TABLE 1. Characteristics of the Study Population

Variable	Cases, n = 581	Controls, n = 793	P Value
Age, y			
Mean 6 SD	72.59 6 8.13	65.99 6 4.33	<0.0001
Range	48–92	60–75	
Sex, *n* (%)			
M	420 (72.3)	326 (41.1)	<0.0001
F	161 (27.7)	467 (58.9)	
Smoking status, *n* (%)			
Never	200 (38.5)	509 (64.3)	<0.0001
Former	195 (37.6)	176 (22.3)	
Current	124 (23.9)	106 (13.4)	

Five hundred eighty-one patients with PCV were recruited from the departments of ophthalmology at Kyoto University Hospital, Fukushima Medical University Hospital, and Kobe City Medical Center General Hospital. The diagnosis of PCV was based on indocyanine green angiography, which showed a branching vascular network terminating in polypoidal swelling (Figure), and was confirmed by three retina specialists (KY, AT, AO); a fourth specialist (NY) was consulted when the diagnosis could not be agreed on by the initial three reviewers. Patients who had both typical CNV and polypoidal lesions were excluded from this study. The control group consisted of 793 unrelated individuals 60 years or older recruited in the Nagahama Prospective Genome Cohort for Comprehensive Human Bioscience (the Nagahama Study).[30] Fundoscopic photographs of both eyes confirmed the absence of any signs of AMD (large drusen or pigment change) using the Age-Related Eye Disease Study[31] severity scale, with grading by two independent ophthalmologists (IN, YAK), followed by grading by a senior reviewer (KY).

We targeted three single-nucleotide polymorphisms (SNPs) of three genes reported to be associated with HDL cholesterol levels in blood, including rs493258 at the hepatic lipase gene (*LIPC*), rs3764261 at the cholesteryl ester transfer protein gene (*CETP*), and rs12678919 at the lipoprotein lipase gene (*LPL*).[32] Genomic DNA was prepared from peripheral blood using a DNA extraction kit (QuickGene-610L; Fujifilm, Minato, Tokyo, Japan). All case samples were genotyped using the Taqman SNP assay with an ABI PRISM 7700 system (Applied Biosystems, Foster City, CA). Controls were genotyped using Human610-Quad BeadChips and HumanOmni2.5 BeadChips (Illumina, Inc., San Diego, CA). *ARMS2* A69S (rs10490924) and *CFH* I62V (rs800292) were also genotyped in the same manner. Fasting serum samples from the control subjects were analyzed for HDL cholesterol level, measured using a direct assay system with the selective inhibitory method on an automatic analyzer (LABOSPECT 008; Hitachi, Ltd., Tokyo, Japan). We did not have HDL cholesterol data for the case samples.

Information on smoking status was obtained via a self-reported questionnaire with three categories of never smoker, former smoker, and current smoker. The never smokers were those who had smoked fewer than 100 cigarettes in the past, current smokers were those who had smoked in the past year, and former smokers were those who had quit smoking more than 1 year earlier.

TABLE 3. Logistic Regression Analysis, Including Major Factors Associated With PCV

Variable	P Value*	OR (95% CI)
Age	<0.0001	1.18 (1.16–1.21)
F:M sex	<0.0001	3.16 (2.20–4.52)
ARMS2 rs10490924 (*G/T*)	<0.0001	2.27 (1.86–2.77)
CFH rs800292 (*A/G*)	<0.0001	1.77 (1.43–2.19)
LIPC rs493258 (*G/A*)	0.689	1.05 (0.82–1.35)
CETP rs3764261 (*C/A*)	0.0013	1.50 (1.17–1.92)
LPL rs12678919 (*A/G*)	0.948	0.99 (0.72–1.35)
Smoking (never, former, or current)	0.0107	1.35 (1.07–1.69)

* A logistic regression model was used for covariate adjustment.

Deviations in genotype distributions from the Hardy-Weinberg equilibrium (HWE) of the controls were assessed with the HWE exact test. Statistical differences in the observed allelic distribution were identified using logistic regression analyses with age and sex adjustments, under the assumption of an additive genetic effect where the genotypes of each SNP are coded numerically as 0, 1, and 2 for the number of minor alleles carried. A linear regression analysis was performed to assess the association between HDL cholesterol level and genotype. R software (http://www.r-project.org/ in the public domain) was used for statistical analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Demographics of the study population are given in Table 1. Genotype and allele frequencies of the three SNPs were analyzed in 581 patients with PCV and compared with those of 793 age-matched individuals without any signs of AMD or PCV. The genotyping of all evaluated SNPs had a success rate exceeding 99.4%.

Table 2 gives details of genotype and allele frequencies and summary statistics. The distributions of the genotypes for all evaluated SNPs were in HWE ($P > 0.05$). We found that *CETP* rs3764261 was significantly associated with the development of PCV; the frequency of the minor allele *A* in the patients with PCV (24.0%) was higher than that in the controls (18.5%) ($P = 0.0025$; odds ratio [OR], 1.41; 95% confidence interval [CI], 1.13–1.75). This significant association remained even after a correction for multiple testing ($P = 0.0075$). *LIPC* rs493258 and *LPL* rs12678919 did not show significant associations with the development of PCV ($P > 0.05$).

Next, we conducted a logistic regression analysis that included the effects of the most robust Japanese variants associated with AMD and PCV, *ARMS2* A69S (rs10490924) and *CFH* I62V (rs800292), as well as age, sex, smoking status, *LIPC*

TABLE 2. Distribution of Genotypes and Results of the Association Tests

		Allele		Cases, n = 581				Controls, n = 793				Association Results*	
Gene	SNP	1	2	11	12	22	MAF	11	12	22	MAF	P Value	OR (95% CI)
LIPC	rs493258	*G*	*A*	32	185	354	0.22	37	259	497	0.21	0.706	1.04 (0.84–1.30)
CETP	rs3764261	*C*	*A*	332	210	33	0.24	528	237	28	0.19	0.0025	1.41 (1.13–1.75)
LPL	rs12678919	*A*	*G*	439	135	3	0.12	602	179	12	0.13	0.883	1.02 (0.77–1.35)

MAF, minor allele frequency.
* Adjusted for age and sex.

rs493258, and *LPL* rs12678919 in the regression model. Table 3 gives the results of the logistic regression analysis. *CETP* rs3764261 remained significant for the development of PCV even after including the effects of these covariates ($P = 0.0013$; OR, 1.50; 95% CI, 1.17–1.92).

Finally, we investigated the role of *CETP* rs3764261 in blood HDL cholesterol level using fasting serum samples from 793 control subjects. The mean 6 SD HDL cholesterol level of the control samples was 61.3 6 16.1 mg/dL. In this analysis, we found that the *A* allele of rs3764261 was associated with the following increases in HDL cholesterol: 59.3 mg/dL for the *CC* genotype, 64.8 mg/dL for the *CA* genotype, and 67.2 mg/dL for the *AA* genotype ($P < 0.0001$).

Discussion

Plasma CETP was first described as a high-molecular-weight protein stimulating the transfer of cholesteryl ester between lipoproteins in plasma of hypercholesterolemic rabbits.[33] Other studies demonstrated various roles of CETP in the lipid pathway: CETP facilitates the transfer of triglycerides and phospholipids[34]; it is an important component of reverse cholesterol transport, which is chiefly characterized by the transport of cholesterol from peripheral tissues to the liver; and it regulates the concentration of HDL cholesterol.[35,36]

After the discovery of the association between HDL cholesterol level and cardiovascular diseases,[37] studies[38,39] evaluated the functional role of the lipid-associated genes that can affect the HDL cholesterol level. Among those genes, the *A* allele of *CETP* rs3764261 was associated with an increase in HDL cholesterol by 5.6 mg/dL among the Japanese population.[40] Herein, we confirmed the role of rs3764261 in increased HDL cholesterol levels among 793 healthy Japanese individuals.

In the present study comparing the allelic distributions of *CETP* variants in a sample of 581 patients with PCV and 793 control subjects, the *A* allele of *CETP* rs3764261 was significantly associated with a risk of developing PCV (OR, 1.41; 95% CI, 1.13–1.75), which indicates a higher level of HDL cholesterol in patients with PCV. In addition, the association of *CETP* variants remained significant even when we adjusted for the effects of other established risk factors for developing AMD and PCV (age, sex, smoking status, and genetic background of *ARMS2* A69S, *CFH* I62V, *LIPC* rs493258, and *LPL* rs12678919). Although the effect of *CETP* variants (OR, 1.50) was not as large as the effects of the major genes associated with AMD and PCV (ORs, 2.27 for *ARMS2* and 1.77 for *CFH*) in this regression analysis, we were able to confirm that *CETP* variants have a significant role in the development of PCV. Our findings for *CETP* rs3764261 were similar to the associations already documented in AMD among Caucasians,[41,42] which suggests that a higher HDL cholesterol level may be a risk factor in both PCV and Caucasian AMD. The hypothesis that a higher level of HDL cholesterol is associated with the development of PCV might appear contradictory to the fact that a lower level of HDL cholesterol is associated with an increased risk of cardiovascular disease. However, despite the well-known antiatherogenic properties of HDL cholesterol, some studies[10,11,43] found elevated levels of HDL cholesterol in Caucasian patients with AMD.

Recently, Zhang et al.[44] reported an investigation of lipid-associated SNPs for PCV and neovascular AMD in a Chinese population. In that article, they showed a significant association of *CETP* with PCV, while no association was found with neovascular AMD. Thus, they concluded that the HDL cholesterol pathway in the pathogenesis of PCV likely differs from that of neovascular AMD. However, the sample size evaluated in the their article was small (204 controls, 250 patients with PCV, and 157 patients with neovascular AMD), which suggests that the negative result of the association between *CETP* and neovascular AMD could have been due to insufficient power to detect the association. To confirm whether the observed association of *CETP* with PCV exists for neovascular AMD as well, we performed an additional analysis using another Japanese cohort of neovascular AMD cases ($n = 452$). In this evaluation, we found a significant association between *CETP* and neovascular AMD ($P = 0.0246$; OR, 1.35).

Adenosine triphosphate-binding cassette, subfamily A member 1 (ABCA1) is also known to be associated with the lipid pathway. Because *ABCA1* has been reported to be another susceptible gene for the development of AMD in Caucasians,[19] we also evaluated whether *ABCA1* rs1883025 has a significant role in the development of PCV but found no significant association with PCV ($P > 0.05$). In previous genome-wide association analyses for HDL cholesterol, the strongest and most consistently associated SNPs have been reported in the *CETP* locus.[45,46] Study[32] findings also suggest that *LIPC* rs493258 and *LPL* rs12678919 are associated with HDL cholesterol level in Caucasians, so the lack of association in the present study could be due to insufficient statistical power or racial/ethnic differences. Further study that includes a larger number of participants is needed to clarify the association between genetic variants of HDL cholesterol-associated genes and the development of PCV.

In the present study, there was a large sex difference between the PCV cases and the general population controls. It remains unknown why there is such a high prevalence of PCV among men. In a previous meta-analysis by Kawasaki et al.,[47] the prevalence of late AMD among Asian women was reported to be much lower than that among Asian men. In contrast, a male predominance was reported in PCV.[4] Considering the high prevalence of PCV among Asian populations, these results suggest that men are more likely to develop PCV. In our study, genetic factors had an enormous influence on whether participants developed PCV (Table 3). However, sex had the largest effect among all covariates on the development of PCV (OR, 3.16). A previous genetic study[23] among Japanese may provide insight into this question because the results suggested that differences in sex would affect phenotypic differences in AMD. Another limitation of the present study was the age difference between cases and controls. Although we enrolled only controls who were 60 years or older, the average age of the control cohort was still younger than that of the case cohort, which means that some of the young controls may develop PCV in the future. To exclude a potential confounder of genetic background with age, a logistic regression analysis adjusting for age and sex was performed in the present study. However, given that the prevalence of late AMD among the Japanese population is reported to be 0.5%,[48] the magnitude of statistical bias of the association analysis is negligible. In addition, considering that case-control association analyses among such subjects are less likely to be statistically significant, our positive results should be acceptable.

Overall, this study provides the first evidence to date that *CETP* variants have a significant role in the risk of developing PCV among the Japanese population. Our study also indicates the same role of HDL cholesterol in both PCV and Caucasian AMD, although the role of fatty acids in Japanese AMD is reported to be different from that in Caucasian AMD.[49] Further studies are needed to increase the understanding of the genetic backgrounds of PCV, as well as the molecular pathogenesis, particularly the role of lipids.

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Appendix

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Investigative Ophthalmology & Visual Science

Prevalence and Characteristics of Age-Related Macular Degeneration in the Japanese Population: The Nagahama Study

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• PURPOSE: To estimate the age- and sex-specific prevalence of early age-related macular degeneration (AMD; drusen and retinal pigment abnormalities) and late AMD (exudative AMD and geographic atrophy) in the Japanese population.

• DESIGN: Community-based, cross-sectional study.

• METHODS: The study was held in Nagahama, Japan, and included 6065 Japanese individuals (aged ≥50 years) recruited in 2008-2010. We graded fundus photographs of both eyes for the AMD phenotype based on drusen size, the presence of retinal pigment abnormalities, and late AMD. The associations between smoking and AMD phenotypes were also evaluated.

• RESULTS: We assessed 5595 subjects (women, 65%) with a gradable macular condition. Early and late AMD prevalence increased from 16.1% and 0.27% at 50-59 years to 31.2% and 0.98%, respectively, at 70-74 years and was predominant in male subjects in each age group. Smoking was associated with both early and late AMD stages and retinal pigment abnormalities ($P < .0001$), but not with drusen ($P = .305$). The prevalence of retinal pigment abnormalities was significantly higher in men ($P < .0001$), which was associated with high rates of cigarette smoking. We found no sex difference for the prevalence of large drusen ($P = .264$).

• CONCLUSIONS: The prevalence of early AMD among adult Japanese persons was similar to the rates in white populations. The prevalence of late AMD in Japanese people aged <70 years was similar to that observed in white populations, whereas that in Japanese people aged ≥70 years was relatively lower. (Am J Ophthalmol 2013;156:1002–1009. © 2013 by Elsevier Inc. All rights reserved.)

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AGE-RELATED MACULAR DEGENERATION (AMD) IS the leading cause of visual impairment in the elderly and is the most common cause of blindness in developed countries.[1] The stages of AMD are categorized as early, in which visual symptoms are inconspicuous,[2] and late, in which severe vision loss is typical. Early AMD is characterized by drusen or by pigment abnormalities of the retinal pigment epithelium (RPE) in the macula, without visible choroidal vessels.[1] The presence or absence of these 2 features is characteristic of AMD and is highly associated with the development of late AMD, especially when the status of both eyes is considered.[3]

To date, the introduction of anti–vascular endothelial growth factor (VEGF) intravitreal injections has offered remarkable clinical benefits for patients with late AMD.[4] However, because these benefits are associated with an increased financial burden of providing care for these patients,[5] determining the precise incidence of AMD and identifying its risk factors are still required in order to develop preventive measures for this disease. In fact, an increasing number of studies have reported the epidemiology of AMD in different racial/ethnic groups over the last 10 years.[6–8] However, although the state of health, food intake, nutritional intake, and lifestyle of the Japanese people have been changing,[9] only 2 small cohorts, the Hisayama study[10] (1998), with 1486 participants aged ≥50 years, and the Funagata study[11] (2000-2002), with 1246 participants aged ≥50 years, have evaluated the prevalence of AMD in the Japanese population.

These 2 population-based studies (the Hisayama study and Funagata study) arrived at similar conclusions regarding the prevalence of late AMD: late AMD is less common among Japanese people (with a reported overall prevalence of 0.87% and 0.6%, respectively) than among white subjects.[10,11] However, these 2 studies arrived at different conclusions regarding the prevalence of early AMD in Japanese. Although the Hisayama study suggested a lower prevalence of early AMD in the Japanese,[10] the Funagata study indicated that the prevalence of early AMD is similar to that reported in the Blue Mountains Eye Study (BMES).[11] A recent meta-analysis in 4 Asian populations reported that the prevalence of early AMD in Asians is lower than that in white populations.[12] It is well known that polypoidal choroidal vasculopathy (PCV) has a higher

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prevalence as a subtype of AMD in Asians than in whites.[13] Therefore, these results showing a lower prevalence of early AMD in Asians were convincing because previous studies reported a lower prevalence of drusen in PCV.[14–16] However, a subsequent clinical study suggested that drusen is not an uncommon feature of PCV.[17–19] Because the small number of participants in previous Japanese studies limits meaningful comparisons of the prevalence between the Japanese and other populations, a study with a larger number of participants is required to estimate the precise prevalence of AMD in the Japanese.

Nagahama is a regional mid-sized city located in the central region of the main island of Japan. The municipality has a population of approximately 126 000 (2010 Japan census). The aim of the present study was to describe the age- and sex-specific prevalence of early and late AMD in a general adult population of Nagahama, Japan.

METHODS

THE NAGAHAMA PROSPECTIVE GENOME COHORT FOR THE Comprehensive Human Bioscience, hereinafter referred to as the Nagahama Study, is a community-based prospective cohort study that aims to determine the prevalence and risk factors of various diseases in a community. At baseline, all participants underwent automatic refractometry (Autorefractor ARK-530; Nidek, Tokyo, Japan), axial length measurement (IOL Master; Carl Zeiss, Jena, Germany), and fundus photography using a digital retinal camera (CR-DG10; Canon, Tokyo, Japan) in a darkened room. For this study, residents of Nagahama City who satisfied the following criteria were recruited as participants and were examined between November 2008 and November 2010: (1) age ≥30 years and ≤74 years; (2) ability to participate on one's own; (3) no significant problems communicating in Japanese; (4) no current serious diseases/symptoms or health issues; and (5) voluntarily decided to participate in this study. Information regarding recruitment was provided through newsletters/homepages of government and citizen organizations, newspaper flyers, and brochures. The goal for the number of participants was set at 10 000 (approximately 15% of the population; age, 30-74 years). All procedures in this study adhered to the tenets of the Declaration of Helsinki. The Kyoto University Graduate School and Faculty of Medicine Ethics Committee, the Ad Hoc Review Board of the Nagahama Cohort Project, and the Nagahama Municipal Review Board of Personal Information Protection approved all protocols and informed consent procedures.

Overall, 6118 healthy Japanese individuals aged ≥50 years participated in the Nagahama Study. In the present study, we evaluated subjects who had nonmydriatic fundus photographs of both eyes showing sufficient quality for grading lesions (Figure 1). Participants with other retinal diseases that would disturb the precise grading for

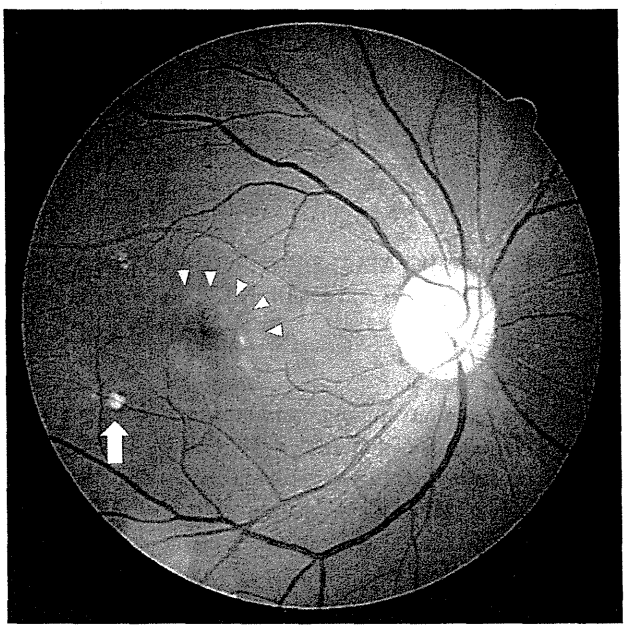

FIGURE 1. Fundus photograph of a 64-year-old Japanese woman with a large drusen (white arrow) and retinal pigment epithelial abnormalities (arrowheads).

macular lesions (such as diabetic retinopathy, retinal vein occlusion, and epiretinal membrane) were excluded from the analysis. Two independent ophthalmologists (I.N. and Y.A. or M.M.) graded each image twice for drusen, RPE abnormalities (hyperpigmentation or hypopigmentation), and late AMD (exudative AMD and geographic atrophy) according to the simplified severity scale for age-related macular degeneration in the Age-Related Eye Disease Study (AREDS).[3] We used the maximum drusen size within the grid (a 3000-μm radius centered on the fovea) at baseline to assess drusen phenotypes. Drusen size was determined using standard circles with diameters corresponding to 63, 125, and 250 μm. Reticular drusen, which were enhanced with the blue channel of the color photograph,[20–22] were considered as soft drusen for the purpose of the analysis.[23] Before grading was initiated for all subjects, intergrader and intragrader agreements were assessed on a random subset of images of 80 eyes of 40 participants. In this initial assessment, the level of agreement between the graders was 1.0 for the presence of late AMD and the agreements between the presence of retinal pigment changes and of drusen size were 0.75 and 0.85-0.90, respectively (crude agreement ratios). The senior reviewers (K.Y. and N.Y.) discussed the cases in which the 2 independent ophthalmologists disagreed and made the final diagnosis. After an agreement had been reached regarding the diagnosis, each photograph was graded twice for all subjects. The level of overall agreement between the grading ophthalmologists was more than 0.94 for most features.

Early AMD was defined by the presence of large drusen (soft distinct and soft indistinct drusen of ≥125 μm in

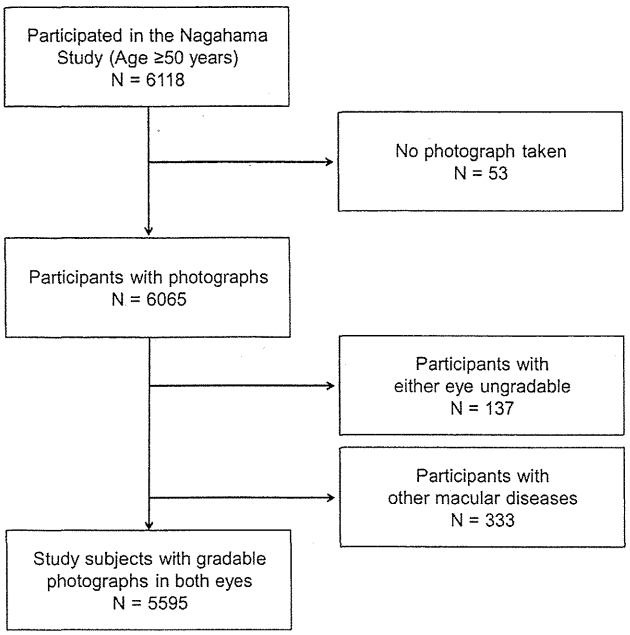

FIGURE 2. Flowchart describing participants from the Nagahama Study who were included and excluded from the analysis for age-related macular degeneration in the Japanese population. Of the 6065 subjects aged ≥50 years, 5595 (92.3%) had gradable fundus photographs in both eyes.

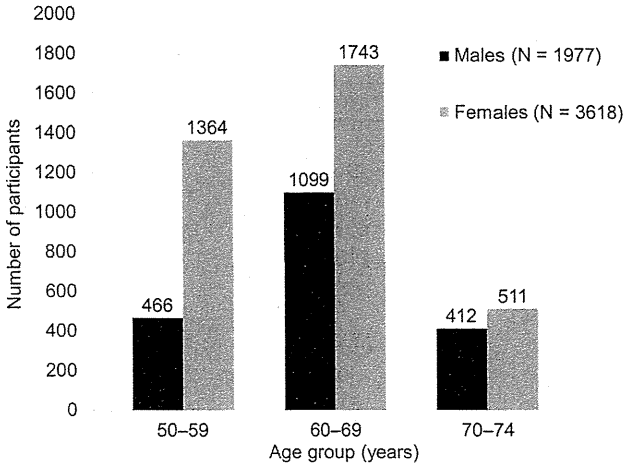

FIGURE 3. Age and sex distribution of the study subjects for age-related macular degeneration in the Japanese population (n = 5595).

diameter) or RPE pigment abnormalities within the grid in the absence of late AMD in either eye.[10,24] Late AMD was defined as the presence of exudative AMD or geographic atrophy (GA). Signs of exudative AMD were retinal pigment epithelial detachment or serous detachment of the sensory retina, subretinal or sub-RPE hemorrhages, and subretinal fibrous scars. GA was defined as a circular discrete area (of at least 175 μm in diameter) of retinal depigmentation with visible choroidal vessels in the absence of exudative AMD.

Information on smoking status was obtained via a self-reported questionnaire. To assess the association between the effect of cigarette smoking and the development of AMD in detail, we used 2 methods of analysis: (1) total cigarette amount using the Brinkman index, which was calculated by the daily number of cigarettes 3 years of smoking[25]; and (2) smoking status, in which the subjects were categorized as never smokers (had smoked less than 100 cigarettes in the past) and ever smokers (had smoked more than 100 cigarettes in the past).

We assessed the age- and sex-specific prevalence of early AMD and late AMD, including the phenotypes of AMD lesions. The age- and sex-adjusted standardized incidences of AMD were calculated using the direct method with reference to the World Health Organization standard population in 2010. We used analysis of variance or the χ^2 test to compare demographic characteristics. P values less than .05 were considered statistically significant.

RESULTS

FUNDUS PHOTOGRAPHS WERE AVAILABLE FOR 6065 subjects aged ≥50 years, and 5595 of these subjects (92.3%) had photographs that were gradable for AMD lesions in both eyes (Figures 2 and 3). Photographs were not taken for 53 participants because of significant media opacities, poor fixation, and/or poor participant cooperation/refusal. Photographs were ungradable in either eye (n = 137) because of media opacities (such as asteroid hyalosis of the vitreous) or poor camera focus. We excluded 333 participants with other macular disease, such as diabetic retinopathy, from the analysis. Thus, a total of 523 participants who had missing or ungradable photographs or who had macular conditions that were inadequate were excluded from this analysis. The participants with gradable photographs (n = 5595) who were included in the analyses were younger (mean age, 62.5 6 6.5 years) than those excluded from the analysis (65.9 6 5.9 years; P < .0001). However, no differences were found in sex between those with gradable and ungradable photographs (P = .588). Thus, the following prevalence data are from 5595 participants with gradable photographs in both eyes.

The summary of the prevalence of phenotypes of AMD in the Nagahama cohort is shown in Table 1. In the study cohort of participants aged ≥50 years, the prevalence of soft drusen (defined as drusen of > 63 μm) was 39.4% (39.2%, standardized) and that of large drusen (defined as drusen of ≥125 μm) was 17.4% (17.5%, standardized). Overall, 22.3% of all subjects had early AMD in at least 1 eye, and the prevalence increased from 16.1% in subjects aged 50-59 years to 31.2% in subjects aged ≥70 years. The overall prevalence of late AMD was 0.52% (0.58%, standardized), which increased from 0.27% in subjects aged 50-59 years to 0.98% in subjects aged 70-74 years.

TABLE 1. Prevalence of Age-Related Macular Degeneration in the Japanese Population

	50-59 Years N = 1830	60-69 Years N = 2842	70-74 Years N = 923	Total N = 5595	Overall Standardized Prevalence[a] (95% CI)
Either eye, n (%)					
Early AMD	294 (16.1)	665 (23.4)	288 (31.2)	1247 (22.3)	22.8 (21.7-24.0)
Late AMD	5 (0.27)	15 (0.53)	9 (0.98)	29 (0.52)	0.58 (0.36-0.80)
Soft drusen	561 (30.7)	1173 (41.3)	468 (50.7)	2202 (39.4)	39.2 (37.9-40.5)
Large drusen	216 (11.8)	516 (18.2)	239 (25.9)	971 (17.4)	17.5 (16.5-18.5)
Pigment abnormality	98 (5.4)	222 (7.8)	71 (7.7)	391 (7.0)	7.6 (6.8-8.3)
Bilateral, n (%)					
Early AMD	57 (3.1)	171 (6.0)	101 (10.9)	329 (5.9)	6.1 (5.5-6.8)
Late AMD	0 (0.00)	3 (0.11)	1 (0.11)	4 (0.07)	0.09 (0.00-0.18)
Soft drusen	61 (3.3)	141 (5.0)	67 (7.3)	269 (4.8)	4.6 (4.0-5.1)
Large drusen	45 (2.5)	127 (4.5)	87 (9.4)	259 (4.6)	4.8 (4.2-5.3)
Pigment abnormality	10 (0.5)	32 (1.1)	22 (2.4)	64 (1.1)	1.3 (1.0-1.7)

AMD = age-related macular degeneration; CI = confidence interval.
[a]The prevalence was standardized to the World Health Organization standard population.

TABLE 2. Prevalence of the Phenotype of Age-Related Macular Degeneration in Japanese According to Sex

	Male N = 1977	Female N = 3618	P Value
Early AMD	491 (24.8)	756 (20.9)	.0007
Late AMD	16 (0.81)	13 (0.36)	.025
Soft drusen	765 (38.7)	1437 (39.7)	.454
Large drusen	357 (18.1)	614 (17.0)	.305
Pigment abnormality	192 (9.7)	199 (5.5)	< .0001

AMD = age-related macular degeneration.
Prevalence shown as n (%).

We found similar tendencies regarding age dependence in other features of AMD (soft drusen, large drusen, and pigment abnormalities). Whereas 10.7% of drusen subjects had pigment abnormalities, 60.4% of subjects with pigment abnormalities also had drusen. We found that subjects with larger drusen tended to have pigment abnormalities ($P < .0001$). Reticular pseudodrusen were present in 38 participants (0.68%), including those that were outside of the grid; 17 cases, including a case with late AMD, were within the grid. The prevalence of reticular pseudodrusen was significantly higher among women ($P = .011$), with women accounting for 32 of the 38 subjects (84.2%) with reticular pseudodrusen.

AMD was present in both eyes in 333 of the 1276 participants (20.7%) with any AMD. The overall prevalence of bilateral early AMD was 5.9% (6.1%, standardized), and this value increased from 3.1% in subjects aged 50-59 years to 10.9% in subjects aged ≥70 years. Bilateral late AMD was present in 4 of the 29 participants (13.8%) with any late AMD.

The prevalence of AMD according to sex is shown in Table 2. The prevalence of early and late AMD was significantly higher in men than in women ($P = .0007$ and $P = .025$, respectively). The subtype analysis revealed that the prevalence of RPE abnormalities was significantly higher in men than in women ($P < .0001$). This tendency was found in all age groups ($P = .0001$ in subjects aged 50-59 years and $P = .0002$ in those aged 60-69 years), although this association failed to reach significance in subjects aged ≥70 years ($P = .0694$). The incidences of soft and large drusen were not significantly different according to sex ($P = .454$ and $P = .305$, respectively).

Finally, we evaluated the association between cigarette smoking and the development of AMD (Table 3). The total amount of cigarette smoking was significantly associated with the development of early and late AMD ($P = .0153$ and $P = .0402$, respectively). Particularly, subjects with a Brinkman index greater than 500 had a significantly higher risk for the incidence of early and late AMD ($P = .011$ and $P = .042$, respectively, Supplemental Table 1, available at AJO.com). Never smokers were less likely to have early and late AMD, although these associations did not reach statistical significance ($P = .120$ and $P = .159$, respectively). In the subgroup phenotype analysis, we found strong associations between the presence of RPE abnormalities and both the total amount ($P < .0001$) and status ($P = .0003$) of cigarette smoking. However, these significant associations diminished when we divided the cohort by sex ($P > .05$, Supplemental Table 2, available at AJO.com). We found no significant association between cigarette smoking and the incidence of soft or large drusen ($P > .05$).

DISCUSSION

ALTHOUGH A RECENT META-ANALYSIS IN 4 ASIAN POPULATIONS suggested that the prevalence of early AMD signs

TABLE 3. Association Between Smoking Status and the Phenotype of Age-Related Macular Degeneration in Japanese

	Brinkman Index[a]			Smoking Status, N (%)		
	N	Mean	P Value	Ever (N = 1853)	Never (N = 3742)	P Value
No AMD	4319	169.9 6 344.3		1405 (75.8)	2914 (77.9)	
Early AMD	1247	197.2 6 368.9	.0153	435 (23.5)	812 (21.7)	.120
Late AMD	29	301.9 6 462.2	.0402	13 (0.70)	16 (0.42)	.159
Soft drusen			.939			.069
Absent	3393	177.0 6 348.8		1155 (62.3)	2238 (59.8)	
Present	2202	176.2 6 354.0		698 (37.7)	1504 (40.2)	
Large drusen			.305			.798
Absent	4624	174.5 6 348.7		1528 (82.5)	3096 (82.7)	
Present	971	187.2 6 360.7		325 (17.5)	646 (17.3)	
Pigment abnormality			< .0001			.0003
Absent	5204	171.6 6 346.4		1691 (91.3)	3513 (93.9)	
Present	391	243.9 6 399.9		162 (8.7)	229 (6.1)	

AMD = age-related macular degeneration.
[a]The Brinkman index was calculated by the daily number of cigarettes 3 years.

TABLE 4. Age-Speci☒ Prevalence of Large Drusen (≥125 μm) in Various Populations

	Nagahama[a]	Los Angeles[23]	Singapore[7]	Blue Mountains[28]	Beaver Dam[29]	Baltimore[27]
Number of Participants	6065	6357	3280	3632	4752	1843
Ethnicity	Japanese	Latino	Malay	White	White	Black
Years Study Conducted	2008-2010	2000-2003	2004-2006	1992-1994	1988-1990	1985-1988
Age, y						
50-59 (95% CI)	11.9 (10.2-13.6)	13.6	38.3	1.9	6.8	4.7
60-69 (95% CI)	18.1 (16.6-19.5)	19.3	48.1	5.2	15.8	8.4
70-79 (95% CI)	25.9 (23.1-28.7)[b]	26.3	46.3	11.6	27.8	7.9
Sex						
Male (95% CI)	15.4 (13.6-17.3)	19.7	43.8	4.3	-	-
Female (95% CI)	16.4 (15.2-17.6)	14.9	34.5	5.5	-	-

CI = con☒dence interval.
[a]The prevalence was standardized to the World Health Organization standard population.
[b]The last age group is 70-74 years.

were lower in Asians than in white populations,[12] a wide consensus regarding the prevalence of AMD in Asians has not been established. Several factors make it difficult to compare the prevalences reported in various studies: the differences in photographic and grading techniques, the definition of early AMD, and the age groups used when reporting age-specific rates. Because the prevalence of AMD is strongly related to age and because the age distributions of different populations are not similar, it is important to compare age-specific rates rather than the overall prevalence. However, the details regarding the age-specific rates of the prevalence of AMD have not been reported in the Japanese population because of the small sample sizes of previous studies.[10,11] Thus, the present study should be more reliable than previous studies for comparing the prevalence of AMD in the Japanese population with that in other populations because it includes the age-specific rates of AMD.

Large drusen is an important component of early AMD that has been shown in many longitudinal studies to be predictive of incident late AMD.[3,26] Because the definition of large drusen (≥125 μm) has been defined similarly and measured in all of the populations, we chose to look at large drusen as a manifestation of intermediate AMD in various populations (Table 4). In this comparison, the age-specific prevalence of large drusen in the Japanese was comparable to that reported in white populations and higher than that reported in the black population among persons aged ≥50 years.[27] Of particular interest, our study found high rates of large drusen in all Japanese age groups, which is comparable to the reported prevalence in the Los Angeles Latino eye study (LALES).[23]

TABLE 5. Age-Specific Prevalence of Late Age-Related Macular Degeneration in Various Populations

	Nagahama[a]	Hisayama[10]	Los Angeles[23]	Singapore[7]	Blue Mountains[28]	Beaver Dam[29]	Baltimore[27]	Barbados[30]
N	6065	1486	6357	3280	3632	4752	1843	3444
Ethnicity	Japanese	Japanese	Latino	Malay	White	White	Black	Black
Years	2008-2010	1998	2000-2003	2004-2006	1992-1994	1988-1990	1985-1988	1988-1992
Age, y								
50-59 (95% CI)	0.39 (0.02-0.77)	0.45	0.22	0.21	0.0	0.2	0.35	0.7
60-69 (95% CI)	0.53 (0.26-0.80)	0.88	0.26	0.39	0.5	0.8	0.42	0.4
70-79 (95% CI)	0.99 (0.35-1.63)[b]	0.51	1.50	2.49	2.6	3.7	0.00	1.0
Sex								
Male (95% CI)	0.73 (0.28-1.18)	1.2	0.53	0.46	1.3	1.2	-	0.36
Female (95% CI)	0.30 (0.13-0.48)	0.34	0.38	0.22	2.4	1.9	-	0.89

CI = confidence interval.

[a]The prevalence was standardized to the World Health Organization standard population.

[b]The last age group is 70-74 years.

The lesions of late AMD have been defined and graded similarly in most population studies. The age-specific prevalence of late AMD in various populations is shown in Table 5. Although the small number of cases in each study limits these comparisons, the age-specific prevalence of late AMD in Japanese subjects aged < 70 years was comparable with that reported in other populations.[7,10,23,27–30] However, the age-specific prevalence of late AMD in subjects aged 70-79 years was relatively lower than that in the other populations. Caution should be exercised when interpreting our data for the oldest age group because we evaluated subjects aged 70-74 years, which would underestimate the prevalence of AMD in elderly Japanese people. However, considering that a recent meta-analysis in whites reported the predicted late AMD prevalence at 70 and 75 years as 1.4% and 2.8%, respectively, the current study suggests that the prevalence of late AMD is lower in elderly Japanese than in elderly whites.[31] This difference among age groups might be linked to the exceptional change in circumstances in Japan that would lead to potential differences in the lifestyles of these groups; for example, participants aged 66 or younger were born after the end of World War II.

In the present study, the prevalence of early and late AMD was higher in men than in women ($P = .0007$ and $P = .025$, respectively). These results are consistent with those of previous studies in Asian populations, which reported a higher prevalence of AMD among men than among women.[7,11,32,33] Although it is speculated that the reason for this disparity is the higher smoking rate in Asian men compared to women, these sex differences remained in this study even after adjusting for smoking status ($P = .0128$). A similar association was found in LALES.[23] The reason for the higher prevalence of AMD in Japanese men is unclear. A previous genetic study in Japanese subjects[34] may provide insight into this observation because this study suggested that sex had the greatest effect on the development of PCV. In this study, we found sex differences in the prevalence of RPE abnormalities in all age groups. Similar results have been consistently found in Asians[7,10,11] but not in whites.[28,29] Given that RPE atrophy was a prevailing finding in the fellow eyes of patients with PCV,[35] this difference between Asians and whites regarding the background of RPE abnormalities may be associated with the higher prevalence of the particular phenotype of AMD, such as PCV, in Asian populations. In contrast, we did not find a sex difference in the prevalence of drusen. These results are consistent with those of many studies in white populations[6,28,36,37] but are inconsistent with those of previous Japanese studies[11,24] that reported a sex difference in the prevalence of drusen.

Cigarette smoking is a consistently identified risk factor for AMD.[38–40] Although several previous reports confirmed a link between current smoking and AMD in the Japanese,[10,32] this association has not been studied in detail. In this study, we showed that smoking is associated with the development of both early and late AMD in the Japanese, and this is particularly dependent on the total amount of cigarettes smoked. This observed association for smoking is consistent with many previous studies that reported a dose-response effect in whites.[36,39,40] In addition, a strong association between smoking and RPE pigment abnormalities has been revealed. This association is consistent with the Beaver Dam Eye Study, which suggested that smoking is associated with the incidence and progression of RPE pigment abnormalities.[39] However, because this association failed to reach significance when we divided the subjects by sex, it must be evaluated in a larger cohort to conclude whether an association exists between smoking and pigment abnormalities. In contrast to late AMD, the association between cigarette smoking and drusen remains controversial because of the limited number of previous studies. In the present study, we did not find any association between smoking and the incidence of drusen, which is consistent with the result of the LALES.[41]

One of the potential limitations of our study is that it included a low percentage of the overall population, which may have introduced selection bias. It is speculated that women who did not work full time were more likely to participate, resulting in the high female-to-male ratio of this study. Because this study recruited persons who were able to participate on their own, the participants may have been highly health conscious. Further, people working in government and citizen organizations may have been more likely to participate in this study. Finally, people who could not read or move on their own would have experienced difficulty participating in this study, and this bias may have resulted in an underestimated prevalence of late AMD in the Japanese population. However, because the symptoms of early AMD are usually not obvious[2] and would not affect study participation, the magnitude of the selection bias on early AMD prevalence should be negligible. Another limitation was the lack of a detailed evaluation for the subtypes of late AMD (ie, PCV) because of the limited examination in our cohort. A study in which further ophthalmic examinations are performed in the general population is required to identify the prevalence and rate of AMD subtypes in the Japanese population.

Previous reports revealed that early signs of AMD are strong predictors of subsequent advanced stage. The reported 5-year-risk estimates for the development of advanced AMD for each of the scores from 0 to 4 are 0.4%, 3.1%, 11.8%, 25.9%, and 47.3%, respectively.[3] In our study, 1.2% of men aged 70-74 years had a score of 4. If our data are generalizable to all Japanese people, we anticipate that an increased number of Japanese individuals, particularly men, will have late AMD (see Supplemental Figure, available at AJO.com). Applying the reported estimates to our data indicates that a total of 3.1% of men aged 70-74 years may develop advanced AMD in 5 years.

In summary, our study involving > 6000 participants aged ≥50 years provides the first evidence of the age-specific prevalence and detailed characteristics of phenotypes of AMD in the Japanese population. We found that the rates of early AMD in the Japanese population are comparable to those of white populations and that the rates of late AMD were comparable to those of white populations aged < 70 years but were relatively lower in those aged ≥70 years. Further, we found a male-dominant prevalence of RPE pigment abnormalities associated with cigarette smoking. In the Nagahama study, follow-up examination will be carried out 5 years after the baseline survey. Further studies with longitudinal progression of phenotypes of AMD are needed to estimate the relative risk of developing late AMD in the Japanese.

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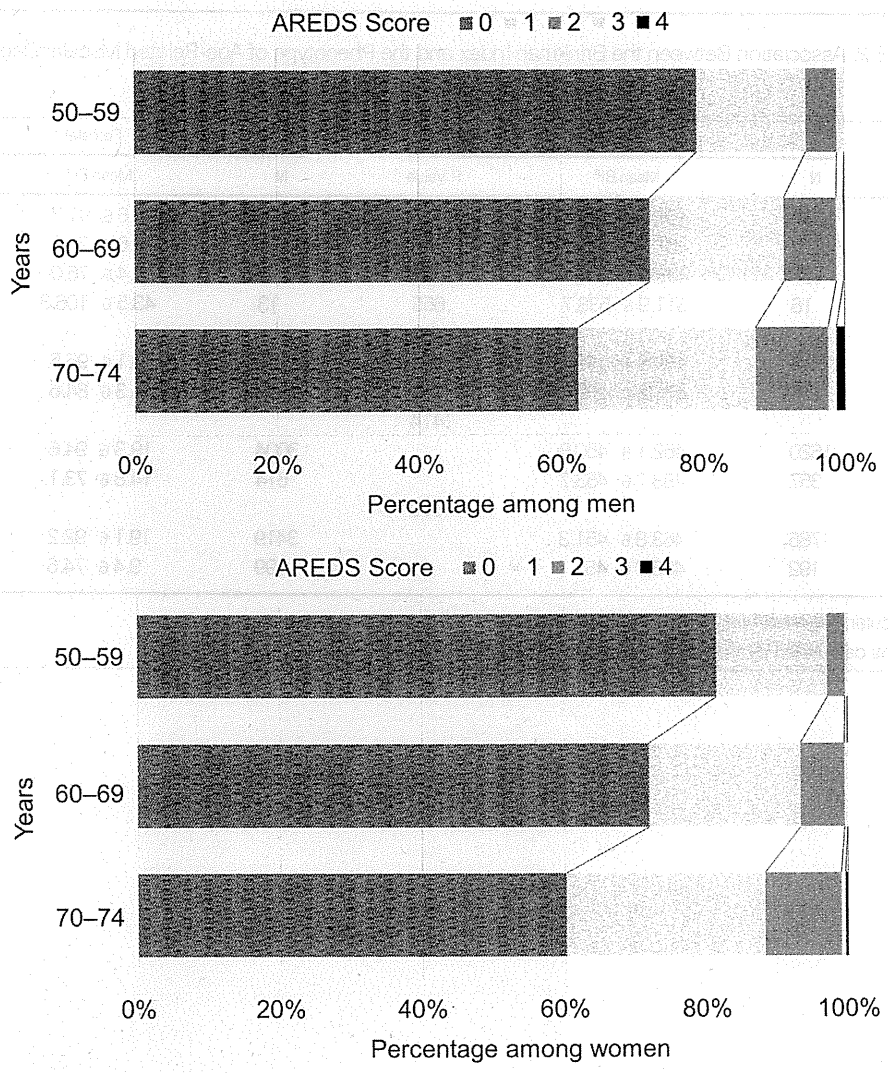

SUPPLEMENTAL FIGURE. Percentages of persons with a risk score for the development of late age-related macular degeneration among men (Top) and women (Bottom) in the Japanese population. Each risk score was calculated by following the severity scale for age-related macular degeneration in the Age-Related Eye Disease Study (AREDS).[3]

SUPPLEMENTAL TABLE 1. Association Between the Brinkman Index and the Risk of Age-Related Macular Degeneration in Japanese

	Brinkman Index[a]			
	Under 500	Over 500	P Value	OR (95% CI)
Early AMD	21.7%	25.5%	.011	1.24 (1.05–1.45)
Late AMD	0.43%	0.97%	.042	2.27 (1.03–5.00)

AMD = age-related macular degeneration; CI = confidence interval; OR = odds ratio.

[a]The Brinkman index was calculated by the daily number of cigarettes 3 years.

SUPPLEMENTAL TABLE 2. Association Between the Brinkman Index and the Phenotype of Age-Related Macular Degeneration in Japanese by Sex

	Male			Female		
	N	Mean BI[a]	P Value	N	Mean BI[a]	P Value
Total	1977	466.0 6 451.3		3618	18.6 6 91.3	
No AMD	1470	461.3 6 449.6		2849	19.6 6 94.9	
Early AMD	491	478.7 6 454.1	.459	756	14.4 6 76.0	.165
Late AMD	16	511.9 6 533.7	.655	13	43.5 6 106.3	.365
Soft drusen			.402			.216
Absent	1212	459.3 6 447.6		2181	20.1 6 95.5	
Present	765	476.8 6 457.1		1437	16.3 6 84.6	
Large drusen			.414			.260
Absent	1620	462.1 6 450.8		3004	19.3 6 94.6	
Present	357	483.7 6 453.7		614	14.8 6 73.1	
Pigment abnormality			.500			.145
Absent	1785	463.8 6 451.3		3419	19.1 6 92.2	
Present	192	486.9 6 451.7		199	9.4 6 74.6	

AMD = age-related macular degeneration; BI = Brinkman index..

[a]The Brinkman index was calculated by the daily number of cigarettes 3 years.

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Genome-wide association study identifies *ZFHX1B* as a susceptibility locus for severe myopia

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Severe myopia (defined as spherical equivalent < −6.0 D) is a predominant problem in Asian countries, resulting in substantial morbidity. We performed a meta-analysis of four genome-wide association studies (GWAS), all of East Asian descent totaling 1603 cases and 3427 controls. Two single nucleotide polymorphisms (SNPs) (rs13382811 from *ZFHX1B* [encoding for ZEB2] and rs6469937 from *SNTB1*) showed highly suggestive evidence of association with disease ($P < 1 \times 10^{-7}$) and were brought forward for replication analysis in a further 1241 severe myopia cases and 3559 controls from a further three independent sample collections. Significant evidence of replication was observed, and both SNP markers surpassed the formal threshold for genome-wide significance upon meta-analysis of both discovery and replication stages ($P = 5.79 \times 10^{-10}$, per-allele odds ratio (OR) = 1.26 for rs13382811 and $P = 2.01 \times 10^{-9}$, per-allele OR = 0.79 for rs6469937). The observation at *SNTB1* is confirmatory of a very recent GWAS on severe myopia. Both genes were expressed in the human retina, sclera, as well as the retinal pigmented epithelium. In an experimental mouse model for myopia, we observed significant alterations to gene and protein expression in the retina and sclera of the unilateral induced myopic eyes for *Zfhx1b* and *Sntb1*. These new data advance our understanding of the molecular pathogenesis of severe myopia.

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INTRODUCTION

Myopia is a world-wide eye disability, suspected to be due to both genetic and environmental influences. The hereditable elements responsible for predisposition to myopia are difficult to identify and dissect due to substantial confounding by environmental factors. This is particularly complicated in studies of common myopia, typically defined as spherical equivalent (SE) of < -0.5 D, in which the robust identification of genetic determinants requires the deployment of very large sample sizes (1–3).

Natural variation in ocular biometric quantitative traits has been shown to be significantly influenced by a large number of genetic sequence variants, each conferring modest effect sizes (4–7). Thus far, three genome-wide association studies (GWAS) on SE quantitative trait have been performed on ever larger sample sizes, revealing very strong evidence of association between multiple single nucleotide polymorphism (SNP) markers and inter-individual SE variation (8–11). Notably, the effect sizes for all of these identified loci are modest, suggesting a multi-factorial etiology and possible presence of unmeasured environmental confounding, in keeping with other common diseases (12). To a more limited extent, GWAS on severe myopia (defined as SE < -6.0 D in either eye) has also been performed (13–16), and robustly associating genetic loci surpassing the formal threshold for genome-wide significance ($P < 5 \times 10^{-8}$) are now beginning to emerge (15,16).

Severe myopia is more common in East Asians than whites, affecting up to 10% of the population aged 40 years and older, and can be associated with vision loss due to myopic macular degeneration, glaucoma and retinal detachment. To date, successful GWAS of severe myopia have been conducted in individuals of Chinese descent from China (15,16), the most recent of which involved 665 cases and 960 controls in the discovery stage (15). To identify further genetic determinants for severe myopia, we conducted a meta-analysis of four genome-wide association studies across four independent Asian collections enrolled from mainland China, Hong Kong, Japan and Singapore (Table 1). The collections from China and Hong Kong have been described in prior publications. Altogether, these total 1603 severe myopia cases and 3427 emmetropic controls. Replication experiments were conducted in a further 1241 severe myopia cases and 3559 controls from Hong Kong, Japan and Singapore.

RESULTS

After stringent quality control filters on samples and SNPs (see Materials and Methods), a total of 494 215 SNPs (see Supplementary Material for summary statistics of the complete GWAS dataset) were observed to be successfully genotyped in two or more sample collections, and 250 531 SNPs were found common across all four study collections. Statistical associations between each SNP genotype and severe myopia were measured using logistic regression, modeling for a trend per-copy effect of the minor allele. A quantile–quantile (QQ) plot of the *P*-values obtained from the four-collection meta-analysis showed low evidence of genomic inflation of the association test statistics ($\lambda_{gc} = 1.057$), giving minimal evidence of cryptic population stratification or differential genotyping success rates between cases and controls which could confound the association results. Observed against this background was several point *P*-values showing extreme deviation at the tail end of the QQ distribution. These data points could represent true positive associations with severe myopia, and reflected two distinct genetic loci ($P < 1 \times 10^{-7}$) (Supplementary Material, Figs S1 and S2). The first locus is rs13382811 mapping within *ZFHX1B* (also known as *ZEB2*; per-allele odds ratio (OR) $= 1.33, P = 7.44 \times 10^{-9}$) (Supplementary Material, Fig. S3). The second marker is rs6469937 mapping within *SNTB1* (per-allele OR $= 0.75, P = 6.08 \times 10^{-8}$)) (Supplementary Material, Fig. S4), which is located within the same linkage disequilibrium region as the three SNP markers recently described as strongly associated with severe myopia (15).

A power calculation based on a per-allele OR of between 1.20 and 1.30 (due to the winner's curse phenomenon), minor allele frequency between 0.20 and 0.30, and a replication sample size of 1232 severe myopia cases and 3559 emmetropic controls showed that only SNPs surpassing $P = 1 \times 10^{-7}$ in the GWAS discovery stage would be sufficiently powered to achieve genome-wide significance upon meta-analysis should the effect be a true positive (Supplementary Material, Table S1). Based on these estimations, we proceeded to conduct replication experiments of both SNPs in a further 1232 severe myopia cases and 3559 emmetropic controls enrolled across three countries (Table 1). Both SNP markers showed significant evidence of replication (Fig. 1A and B, Supplementary Material, Table S2a and b), with minimal to no heterogeneity across the three study collections ($0\% \leq I^2$ index $< 25\%$). Meta-analyzing data from all seven collections, we note genome-wide significant association with severe myopia ($P = 5.79 \times 10^{-10}$, per-allele OR $= 1.26$ for rs13382811 and $P = 2.01 \times 10^{-9}$, per-allele OR $= 0.79$ for rs6469937) for both SNP markers, again with no evidence of inter-collection heterogeneity.

Differential gene expressions for *ZEB2* and *SNTB1* from the tissues of myopic (with SE < -5.0 D) and fellow non-occluded eyes of the experimental mice were compared with age-matched control tissues (Fig. 2). The mRNA levels of *ZEB2* and *SNTB1* were significantly downregulated in myopic retina/RPE compared with naive control retina/RPE and this was upregulated in the myopic sclera. Fold change for ZEB2 in retina/RPE/sclera $-3.1/-7.8/2$; $P = 0.00004$; $P = 0.0002$ and $P = 0.0003$, respectively. Fold change for SNTB1 in retina/RPE/sclera $-5.6/-22/17.4$; $P = 0.0001$; $P = 0.0008$ and $P = 0.00006$, respectively.

We performed additional analysis assessing for association within the current severe myopia dataset for previously reported severe myopia loci (Supplementary Material, Table S3), as well as previously reported loci influencing SE (Supplementary Material, Table S4) identified via the GWAS approach. All SNP markers showing evidence of association surpassing $P < 1 \times 10^{-4}$ in the GWAS meta-analysis discovery stage are shown in Supplementary Material, Table S5.

DISCUSSION

Severe myopia is much more extreme phenotype compared with common myopia (defined as SE < -0.5 D) and the study of individuals at the more severe end of the quantitative phenotype spectrum has documented usefulness (17,18). Furthermore, severe myopia can cause significant visual impairment via myopic macular degeneration, retinal detachment and glaucoma. Severe myopia is more common in East Asians, affecting 5–10% of persons 40 years and older. In an attempt to minimize

Table 1. Sample collections used in both stages of the study.

Collection	Cases	Age (mean; [range])	Controls	Age (mean; [range])	Genotyping platform
GWAS stage					
China (Sichuan)	419	34.3 [12–76]	669	32.7 [22–55]	Illumina 610K
Hong Kong	232	50.6 [12–87]	244	69.0 [39–94]	Illumina 370K
Japan	500	57.8 [14–91]	1194	50.0 [20–79]	Illumina 550K
Singapore	452	40.9 [10–75]	1320	43.5 [10–85]	Illumina 610K
Total GWAS	1603		3427		
Replication stage					
Hong Kong	106	56.2 [21–85]	178	75.0 [55–99]	
Japan	728	56.6 [11–86]	3248	52.2 [30–75]	
Singapore	407	19.5 [16–37]	133	18.7 [16–25]	
Total replication	1241		3559		
Total samples	2844		6986		

spurious associations due to population stratification or environmental effects (as >70% Asians are myopic to some degree), we utilized emmetropic adults (-0.5 D < SE < $+1.0$ D) as far as possible, adjusted for genetic stratification, and included multiple populations in the study design. Also, we only considered markers showing low inter-cohort heterogeneity, in order to avoid genotyping artifacts driven by specific sample collections. This current study in 2844 severe myopia cases and 6986 controls identified *ZFHX1B* as a new susceptibility locus for severe myopia, and confirmed a recent observation at *SNTB1* where the minor allele of several SNP markers were significantly associated with decreased susceptibility towards severe myopia.

Many different experimental animal models (chick, rabbit, tree shrew, macaque and tree shrew) (19,20) had been used for the studies of emmetropization and myopia generation. These animal models were used to characterize the optical parameters of and study the mechanisms of induced myopia (21). Studies of the chick eye have formed the basis for several hypotheses of myopic development, but the chick does not possess a fovea or retinal blood supply. It is thus unclear whether these differences alter the pathways of emmetropization. Even closely related primate species can exhibit different responses to form deprivation conditions, suggesting differing mechanisms of eye growth control. Monocular occlusion of the rhesus macaque, for instance, results in myopia when the ciliary muscle is paralyzed or the optic nerve cut, but does not in the stump tailed macaque, suggesting a role of excessive accommodation in the development of myopia in the stump tail but not the rhesus.

Given such variability in the models, a persisting element of continued myopia research must be an evaluation of the relevance of any given model to the human condition. In this regard, the study of changing patterns of gene expression within and among species during emmetropization and myopic progression may offer a productive avenue for future research. Experimental myopia has been induced in the mouse by us and others (22–24). The mouse myopia model was developed and assayed because of the availability of the whole-genome sequence, comprehensive protein database and more importantly, the availability of molecular tools like whole-genome gene chip. With our mouse models and noninvasive methods for measuring and monitoring axial length, we were able to monitor the progress of myopia in the same animal without the need to sacrifice it.

ZFHX1B encodes for Zinc finger E-box-binding homeobox 2, also known as SMAD-interacting protein-1 (SMADIP1). It functions as a transcriptional co-repressor involved in the transforming growth factor-β (TGF-β) signaling pathway, and has also been implicated in Mowat–Wilson syndrome (25,26). Members of this TGF-β signaling pathway, such as *BMP2* and *BMP3*, have only recently been implicated in a large multiethnic GWAS ($N > 45\,000$) as quantitative trait loci for SE (27). Notably, multiple genes encoding for zinc finger proteins (including *ZIC2* (27), *ZC3H11B* (28) and *ZNF644* (29)) have been shown to associate with refractive error in recent GWAS and exome sequencing studies. Mowat–Wilson syndrome is a genetic condition that could affect multiple distinct parts of the body. Common manifestations of this disorder frequently include characteristic facial features, intellectual disability, delayed development, Hirschsprung disease and other associated birth defects. This syndrome is often associated with an unusually small head (microcephaly), structural brain abnormalities and intellectual disability ranging from moderate to severe. Although ocular disorders are not particularly pronounced in patients with Mowat–Wilson syndrome apart from wide-set eyes, DNA sequencing experiments performed on a patient presenting with Down syndrome, Hirschsprung disease, high myopia and ocular coloboma revealed a non-synonymous amino acid substitution (953Arg→Gly) which was not present in 200 matched normal chromosomes (30). This significant association observed between natural genetic variation within *ZFHX1B* and severe myopia is thus in keeping with current knowledge on the biological pathways for refractive errors. The second locus, *SNTB1*, has only been recently described as a susceptibility locus for severe myopia [15]. It encodes for Beta-1 syntrophin. The Syntrophin family of proteins associate with ion channel and signaling proteins of the dystrophin-associated protein complex. Syntrophins have a diverse role of acting as molecular adaptors for many cellular signaling pathways (31,32). Beta-1 syntrophin, one of the homologous isoforms, has been implicated in the regulation of ABCA1 protein levels in human fibroblast cell lines (33).

We were able to show nominal evidence of association ($0.001 < P < 0.05$) at some of the previously reported GWAS loci for high myopia, namely *CTNND2*, *MIPEP-C1QTNF9B* on Chromosome 13q12 and *VIPR2* (Supplementary Material, Table S1). For the previously reported SE loci, we could observe this level of association at directly genotyped SNPs found at Chromosome 15q14 and 15q25, which were the first SE loci identified via GWAS. The allele associated with

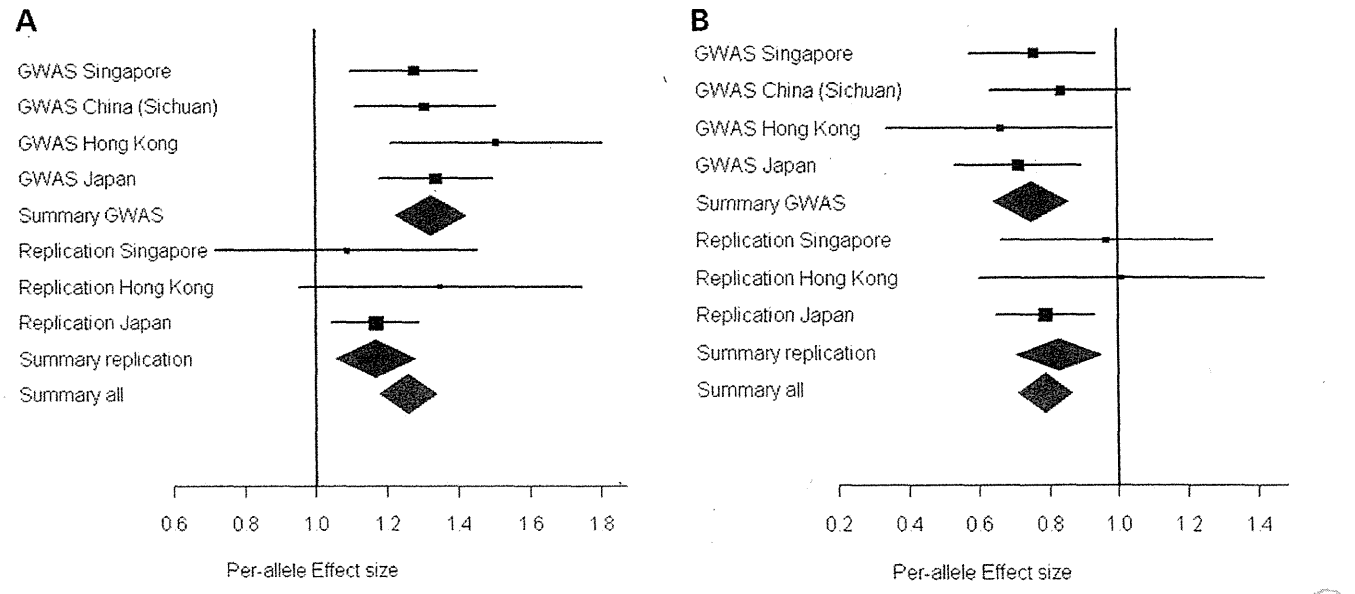

Figure 1. (**A**) Forest plot of the meta-analysis for all sample collections at *ZFHX1B* rs13382811. (**B**) Forest plot of the meta-analysis for all sample collections at *SN* rs6469937.

increased negativity of SE in quantitative trait analysis (8,9) was also found to be associated with the increased risk of severe myopia in this current study.

In summary, our GWAS on seven independent sample collections of East Asians (five Chinese and two Japanese) identify *ZFHX1B* as a new susceptibility locus for severe myopia. It offers new information into the pathogenesis of severe myopia in Chinese and Japanese, and further implicates the TGF-β biological pathway as an important determinant of severe extreme of refractive error disorders.

MATERIALS AND METHODS

Samples for GWAS stage

Details of the GWAS from Sichuan (419 severe myopia cases and 669 emmetropic controls) and Hong Kong (232 severe myopia cases and 244 emmetropic controls) have been described elsewhere (15,16). The 452 severe myopia cases from Singapore are of Chinese descent, and were enrolled from the SCORM, SP2 and SCES collection. The emmetropic controls were enrolled from the same studies. For Japan, a total of 500 severe myopia cases with axial length ≥28 mm in both eyes and 1194 controls were enrolled for this study.

Sample for replication stage

We enrolled a further 1232 severe myopia cases and 3559 controls from Hong Kong, Japan and Singapore.

Hong Kong

A total of 338 unrelated individuals with severe myopia (refractive error in at least one eye ≤ −6 D and axial length in at least one eye ≥26 mm) and 422 unrelated emmetropic control individuals were recruited from the CUHK Eye Centre (Chinese University of Hong Kong), Hong Kong Eye Hospital and the eye clinics of the Prince of Wales Hospital, Hong Kong. As 232 severe myopia cases and 244 emmetropic controls have undergone GWAS genotyping and were included in the discovery stage, there remained 106 severe myopia cases and 178 emmetropic controls for the replication stage.

Japan

The 728 individuals with severe myopia (axial length ≥26 mm in both eyes) were recruited from Kyoto University Hospital, and Tokyo Medical and Dental University Hospital. For the controls, we used 3248 unrelated individuals recruited from the Nagahama Prospective Genome Cohort for the Comprehensive Human Bioscience (The Nagahama Study).

Singapore

A total of 407 individuals with severe myopia and 133 emmetrophic controls were volunteers recruited by DSO National Laboratories from military personnel. The severe myopia l[illegible] spherically equivalent refractive error of at least −6 D in either eye. All subjects were of self-declared Chinese ancestry (all four grandparents), with an age range of between 16 and 25 years.

Genotyping and quality checks

Genome-wide genotyping for the Sichuan (China) and Hong Kong sample collections were performed as previously described (15). The Japanese severe myopia cases were genotyped using the Illumina 550K and 660 W Bead chips, whilst the Japanese controls were genotyped using the Illumina 610K Bead chip. The Singaporean severe myopia collections were genotyped using the Illumina 610K platform according to manufacturer's instructions. Post-genotyping quality checks were conducted on a per-SNP and per-sample basis. SNP markers showing any one of these characteristics were removed from further analysis:

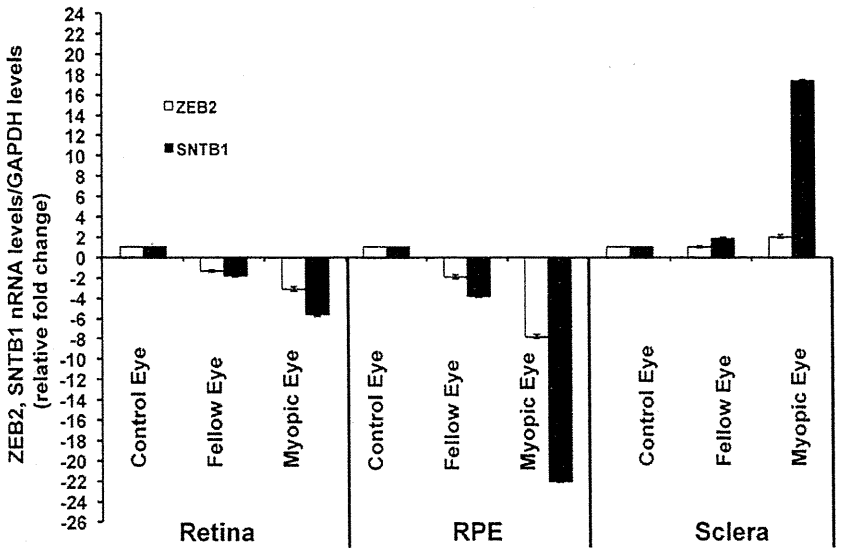

Figure 2. Transcription quantification of *ZEB2* and *SNTB1* in mouse retina, RPE and sclera in induced myopic eyes, fellow eyes and independent control eyes. (transcription quantification of *ZEB2* and *SNTB1* in mouse retina, RPE and sclera in induced myopic eyes, fellow eyes and independent control eyes. Myopia was induced using − 15 D negative lenses in the right eye of mice for 6 weeks. Uncovered left eyes were served as fellow eyes and age-matched naive mice eyes were controls. Quantification of mRNA expression in mice retina, RPE and sclera is via qRT-PCR. The bar represents the fold changes of mRNA for *ZEB2* and *SNTB1* genes after normalization using *GAPDH* as reference. The mRNA levels of *ZEB2* and *SNTB1* in myopic and fellow retina, RPE and sclera are compared with independent controls).

(i) call rate < 95%,
(ii) minor allele frequency < 1% and
(iii) significant deviation from Hardy–Weinberg equilibrium ($P < 10^{-6}$).

Samples showing any of the following characteristics were removed from further analysis:

(i) call rate < 95%,
(ii) extremes of heterozygosity,
(iii) significant ancestral outlier on principal component analysis (PCA) and
(iv) first degree relatives in which case the sample with the lower call rate within the pair would be excluded.

Replication genotyping was performed using the Taqman allelic discrimination assay (Applied Biosystems) according to maufacturer's instructions in all three sample collections.

Statistical analysis

Statistical analyses were conducted using PLINK version 1.07 (34) and the R statistical software package (http://www.r-project.org/). SNP association analysis with severe myopia was performed using logistic regression testing for a trend per-copy of the minor allele, within a multiplicative model for the OR. Additional adjustments compensating for the significant axes of genetic stratification were also performed, when data are available (e.g. in the GWAS stage). PCA and quantile–quantile distributions were plotted using the R statistical software package. PCA assessing for genetic stratification was performed using EIGENSTRAT, and has been described previously for the Chinese (Sichuan) and Hong Kong collections (15,16). The plots contrasting principal component scores between severe myopia cases and controls for each collection are presented in Supplementary Material, Figures S5–S8. Good genetic matching between cases and controls was observed along the top five axes of variation of genetic ancestry.

Ethical approval for animal study

Animal study approval was obtained from the Sing Health IACUC (AAALAC accredited). All procedures performed in this study complied with the Association of Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmology and Vision Research.

Differential gene expression in a mouse model of myopia

Experimental myopia was induced in B6 wild-type mice ($n = 36$) by applying a − 15.00 D spectacle lens on the right eye (experimental eye) for 6 weeks since post-natal Day 10. The left eyes were uncovered and served as contra-lateral fellow eyes. Age-matched naive mice eyes were used as independent control eyes ($n = 36$). Eye biometry, refraction and data analysis were followed as described previously (22,35).

We used quantitative real-time PCR (qRT-PCR) to validate the gene expression. Tissue collection, RNA extraction, qRT-PCR methods and analysis were followed as described previously (28). qRT-PCR primers were designed using Probe Finder 2.45 (Roche Applied Science, Indianapolis, IN, USA) and this was performed using a Lightcycler 480 Probe Master (Roche Applied Science). The primer sequences for ZEB2 and SNTB1 were forward: 5′-ccagaggaaacaaggatttcag-3′ and reverse: 5′-aggcctgacatgtagtcttgtg-3′ (NM_015753.3) and forward: 5′-ttt ggaggcaaagaaggaga-3′ and reverse: 5′-aggagtggatgatgaaaacga-3′ (NM_016667.3), respectively.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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