolute pressure of the late systolic peak of the waveform. Differences in central and brachial pulse pressure (PP) were considered to represent PP amplification. PP amplification differed significantly among serum FFA level quartiles (Q1, 7.8±5.3; Q2, 8.6±5.0; Q3, 9.3±5.7; Q4, 10.3±6.1 mm Hg; $P<0.001$), and the maximum difference in combination with diabetes mellitus status was 4.9 mm Hg. Multivariate analysis adjusted for major covariates indicated that higher serum FFA was an independent determinant for higher PP amplification ($\beta=0.145$, $P<0.001$) and lower augmentation index ($\beta=-0.122$, $P<0.001$) and central systolic BP ($\beta=-0.044$, $P<0.001$), whereas the association between FFA and PP amplification significantly decreased ($\beta=0.022$, $P<0.001$) after further adjustment for augmentation index. Serum FFA is an overlooked factor favorably influencing central hemodynamics. A low-magnitude reflection pressure wave might be involved in this paradoxical relationship. (***Hypertension*. 2014;64:1212-1218.**) • Online Data Supplement

Key Words: aortic blood pressure ■ free fatty acid ■ insulin resistance ■ pulse wave analysis

Hypertension is a leading cause of cardiovascular disease, with brachial blood pressure (BP) being a standard measure in the assessment of arterial pressure load. However, central BP estimated from the radial arterial waveform has recently been suggested to be more closely associated with cardiovascular outcomes than brachial BP.[1–3] In addition to these epidemiological findings, clinical studies from several groups[4–6] and our own[7] have suggested that antihypertensive drugs might exert different effects on arterial waveform and central BP, possibly resulting in different cardiovascular outcomes. The Conduit Artery Function Evaluation substudy[4] of the Anglo-Scandinavian Cardiac Outcomes Trial demonstrated that calcium channel blockers were superior to β-blockers for reducing cardiovascular events. This effect was presumably because of the central systolic BP (SBP) being lower in the calcium channel blocker treatment arm, whereas no class-specific effects were observed regarding brachial SBP. The apparent influence of central BP on cardiac outcomes highlights the importance of identifying factors that might affect central BP levels.

Several factors have been reported to influence central BP levels by altering the arterial pressure waveform,[8] a composite waveform of the forward pressure wave generated by cardiac ejection and the backward pressure wave reflected at peripheral sites. Arterial stiffness causes the early return of reflection pressure waves from peripheral sites and thus increases overlaps between forward and reflection pressure waves at the aorta, which increase central SBP. Other factors also influence arterial waveform, such as tall stature greatly decreasing the overlap of the 2 waveforms by delaying the arrival of the

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reflection pressure wave and increased heart rate (HR) reducing the overlap by shortening the cardiac ejection period.

Curiously, type 2 diabetes mellitus and insulin resistance have been favorably associated with central hemodynamics. Several groups[9,10] and our own[11] have shown that individuals with type 2 diabetes mellitus had relatively low central SBP, despite the well-established pathogenicity of diabetes mellitus for arterial stiffness and cardiovascular diseases. Although the mechanisms behind this paradoxical relationship are unclear, a possible explanation is reduced magnitude of the reflection pressure wave[12] because of a stiffer aortic artery and consequently larger penetration of pulsatile energy into the microcirculation.[13,14] Insulin-mediated vasoconstriction under insulin-resistant conditions[15] might also be involved in the increased transmission of pulsatile energy.

Free fatty acid (FFA) is a major initiator of insulin resistance.[15,16] FFA blocks insulin signaling via phosphorylation of insulin receptor substrate 1, which inhibits translocation of glucose transporter to the cell membrane and reduces glucose uptake.[15] Further, FFA has been shown to reduce endothelium-dependent vasodilation by decreasing endothelial nitric oxide production.[14] Given these molecular bases of FFA in initiation of insulin resistance, we hypothesized that serum FFA levels might also be associated with central hemodynamics. Proving our hypothesis would further support the involvement of insulin signaling in central hemodynamic control and would help to further understand the basis of paradoxical relationship between insulin resistance and better central hemodynamic profile.

Here, we investigated our hypothesis using a data set from the Nagahama Prospective Cohort for Comprehensive Human Bioscience (the Nagahama Study), a large-scale population-based cohort study in Japan.

Methods

Study Subjects

Study subjects were 9393 apparently healthy middle-aged to elderly citizens who had participated in the Nagahama Study. This study cohort was recruited from 2008 to 2010 from the general population of Nagahama City, a largely suburban city of 125 000 inhabitants in central Japan. Community residents aged 30 to 74 years, living independently and with no physical impairment or dysfunction, were recruited. Of 9804 total subjects, those meeting any of the following conditions were excluded from this study: history of symptomatic cardiovascular diseases (n=266), taking insulin therapy (n=22), unsuccessful assessment of clinical parameters required for this study (n=80), and pregnant women (n=43).

Of the 9393 subjects remaining after exclusion, individuals with available fasting blood specimens (>11 hours) were used as the study panel (n=4322), whereas those with peripheral blood samples drawn within 10 hours of their last meal were used as the replication panel (n=5071).

All study procedures were approved by the ethics committee of Kyoto University Graduate School of Medicine and the Nagahama Municipal Review Board. Written informed consent was obtained from all participants.

BP Measurement

Radial arterial waveform, brachial BP, and HR were measured simultaneously (HEM-9000AI; Omron Healthcare, Kyoto, Japan) after 5 minutes resting in the sitting position. Briefly, brachial BP was measured at the right upper arm using a cuff-oscillometric device, and the radial arterial waveform was simultaneously obtained from the left wrist using a multielement tonometry sensor. The augmentation index (AIx) was calculated from the radial arterial waveform as the ratio of the height of the late systolic peak (SBP2) to the first systolic peak. The absolute pressure of SBP2 obtained by calibrating the first systolic peak with brachial SBP was considered to represent the central SBP. Pulse pressure (PP) amplification was calculated by subtracting central PP from brachial PP and expressed in absolute values (mm Hg). Measurements were taken twice, and the mean value of these measurements was used in analysis. The validity of SBP2 in estimating central SBP has been demonstrated by invasive simultaneous measurement of the ascending aorta and radial artery pressure.[17,18] We also previously reported that radial SBP2 was closely related to the central SBP calculated by the widely used generalized transfer function.[19] Mean BP was calculated using the following formula: Mean BP=diastolic BP +(SBP−diastolic BP)/3.

Clinical Parameters

Basic clinical parameters were measured at the baseline examination of the Nagahama cohort study. Serum FFA levels were quantified using an enzymatic assay (NEFA-HR; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Intra- and interassay coefficients of variation in FFA measurements were 1.42% and 1.79%, respectively. Homeostasis model assessment of insulin resistance was calculated as an index of insulin resistance using the following formula: [insulin (IU/l)×glucose (mg/dL)]/405.

Assessment of Arterial Stiffness

Arterial stiffness was assessed by pulse wave velocity (PWV) measured between the brachia and ankle (baPWV). Briefly, cuffs were applied to both brachia and ankles, and BP was measured simultaneously in the supine position using a cuff-oscillometric device (Vasera-1500; Fukuda Denshi, Tokyo, Japan). Pulse volume waveforms were also recorded simultaneously using a plethysmographic sensor connected to the cuffs. The baPWV was calculated from the time interval between the wave fronts of the brachial and ankle waveforms and the path length from the brachia to ankle (0.597×height+14.4014).[20] The colinearity of baPWV with a carotid-to-femoral PWV, a standard measure of arterial stiffness, has been previously reported.[21]

Statistical Analysis

Quartile of PP amplification and serum FFA level was calculated for each sex and then combined to avoid potential sex differences. Differences in numeric parameters among subgroups were assessed by analysis of variance, whereas the frequency of differences among subgroups was evaluated using a χ^2 test. Factors independently associated with PP amplification and AIx were assessed by multiple linear regression analysis. Statistical analysis was performed using JMP 9.0.3 software (SAS Institute, Cary, NC, USA). $P<0.05$ indicated statistical significance.

Results

Clinical characteristics of study subjects are summarized in Table 1. Plasma levels of triglycerides, insulin, and FFA were slightly higher in the replication panel than in the study panel ($P<0.001$), whereas no marked differences were observed for other parameters.

Table 2 shows the differences in metabolic parameters among quartiles of PP amplification. Subjects with larger PP amplification were markedly younger and taller and had faster HR than those with less amplification. Although several clinical parameters significantly differed among quartiles in crude analysis, parameters for insulin resistance, including FFA levels, remained significant even after adjustment for major covariates.

As a whole, women had significantly higher FFA levels than men (Figure 1). Older age ($r=0.087$, $P<0.001$), lower body mass index ($r=-0.084$, $P<0.001$), increased high-density lipoprotein