

took PROGRAF (Astellas Pharma Inc., Tokyo, Japan) capsules containing 1 or 0.5 mg TAC. CTD patients fulfilled criteria for each disease, namely, American College of Rheumatology (ACR) criteria for RA in 1987¹² or ACR/EULAR criteria in 2010,¹³ for systemic lupus erythematosus,¹⁴ polymyositis and dermatomyositis¹⁵ and for polyarteritis nodosa and microscopic polyangiitis.¹⁶ Information of age, sex, weight, serum creatinine and date of prescription, dosage, and blood concentration of TAC were obtained from clinical charts retrospectively by the system previously described.¹⁷ Information of prescription and dosage of corticosteroid was also obtained. TAC concentration data were obtained at least one week after prescription was initiated or changed. The data of concurrent use of cyclosporine or bosentan, contraindication due to interaction with TAC, was excluded. Estimated glomerular filtration ratio (eGFR) was inferred by serum creatinine, age and sex. The blood trough TAC concentration with 3 mg TAC (around 12 h after taking TAC) was used. TAC concentrations were quantified by two measurements according to measuring time; namely, microparticle enzyme immunoassay (MEIA, IMxTM-TACRO II, Abbott Laboratories, Green Oaks, IL, USA) until May 2009 and chemiluminescent immunoassay (CLIA, ARCHITECT_TACRO, Abbott Laboratories) from May 2009. These measurements can quantify even low TAC concentrations (~ 1.5 and ~ 0.5 ng ml⁻¹, respectively). When TAC concentrations were available for both measurements in a patient, data of CLIA with lower measuring limit of TAC concentrations was used. A total of 63 and 9 patients were quantified by CLIA and MEIA, respectively (hereafter termed as CLIA group and MEIA group, respectively). When multiple TAC concentrations were available, the mean of them was adopted. Calculations were performed on the basis of logarithm of TAC concentrations to obtain normal distribution and to avoid excess influence of extreme data.

The summary of the subjects in the current study is shown in Table 1. Log-transformation of TAC concentration supported applying linear regression analysis. When we analyzed correlations between TAC concentration and age, sex, weight, eGFR, dosage or usage of corticosteroid or the presence of RA or systemic lupus erythematosus by single linear regression analysis, none of them displayed overall significant associations ($P \geq 0.083$). However, because presence of RA showed a suggestive association in MEIA group ($P = 0.0091$), we used presence of RA as a covariate. Rs776746, whose G allele determined CYP3A5*3, was selected on the basis of previous studies and genotyped by the Taqman assay (Applied Biosystems Inc, Foster city, CA, USA). As a result, no deviation from Hardy–Weinberg equilibrium was observed ($P = 0.13$). Although previous reports comparing different measurement methods of blood concentration of TAC showed good correlations ($r \geq 0.84$) and did not detect discrepancy even in ranges of low concentrations,^{18,19} MEIA was suggested to underestimate TAC concentrations in low levels.¹⁹ Thus we analyzed the associations in CLIA and MEIA groups separately, and the overall

associations were estimated by meta-analysis using inverse-variance method. We found a significant decreasing effect of the A allele of rs776746 on TAC concentration ($P = 0.0038$, Figure 1). Both MEIA and CLIA groups showed the comparable effect sizes, supporting the accuracy of the result (Table 2). Patients who carried A allele had 31.0% lower mean concentration than those who were homozygote for G allele. Although the current and the previous studies¹¹ showed a good fit of the dose-dependent model of rs776746, there are also conflicting reports.^{6,20} Considering the limited number of subjects with AA genotype, the dose-dependent effect of rs776746 should be regarded as inconclusive. Meta-analysis of the recessive model resulted in a comparable result ($P = 0.0035$, AA + GA vs GG).

The current study provided evidence that TAC concentration was strongly influenced by CYP3A5 in patients with CTD even taking a small amount of TAC. Our results showed the same direction of A allele of rs776746 and comparable effect sizes in the previous studies using patients of solid organ transplantation.^{5,6} Disease-specific influence on TAC concentrations was not clear. As this study contained relatively small number of subjects and low TAC concentrations around the measurement limits might be associated with diminished accuracy, these results should be replicated by a larger number of patients with CTD, also including other populations. Because the predictive model of TAC concentration is proposed in

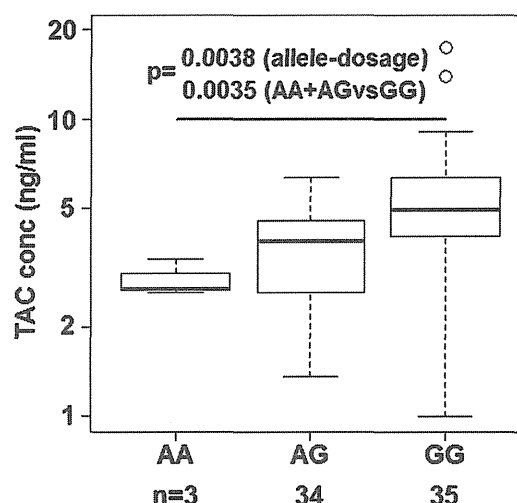


Figure 1 Association between TAC concentration and the polymorphism in CYP3A5 in patients with CTD. The obtained or inferred TAC concentrations adjusted for 3mg TAC are shown according to rs776746 genotypes. Y axis is shown in log scale. The mean concentrations are 2.88, 3.57 and 5.10 ng ml⁻¹ for AA, AG and GG genotypes, respectively. TAC concentrations were adjusted for MEIA group.

Table 1 Summary of subjects in the current study

	Study subjects
Age ^a	48.94 ± 17.24
Sex	Male 13, female 59
Disease	RA: 22, SLE: 43, DM: 3, PM: 2, PAN: 1, mPA: 1

Abbreviations: DM, dermatomyositis; mPA, microscopic polyangiitis; PAN, polyarteritis nodosa; PM, polymyositis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

^amean ± s.d.

Table 2 Association between rs776746 and TAC concentration in multiple regression analysis

	Number	Beta	s.e.	P-value
CLIA	63	0.106	0.041	0.012
MEIA	9	0.161	0.12	0.22
Overall	72	0.112	0.039	0.0038

Abbreviations: CLIA, chemiluminescent immunoassay; MEIA, microparticle enzyme immunoassay; TAC, tacrolimus.

Statistics adjusted by rheumatoid arthritis presence.

patients of organ transplantation, it will be interesting to construct a predictive model of TAC concentration in patients with CTD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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BRIEF COMMUNICATION

Novel Germline Mutation in the Transmembrane Domain of *HER2* in Familial Lung Adenocarcinomas

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We encountered a family of Japanese descent in which multiple members developed lung cancer. Using whole-exome sequencing, we identified a novel germline mutation in the transmembrane domain of the human epidermal growth factor receptor 2 (*HER2*) gene (G660D). A novel somatic mutation (V659E) was also detected in the transmembrane domain of *HER2* in one of 253 sporadic lung adenocarcinomas. Because the transmembrane domain of *HER2* is considered to be responsible for the dimerization and subsequent activation of the *HER* family and downstream signaling pathways, we performed functional analyses of these *HER2* mutants. Mutant *HER2* G660D and V659E proteins were more stable than wild-type protein. Both the G660D and V659E mutants activated Akt. In addition, they activated p38, which is thought to promote cell proliferation in lung adenocarcinoma. Our findings strongly suggest that mutations in the transmembrane domain of *HER2* may be oncogenic, causing hereditary and sporadic lung adenocarcinomas.

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Familial lung cancers are rare among human malignancies. Recent studies have reported that germline mutations in the epidermal growth factor receptor (*EGFR*) gene predispose the development of lung cancer. Reported familial lung adenocarcinomas with a germline *EGFR* mutation, such as T790M, carry secondary somatic *EGFR* mutations, including exon 19 deletion and exon 21 L858R mutation (1-4). We encountered a family of Japanese descent in which multiple members developed lung cancer (Figure 1). The proband (III-4) was a 53-year-old woman with multiple lung adenocarcinomas in bilateral lungs. She was a light smoker with a 1.2-pack-year history of smoking. She had undergone a left lower lobectomy for multiple lung adenocarcinomas at the age of 44 years. Her mother (II-4), a never smoker, also had multiple lung adenocarcinomas. Partial pulmonary resections of two tumors were performed for II-4 for the purpose of diagnosis after pleural dissemination was found during surgery, and multiple lesions were removed in a lobectomy or partial resections in III-4. A histological examination of the resected tumors in II-4 revealed nonmucinous adenocarcinoma in situ and nonmucinous minimally invasive adenocarcinoma, whereas

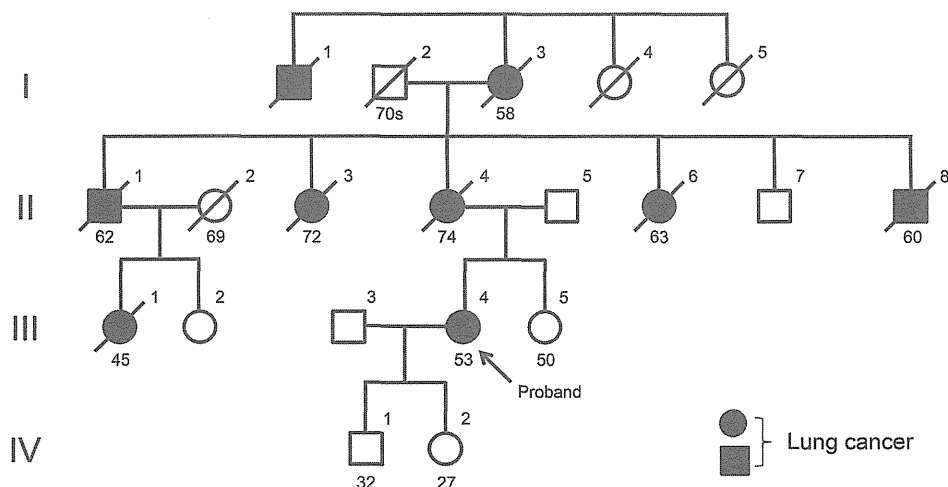


Figure 1. Pedigree chart of a Japanese family in which multiple members developed lung cancer. The boxes and circles indicate men and women, respectively. The numbers at the bottom of each member indicate the age at the time of death or the time of the analysis. An oblique line shows deceased family members. The proband (III-4) had multiple lung adenocarcinomas (arrow). Tumor tissue, nonmalignant lung tissue, and peripheral blood samples were obtained from III-4. The proband's

mother (II-4) also had multiple lung adenocarcinomas, and tumor and nonmalignant lung tissue samples were available. The proband's father (II-5) and sister (III-5) were both unaffected, and peripheral blood samples were obtained from these individuals. Some family members who were not considered as critical for this study were excluded from the pedigree chart to preserve confidentiality. Whole-exome sequencing was performed for individuals II-4, II-5, III-4, and III-5.

the histological findings of pleural dissemination indicated mucus-containing adenocarcinoma. Those of III-4 contained various subtypes of adenocarcinoma, including non-mucinous and mucinous adenocarcinoma in situ and invasive mucinous adenocarcinoma. In addition, normal-appearing lung parenchyma obtained from a lobectomy in III-4 revealed innumerable small pre-invasive lesions, implying the presence of precancerous changes throughout the lung (Supplementary Figure 1, available online). Sequencing analyses of *EGFR* exons 18 to 21 and *KRAS* as well as an immunohistochemical staining for ALK protein in the resected tumors indicated no genetic alterations in these genes. The pedigree chart

suggested that lung cancer was inherited in an autosomal dominant manner.

After obtaining permission from the Institutional Review Board at Okayama University Hospital and informed consent from the patients and other family members, we performed a whole-exome sequencing study. Tumor DNA samples from II-4, tumor and peripheral blood DNA samples from III-4, and peripheral blood DNA samples from two unaffected family members (II-5 and III-5) were used for the analysis. The candidate germline alterations were restricted to 29 variants by comparing the whole-exome sequencing results between the patients and the unaffected family members. Among them, we focused on a point mutation in the

human epidermal growth factor receptor 2 (*HER2/neu*) gene (NM_004448, G660D, GGC to GAC), which was located in exon 17 encoding the transmembrane domain of *HER2* (Supplementary Tables 1–3). This alteration was confirmed by direct sequencing (Figure 2A). We also confirmed that there was no copy number gain of *HER2* in the examined tumors based on the degree of read-depth in the whole-exome sequencing results. Of note, no mutations in genes known to cause lung cancers were detected for tumors from III-4 and II-4.

We considered that somatic mutations in the *HER2* transmembrane domain might act as driver mutations in lung cancer. Hence, we sequenced exon 17 of the *HER2*

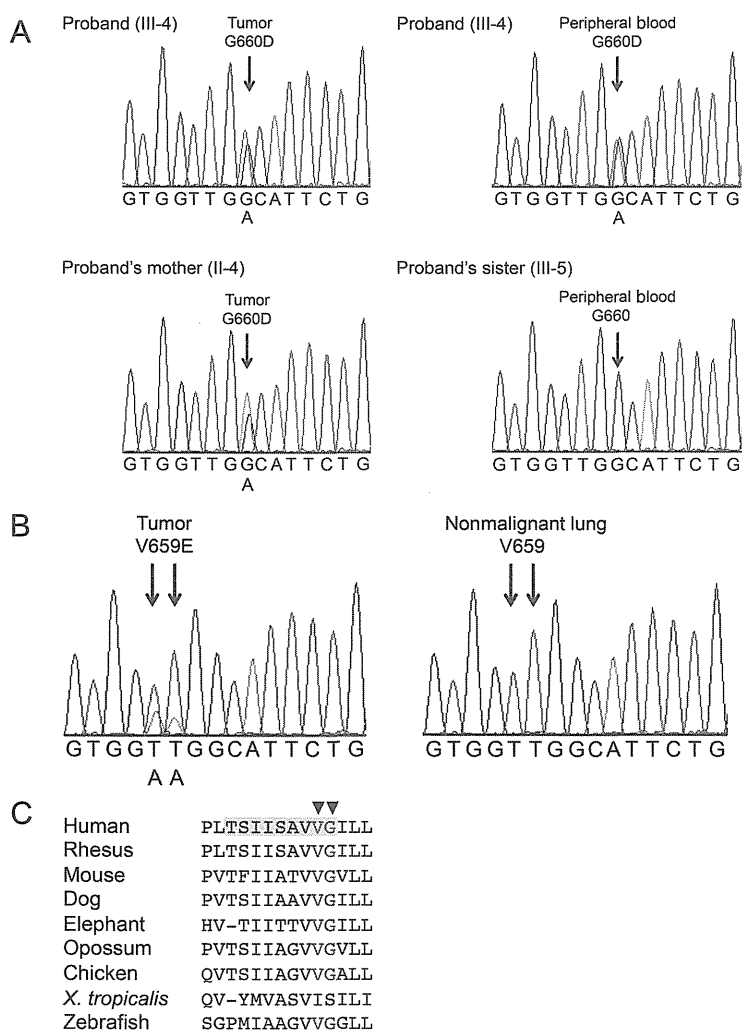


Figure 2. DNA and amino acid sequences in the transmembrane domain of *HER2*. **A**) Direct Sanger sequencing of the proband (III-4), her affected mother (II-4), and her unaffected sister (III-5). The results indicated that G660D was a germline mutation. **B**) Direct sequencing of a sporadic lung adenocarcinoma with a *HER2* V659E mutation. V659E was found to be of somatic origin based on the sequencing results of the peritumoral lung tissue from the same specimen. All the sequence variants were confirmed by independent

polymerase chain reaction amplifications and were sequenced in both directions. **C**) Interspecies conservation of the transmembrane domain of *HER2* (UCSC Genome Browser, <http://genome.ucsc.edu>, accessed September 12, 2013). The yellow highlight indicates the N-terminal glycine zipper motif Thr⁶⁵²-X₃-Ser⁶⁵⁶-X₃-Gly⁶⁶⁰, a tandem variant of a GG4-like motif of human *HER2*. Codons 659 and 660 in human *HER2* are highly conserved among the listed vertebrate species (shown in red). *X. tropicalis* = *Xenopus tropicalis*.

in the tumor samples of 315 sporadic non-small cell lung cancer patients, of which 253 were adenocarcinomas. Although the *HER2* G660D mutation was not detected, a novel nonsynonymous mutation, V659E (GTT to GAA), next to codon 660 was identified in one of these patients. This patient was histologically diagnosed as nonmucinous adenocarcinoma in situ, and the patient had neither smoking history nor apparent family history of lung cancer. This V659E mutation was certainly a somatic mutation because it was not identified in the peritumoral lung tissue of the same patient (Figure 2B). The alignment of *HER2* amino acid sequences showed high conservation of valine 659 and glycine 660 among vertebrates (Figure 2C).

HER2 somatic mutations have been reported in 2% to 4% of lung adenocarcinomas (5–7). However, all reported mutations were restricted to its tyrosine kinase domain (6,7). According to the cBioPortal for Cancer Genomics (<http://www.cbioportal.org/public-portal/>, accessed September 12, 2013), the same genetic mutation in the *HER2* has not been reported in any type of cancer. Interestingly, a previous study reported that a mutation in the transmembrane domain (V664E) of the rat *neu* gene, which corresponds to V659E in its human homolog *HER2*, induced oncogenic transformation (8). In addition, in vivo experiments showed that the *HER2* V659E mutation contributed to the stability of *HER2* dimers, resulting in the dysregulated receptor activation and subsequent cell transformation (9,10). Furthermore, the novel mutations were located within the glycine zipper motif Thr⁶⁵²-X₃-Ser⁶⁵⁶-X₃-Gly⁶⁶⁰, a tandem variant of the GG4-like motif, at the N-terminal portion of the transmembrane domain, which was critically related to the dimerization of *HER2* (Figure 2C) (9,11). Accordingly, we performed a functional analysis of the mutant *HER2* proteins. We found that the degradation of *HER2* protein after the administration of cycloheximide was slower in G660D and V659E mutants as compared with wild-type (Supplementary Figure 2A), indicating the higher stability of the mutant proteins than wild-type protein. In addition, results of a phospho-mitogen-activated protein kinase array indicated the activation of Akt and p38 α (data not shown). Indeed, Akt is known

to be activated by *HER2* by phosphatidylinositol 3-kinase and leads to increased cell growth and survival (12,13). Also, the activation of p38 was shown to contribute to the viability of lung adenocarcinoma cells derived from never or light smokers (14,15). A western blot analysis for Akt and p38 successfully confirmed the upregulation of both phospho-Akt and phospho-p38 expression in the mutant *HER2* transfectants (Supplementary Figure 2B).

Because the G660D alteration in *HER2* might have been the cause of the lung cancer in the pedigree studied, we investigated whether familial aggregation of cancer in other organs could be seen in this pedigree. We found that II-1 and II-6 developed renal and gastric cancers, respectively; however, both of them also had lung cancer. The reason why other types of clinically apparent malignancies were rarely found in this pedigree is unclear. The G660D germline mutation may be tolerated in organs other than the lung.

This study had some limitations. First, the carcinogenic potential of the *HER2* mutation at the transmembrane domain should be confirmed in other models such as transgenic mice. Second, the rarity of these mutations in sporadic lung cancers may be the limitation for generalizability to other cases even if targeting therapies for similar types of *HER2* mutation were developed.

In conclusion, we identified a novel germline mutation in the transmembrane domain of the *HER2* in familial lung adenocarcinomas. Somatic mutation in the *HER2* transmembrane domain may be a possible cause of sporadic lung adenocarcinomas.

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Note

H. Yamamoto, J. Soh, S. Miyoshi, and S. Toyooka conceived the project. K. Higasa, M. Sakaguchi, K. Shien, and K. Ichimura performed the experiments. H. Yamamoto, J. Soh, M. Furukawa, S. Hashida, N. Takigawa, K. Kiura, K. Tsukuda, and S. Toyooka collected the samples and assisted with the experiments. H. Yamamoto, K. Higasa, K. Shien, and K. Matsuo analyzed the data. H. Yamamoto, K. Higasa, M. Sakaguchi, F. Matsuda, and S. Toyooka prepared the manuscript with input from the other authors. S. Miyoshi, F. Matsuda, and S. Toyooka supervised the project. The authors declared no conflicts of interest.

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

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

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

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

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

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

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

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

Previous studies have indicated the involvement of genetic and environmental factors in the progression of myopia.¹³⁻¹⁶ Family-based linkage analyses and twin studies have identified MYP1-19 loci and several candidate genes,^{17,18} but genetic screening studies have achieved limited success. Since 2009, several genome-wide association studies (GWAS) have reported candidate genes for myopia,¹⁹⁻²⁶ but none of the reported genes or loci, except for the 15q14 locus, showed a consistent association with either common or high myopia in later studies.²⁷⁻³⁰ Moreover, although some loci were reported to

be associated with common and high myopia,^{25,27,31} it still is not clear whether common myopia and high myopia share the same genetic background.

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

METHODS

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

TABLE 1. Characteristics of the Study Population

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

* Axial length of ≥ 26.0 mm in eyes.

† Healthy individuals recruited from Nagaha cohort study.

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

Patients and Controls

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

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

SNP Selection

Verhoeven et al.³² reported 26 loci (29 potential candidate genes) associated with refractive error and common myopia in a large-scale multi-ethnic GWAS. Of these loci, 8 also were reported to be associated with myopia and replicated in another recent large-scale GWAS including Caucasian participants.³³ For our analysis, we selected 8 SNPs in these 8 loci that were evaluated in the original report. Among these 8 SNPs, 3 showed extremely low minor allele frequency (MAF) in the Japanese population according to the HapMap data (rs12205363 in *LAMA2*, rs1656404 near *PRSS56*, and rs1960445 near *BMP3*; MAF 0.00, 0.01, and 0.02, respectively).

In addition to these 3 SNPs, rs524952 in *GJD2* also was excluded as we had confirmed its association previously with high myopia.²⁷ Selected SNPs included rs7837791 near *TOX*, rs3138144 in *RDH5*, rs8000973 near *ZIC2*, and rs2969180 in *SHISA6*. Although negated by Kiefer et al.³³ at the replication stage ($P = 0.08$), rs4778879 in *RASGRF1* was included because its association with myopia still is disputed despite numerous replication studies.

Genotyping

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

Statistical Analyses

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

RESULTS

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

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

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

Chr, chromosome; CI, confidence interval.

* Differences in the observed genotypic distribution were examined by χ^2 test for trend.

† Age and sex adjustment was performed based on a logistic regression model.

‡ Number of control subjects who were genotyped directly.

§ The HWE test results for control subjects who were genotyped directly.

|| Data of SNP rs6495367, whose linkage disequilibrium value (r^2) is 1.0 compared to SNP rs4778879.

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

Genotype counts, associations examined using the χ^2 test for trend analysis, odds ratios for the 5 SNPs between high myopia cases and controls, number of control subjects who were genotyped directly, and the results of the HWE exact test in controls are shown in Table 2. The SNPs rs8000973 near ZIC2, rs4778879 in RASGRF1, and rs2969180 in SHISA6

showed significant association with high myopia ($P = 7.16 \times 10^{-7}$, 3.40×10^{-7} , and 0.033, respectively). The odds ratios (95% confidence intervals) were 1.33 (1.19-1.49) for the rs8000973 C allele, 0.78 (0.71-0.86) for the rs4778879 A allele, and 1.11 (1.01-1.22) for the rs2969180 G allele. The effect of the rs2969180 allele G was contrasting to that obtained in the previous study, whereas the other 2 SNPs showed the same trend as that observed in the original report. The distributions of the genotypes for all the five SNPs were in HWE. When control group 2 was evaluated, the SNPs rs8000973 and rs4778879 showed significant association with high myopia

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

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

* Healthy individuals with spherical equivalent between -1.00 and +1.00 in eyes.

† Differences in the observed genotypic distribution were examined by χ^2 test for trend.

‡ Age and sex adjustment was performed based on a logistic regression model.

§ Number of control subjects who were genotyped directly.

|| Data of SNP rs6495367 whose linkage disequilibrium value (r^2) is 1.0 compared to SNP rs4778879.

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

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

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

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

DISCUSSION

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

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

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

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

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

* Differences in the observed genotypic distribution were examined by χ^2 test for trend.

† Age and sex adjustment was performed based on a logistic regression model.

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

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

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

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

In conclusion, we showed that genetic variants of SNP rs8000973 near the *ZIC2* gene and rs4778879 in the *RASGRF1*

gene are associated with high myopia in Japanese subjects. This result, together with previous GWAS, implied that these SNPs may be the susceptibility loci for myopia and high myopia. However, we were not able to identify genetic factors influencing CNV risk in high myopic patients among these 5 SNPs.

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APPENDIX

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Nine Loci for Ocular Axial Length Identified through Genome-wide Association Studies, Including Shared Loci with Refractive Error

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Refractive errors are common eye disorders of public health importance worldwide. Ocular axial length (AL) is the major determinant of refraction and thus of myopia and hyperopia. We conducted a meta-analysis of genome-wide association studies for AL, combining 12,531 Europeans and 8,216 Asians. We identified eight genome-wide significant loci for AL (*RSPO1*, *C3orf26*, *LAMA2*, *GJD2*, *ZNRF3*, *CD55*, *MIP*, and *ALPL2*) and confirmed one previously reported AL locus (*ZC3H11B*). Of the nine loci, five (*LAMA2*, *GJD2*, *CD55*, *ALPL2*, and *ZC3H11B*) were associated with refraction in 18 independent cohorts (n = 23,591). Differential gene expression was observed for these loci in minus-lens-induced myopia mouse experiments and human ocular tissues. Two of the AL genes, *RSPO1* and *ZNRF3*, are involved in Wnt signaling, a pathway playing a major role in the regulation of eyeball size. This study provides evidence of shared genes between AL and refraction, but importantly also suggests that these traits may have unique pathways.

Introduction

Myopia (nearsightedness), the most common form of refractive errors, is an ocular disorder of major public health importance worldwide, particularly in Asia. About 40% of adults and 80%–90% of children completing high school are myopic in urban areas in East Asian countries, and 10%–20% of them have high myopia.^{1,2} Uncorrected myopia and refractive errors are leading causes of visual impairment.^{3–6} Furthermore, adults with high myopia are at a substantially higher risk of potentially blinding pathologies, including glaucoma, retinal detachment, and myopic maculopathy.⁷ The correction of myopia and refractive errors in general by spectacles, contact lenses, or refractive surgery can entail substantial socioeconomic costs^{8,9} and does not treat the underlying mechanism of disease.

Myopia develops primarily from an eye that is excessively elongated axially and thus ocular axial length (AL) is an attractive endophenotype to investigate for several reasons. First, AL alone accounts for more than 40% of variation in refractive errors.^{10–12} MRI studies of the orbit have also demonstrated that extremely highly myopic eyes are generally prolate in shape with unusually long ALs, leading to associated visually disabling complications such as posterior staphylomas.^{13,14} Second,

the heritability of AL (67% to 94%) is consistently higher than that for refraction.^{15–18} Furthermore, the measurement of AL (in mm) is more objective, precise, and reproducible compared to assessments of refractive status.

Although more than 30 myopia loci have been implicated in previous linkage and genome-wide association studies (GWASs), there have been few reports of AL-specific loci. A recent GWAS identified an association at *ZC3H11B* for both AL and high myopia in Asians.¹⁹ To identify additional genetic variants that modulate AL, we conducted the largest international GWAS meta-analysis of AL to date in cohorts participating in the Consortium for Refractive Error and Myopia (CREAM).^{20,21}

Subjects and Methods

We used a three-stage approach.²⁰ First, we performed a GWAS meta-analysis in 12,531 European ancestry individuals (stage 1). Second, we tested the cross-ethnic transferability of the associations from this first stage in 8,216 Asian ancestry individuals (stage 2). Lastly, we conducted a meta-analysis combining individuals of European and Asian ancestry, totaling 20,747 individuals (stage 3). We subsequently examined the effect of the associated AL loci on spherical equivalent (SE) in 23,591 individuals from 18 other independent cohorts.

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Table 1. Study Cohorts and Summary of Axial Length Measures

Ethnicity	n	Study	Mean Age (SD), Years	Men, %	Axial Length		Methods of Measurement
					Mean (SD), mm	Range, mm	
European	2,069	ALSPAC Children	15.5 (0.3)	46.5	23.41 (0.87)	20.49–26.57	IOLmaster
	1,316	BATS/TEST	24.6 (11.9)	43.2	23.25 (0.87)	20.03–28.25	IOLmaster
	1,030	BMES	73.8 (7.8)	59.5	23.45 (1.04)	19.94–29.86	IOLmaster
	826	Croatia-Korcula	55.8 (13.4)	35.1	23.19 (1.06)	18.55–28.24	Echoscan US-1800
	352	Croatia-Split	50.0 (14.2)	44.3	23.39 (0.90)	20.98–27.3	Echoscan US-1800
	552	Croatia-Vis	56.0 (14.0)	39.7	23.08 (0.90)	20.09–26.48	Echoscan US-1800
	2,397	ERF4	48.7 (14.2)	55.5	23.22 (1.04)	19.79–27.30	A scan
	503	ORCADES	57.6 (13.7)	43.3	23.70 (1.08)	20.69–28.00	IOLmaster
	1,011	Raine	20.1 (0.4)	51.6	23.56 (0.89)	20.36–27.94	IOLmaster
	676	RS1	78.4 (4.4)	49.0	23.52 (1.06)	20.44–27.72	Lenstar LS900
	1,085	RS2	72.0 (4.7)	47.2	23.50 (1.14)	19.87–28.00	Lenstar LS900
	714	RS3	59.3 (5.8)	42.6	23.56 (1.27)	19.79–28.45	Lenstar LS900 and A scan
	Asian	564	BES	62.05 (8.4)	35.5	23.07 (1.15)	19.90–30.36
1,720		SCES	57.6 (9.0)	51.7	23.95 (1.31)	20.87–32.66	IOLmaster
926		SCORM	10.8 (0.8)	51.7	24.13 (1.12)	21.05–28.20	Echoscan US-800
2,141		SiMES	57.6 (10.7)	49.3	23.57 (1.04)	20.48–31.11	IOLmaster
2,120		SINDI	55.9 (8.8)	51.4	23.41 (1.08)	19.07–31.59	IOLmaster
745		STARS Parents	38.8 (5.3)	51.0	24.64 (1.51)	21.66–31.57	IOLmaster

Abbreviations are as follows: ALSPAC, Avon Longitudinal Study of Parents and Children; BATS, Brisbane Adolescent Twins Study; TEST, Twins Eye Study in Tasmania; BMES, Blue Mountains Eye Study; ERF, Erasmus Rucphen Family Study; ORCADES, Orkney Complex Disease Study; RS, Rotterdam Study; BES, Beijing Eye Study; SCES, Singapore Chinese Eye Study Singapore; SCORM, Singapore Cohort Study of the Risk Factors for Myopia; SiMES, Singapore Malay Eye Study; SINDI, Singapore Indian Eye Study; STARS, Strabismus, Amblyopia, and Refractive Error Study of Preschool Children; SD, standard deviation.

Study Populations in CREAM

All studies participating in this meta-analysis are part of the CREAM.^{20,21} The discovery cohorts included 12,531 European ancestry individuals from 18 studies (Table 1), including ALSPAC Children,²² BATS/TEST,²³ BMES,^{24,25} Croatia-Korcula, Croatia-Split, Croatia-Vis,²⁶ ERF,^{27,28} RS1, RS2, RS3,²⁹ ORCADES,³⁰ and RAINE.^{31–33} In addition, 8,216 Asian ancestry individuals from six cohorts (Table 1) (BES,³⁴ SCES,³⁵ SCORM,³⁶ SiMES,³⁷ SINDI,³⁵ and STARS Parents³⁸) were included in the replication stage. General methods, demographics, and phenotyping of the study cohorts have previously been described extensively and are provided in brief in Table 1. All studies were performed with the approval of their local Medical Ethics Committee, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Independent Populations in CREAM

To examine whether the loci affecting AL contributed to SE, we studied associations with SE in an additional 18 studies (Table S1 available online): 1958 British Birth Cohort,³⁹ ALSPAC Mothers,⁴⁰ ANZRAG,⁴¹ AREDS 1a1b, AREDS 1c,^{15,16} DCCT,⁴² EGCUT,⁴³ FECD,⁴⁴ FES,⁴⁵ FITSA,⁴⁶ GHS 1, GHS 2, KORA,^{47–50} OGP Talana,⁵¹ SP2,⁵² TwinsUK,⁵³ WESDR,⁵⁴ and Young Finns Study.⁵⁵ Only SE (not AL) measures were available in these additional 18 CREAM studies. Detailed study design and methodology of these studies have been published elsewhere. Descriptive data

on demographics and phenotypes of these cohorts are shown in brief in Table S1.

Phenotype Measurements

All studies used a similar protocol for ocular phenotype measurements. Eligible participants underwent an ophthalmologic examination including measurements of AL and refraction of both eyes. AL was measured with either optical laser interferometry or A-scan ultrasound biometry (Table 1). Refraction was measured by autorefractor and/or subjective refraction (Table S1). SE was calculated according to the standard formula (SE = sphere + 1/2 cylinder).

Genotyping and Imputation

The study samples were genotyped on either the Illumina or Affymetrix platforms. Each study performed SNP imputation with the genotype data, together with the HapMap Phase II ethnically matched reference panels (CEU, JPT+CHB, or the four HapMap populations) on the basis of HapMap build 36 databases (release 22 or 24). The Markov Chain Haplotyping software, IMPUTE^{56,57} or MACH,⁵⁸ were adopted for imputation. A detailed description regarding genotyping platforms and imputation procedures for each study is provided in Tables S2 and S3.

Stringent quality control of genotype data was applied in each cohort. Samples with low call rates (<95%) or with gender discrepancies were excluded. Cryptically related samples and outliers in population structure from principal component analyses were

also excluded. SNPs flagged with missingness >5%, gross departure from Hardy-Weinberg equilibrium (p value < 10^{-6} , except in the ALSPAC study where a threshold of < 10^{-7} was used), and minor allele frequency (MAF) <1% were removed from further analyses.

Statistical Analysis

For each study, an allele-dosage regression model at each genotyped or imputed SNP was conducted to determine its association with AL as a quantitative trait as well as its association with SE. Individuals with prior refractive or cataract surgery or other intra-ocular procedures that could alter refraction were excluded. The mean of the right and left eyes was taken. When data from only one eye were available, the AL or SE of this eye was used. Sample outliers with AL value exceeding four standard deviations from the mean were excluded at the study level. We assumed an additive genetic model where the dosage of each SNP is a continuous variable ranging from 0 to 2 for minor alleles carried. Primary analysis for AL was adjusted for age, sex, and height (because height was consistently correlated with AL^{59,60}) and in the case of SE for age and sex. Additional adjustment for principal components was carried out according to the population substructure in each individual study.

The per-SNP meta-analyses were performed by METAL software with weighted inverse-variance approach, assuming fixed effects, because for initial discovery purposes, the fixed-effects model is preferred for increased statistical power.⁶¹ A Cochran's Q test was used to assess heterogeneity across studies.⁶² Imputation quality scores were reviewed for the top SNPs reported to ensure good imputation quality (proper-info of IMPUTE or R^2 of MACH > 0.3).

Gene-based testing was conducted with VEGAS software⁶³ on the European ancestry and Asian ancestry meta-analysis results separately. VEGAS incorporates information from the full set of markers within a gene and thus can be more powerful than tests of individual SNPs if there are multiple risk variants within a gene. VEGAS corrects for LD and gene size by conducting simulations based on the LD structure in the population of interest (here, European or Asian ancestry). VEGAS was therefore run separately on all the European and Asian GWAS data, with results for each gene combined at the end by meta-analysis on the two sets of gene-based p values by Fisher's methods. For samples of European descent, we used the HapMap 2 CEU population as the reference to estimate patterns of LD. For Asian ancestry groups, we used the combined HapMap 2 JPT and CHB populations as the reference population to approximate linkage disequilibrium (LD) patterns. To include gene regulatory regions, SNPs were included if they fell within 50 kb of a gene.

VEGAS-Pathway analysis^{63,64} was carried out with prespecified pathways from Gene Ontology. Pathways with 10 to 1,000 components were selected, yielding 4,628 pathways. Pathway analysis was based on combining gene-based test results from VEGAS. Pathway p values were computed by summing χ^2 test statistics derived from VEGAS p values. Empirical VEGAS-Pathway p values for each pathway were computed by comparing the summed χ^2 test statistics from real data with those generated in 500,000 simulations where the relevant number (according to the size of the pathway) of randomly drawn χ^2 test statistics was summed. To ensure that clusters of genes did not adversely affect results, within each pathway, gene sets were pruned such that each gene was >500 kb away from all other genes in the pathway. Where required, all but one of the clustered genes was dropped at random when genes were clustered. We

performed meta-analysis on the two sets of pathway p values by Fisher's method.

Differential Gene Expression in a Mouse Model of Myopia

Animal study approval was obtained from the SingHealth Institutional Animal Care and Use Committee (AAALAC accredited). All procedures performed in this study complied with the Association of Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmology and Vision Research. Experimental myopia was induced in B6 wild-type (WT) mice ($n = 36$) by applying a -15.0 diopter spectacle lens on the right eye (experimental eye) for 6 weeks from postnatal day 10. The left uncovered eye served as the contra-lateral control eye. Age-matched naive mice eyes were also used as independent control eyes ($n = 36$).^{65,66} Eye biometry, refraction, tissue collection, RNA extraction, real-time polymerase chain reaction (PCR) qRT-PCR methods, and analysis were followed as described previously.¹⁹ qRT-PCR primers (Table S4) were designed with ProbeFinder 2.45 (Roche Applied Science) and performed with a Lightcycler 480 Probe Master (Roche Applied Science). The experiments were repeated in triplicate. Gene expression of all identified genes in the control and experimental groups was quantified by the $2^{-\Delta\Delta C_t}$ method.⁶⁷ Student's t test was performed to determine the significance of the relative fold difference of mRNA between the myopic eyes of the experimental mice and the age-matched controls.

Gene Expression in Human Tissues

Adult ocular samples were obtained from normal eyes of an 82-year-old female of European ancestry from the North Carolina Eye Bank (Winston-Salem, NC). All adult ocular samples were stored in QIAGEN's RNAlater within 6.5 hr of collection and shipped on dry ice overnight to the lab. Isolated tissues were snap-frozen and stored at -280°C until RNA extraction. RNA was extracted from each tissue sample independently by the Ambion *mirVana* total RNA extraction kit. The tissue samples were homogenized in Ambion lysis buffer with an Omni Bead Ruptor Tissue Homogenizer per protocol. Reverse transcription reactions were performed with Invitrogen SuperScript III First-Strand Synthesis kit. The expression of the identified genes was assessed by running 10 μl reactions with QIAGEN's PCR products consisting of 1.26 μl H₂O, 1.0 μl $10\times$ buffer, 1.0 μl dNTPs, 0.3 μl MgCl, 2.0 μl Q-Solution, 0.06 μl taq polymerase, 1.0 μl forward primer, 1.0 μl reverse primer, and 1.5.0 μl cDNA. The reactions were run on a Eppendorf Mastercycler Pro S thermocycler with touchdown PCR ramping down 1°C per cycle from 72°C to 55°C followed by 50 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final elongation of 7 min at 72°C . All primer sets were designed by Primer3.⁶⁸ Products were run on a 2% agarose gel at 70 V for 35 min. Primer sets were run on a custom tissue panel including Clontech's Human MTC Panel I, Fetal MTC Panel I, and an ocular tissue panel.

Results

We analyzed 2.5 million genotyped and imputed SNPs (Table S2). The genomic control inflation factor (λ) for individual studies (Table S2) as well as for the meta-analysis ($\lambda_{GC} = 1.06$) and quantile-quantile plots (Figure S1) showed little evidence for inflation.

Table 2. Associations with Ocular Axial Length in the European Ancestry Cohorts with Results in the Asian Cohorts and Combined Analysis

Lead SNP ^a	Chr	Position ^b	European Ancestry Cohorts (Stage 1, n = 12,531)				Asian Cohorts (Stage 2, n = 8,216)				Combined (Stage 3, n = 20,747)				Localization Relative to Protein-Coding Genes ^b	
			EA	EAF	Beta ^c	SEM	p Value	EAF	Beta ^c	SEM	p Value	EAF	Beta ^c	SEM		p Value
rs4074961	1	37865310	T	0.43	0.06	0.01	6.6 × 10 ⁻⁶	0.45	0.10	0.02	1.1 × 10 ⁻⁹	0.44	0.07	0.01	4.0 × 10 ⁻¹³	intron 4 of <i>RSPO1</i> (MIM 609595)
rs994767	1	217842055	A	0.45	-0.06	0.01	1.2 × 10 ⁻⁶	0.32	-0.10	0.02	4.4 × 10 ⁻⁷	0.41	-0.07	0.01	9.6 × 10 ⁻¹²	7 kb upstream of <i>ZC3H11B</i>
rs9811920	3	101326983	A	0.41	0.07	0.01	3.0 × 10 ⁻⁷	0.36	0.13	0.03	6.0 × 10 ⁻⁶	0.40	0.08	0.01	4.9 × 10 ⁻¹¹	intron 1 of <i>C3orf26</i>
rs12193446	6	129861731	A	0.91	0.12	0.02	1.1 × 10 ⁻⁷	0.98	0.28	0.11	1.2 × 10 ⁻²	0.91	0.12	0.02	1.2 × 10 ⁻⁸	intron 58 of <i>LAMA2</i> (MIM 156225)
rs11073058	15	32776918	T	0.43	0.07	0.01	2.0 × 10 ⁻⁸	0.50	0.06	0.02	4.7 × 10 ⁻⁴	0.45	0.07	0.01	4.3 × 10 ⁻¹¹	57 kb upstream of <i>GJD2</i> (MIM 607058)
Additional loci identified through the combined analysis of European and Asian cohorts																
rs12321	22	27783183	C	0.44	-0.05	0.01	1.1 × 10 ⁻⁵	0.49	-0.06	0.02	9.9 × 10 ⁻⁴	0.46	-0.05	0.01	4.1 × 10 ⁻⁸	3' UTR of <i>ZNRF3</i> (MIM 612062)

SNPs with $p < 1 \times 10^{-5}$ in European ancestry cohorts were brought for replication in Asians. Genome-wide significance is defined as $p < 5.0 \times 10^{-8}$. The following abbreviations are used: SNP, single-nucleotide polymorphism; Chr, chromosome; EA, effect allele; EAF, effect allele frequency; SEM, standard error of the mean.

^aLead SNPs of each locus identified in the combined meta-analysis (stage 3) are presented. The lead SNPs in the combined meta-analysis are the same as the lead SNPs in the European-only analysis (stage 1) for all loci, except for the 1q41 locus near *ZC3H11B*, where the lead SNP in European-only analysis is rs10863469 (position, 217844091; frequency of effect allele T = 0.47; Beta = 0.47, $p = 1.2 \times 10^{-6}$), being in high LD ($r^2 = 0.84$) with rs994767.

^bPosition is based on NCBI human genome build 36.

^cEffect sizes on axial length are in mm.

Per-SNP Meta-analysis

In the first stage, a total of 177 SNPs, representing 24 physically distinct loci, were associated with $p < 1 \times 10^{-5}$ in the European ancestry discovery cohort (Table S5). Of them, we identified one locus at chromosome 15q14 in the proximity of *GJD2* (MIM 607058; rs11073058, $p = 2.0 \times 10^{-8}$) exceeding genome-wide significance level ($p < 5 \times 10^{-8}$; Table 2), which was previously reported to be associated with refractive errors.⁶⁹ We took the 177 SNPs forward for replication in the Asian cohorts (stage 2). Five regions showed significant evidence of replication ($1.12 \times 10^{-9} \leq p \leq 1.18 \times 10^{-2}$; Table 2): *RSPO1* (MIM 609595), *C3orf26*, *LAMA2* (MIM 156225), and regions close to *ZC3H11B* and *GJD2*. In the combined meta-analysis of all 18 European and Asian cohorts (stage 3, $n = 20,747$), all five loci surpassed genome-wide significance level ($3.97 \times 10^{-13} \leq p \leq 1.24 \times 10^{-8}$; Table 2 and Figure 1). Furthermore, in stage 3 we detected an additional genome-wide significant locus at *ZNRF3* (MIM 612062, $p = 4.08 \times 10^{-8}$; Table 2).

Overall, the significant regions included six loci for AL: *RSPO1*, *C3orf26*, *LAMA2*, *GJD2*, *ZNRF3*, and one previously identified locus for AL at 1q41 close to *ZC3H11B*.¹⁹ A common SNP in *RSPO1* displayed the strongest evidence for association (rs4074961, $\beta = 0.07$ mm per copy of risk allele, $p = 3.97 \times 10^{-13}$), with no evidence of heterogeneity ($I^2 = 0\%$, $p = 0.78$) across the 18 AL cohorts (Table S6), although the strongest effect was observed for the rarer intronic variant in *LAMA2* (rs12193446, $\beta = 0.12$ mm, $p = 1.24 \times 10^{-8}$). Figure 2 shows the regional association plots for the six loci significant in single SNP tests. Forest plots showing the effect sizes across cohorts are provided in Figure S2. We constructed a multilocus genetic risk score to evaluate the combined effects of the AL SNPs in the Blue Mountains Eye Study^{24,25} and the Singapore Chinese Eye Study,³⁵ both of which were part of the 18 AL discovery cohorts. Figure S3 shows that the odd ratios for longer AL (Tertile 3 versus Tertile 1) were higher with increasing genetic risk scores.

Gene-Based Meta-analysis

In addition to per-SNP meta-analysis, we applied gene-based tests with VEGAS,²⁵ with genome-wide significance declared if $p_{\text{gene-based}} < 0.05/17,872 = 2.8 \times 10^{-6}$ (17,872 genes tested). Over and above the loci found in per-SNP tests, three additional genomic regions were genome-wide significantly associated with AL via gene-based tests (Table 3): *CD55* (MIM 125240), *ALPPL2* (MIM 171810), and *TIMELESS/MIP/SPRYD4/GLS2* (MIM 603887 for *TIMELESS*). Figure S4 shows the regional association for the three loci significant in gene-based tests.

Association with Refraction

We subsequently assessed the association of these AL SNPs and genes with SE in 23,591 individuals from 18 independent studies in CREAM that had SE but no AL measures (Tables S1 and S3). We found associations ($p < 0.05$) with SE for three of the six AL SNPs (Table 4 and Figure S5)

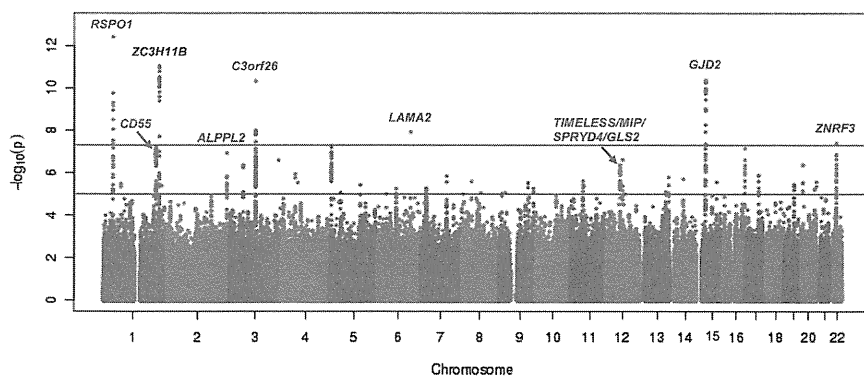


Figure 1. Summary of Meta-analysis Results for Genome-wide Association to Ocular Axial Length

Data of both directly genotyped and imputed SNPs are presented in the Manhattan plot. The y axis represents $-\log_{10} p$ values for association with axial length, and the x axis represents chromosomes and base-pair positions based on human genome build 36. The horizontal red line indicates the genome-wide significance level of $p < 5.0 \times 10^{-8}$. The horizontal blue line indicates the suggestive significance level of $p < 1.0 \times 10^{-5}$. The previously described locus for axial length is labeled in black. Other loci reaching genome-wide significance identified from the per-SNP meta-analysis are labeled in red. The genes identified in gene-based tests are labeled in blue.

(rs994767 [*ZC3H11B*, $p = 0.013$], rs11073058 [*GJD2*, $p = 1.66 \times 10^{-8}$], and rs12193446 [*LAMA2*, $p = 3.58 \times 10^{-10}$]), with directions of the SE association being consistent with AL. For example, the risk allele T of rs11073058 in *GJD2* was associated with both longer AL and more myopia (more negative SE). In gene-based tests, only *CD55* ($p = 4.5 \times 10^{-6}$) and *ALPPL2* ($p = 8.3 \times 10^{-3}$) were associated with SE (Table 5).

SNPs close to *CD55* had reached genome-wide significant association with SE in the meta-analysis of all CREAM cohorts (i.e., with and without AL measures).²⁰ There was an association with SE at *CHRNA2*, along with a less significant independent hit near *ALPPL2* (125 kb away).²⁰ Our AL gene-based results showed a genome-wide significant signal at *ALPPL2* but not at *CHRNA2*. There was also an association with SE at *RDH5*,²⁰ on the same chromosomal band as the AL signal at *MIP* (MIM 154050), but *RDH5* and *MIP* are 727 kb apart without LD between them, suggesting that they are independent signals.

Pathway Analysis

We conducted pathway analysis with VEGAS-Pathway^{63,64} by combining the gene-based p values for 4,628 prespecified pathways. The most significant pathway was the “Wnt receptor signaling” pathway ($p = 2.9 \times 10^{-5}$). The Bonferroni corrected p value was 0.13 (for the total number of 4,628 pathways tested). However, Bonferroni correction is an overcorrection, because many of the pathways have overlapping genes. The identification of the Wnt signaling pathway, even if only nominally associated, is of interest because the pathway involves two genes identified from the per-SNP tests. Also among the top ten pathways were “lens development in camera-type eye” ($p = 2.4 \times 10^{-4}$) and “collagen” ($p = 5.1 \times 10^{-4}$) pathways (Table S7). The collagen pathway was implicated in a recent meta-analysis of corneal thickness.⁶⁴

Gene Expression

Differential expression of the nearest genes in the six implicated loci from per-SNP meta-analysis (Table S4) was

assessed by measuring mRNA levels in minus-lens-induced myopia mouse models.^{65,66} The mRNA levels of all six genes had a 2-fold difference in the induced myopic eyes as compared to the control eyes in most of the tissues tested: sclera, retinal pigment epithelium (RPE), and neural retina (Figure S6).

In human ocular tissue, we have previously shown that *ZC3H11B* is expressed in neural retina, RPE, and sclera,¹⁹ *LAMA2* is expressed in sclera and optic nerve, and *CD55* is expressed in retina, choroid, and cornea, and *GJD2* is less abundant in sclera and other ocular tissues.²⁰ In this study, we measured the mRNA expression levels of the other genes in adult ocular tissues via reverse-transcriptase PCR. We found that *C3orf26*, *ZNRF3*, and *TIMELESS* were expressed in most ocular tissues and the expression of *RSPO1*, *ALPPL2*, and *MIP* was less strong and/or more restricted (Table S8).

Discussion

We identified five AL loci (*RSPO1*, *C3orf26*, *LAMA2*, *GJD2*, and *ZNRF3*) and confirmed the previously described locus (*ZC3H11B*) via per-SNP tests. In addition, three loci (*CD55*, *ALPPL2*, and *TIMELESS/MIP/SPRYD4/GLS2*) were identified by gene-based tests. Therefore, a total of nine AL loci were identified in this meta-analysis. Seven of the nine AL loci are located within the genomic region of protein-coding genes (Tables 2 and 3). Of note, two of them (*RSPO1* and *ZNRF3*) encode proteins that are directly involved in the Wnt signaling pathway. *RSPO1* is a member of a family of secreted proteins that act as stem-cell growth factors by enhancing the Wnt signaling pathway.⁷⁰ On the other hand, *ZNRF3* is a membrane-bound protein that acts as a negative regulator of the Wnt signaling pathway by mediating degradation of the Wnt receptor complex components Frizzled and LRP6.⁷¹ The two proteins have recently been shown to interact, *RSPO1* enhancing Wnt signaling through inhibition of *ZNRF3*.⁷¹ The Wnt signaling was the most significant pathway in our analysis, further

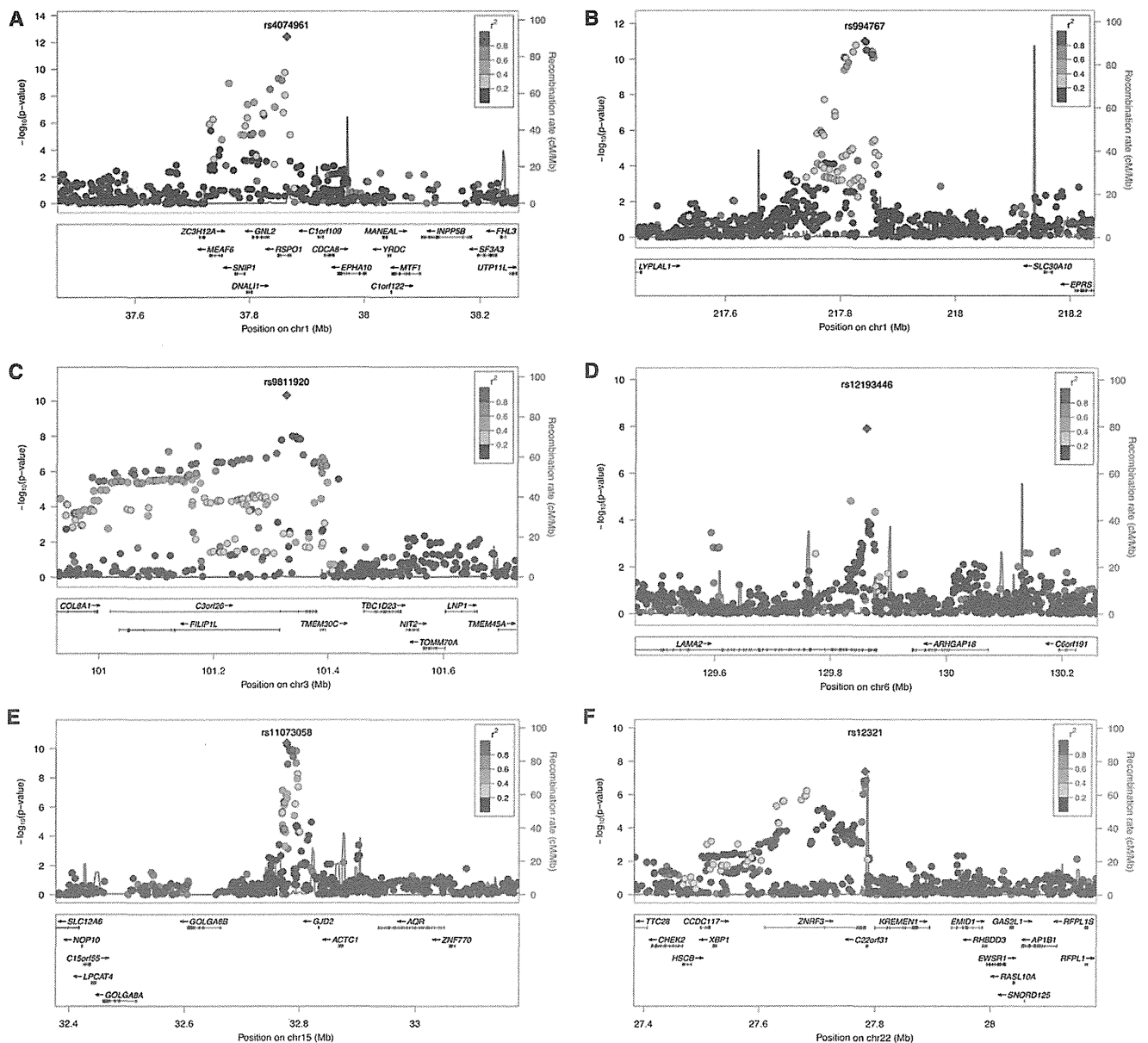


Figure 2. Regional Association Plots and Recombination Rates of the Loci Associated with Ocular Axial Length
 Data are shown for association at chromosome (A) 1p34.3 (*RSP01*), (B) 1q41 (*ZC3H11B*), (C) 3q12.1 (*C3orf26*), (D) 6q22.33 (*LAMA2*), (E) 15q14 (*GJD2*), and (F) 22q12.1 (*ZNRf3*) in the combined meta-analysis. Data of both directly genotyped and imputed SNPs are presented. In each panel, the genotyped SNP with the most significant association is denoted with a purple diamond. The color coding of all other SNPs indicates LD with the lead SNP, estimated by CEU r^2 from phase II HapMap: red, $r^2 \geq 0.8$; yellow, $0.6 \leq r^2 < 0.8$; green, $0.4 \leq r^2 < 0.6$; cyan, $0.2 \leq r^2 < 0.4$; blue, $r^2 < 0.2$; and gray, r^2 unknown. The left y axis represents $-\log_{10} p$ values for association with axial length, the right y axis represents the recombination rate, estimated from the International HapMap Project, and the x axis represents base-pair positions along the chromosome based on human genome build 36. Gene annotations are taken from the University of California Santa Cruz (UCSC) genome browser. The plots were created with LocusZoom.

supporting its prominent role in vertebrate eye development.⁷² Indeed, overexpression of a dominant-negative variant of human *ZNRf3* in zebrafish embryos induces small eye or loss of eyes.⁷¹

Remodeling of extracellular matrix in sclera plays an important role in changes of eye size during myopia development. *LAMA2* encodes the alpha 2 chain of laminin, a major extracellular protein of the basement membrane. We used HaploReg⁷³ to search for evidence of a functional

role for variants at the *LAMA2* locus, because it has the largest per-allele effect on AL. The intronic lead SNP rs12193446 lies within the promoter and enhancer histone marks as well as DNase hypersensitive sites. Analysis with RegulomeDB⁷⁴ suggested that rs12193446 occurs in a region that binds EP300, TCF4, STAT3, GATA2, and RFX4. Four of these interactions (EP300, TCF4, STAT3, and GATA2) were predicted by HaploReg⁷³ to be affected by the genotype at rs12193446. Mutations in the cognate

Table 3. Loci Associated with Ocular Axial Length in Gene-Based Tests

Gene	MIM Number	Chr	Start Position ^a	End Position ^a	P _{gene-based} Value		
					European Ancestry Cohorts	Asian Cohorts	Combined ^b
<i>CD55</i>	125240	1	205561439	205600934	1.3×10^{-5}	9.6×10^{-4}	2.3×10^{-7}
<i>ALPL2</i>	171810	2	232979795	232983669	6.4×10^{-5}	1.7×10^{-3}	1.8×10^{-6}
<i>TIMELESS/MIP/SPRYD4/GLS2^c</i>	603887	12	55097173	55168448	2.0×10^{-7}	7.3×10^{-2}	2.8×10^{-7}

The following abbreviation is used: Chr, chromosome.

^aPosition is based on NCBI human genome build 36. Note this is the start and stop position of the gene. For gene-based tests, 50 kb was added to either side to account for possible regulatory variants that fall outside the gene boundaries.

^bGene-based genome-wide significance was defined as $p < 2.80 \times 10^{-6}$. Only loci that were genome-wide significant in gene-based testing but not genome-wide significant in per-SNP testing are shown.

^c*TIMELESS* was the most significant gene in the region. Because of the ± 50 kb added to the definition for each gene and the close proximity of the genes, *MIP*, *SPRYD4*, *GLS2*, and *TIMELESS* all had similar gene-based p values (ranged from 1.4×10^{-6} to 2.8×10^{-7} for the combined analysis), and thus p value and MIM number for only *TIMELESS* is presented.

gene for *TCF4* cause Pitt-Hopkins syndrome (PTHS [MIM 610954]), the predominant ocular feature of which is high-grade myopia.⁷⁵ Interestingly, common genetic variants in *TCF4* (MIM 602272) have also been associated with Fuchs corneal dystrophy, suggesting the pleiotropic effects of *TCF4* on ocular diseases.⁷⁶

Gene-based testing implicated the *TIMELESS/MIP/SPRYD4/GLS2* region, although determining which of these genes are functionally relevant is difficult because there are multiple association signals in the region. *MIP* is an interesting candidate gene because it is expressed in the ocular lens and is required for correct lens function.⁷⁷ *CD55*, implicated here in AL and previously in SE,²⁰ is known to elevate cytosolic calcium ion concentration.

For all six of the genes identified in our per-SNP meta-analysis, we found evidence for differential expression in a mouse model of myopia. Differential expression was observed in the mouse sclera and retina as well as RPE cells, suggesting a role for these genes in myopia. Further strengthening our results, the expression data showed that all but one of these genes expressed in the

adult human eye. These data potentially provide insights into the complexity of AL elongation and myopia at the biological level. Some genes, namely *ZC3H11A*, *GJD2*, and *LAMA2*, showed changes in expression that are consistently in the same direction across the different eye sections analyzed, whereas others, namely *RSPO1*, *C3orf26*, and *ZNRF3*, showed variable directions of differential expression. These results, together with the pathway analysis results, suggest that the genetic mechanisms of myopia are complex, involving more than one eye component.

We have previously shown that up to 50% of the variation in SE is due to shared genetic factors with AL.⁷⁸ Thus, we undertook further analyses and found that five of the nine AL loci are also associated with SE. Furthermore, we looked up the association of AL with the SNPs discovered from the recent CREAM GWAS meta-analysis on SE in 32 cohorts²⁰ and observed that 23 of the 29 SNPs identified with SE have significant effects on AL ($p < 0.05$; Table S9). This has important implications. First, it confirms the previous findings in twins⁷⁸ that there are common genetic determinants of the two traits, such as variants in *GJD2*, *LAMA2*, *CD55*, and *ALPL2*. Second, it indicates that some genetic variants for AL do not influence SE, suggesting that they regulate the coordinated scaling of eye size.⁷⁹ For example, the SNP in *RSPO1* showed the strongest evidence of association with AL, yet it had no association with refractive error. In eyes without refractive error, AL and corneal curvature are carefully scaled relative to one another and have a high phenotypic correlation between them.⁸⁰ Therefore, genes like *RSPO1* might mediate a compensatory mechanism through changes in corneal curvature or optical power, thereby balancing their effects on SE.

Shorter axial length is a major risk factor for angle closure glaucoma. A recent GWAS on primary angle closure glaucoma identified three genome-wide significant loci located at *PLEKHA7* (MIM 612686), *COL11A1* (MIM 120280), and *PCMTD1-ST18*.⁸¹ However, none of the common variants in the three loci were significantly associated with AL in our meta-analysis (Table S10). This suggests that

Table 4. Association with Spherical Equivalent of the SNPs Most Strongly Associated with Axial Length in Each Genomic Locus in Independent Cohorts

Lead SNP	Nearest Gene	Effect Allele	Beta ^a	SEM	p Value
rs4074961	<i>RSPO1</i> (MIM 609595)	T	0.004	0.023	0.84
rs994767	<i>ZC3H11B</i>	A	0.054	0.022	1.3×10^{-2}
rs9811920	<i>C3orf26</i>	A	-0.022	0.022	0.31
rs12193446	<i>LAMA2</i> (MIM 156225)	A	-0.242	0.039	3.6×10^{-10}
rs11073058	<i>GJD2</i> (MIM 607058)	T	-0.121	0.022	1.7×10^{-8}
rs12321	<i>ZNRF3</i> (MIM 612062)	C	-0.004	0.021	0.86

Abbreviations are as follows: SNP, single-nucleotide polymorphism; SEM, standard error of the mean.

^aEffect sizes on spherical equivalent are in diopters.