CLINICAL INVESTIGATIONS

# Tooth Loss and Atherosclerosis: The Nagahama Study

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Abstract: *Several epidemiologic studies have suggested that oral disease is a risk factor for cardiovascular disease (CVD). However, whether a clinically significant association exists between the 2 disorders remains controversial. Here, we investigated the association between tooth loss, as an indicator of oral disease, and arterial stiffness, as a marker of atherosclerosis, in Japanese adults. Cross-sectional data were collected for 8,124 persons aged 30 to 75 y with no history of tooth loss for non-inflammatory reasons, such as orthodontic treatment, malposition, and trauma. Participants received a comprehensive dental examination and extensive in-person measurements of CVD risk factors, and arterial stiffness was evaluated using the cardio-ankle vascular index (CAVI). We examined the association between CAVI and tooth loss using general linear models with adjustment for age, sex, body mass index, smoking status, hemoglobin A1c, and a history of insulin or hypoglycemic medication depending on the model. In addition, we performed an analysis that included interaction terms of the centered variables tooth loss, sex, and age. The results of the multiple regression analysis that included the interaction terms detected that the relationship between CAVI and tooth loss was dependent on sex, with only men showing a positive correlation (β for interaction = 0.04; 95% confidence interval, 0.02–0.06). The findings from this study suggest that a linear relationship exists between tooth loss and degree of arterial stiffness and that the association differed depending on sex.*

Key Words: arterial stiffness, epidemiology, inflammation, periodontal disease, cross-sectional analysis, cardiovascular diseases.

## Introduction

Cardiovascular disease (CVD) is the most common cause of death and disability in industrialized nations and has a high cost to society. The coincidence of cardiovascular and oral disease is relatively high, and numerous studies have reported that a positive association exists between these 2 diseases (Beck et al. 1998; Beck et al. 2001; Hujoel et al. 2001; Desvarieux et al. 2003; Pussinen et al. 2003; Desvarieux et al. 2004; Ylostalo et al. 2006; Tonetti et al. 2007; Tu et al. 2007; Dietrich et al. 2008; Senba et al. 2008; Choe et al. 2009; de Oliveira et al. 2010; Kim et al. 2010). However, as a significant relationship was not detected in several studies (Hujoel et al. 2000; Lavelle 2002; Colhoun et al. 2008), it remains controversial whether a clinically significant association exists between the 2 diseases (Hujoel et al. 2000; Lavelle 2002; Lockhart et al. 2012).

Inflammation plays an important role in the pathogenesis of CVD. Systemic inflammation may represent the underlying mechanism that links oral and cardiovascular diseases. Oral disease, such as periodontal disease, is characterized by chronic systemic inflammation and often results in tooth loss due to the breakdown of periodontal tissue. Therefore, tooth loss is a useful proxy for the accumulated burden of inflammatory disease (Desvarieux et al. 2003; Houshmand et al. 2012).

Elderly people and men carry a disproportionate burden of CVD and oral disease. Therefore, age and sex adjustment must be performed when evaluating the potential link between oral disease and CVD. Moreover, as oral disease and CVD share common risk factors, including smoking, diabetes, hypertension, and obesity, the potential for confounding is substantial. Thus, the power for detecting effect modification is limited in small population studies.

Arterial stiffness, as assessed by the cardio-ankle vascular index (CAVI), is

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a measure of CVD (Kadota et al. 2008). CAVI was developed to overcome the dependency of pulse-wave velocity (PWV) measurements on blood pressure. The underlying principle of CAVI measurement is based on the stiffness parameter β and is calculated using the Bramwell-Hill formula, which is basically independent of blood pressure (Yambe et al. 2004; Shirai et al. 2006; Takaki et al. 2008; Shirai et al. 2011). Thus, CAVI is an easily administered arterial stiffness screening test that ensures good reproducibility as a diagnostic tool for CVD. Here, we investigated the relationship between tooth loss and arterial stiffness using baseline survey data in a population-based cohort.

## Methods

### Ethics Statement

This study was approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee, the Ad Hoc Review Board of the Nagahama Study, and the Nagahama Municipal Review Board of Personal Information Protection. Appointments for health examinations were made by telephone by the municipal government staff, and participant registration was performed at the site of the health examinations. Written informed consent was also obtained from all participants prior to their health examination.

### Study Design and Population

The Nagahama Prospective Genome Cohort for the Comprehensive Human Bioscience (the Nagahama Study) is a population-based prospective cohort study of a broad range of chronic illnesses and was conducted in Nagahama City, Shiga Prefecture, Japan (Tabara et al. 2013). The present study is a prospective study that collected data by means of questionnaires, anthropometric and physiologic measures, biochemical measurements of blood samples, genomic information, and oral examinations. The Nagahama Study participants were recruited from apparently healthy community residents living in Nagahama City, a largely rural city of approximately 125,000 inhabitants in Shiga Prefecture, which is located in central Japan. A baseline survey was conducted between fiscal years 2008 and 2010. Information on the project was provided to potential participants by newsletters, newspaper flyers, and brochures and on the homepages of the local government and citizen organizations. Information sessions for the residents were also held by researchers and city employees, who explained the project to interested residents. Residents of Nagahama City who fulfilled the following criteria were recruited for the cohort study: 1) age between 30 and 74 years old at the time of recruitment, 2) able to participate in the health examinations independently, 3) no difficulties in communication in Japanese, 4) no serious diseases/symptoms or health issues, and 5) voluntarily decided to participate in the project. We performed a complete case analysis, so only participants with complete information for subjective measures of tooth loss, CAVI, and all other examined covariates were included in the analytical sample. As no variables were missing from more than 5% of the total number of cases, and the missing data were random, the missing data are not considered to have markedly influenced the outcomes of the analysis. A total of 1,670 participants were excluded from the adjusted analyses because of missing data, and participants who reported that tooth loss was due to orthodontic treatment, malpositioned teeth, or trauma were also excluded. Therefore, the analyses reported in the present study included a total of 8,124 participants.

### Risk Factor Assessment

Trained physicians and research assistants administered standardized questionnaires, performed anthropomorphic measurements, and collected fasting blood specimens using standardized protocols. Subjects were interviewed and completed a questionnaire regarding sex, age, cardiovascular risk factors, and other medical conditions.

Height and weight measurements were determined with calibrated scales. Body mass index (BMI) was calculated using the obtained height and weight data. Trained nurses measured blood pressure using a calibrated automated sphygmomanometer (HEM-9000; Omron Healthcare Co., Ltd., Kyoto, Japan). All measurements were taken at least twice in a sitting position, and the last measurement among the data measured without error was used in the analysis. Fasting blood glucose level, high-density lipoprotein (HDL) cholesterol (HDL-C), low-density lipoprotein (LDL) cholesterol (LDL-C), and hemoglobin A1c (HbA1c) were measured using the collected blood samples from all subjects. Participants were categorized as current smokers, former smokers, or never smokers based on self-report.

Hypertension was defined as a systolic blood pressure (SBP) of ≥140 mm Hg or a diastolic blood pressure (DBP) of ≥90 mm Hg, or the self-report of history of antihypertensive drug use. HbA1c values (%) are reported according to the National Glycohemoglobin Standardization Program. Diabetes mellitus was defined by a history of insulin or hypoglycemic medication, or a fasting glucose level ≥126 mg/dL or random plasma glucose level ≥200 mg/dL, or HbA1c ≥6.5 (HbA1c ≥6.1%), according to the Japan Diabetes Society criteria (Seino et al. 2010).

### Measurement of CAVI

CAVI was recorded using a Vasera VS-1500 vascular screening system (Fukuda Denshi Ltd., Tokyo, Japan) with the participant resting in the supine position, as described in a previous report (Shirai et al. 2006). Briefly, electrocardiograph electrodes were placed on both wrists, a microphone for detecting heart sounds was placed on the sternum, and cuffs were wrapped around both arms and ankles. After automatic measurements, obtained data were analyzed using VSS-10 software (Fukuda Denshi Ltd.), and values of the right and left CAVI were calculated. Averages of the right and left CAVI were used for analysis.

### Dental History and Oral Examination

At baseline, subjects were interviewed and underwent a complete examination of the oral cavity administered by 1 of 2

**Table 1.**
Characteristics of Study Participants.

| Variable | Men ($n$ = 2680) | Women ($n$ = 5444) | All Participants ($n$ = 8124) |
|---|---|---|---|
| Age, y | 56.0 (28.9–83.1) | 53.3 (27.2–79.5) | 54.2 (53.9–54.5) |
| Tooth loss | 4.3 (0–18.3) | 3.2 (0–14.4) | 3.6 (0–15.8) |
| CAVI | 7.9 (5.4–10.3) | 7.2 (5.2–9.3) | 7.4 (5.2–9.7) |
| Hypertension | 1,066 (39.8) | 1,549 (23.5) | 2,420 (29.8) |
| Diabetes | 226 (8.4) | 170 (2.6) | 377 (4.6) |
| Former smoker | 1,184 (44.2) | 454 (8.3) | 1,638 (20.2) |
| Current smoker | 827 (30.9) | 345 (6.4) | 1,172 (14.4) |
| BMI, kg/m$^2$ | 23.4 (17.3–29.5) | 21.8 (15.3–28.3) | 22.3 (15.8–28.9) |
| SBP, mm Hg | 128.6 (91.9–160.3) | 119.4 (85.6–153.3) | 122.4 (88.2–156.7) |
| DBP, mm Hg | 79.9 (58.4–101.3) | 73.5 (52.1–94.9) | 75.6 (53.3–97.8) |
| HDL-C, mg/dL | 57.7 (26.2–89.2) | 68.7 (36.3–101.2) | 65.1 (31.3–98.8) |
| LDL-C, mg/dL | 123.0 (60.4–185.7) | 123.8 (61.36–186.2) | 123.5 (61.1–181.0) |
| HbA1c | 5.2 (3.9–6.5) | 5.1 (4.2–6.0) | 5.1 (4.0–6.2) |
| Glucose, mg/dL | 94.3 (51.9–136.7) | 88.8 (63.2–114.4) | 90.6 (58.1–123.2) |
| Antihypertensive medication | 598 (22.3) | 843 (15.5) | 1,441 (17.7) |
| Hypoglycemic medication | 141 (5.3) | 88 (1.6) | 229 (2.8) |
| Insulin | 14 (0.5) | 14 (0.3) | 28 (0.3) |

Values are presented as the mean (reference range: mean −2 SD to mean +2 SD) or $n$ (%).
BMI, body mass index; CAVI, cardio-ankle vascular index; DBP, diastolic blood pressure; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure.

trained, calibrated dentists, who were randomly assigned to subjects. The same 2 dentists performed the dental examinations during the study period. During the oral examination, the number of missing teeth was counted. Congenitally missing and impacted teeth were excluded from the count of tooth loss. Third molars were excluded from counts of tooth loss, because third molars tend to be completely impacted or congenitally missing.

### Statistical Analysis

Continuous variables are reported as means (reference range: mean −2 SD to mean +2 SD), and categorical variables are given as counts (percentages). The number of teeth showed right-skewed distributions and was therefore logarithmically transformed before analyses and back transformed to the original scale when presented. We examined the association between CAVI and tooth loss using general linear models with adjustment for age, sex, BMI, smoking status, HbA1c, and a history of insulin or hypoglycemic medication depending on the model, having excluded the presence of multicollinearity. In addition, the models were compared with and without interaction terms. To investigate possible effect modification with sex, age, and tooth loss, we added interaction terms using the centered variables tooth loss, sex, and age (<60 and ≥60 years). We checked the linear relationship identified in this multiple regression model by visual examination of plots of standardized residuals.

Probability values of less than 0.05 were considered indicative of statistical significance. Statistical analyses were performed using the STATA version 11 software package (Stata Corp., College Station, TX).

## Results

### General Characteristics

Table 1 lists demographic information of the study participants, including data for the known risk factors of arteriosclerosis and tooth loss. The mean (reference range) age of the 8124 participants was 54.2 y (27.7–80.8 y), and 67.0% were women. Men were significantly older than women (56.0 [28.9–83.1] vs. 53.3 [27.2–79.4] y) and had a high prevalence of arteriosclerosis risk factors, including hypertension, diabetes, smoking status, and obesity. CAVI values were higher for men than for women (7.9 [5.4–10.3] vs. 7.2 [5.2–9.3], respectively). The mean

**Table 2.**
Multivariable Linear Regression Models for CAVI and Tooth Loss Adjusted for Age, Sex, BMI, Smoking Status, Hemoglobin A1c, a History of Insulin or Hypoglycemic Medication, and Interactions.

| | Unadjusted Model | | Adjusted Model[a] | | | |
|---|---|---|---|---|---|---|
| | | | Model 1 | | Model 2 | |
| | β | 95% CI | β | 95% CI | β | 95% CI |
| Tooth loss | 0.47 | (0.45 to 0.50) | 0.04 | (0.02 to 0.06) | 0.03 | (0.01 to 0.06) |
| Sex | −0.65 | (−0.70 to −0.60) | −0.48 | (−0.53 to −0.43) | −0.48 | (−0.53 to −0.43) |
| Age | 0.06 | (0.06 to 0.06) | 0.06 | (0.05 to 0.06) | 0.06 | (0.05 to 0.06) |
| Tooth loss × sex[b] | | | | | 0.05 | (0.02 to 0.09) |
| Tooth loss × age category[c] | | | | | −0.04 | (−0.08 to 0.01) |

Tooth loss was logarithmically transformed.
CAVI, cardio-ankle vascular index; CI, confidence interval; BMI, body mass index.
[a]Multivariable linear regression analysis, adjusting for body mass index, smoking status, hemoglobin A1c, and a history of insulin or hypoglycemic medication.
[b]Sex (0: male, 1: female).
[c]Age category (0: <60 years, 1: ≥60 years).

(reference range) tooth loss was higher in men 4.3 (0–18.3) than in women 3.2 (0–14.3).

### Association between Tooth Loss and CAVI

Table 2 shows the results of the regression modeling for the association between tooth loss and CAVI, as reported by coefficient β (β) and 95% confidence intervals (CIs). For the unadjusted analysis, a significant relationship was detected between tooth loss and CAVI (β = 0.47; 95% CI, 0.45–0.50).

Multiple regression modeling after adjustment for age, sex, BMI, smoking status, HbA1c, and a history of insulin or hypoglycemic medication was also performed (model 1, Table 2). The adjusted multiple regression analysis detected a significant positive association between CAVI and tooth loss (β = 0.04; 95% CI, 0.02–0.6). As the residuals were randomly scattered around 0 (horizontal line) and exhibited a relatively even distribution, the association between CAVI and tooth loss appeared to be linear (data not shown).

### Interaction Effects of Sex, Age, and Tooth Loss

We introduced the interaction between sex, age, and tooth loss in the multiple regression analysis by examining the association between CAVI and tooth loss (model 2, Table 2). The analysis revealed that the relationship between CAVI and tooth loss differed depending on sex, with only men showing a positive correlation (β for interaction = 0.04; 95% CI, 0.02–0.6).

## Discussion

The present large-scale epidemiologic study has identified that a significant positive correlation exists between tooth loss and arterial stiffness, even after adjustment for age, sex, and other confounding factors. Notably, we found an association between tooth loss and CAVI, although the association differed depending on sex. Our data provide evidence that oral disease and CVD may be positively related in men, who had higher rates of tooth loss and arterial stiffness than did women.

Previous reports examining the association between tooth loss and CVD have treated tooth loss as a nominal variable and have primarily focused on the presence or absence of CVD (Choe et al. 2009; Desvarieux et al. 2004). In contrast, here we treated both tooth loss and the primary outcome of atherosclerosis, arterial stiffness, as numeric variables. Our analysis revealed that the severity of atherosclerosis is linearly related to tooth loss, which often results from the breakdown of periodontal tissue caused by periodontal disease. As tooth loss is a marker of current and long-term cumulative effects of periodontal disease (Desvarieux et al. 2003; Houshmand et al. 2012), our findings suggest that periodontal disease may play a role in the pathogenesis of atherosclerosis progression. In this study, we excluded noninflammatory reasons for tooth loss such as traumatic or orthodontic procedures, because we considered that noninflammatory tooth loss may bias the association between tooth loss and CAVI. However, we considered that the influence of excluding noninflammatory tooth loss on the findings from this study was not significant, because the number of individuals who were excluded for noninflammatory tooth loss was small.

Several potential mechanisms have been proposed in the literature for the association between periodontal disease, including tooth loss, and CVD. The findings from animal and epidemiologic studies suggest that infectious agents, including those associated with periodontal disease, increase

inflammatory cytokine production and platelet aggregation (Herzberg and Meyer 1996), which contribute to arteriosclerosis and thrombosis. In the present study cohort, age was the most important covariate for the relationship between tooth loss and arterial stiffness. Elderly people are more likely to develop periodontal disease and ensuing tooth loss, and they have increased arterial stiffness. We detected a positive association between tooth loss and CAVI and also found that the association differed depending on sex.

We investigated the factors of sex, age, and tooth loss as potential effect modifiers and found that sex that appeared to modify the association of interest. The identified sex difference in the association between clinical periodontal disease, tooth loss, and systemic disease has several potential explanations (Desvarieux et al. 2004; Demmer et al. 2008). Findings from clinical studies and laboratory research have suggested that estrogen is associated with beneficial cardiovascular effects in women (Kannel et al. 1976; Barrett-Connor and Bush 1991) as it reduces the development of atherosclerotic plaque. However, it is also possible that oral inflammation has little or no causal relationship with arteriosclerosis in women.

Progression of atherosclerosis is closely related with increased pulse pressure (Nichols et al. 1985; Sako et al. 2009), which therefore represents an important surrogate marker of arterial stiffness. Pulse pressure is a function of SBP and DBP (pulse pressure [$P$] = SBP – DBP) and was incorporated into the equation used to calculate CAVI as follows: CAVI = $a\{(2\rho/\Delta P) \times \ln (SBP/DBP)PWV^2\} + b$, where $\Delta P$ = SBP – DBP, $\rho$ is blood density, $a$ and $b$ are constants to match aortic PWV. Therefore, pulse pressure is an independent determinant of CAVI (Okura et al. 2007). In CAVI, the rate of increase is reportedly approximately 0.05 per year (Shirai et al. 2011). In the present study, the coefficient $\beta$ of the multiple regression analysis was 0.04; the loss of 5 teeth corresponds to the amount that CAVI increases in a 4-y period. This finding suggests that the relationship between CAVI and tooth loss is clinically significant.

This study has several limitations that are inherent to cross-sectional analyses. First, the relationships reported here, while robust, should not be interpreted as causal. Our cross-sectional study design lacked information on the time sequence of events and therefore did not permit identification of causal relationships. To confirm the relationship between tooth loss and subclinical atherosclerosis, it is necessary to follow a cohort of middle-age adults until death. Second, we did not measure dietary habits. Increased tooth loss often leads to decreased masticatory performance and a change of dietary habit, which is related to risk factors of atherosclerosis, such as diabetes and hypertension. For this reason, we conducted multivariate analyses with adjustment for potential confounding factors, including hypertension and diabetes. Finally, information related to socioeconomic factors, such as education and income, were not collected in this cohort. Although previous studies have attempted to delineate the influence of socioeconomic differences on mortality, morbidity, and risk factors of disease, the Japanese population may not necessarily reflect the same pattern of relationships observed in other developed countries (Kagamimori et al. 2009). For example, an association between higher education and health is not strongly expressed among the Japanese population (Kagamimori et al. 2009; Lahelma et al. 2010). This may be partly due to the fact that a compulsory insurance system covers all people living in Japan, thereby minimizing differences in access to health care based on socioeconomic status.

In conclusion, our results suggest that the progression of atherosclerosis is linearly related to increased tooth loss and further strengthen the suggested association between these 2 factors. Notably, the age and sex differences in atherosclerosis prevalence seemed to be related to not only the distribution but also the differing contributions of oral inflammatory disease to atherosclerosis across sexes. These findings have profound clinical and public health implications, as they provide further evidence that implementing strategies for preventing periodontal disease, which is both preventable and treatable, might help prevent atherosclerosis. Preventable and treatable contributors of CVD would add to the existing options available to clinicians and public health practitioners for the control of CVD. Educating patients in methods for preventing periodontal disease and improving personal oral hygiene is expected to benefit not only their oral but also their systemic health.

## Author Contributions

K. Asai, contributed to conception and design, performed the experiments, contributed to data analysis, drafted manuscript; M. Yamori, contributed to conception and design, performed the experiments, drafted manuscript, initially revised manuscript; T. Yamazaki, contributed to conception and design, performed the experiments, contributed to data analysis, initially revised manuscript; A. Yamaguchi, S. Kosugi, contributed to conception and design, critically revised manuscript; K. Takahashi, contributed to conception and design, performed the experiments, critically revised manuscript; A. Sekine, contributed to conception and design, contributed reagents/materials/tools for analysis, critically revised manuscript; F. Matsuda, T. Nakayama, contributed to conception and design, performed the experiments, initially revised manuscript; K. Bessho, contributed to conception and design, critically revised manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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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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**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

A

| 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 |

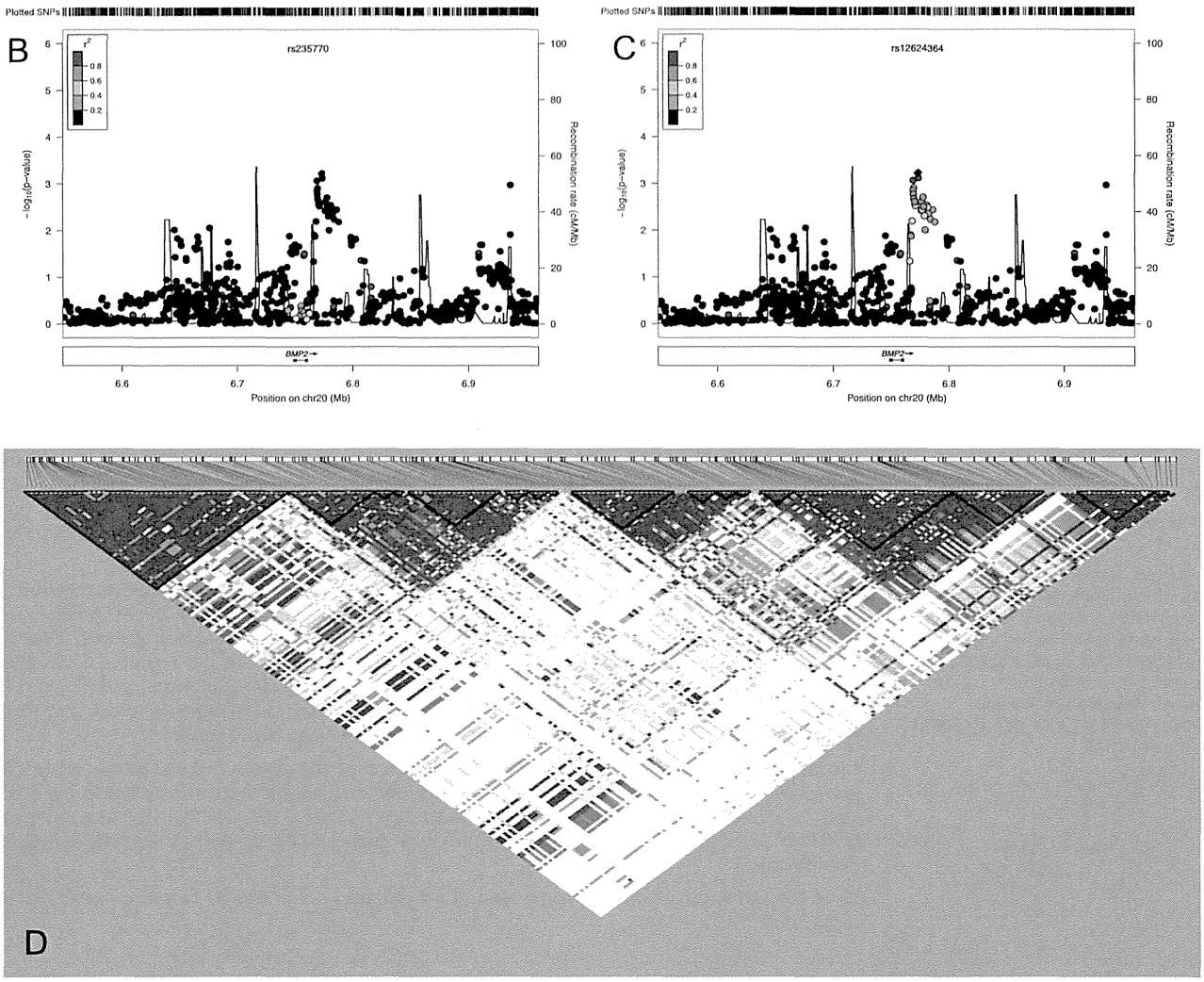


FIGURE 1. Description and illustration of three replication methods used in our analysis, using *BMP2* as an example. (**A**) Definitions of the three methods are summarized. (**B**, **C**) Association plots of the SNPs near *BMP2* in our dataset, showing the target SNPs of per-SNP analysis (**B**) and 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



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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 ($\lambda$) 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.

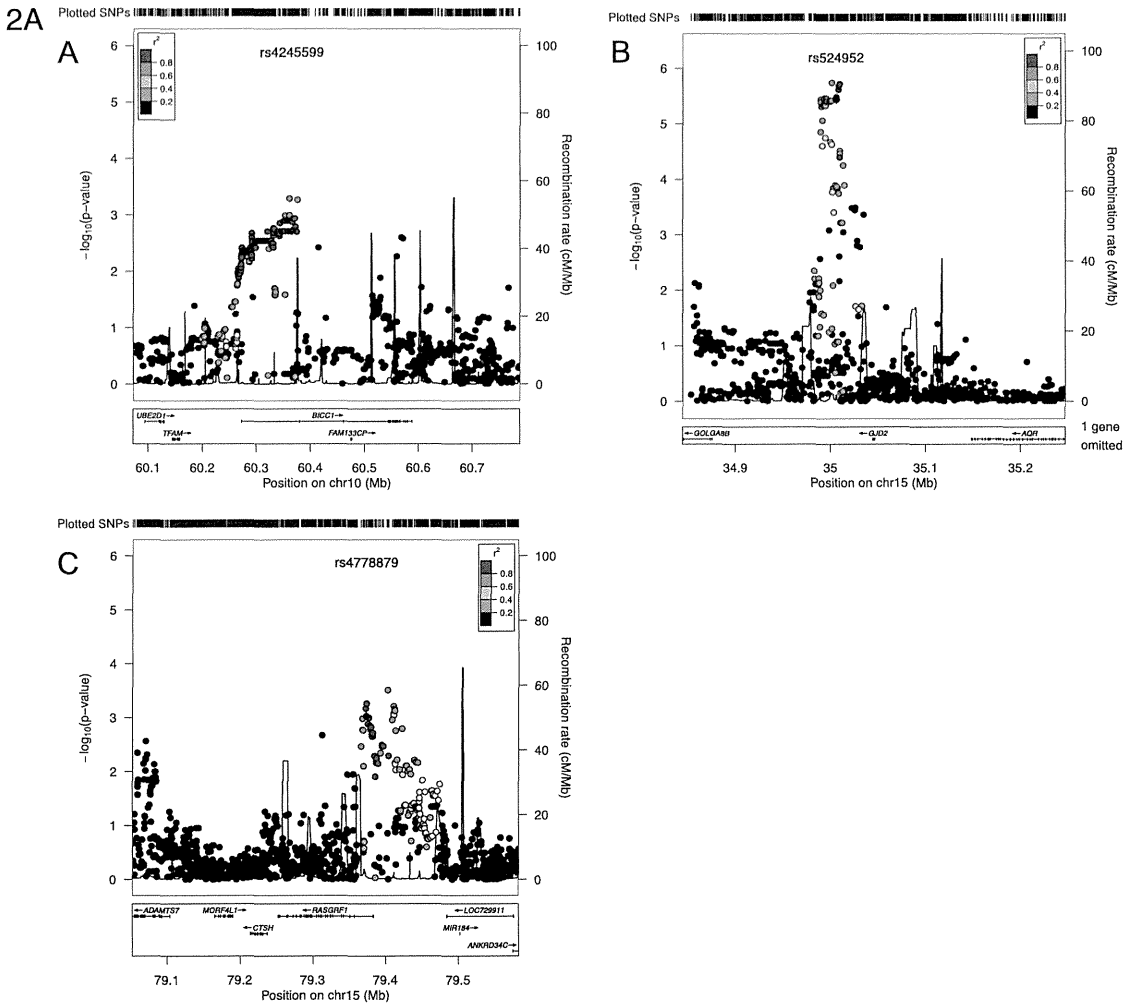


FIGURE 2.

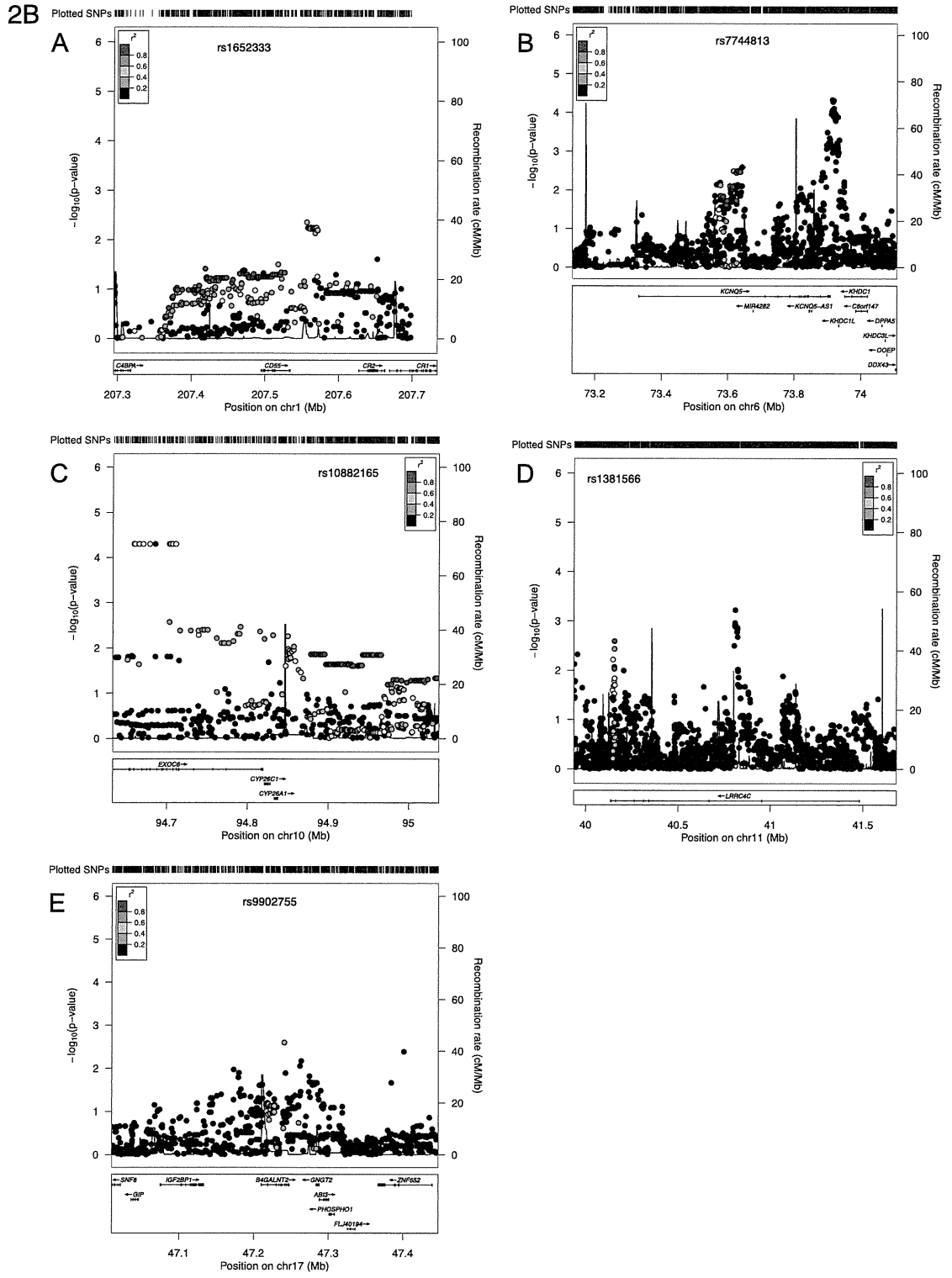


**Figure 2. Continued** Association plots of the eight genes that were significantly replicated in our per-SNP analysis. Reported SNPs near *BICC1, GJD2,* and *RASGRF1* showed strong associations with MSE and composed one of the peak signals in our dataset (**A**, *A–C*). In contrast, association signals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

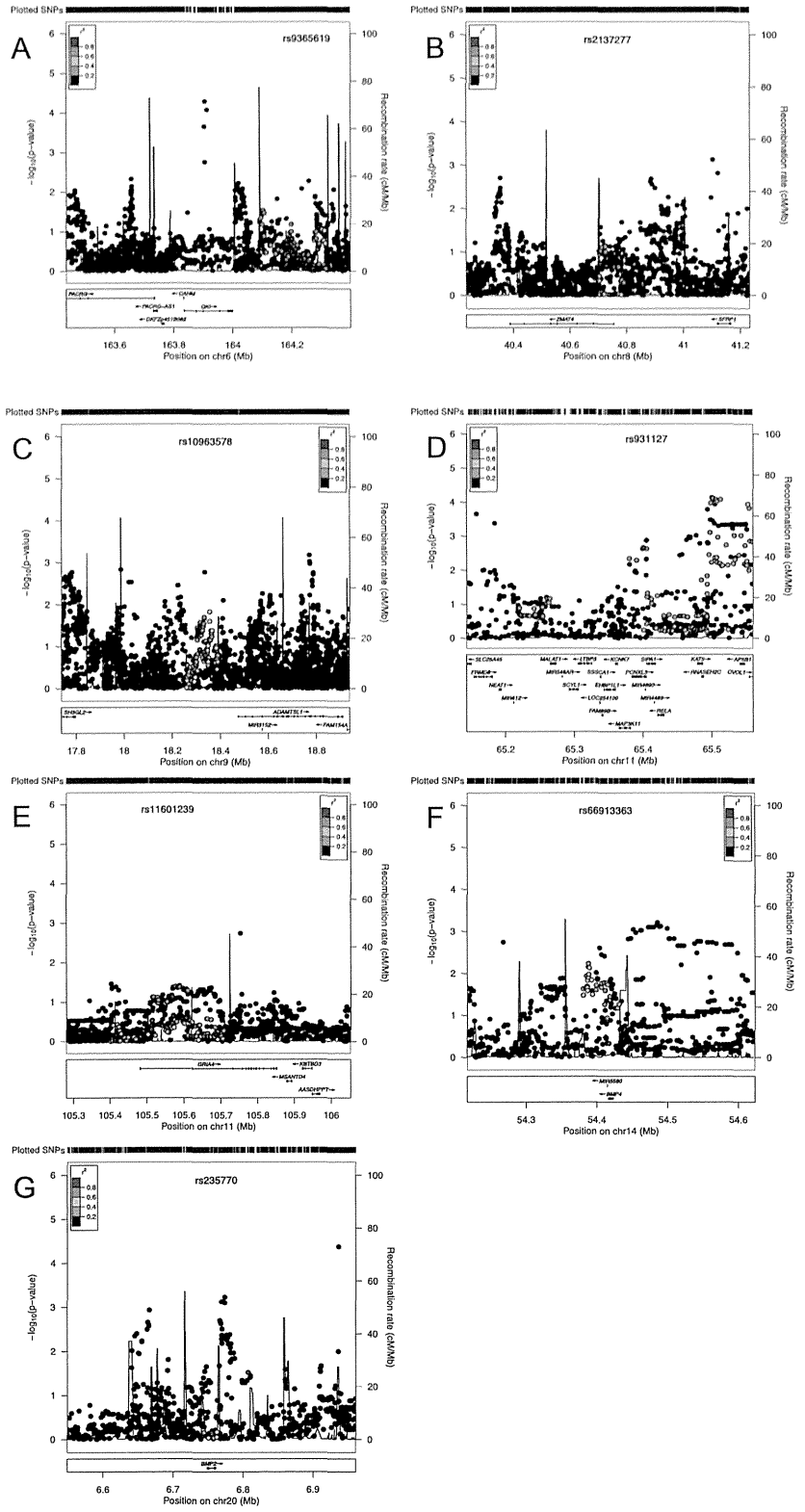


**Figure 3.** Association plots of the SNPs within seven genetic regions near *QKI, SFRP1, SH3GL2, EHBP1L1, GRIA4, BMP4*, and *BMP2* that were replicated in our gene-based analyses but failed to be replicated in our per-SNP analysis. Reported SNPs are highlighted in *purple* and SNPs within high LD to 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).

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# Three-dimensional reconstruction of rat knee joint using episcopic fluorescence image capture

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SUMMARY

*Objective:* Development of the knee joint was morphologically investigated, and the process of cavitation was analyzed by using episcopic fluorescence image capture (EFIC) to create spatial and temporal three-dimensional (3D) reconstructions.
*Methods:* Knee joints of Wister rat embryos between embryonic day (E)14 and E20 were investigated. Samples were sectioned and visualized using an EFIC. Then, two-dimensional image stacks were reconstructed using OsiriX software, and 3D reconstructions were generated using Amira software.
*Results:* Cavitations of the knee joint were constructed from five divided portions. Cavity formation initiated at multiple sites at E17; among them, the femoropatellar cavity (FPC) was the first. Cavitations of the medial side preceded those of the lateral side. Each cavity connected at E20 when cavitations around the anterior cruciate ligament (ACL) and posterior cruciate ligament (PCL) were completed.
*Conclusion:* Cavity formation initiated from six portions. In each portion, development proceeded asymmetrically. These results concerning anatomical development of the knee joint using EFIC contribute to a better understanding of the structural feature of the knee joint.


## Introduction

The synovial joint is a complex multi-tissued organ that is essential for skeletal function[1]. Synovial joints arise through two main processes. In long bone elements, cartilaginous differentiation occurs across the location of the prospective joint that then segments secondarily[1–3]. Cavitation of the joint follows, driven by selective high-level synthesis of hyaluronan by interzone cells and presumptive synovial cells[4]. This process has fascinated developmental biologists for decades[5–7].

The knee joint—one of the largest synovial joints—consists of distinct tissues including bones, articular cartilages, ligaments, synovial membrane, cruciate ligaments, menisci, and other components that interact to mechanically stabilize the joint and allow smooth motion[5]. The joint cavity of the knee is anatomically complicated and involves the space between the tibial plateaus, two femoral condyles, and the patella. The cavity is divided into at least five parts during the developmental stage, including the femoropatellar cavity (FPC), medial femoromeniscal cavity (mFMC), lateral femoromeniscal cavity (lFMC), medial meniscotibial cavity (mMTC), and lateral meniscotibial cavity (lMTC)[7].

The initiation, and spatial and temporal formation of the cavity is an important issue in joint development. Development of the joint cavity has been described in several different species, including rats[8] and humans[9]. However, the timing of cavity formation is ambiguous and discrepant, and the schedule of formation of the five parts has not been fully investigated. Ito and Kida reported that formation of the knee joint cavity in rats seemed to start at embryonic day (E)16.5 in paraffin-embedded sections, but that lacunar spaces were confirmed between spindle cells at E18.5 in resin-embedded sections[8]. Their study indicates that an artificial cleft during histologic preparation may interfere with the judgment of joint cavity formation.

*Abbreviations:* femoropatellar cavity, FPC; medial femoromeniscal cavity, mFMC; lateral femoromeniscal cavity, lFMC; medial meniscotibial cavity, mMTC; lateral meniscotibial cavity, lMTC; circumligament cavity, CLC; anterior cruciate ligament, ACL; posterior cruciate ligament, PCL; medial meniscus, MM; lateral meniscus, LM; episcopic fluorescence image capture, EFIC; embryonic day, E; three-dimensional, 3D; hematoxylin and eosin, H&E; confidence interval, CI.

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