Association of *ARMS2* Genotype With Bilateral Involvement of Exudative Age-Related Macular Degeneration

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• PURPOSE: To study the association of ARMS2 A69S genotype with the development of exudative age-related macular degeneration (AMD) in the unaffected fellow eye and to estimate the duration until the development of AMD in the second eye.

• DESIGN: Retrospective cohort study.

• METHODS: We retrospectively reviewed 326 patients who had exudative AMD in at least 1 eye, genotyping of ARMS2 A69S, and a minimum follow-up of 2 years. Survival analysis and Cox proportional hazard regression analysis were used to examine the association between candidate factors and the duration until the development of AMD in the second eye.

• RESULTS: One hundred nineteen patients (36.5%) had bilateral exudative AMD at the initial visit. A risk allele of ARMS2 A69S was more frequently seen in patients with bilateral AMD ($P = .0270$) than in those with unilateral AMD. Of the 207 unilateral AMD patients, 23 (11.1%) had AMD in the fellow eye after a mean duration of 56.3 ± 40.4 months. Fellow-eye involvement was associated with ARMS2 A69S genotype (hazard ratio [HR], 2.673; $P = .0013$), age (HR, 1.102; $P = .0005$), and smoking history (HR, 0.680; $P = .3663$). As HRs indicate, correlation of genotype (2.673) was as high as that of 10-year aging ($1.102^{10} = 2.641$). Survival analysis revealed that patients with risk homozygous (TT) genotype had second-eye involvement significantly earlier than those with other genotypes ($P = .0028$). When the observation duration reached 120 months, second-eye involvement had developed in 50%, 6.6%, and 11.2% of the TT, GT, and GG cohorts, respectively.

• CONCLUSION: ARMS2 A69S genotype is associated with second-eye involvement of exudative AMD and with the period between first- and second-eye involvements. (Am J Ophthalmol 2012;154:542–548.

See Accompanying Editorial on page 427.
Accepted for publication Mar 28, 2012.
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EXUDATIVE AGE-RELATED MACULAR DEGENERATION (AMD) is one of the most common vision-threatening eye diseases currently seen in developed countries. Although its exact pathogenesis remains unknown, authors of population-based studies have reported various factors associated with the development of exudative AMD, including age, cataract, sunbathing, sex, history of smoking, hypertension, and soft drusen.[1,2] In the clinical setting, some patients with unilateral exudative AMD maintain good visual function in the fellow eye for a long time, while others have development of exudative AMD in the fellow eye. When visual disturbance attributable to AMD is seen in 1 eye, the impairment of quality of life (QOL) may be limited, but the involvement of exudative AMD in the second eye, when accompanied by a visual disturbance, often causes a severe decrease in QOL. The rate of bilateral involvement of exudative AMD in whites has been reported to vary from 6% to 9% annually.[2–4] In Japanese patients, the rate is relatively low, with a cumulative incidence of only 11% to 12% over 5 years having been documented.[5–8]

Recently, many genetic factors have been reported in the development of exudative AMD, including *ARMS2/HTRA1*, *CFH*, and C2/CFB.[9–14] Although *CFH* is the most prevalent susceptibility gene in whites, *ARMS2/HTRA1* is the most prevalent gene associated with AMD in Asians.[15–17] Andreoli and associates have shown that *ARMS2/HTRA1* is associated with phenotypic attributes of AMD, while *CFH* is not.[18] A higher risk for bilateral advanced disease has been shown in several articles,[13,14] and a higher risk of *ARMS2/HTRA1* for exudative disease than for atrophy has also been described.[19] An increasing number of reports have shown that *ARMS2* A69S is strongly associated with exudative AMD as well as with typical AMD and polypoidal choroidal vasculopathy (PCV). In addition, *HTRA1* polymorphism has been significantly associated with bilateral involvement of exudative AMD,[20] and Sakurada and associates recently reported a significant association between *ARMS2* A69S polymorphism and bilaterality of PCV.[21] Accordingly, it might follow that patients with unilateral exudative AMD have a higher risk for the development of exudative AMD in the fellow eye if they have a risk allele of

0002-9394/$36.00
http://dx.doi.org/10.1016/j.ajo.2012.03.042

ARMS2 A69S. It would be a great help for both physicians and patients to be better able to estimate the risk of fellow-eye involvement by exudative AMD in order to determine visit frequency and treatment strategy. However, limited information is available about genetic risk factors for fellow-eye involvement of exudative AMD. In the study described herein, we assessed the association of the genotype of *ARMS2* A69S and fellow-eye involvement by exudative AMD. In addition, survival analysis was conducted to estimate the elapsed time from the initial visit for first-eye involvement until second-eye involvement, depending on the particular genotype of ARMS2 A69S.

PATIENTS AND METHODS

FOR THIS OBSERVATIONAL CASE STUDY, WE REVIEWED retrospectively the medical records of 326 patients with exudative AMD who visited the Macular Service of the Department of Ophthalmology at Kyoto University Hospital between May 1, 2004 and April 30, 2007. Inclusion criteria of this study were 1) exudative AMD in at least 1 eye, 2) initial comprehensive ophthalmic examination of both eyes, and 3) minimum follow-up of 2 years after the initial presentation. The diagnosis of exudative AMD was based primarily on indirect ophthalmoscopy and fluorescein angiography, according to the definition of the International Classification System for Age-Related Maculopathy,[22] but we also used indocyanine angiography and optical coherence tomography (OCT) to make the diagnosis. The current study of AMD included patients with PCV and retinal angiomatous proliferation (RAP). However, patients with other macular abnormalities (ie, pathologic myopia, idiopathic choroidal neovascularization [CNV], presumed ocular histoplasmosis, angioid streaks, and other secondary CNV) were excluded from the study. If detailed examination of either eye was difficult because of ocular disease other than AMD, the patient was also excluded from the study.

Baseline characteristics of the patients were obtained from their medical charts, including age, sex, presence of hypertension and diabetes, and history of smoking. Each patient's smoking status was categorized into never smoker, former smoker, and current smoker, according to the classification by Nakanishi and associates.[23] At the initial visit, each patient underwent a comprehensive ophthalmic examination, including determination of best-corrected visual acuity (VA), intraocular pressure measurement, indirect ophthalmoscopy, slit-lamp biomicroscopy with a contact lens, and OCT examination. After fundus photographs were taken, fluorescein angiography and indocyanine green angiography were performed on each patient, using a confocal laser scanning system (HRA-2; Heidelberg Engineering, Dossenheim, Germany). At each scheduled follow-up visit, each patient underwent a complete ophthalmic examination, including VA measurement, slit-lamp biomicroscopy, indirect fundus ophthalmoscopy, and OCT examination. Fluorescein and indocyanine green angiography was performed if necessary.

TABLE 1. General and Fundus Characteristics in Eyes With Unilateral or Bilateral Exudative Age-Related Macular Degeneration at Initial Presentation.

	Unilateral n = 207	Bilateral n = 119	*P* Value
Sex, n (%)			.1987
Female	68 (32.9)	31 (26.1)	
Male	139 (67.1)	88 (73.9)	
Age (y; mean ± standard deviation)	70.1 ± 7.9	74.0 ± 7.7	<.0001
Smoking, n (%)			.0076
None	97 (46.9)	40 (33.6)	
Former	51 (24.6)	49 (41.2)	
Current	42 (20.3)	28 (23.5)	
Diabetes mellitus, n (%)	20 (9.7)	8 (6.7)	.4798
Hypertension, n (%)	49 (23.7)	24 (20.2)	.4650
Genotype of *ARMS2* A69S (GG/TG/TT)			.0270
GG	43 (20.8)	22 (18.5)	
TG	88 (42.5)	33 (27.7)	
TT	76 (36.7)	64 (53.8)	
Polypoidal lesion in either eye, n (%)	144 (69.6)	65 (54.6)	.0068

TABLE 2. General and Fundus Characteristics in Patients With a New Development of Age-Related Macular Degeneration in the Fellow Eye

	Fellow-Eye Involvement (+) n = 23	Fellow-Eye Involvement (−) n = 184	*P* Value
Sex			.6192
Female	6 (26.1)	62 (33.7)	
Male	17 (73.9)	122 (66.3)	
Age (y; mean ± standard deviation)	69.8 ± 7.9	72.4 ± 7.7	.0110
Smoking (none/former/current)			.0619
None	13 (56.5)	84 (45.7)	
Former	10 (43.5)	51 (27.7)	
Current	0 (0)	42 (22.8)	
Diabetes mellitus	3 (13.0)	17 (9.2)	.8353
Hypertension	6 (26.1)	43 (23.4)	.9769
Polypoidal lesion in the first eye	15 (65.2)	130 (70.7)	.7679
ARMS2 A69S genotype (GG/TG/TT)			.0054
GG	3 (13.0)	40 (21.7)	
TG	4 (17.4)	84 (45.7)	
TT	16 (69.6)	60 (32.6)	

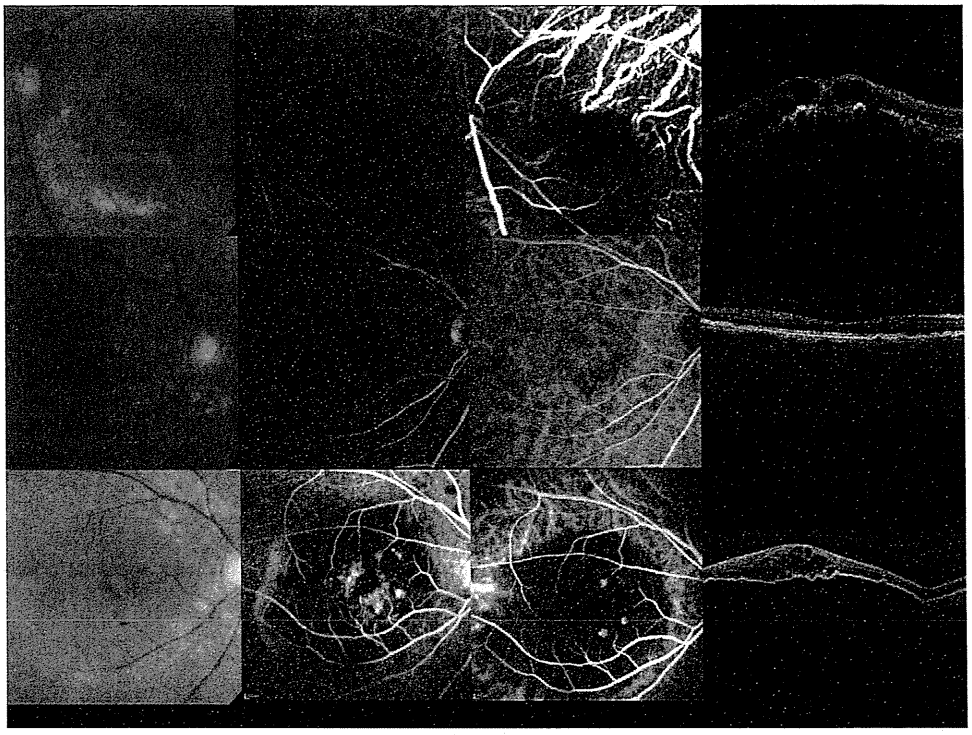

FIGURE 1. Development of exudative age-related macular degeneration in the fellow eye. An 83-year-old woman was referred to our clinic with a 6-month history of metamorphopsia and visual acuity loss in the left eye. At the initial visit, her visual acuity was 20/2000 OS. (Top left) Initial fundus photograph of the left eye shows a grayish lesion with subretinal hemorrhage and hard exudate. (Top, second from left) Fluorescein angiography (FA) shows minimally classic choroidal neovascularization (CNV). (Top, second from right) Indocyanine green angiography (IA) shows blocked fluorescence. (Top right) A sectional image with optical coherence tomography (OCT) shows a pigment epithelial detachment and cystoid macular edema. (Middle left) Initial fundus photograph of the right eye shows only soft drusen in the macular area. No CNV was seen, even by (Middle, second from left) FA, (Middle, second from right) IA, or (Middle right) OCT. Her visual acuity was 20/30 in this eye. (Bottom left) Thirty months after the initial visit, fundus photograph of the right eye shows a grayish exudate and subretinal hemorrhage with a large pigment epithelial detachment. Visual acuity had decreased to 20/130 OD. (Bottom, second from left) FA shows minimally classic CNV corresponding to the lesion seen on fundus photograph. (Bottom, second from right) IA shows retinal angiomatous proliferation. (Bottom right) A sectional image with OCT shows a large pigment epithelial detachment with cystoid macular edema. The genotype of *ARMS2* A69S was identified as TT. She had no smoking history and had no known systemic disease.

Preparation of genomic DNA was carried out from peripheral blood using a DNA extraction kit (QuickGene-610L, Fujifilm, Minato, Tokyo, Japan). *CFH* Y402H rs1061170, I62V rs 800292, and *ARMS2* A69S rs10490924 were genotyped via the Taqman SNP assay with the ABI PRISM 7700 system (Applied Biosystems, Foster City, California, USA).

All values are presented as mean ± standard deviation. Statistical analysis among genotypes was performed using χ^2 test for trend or its exact counterpart. In the current study, the date of occurrence of exudative AMD in the second eye was regarded as the date when the physicians documented AMD newly developing in the fellow eye. A Cox proportional hazard regression analysis was conducted to analyze the association between genotype, smoking history, or age with involvement of this fellow eye. In the current study, survival analysis, with the AMD-free period in the better eye after initial visit, was conducted using Kaplan-Meier methods to analyze the relationship between genotype and second-eye involvement. Of the 207 patients, 29 (14%) were lost to follow-up. A difference was considered statistically significant when the *P* value was less than .05.

RESULTS

IN THE CURRENT STUDY, WE EXAMINED 326 PATIENTS (227 male and 99 female) with exudative AMD. The patients ranged in age from 50 to 90 years (71.6 ± 8.0 years) and all were Japanese. Of the 326 patients, 119 (36.5%) were diagnosed as having bilateral exudative AMD at the initial visit. Table 1 shows the general and ocular characteristics of patients with either unilateral or bilateral AMD at the initial visit. There was no significant difference in sex distribution or in coexisting diabetes mellitus or hypertension between patients with unilateral AMD and those with bilateral AMD ($P = .1987$, $P = .4798$, and $P = .4650$). The mean age of patients with bilateral AMD was signif-

TABLE 3. Cox Proportional Hazard Regression Analysis of Relationship Between Genotype, Smoking History, or Age and Duration From Initial Visit to Second-Eye Involvement of Age-Related Macular Degeneration

Variables	Fellow-Eye Involvement (+)	Fellow-Eye Involvement (−)	Hazard Ratio	95% CI	*P* Value
Genotype			2.673	1.443–5.489	.0013
GG	3	40			
TG	4	84			
TT	16	60			
Smoking	(Never & Former) vs Current		0.680	0.286–1.573	.3663
Never & former	23	135			
Current	0	42			
Age (y), mean ± standard deviation	69.8 ± 7.9	72.4 ± 7.7	1.102	1.043–1.169	.0005

CI = confidence interval; GG = non-risk homozygous; TG = heterozygous; TT = risk homozygous.

icantly higher than that of patients with unilateral AMD ($P < .0001$), and the proportion of current smokers among bilateral AMD patients was significantly greater than in unilateral AMD patients ($P = .0076$). A risk allele of *ARMS2* A69S was associated significantly with bilaterality of AMD ($P = .027$). In addition, polypoidal lesions were more commonly seen in patients with unilateral AMD than in those with bilateral AMD ($P = .0068$) at the initial visit.

To determine those factors associated with fellow-eye involvement, we further examined 207 patients (139 male and 68 female) with unilateral AMD at the initial visit (Table 2). The mean follow-up duration was 56.0 ± 30.2 months (range, 24–182 months). In 23 of these 207 patients (11.1%), exudative AMD developed in the fellow eye during the follow-up period (Fig. 1). The mean elapsed time from the initial visit until the development of exudative AMD in the fellow eye was 56.3 ± 40.4 months (range, 2–149 months). Table 3 shows general and ocular characteristics of patients with and without fellow-eye involvement. There was no significant difference in sex distribution, smoking, coexisting diabetes mellitus or hypertension, or detection of polypoidal lesion in the first eye between the 2 groups ($P = .6192$, $P = .8353$, $P = .9769$, and $P = .7679$, respectively). The mean age of the fellow eye (−) group was higher than that in the fellow-eye involvement (+) group ($P = .0110$). Regarding the distribution of *ARMS2* A69S genotypes, the GG, TG, and TT genotypes were seen in 3, 4, and 16 patients with fellow-eye involvement, respectively, while seen in 40, 84, 60 patients without fellow-eye involvement, respectively. The risk allele of *ARMS2* A69S was significantly associated with fellow-eye involvement ($P = .0054$). In contrast, no association was observed with *CFH* Y402H rs1061170 or I62V rs 800292 in the current study.

Fellow-eye involvement was associated with *ARMS2* A69S genotype (hazard ratio, 2.673; 95% CI, 1.443–5.489; $P = .0013$), age (hazard ratio, 1.102; 95% CI, 1.043–1.169; $P = .0005$), and smoking history (hazard ratio, 0.680; 95% CI, 0.286–1.573; $P = .3663$), in decreasing order (Table 3). As hazard ratios indicate, correlation of genotype (2.673) was as high as that seen with 10 years of aging ($1.102^{10} = 2.641$).

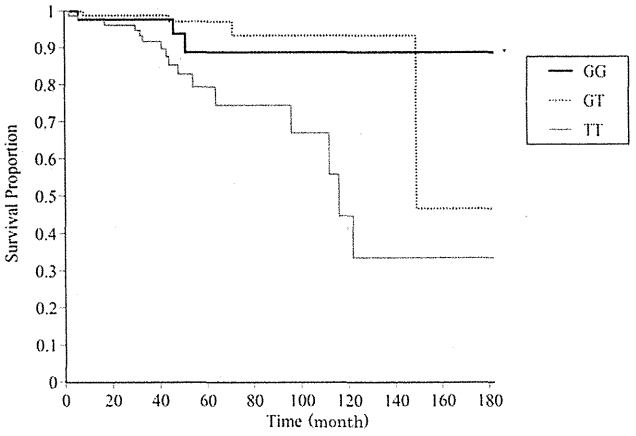

FIGURE 2. Overall survival analysis curve of the period free from second-eye involvement by age-related macular degeneration among patients with discrete genotypes of *ARMS2* A69S. Patients with the risk homozygous genotype (TT) experienced second-eye involvement in a significantly shorter period of time than did those with other genotypes ($P = .0028$). At 120 months after the initial visit, 50% of TT patients presented with second-eye involvement, while only 6.6% of GT patients and 11.2% of GG patients had second-eye involvement.

Survival analysis for the AMD-free duration in the second eye revealed that the risk homozygous TT genotype caused second-eye involvement significantly earlier than other genotypes ($P = .0028$). The median survival time was 120 months for the TT cohort, was 150 months for the TG cohort, and was not determined for the GG cohort. When the observation duration reached 120 months, second-eye involvement was seen in 50% of the TT cohort, compared with 6.6% of the GT cohort and 11.2% of the GG cohort (Figure 2).

DISCUSSION

TO DATE, VARIOUS RISK FACTORS FOR AMD HAVE BEEN seen in cohort studies, including the Age-Related Eye Disease Study (AREDS), the Beaver Dam Eye Study, the Rotterdam Study, and the Blue Mountains Eye Study.[1,2] From these reports, it is generally recognized that smoking and age are common risk factors for any type of AMD.[1] The AREDS recommended supplementation, a combination of zinc and antioxidants (β-carotene, vitamin C, and vitamin E); this produced a 25% reduction in the incidence of advanced AMD over 5 years and a 19% reduction in severe vision loss in those deemed to be at high risk of having an advanced form of the disease.[2] However, dietary supplementation cannot completely prevent AMD or its fellow-eye involvement. Furthermore, the response to this AREDS supplementation is reported to be related to genotypes.[24]

Both in whites and in Asians, *CFH* and *ARMS2/HTRA1* genes seem to be the major susceptibility genes for AMD.[9,10,13,14] Although in whites, *CFH* is the most significantly associated gene, followed by *ARMS/HTRA1*, AMD in Asian patients showed a stronger association with *AMRS2/HTRA1* than with *CFH*.[17,25] A phenotypic study for AMD revealed that *ARMS2/HTRA1* is associated with visual acuity, RPE hyperpigmentation, drusen size, and CNV size, while *CFH* is not associated—at least in the Japanese population.[25] We have also demonstrated that, unlike *CFH*, *ARMS2/HTRA1* is associated with CNV size in both AMD and PCV,[16] and is also significantly associated with bilaterality of these conditions.[13,20,21] Furthermore, recent reports have shown that the *ARMS2/HTRA1* genotype affects visual prognosis of AMD and PCV—even after photodynamic therapy.[26–28]

In the current study, a risk allele (T) of *ARMS2* A69S was more frequently seen in patients having bilateral AMD at the initial presentation than in those having unilateral presentation. However, even in patients with unilateral AMD at the initial visit, the *ARMS2* A69S risk allele is associated with a higher risk for the development of exudative AMD in the fellow eye. As far as our literature survey could ascertain, there have been no reports on the relationship between *ARMS2* and the AMD-free period in the second eye after the initial presentation. Survival analysis revealed that patients with the TT homozygous genotype presented with second-eye involvement significantly earlier than did patients with other genotypes. When the observation duration reached 120 months, second-eye involvement was evident in 50% of the TT cohort.

The current study also showed that patients with other genotypes of *ARMS2* A69S had a lower risk for bilateral AMD. Patients that do not have risk homozygous *ARMS2* A69S are estimated to have about a 10% risk of having fellow-eye involvement by AMD in 10 years, which may be of help to physicians who are determining the endpoint of treatment of the first eye with advanced AMD, especially when visual function is poor. If visual disturbance is limited to 1 eye because of AMD and other ocular diseases, the quality of life may be not impaired greatly, but once the second eye is also involved and the visual function of both eyes is impaired, QOL will be significantly damaged.[29] These academic discussions have been applied already to clinical practice, as is clear in the assessment for amblyopia screening in Health Technology Assessment.[30]

Smoking status and age at the initial visit are also risk factors for bilateral AMD. In the EUREYE study, patients with bilateral AMD tended to have a heavier smoking history than did those with unilateral involvement.[31] On the other hand, Sakurada and associates did not report any association of smoking history with bilateral development of PCV.[21] In the current study, smoking status had a significant association if bilateral AMD was diagnosed at the initial visit, but had no significant association with second-eye involvement by AMD or with the duration until second-eye involvement. Of smokers at the initial visit, a considerable proportion stopped smoking after being informed that smoking is the major risk factor for AMD. Thus smoking status at the initial visit may not be the best explanatory variable for the second-eye involvement model. There remains conflicting evidence about the relationship between smoking and second-eye involvement by AMD, and the influence of smoking seems to require more investigation with a larger body of data, although in the current study, aging was correlated significantly with second-eye involvement by AMD, which is consistent with previous findings.[32] As the hazard ratios indicate that the correlation of genotype to second-eye involvement (2.673) was as high as that of 10 years of aging ($1.102^{10} = 2.641$), the genotype of *ARMS2* A69S has as strong an association with second-eye involvement as 10 years of aging.

The current study has several limitations that need to be pointed out. First, this investigation was conducted as a retrospective study of relatively small size. Second, elderly patients (over 80 years of age at the initial visit) were included in the current study, and it might be inappropriate to include such elderly patients for estimation of the future occurrence of AMD in the second eye. Third, exudative AMD includes subgroups such as PCV and RAP. It has been reported that typical AMD and PCV have a similar probability of involvement of the fellow eye in unilaterally affected Japanese patients, even though PCV and RAP have different clinical presentations. Finally, dietary supplementation was not considered in the current study, and it is possible that such supplements may contribute to the avoidance of second-eye affection.

In the current research, we reconfirmed the association of *ARMS2* A69S genotype with second-eye involvement of

AMD and found an association with elapsed time until second-eye involvement. However, future research involving more candidate genes and other possible factors may reveal more precisely the future risks of fellow-eye involvement by AMD.

ALL AUTHORS HAVE COMPLETED AND SUBMITTED THE ICMJE FORM FOR DISCLOSURE OF POTENTIAL CONFLICTS OF Interest. A. Tsujikawa has received grant support from Pfizer; N. Yoshimura is a consultant for Nidek and has received grant support and lecture fees from Topcon Corporation, Nidek, and Canon. Publication of this article was supported in part by grants-in-aid for scientific research (Nos. 22791655) from the Japan Society for the Promotion of Science, Tokyo, Japan; and the Japan National Society for the Prevention of Blindness, Tokyo, Japan. The funding organizations had no role in the design or conduct of this research. Involved in conception and design of study (H.T., A.T., K.Y., N.Y.); analysis and interpretation (H.T., A.T., K.Y., Y.A.-K., I.N.); writing of the article (H.T., K.Y.); critical revision of the article (A.T., S.O., K.Y., N.Y.); final approval of the article (H.T., A.T., K.Y., Y.A.-K., I.N., H.N., H.H., S.O., A.O., N.Y.); and data collection (H.T., Y.A.-K., I.N., H.N., H.H.). Both prospective protocol to collect DNA and retrospective protocol for the review of selected patients in the current study were approved by the Institutional Review Board (IRB) of Kyoto University Hospital and Kyoto University Graduate School of Medicine. All patients were fully informed of the purpose and procedure of this study, and written consent was obtained from each patient included in this study. All investigations in the current study adhered to the tenets of the Declaration of Helsinki.

The authors thank Dr Francis Cook, of the Harvard School of Public Health, for epidemiologic advice, and Dr John Orav, also of the Harvard School of Public Health, for biostatistical advice.

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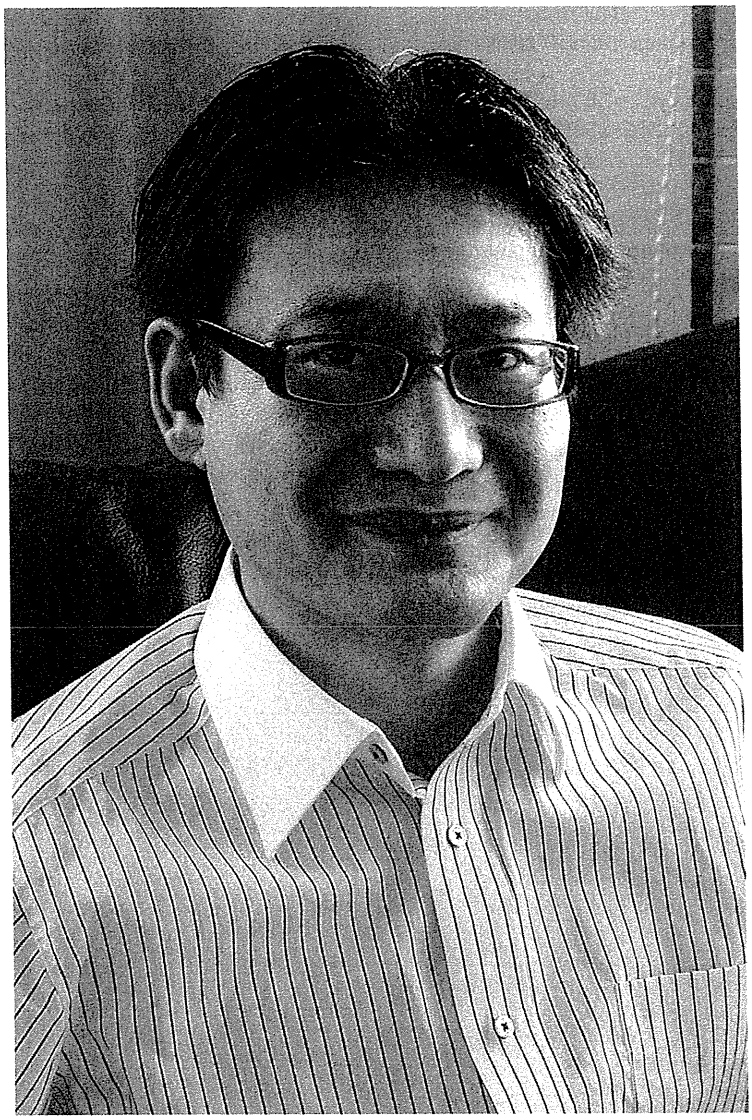

Biosketch

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ARTICLE

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 *ALPPL2*) and confirmed one previously reported AL locus (*ZC3H11B*). Of the nine loci, five (*LAMA2, GJD2, CD55, ALPPL2,* 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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http://dx.doi.org/10.1016/j.ajhg.2013.06.016.

Table 1. Study Cohorts and Summary of Axial Length Measures

Ethnicity	n	Study	Mean Age (SD), Years	Men, %	Axial Length		
					Mean (SD), mm	Range, mm	Methods of Measurement
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	Echoşcan 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	Lenstar LS900
	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 Parents[38]) 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 intraocular 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 software[63] 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 c^2 test statistics derived from VEGAS p values. Empirical VEGAS-Pathway p values for each pathway were computed by comparing the summed c^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 c^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^{-DDCt} 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 RNA*later* 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 *mir*Vana 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 mi reactions with QIAGEN's PCR products consisting of 1.26 mi H_2O, 1.0 mi 103 buffer, 1.0 mi dNTPs, 0.3 mi MgCl, 2.0 mi Q-Solution, 0.06 mi taq polymerase, 1.0 mi forward primer, 1.0 mi reverse primer, and 1.5.0 mi 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 (λ_{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

				European Ancestry Cohorts (Stage 1, n = 12,531)				Asian Cohorts (Stage 2, n = 8,216)				Combined (Stage 3, n = 20,747)				
Lead SNP[a]	Chr	Position[b]	EA	EAF	Beta[c]	SEM	p Value	EAF	Beta[c]	SEM	p Value	EAF	Beta[c]	SEM	p Value	Localization Relative to Protein-Coding Genes[b]
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 *RSPO1* (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 *ZC3H11B*
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 *C3orf26*
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 *LAMA2* (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 *GJD2* (MIM 607058)
Additional locus 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 *ZNRF3* (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.

[a]Lead 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.

[b]Position is based on NCBI human genome build 36.

[c]Effect 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, b = 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, b = 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 $p_{gene\text{-}based} < 0.05/17{,}872 = 2.8 \times 10^{-6}$ (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)

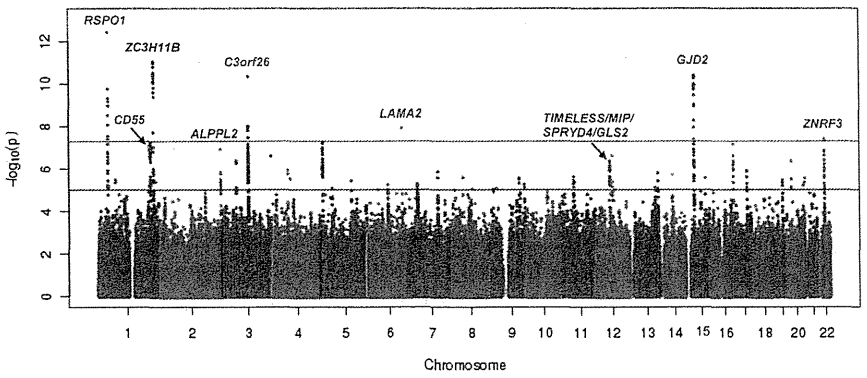

Figure 1. Summary of Meta-analysis Results for Genome-wide Association to Ocular Axial Length

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 *CHRNG*, 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 *CHRNG*. 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

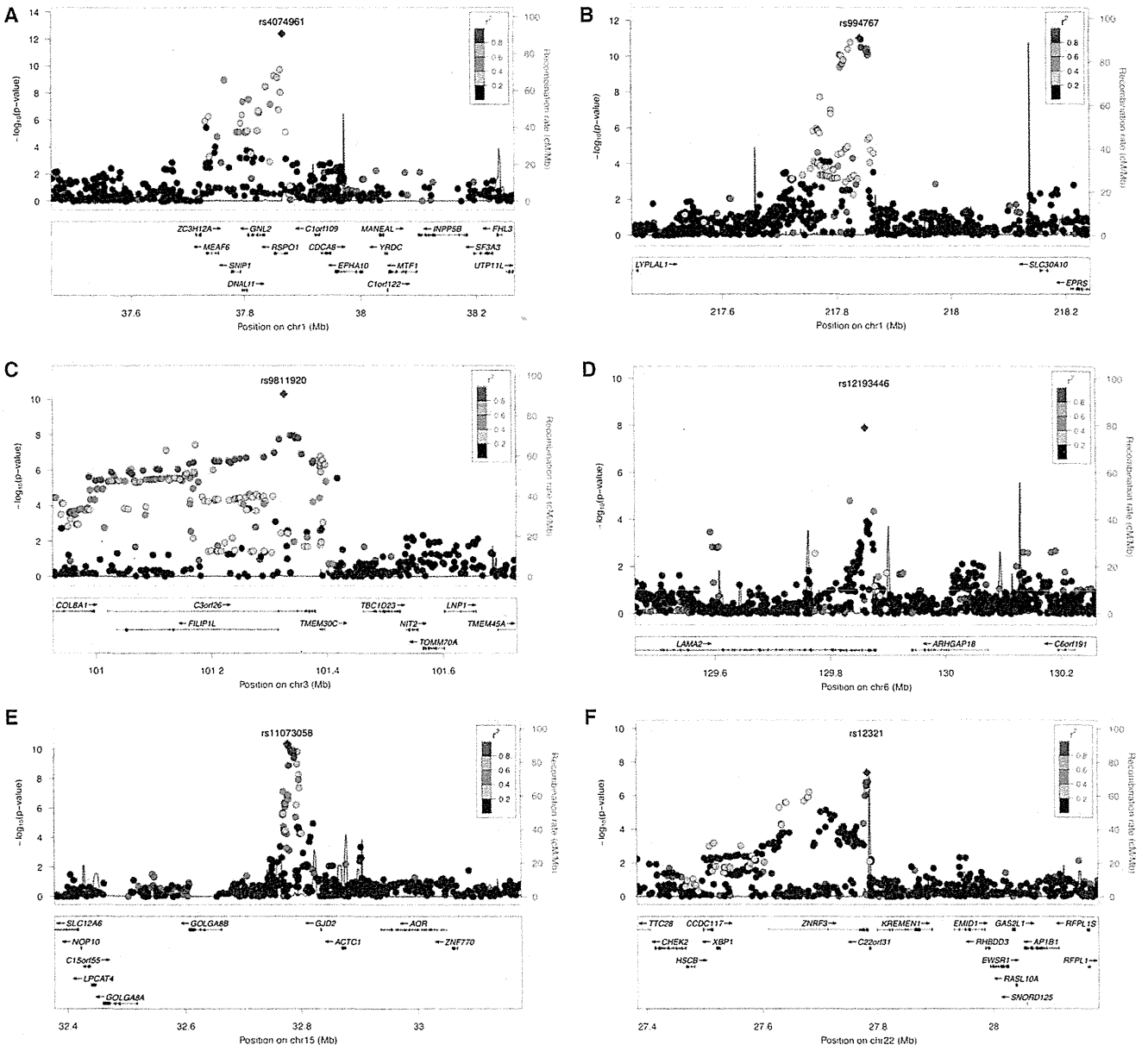

Figure 2. Regional Association Plots and Recombination Rates of the Loci Associated with Ocular Axial Length
Data are shown for association at chromosome (A) 1p34.3 (*RSPO1*), (B) 1q41 (*ZC3H11B*), (C) 3q12.1 (*C3orf26*), (D) 6q22.33 (*LAMA2*), (E) 15q14 (*GJD2*), and (F) 22q12.1 (*ZNRF3*) in the combined 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 R 0.8; yellow, 0.6 % r^2 < 0.8; green, 0.4 % r^2 < 0.6; cyan, 0.2 % 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 HaploReg[73] 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 RegulomeDB2[74] 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 HaploReg[73] 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]	$p_{gene\text{-}based}$ Value		
					European Ancestry Cohorts	Asian Cohorts	Combined[b]
CD55	125240	1	205561439	205600934	1.3×10^{-5}	9.6×10^{-4}	2.3×10^{-7}
ALPPL2	171810	2	232979795	232983669	6.4×10^{-5}	1.7×10^{-3}	1.8×10^{-6}
TIMELESS/MIP/SPRYD4/GLS2[c]	603887	12	55097173	55168448	2.0×10^{-7}	7.3×10^{-2}	2.8×10^{-7}

The following abbreviation is used: Chr, chromosome.
[a]Position 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.
[b]Gene-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 5 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 cohorts[20] 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 twins[78] that there are common genetic determinants of the two traits, such as variants in *GJD2, LAMA2, CD55*, and *ALPPL2*. 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	*RSPO1* (MIM 609595)	T	0.004	0.023	0.84
rs994767	*ZC3H11B*	A	0.054	0.022	1.3×10^{-2}
rs9811920	*C3orf26*	A	−0.022	0.022	0.31
rs12193446	*LAMA2* (MIM 156225)	A	−0.242	0.039	3.6×10^{-10}
rs11073058	*GJD2* (MIM 607058)	T	−0.121	0.022	1.7×10^{-8}
rs12321	*ZNRF3* (MIM 612062)	C	−0.004	0.021	0.86

Abbreviations are as follows: SNP, single-nucleotide polymorphism; SEM, standard error of the mean.
[a]Effect sizes on spherical equivalent are in diopters.

Table 5. Association of the Axial Length Genes Identified in Gene-Based Tests with Spherical Equivalent in Independent Cohorts

Gene	MIM Number	Chr	$p_{gene\text{-}based}$ Value[a]
CD55	125240	1	4.5×10^{-6}
ALPPL2	171810	2	8.3×10^{-3}
TIMELESS/MIP/SPRYD4/GLS2[b]	603887	12	0.14

Abbreviation is as follows: Chr, chromosome.
[a]The association with spherical equivalent was assessed in 17 European ancestry cohorts of the 18 independent cohorts, with the HapMap 2 CEU population as the reference to estimate patterns of LD.
[b]Because of the 5 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 0.14 to 0.20 for the combined analysis), and thus p value and MIM number for *TIMELESS* only is presented.

susceptibility genes do not overlap between primary angle closure glaucoma and eyes with shorter axial length.

In summary, we identified nine loci associated with AL. They fall into two groups, one also influencing common refractive error variation, and the other, which includes two genes in the Wnt signaling pathway, uniquely determining eye size with little effect on natural refractive status. Further elucidation and characterization of the causal variants underlying the growth of ocular component dimensions and the development of myopia may enable new pathway and target identification, leading to potential prevention and treatment development.

Supplemental Data

Supplemental Data include six figures, ten tables, and Supplemental Acknowledgments and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments

The authors thank the staff and participants of all studies for their important contributions. Complete funding information and acknowledgements are provided in the Supplemental Data. ALSPAC thanks 23andMe for funding the generation of the ALSPAC GWA data.

Received: March 15, 2013
Revised: April 17, 2013
Accepted: June 12, 2013
Published: August 8, 2013

Web Resources

The URLs for data presented herein are as follows:

HaploReg, http://www.broadinstitute.org/mammals/haploreg/haploreg.php
IMPUTE2, http://mathgen.stats.ox.ac.uk/impute/impute_v2.html
International HapMap Project, http://hapmap.ncbi.nlm.nih.gov/
LocusZoom, http://csg.sph.umich.edu/locuszoom/
MACH, http://www.sph.umich.edu/csg/abecasis/MACH/
METAL, http://www.sph.umich.edu/csg/abecasis/metal/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
RegulomeDB, http://RegulomeDB.org/
UCSC Genome Browser, http://genome.ucsc.edu
VEGAS, http://gump.qimr.edu.au/VEGAS/

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