have revealed several genes that are linked to the development of PCV.[17–20,25] However, because PCV has many similarities with AMD, including genetic factors, demography, pathology, and clinical manifestation, it remains controversial whether PCV is a subtype of AMD or a distinct clinical entity.[16,22,24,26]

Recently, Arakawa et al.[27] suggested that two new loci (rs13278062 at *TNFRSF10A-LOC389641* on chromosome 8p21 and rs1713985 at *REST-C4orf14-POLR2B-IGFBP7* on chromosome 4q12) conferred risk for the development of AMD-CNV in case-control studies involving a Japanese population. They noted that *TNFRSF10A* on 8p21 and all four genes on 4q12 were expressed in human adult retinal pigment epithelium (RPE) and rod photoreceptors in mice, which suggests that these loci have functional roles in the development of AMD. To accurately evaluate the gene–disease association, it is important to replicate the positive association reported in previous studies using the same or different ethnic cohorts. In addition, it is important to evaluate the significance of these two variants for typical AMD-CNV and PCV in a larger number of cases, because the original study by Arakawa et al.[27] involved subgroup analysis of AMD with relatively small numbers of patients (298 typical AMD-CNV and 480 PCV).

The aim of this study was to investigate whether the two suggested loci play a significant role in the development of AMD in Asians and its subtypes, typical AMD-CNV and PCV, by assessing 6000 participants from Japanese and Chinese populations.

Methods

We conducted a case-control study with meta-analysis for 2360 patients with neovascular AMD (1013 typical AMD-CNV and 1282 PCV) and 3598 controls from four cohorts.

All procedures in this study adhered to the tenets of the Declaration of Helsinki. This study was approved by the ethics committee of each institute involved (Kyoto University Graduate School and Faculty of Medicine, ethics committee, the ethical committee of Fukushima Medical University, the ethical committee of Kobe City Medical Center General Hospital, the ethical committee at Aichi Cancer Center, the Ethics and Gene Analysis Committee in the Faculty of Medicine, University of Yamanashi, the Singapore Eye Research Institute (SERI) institutional review board, and the Ethics Committee on Human Research, the Chinese University of Hong Kong). All the patients were fully informed about the purpose and procedures of this study, and written consent was obtained from each.

Patients and Controls

Kyoto Cohort. The Kyoto cohort consisted of 1364 neovascular AMD cases and 3057 general healthy population controls. Neovascular AMD patients were recruited from the Department of Ophthalmology at Kyoto University Hospital, Fukushima Medical University Hospital, and Kobe City Medical Center General Hospital. The diagnoses of AMD were based on the International Classification System for age-related maculopathy.[28] Of these patients, 720 were diagnosed as PCV. As proposed by the Japanese Study Group of PCV,[29] the diagnoses were based on indocyanine green angiography, which showed a branching vascular network terminating in polypoidal swelling. Typical AMD-CNV showed classic CNV, occult CNV, or mixed CNV with clear images of vascular CNV networks or diffuse staining of the CNV membrane without polypoidal lesions in indocyanine green angiography.

Patients displaying any of the following characteristics were excluded from the study: (1) high myopia (spherical equivalent < −6.00 diopters [D]), (2) geographic atrophy or drusen only, (3) patients with one eye affected by typical choroidal neovascularization and the other with polypoidal lesions, or (4) an old lesion without a clear diagnosis. The healthy Japanese individuals were obtained from the three studies, which have been reported elsewhere: the Pharma SNP Consortium (PSC),[30] the Aichi Cancer Center Research Institute (with patients confirmed not to have cancer according to the cancer registry, medical record, and self-reporting)[31] and the Japanese Single Nucleotide polymorphism (JSNP) database.[32] We recruited them without ophthalmic data, and they served as general population controls. All subjects in this cohort were unrelated and of Japanese ethnicity.

Yamanashi Cohort. The Yamanashi cohort consisted of 323 neovascular AMD cases and 115 controls. All participants were recruited from the Macular Clinic, Department of Ophthalmology, University of Yamanashi Hospital. Of the patients, 211 patients were diagnosed with PCV. All diagnoses were made as described for the Kyoto cohort. All control subjects were confirmed not to have any signs of AMD by funduscopic examination. All subjects in this cohort were unrelated and of Japanese ethnicity.

Singapore Cohort. The Singapore cohort consisted of 240 neovascular AMD cases and 151 controls. The AMD cases were recruited from a tertiary eye hospital, the Singapore National Eye Center, between September 2007 and April 2008.[33] Controls comprised participants from the Singapore Chinese Eye Study[15] without any sign of AMD.[34] Of the AMD patients, 118 were diagnosed with PCV. All diagnoses were based on criteria as described for the Kyoto cohort. All subjects in this cohort were unrelated and of Chinese ethnicity.

Hong Kong Cohort. The Hong Kong cohort consisted of 433 neovascular AMD cases and 275 controls. All participants were recruited from two tertiary ophthalmic centers in Hong Kong, the Hong Kong Eye Hospital and the Prince of Wales Hospital. Of these patients, 233 were diagnosed with PCV. All diagnoses were made as described for the Kyoto cohort. All control subjects were confirmed not to have any signs of AMD by funduscopic examination. All subjects in this cohort were unrelated and of Chinese ethnicity.

Genotyping

We targeted rs13278062 at *TNFRSF10A-LOC389641* on chromosome 8p21 and rs1713985 at *REST-C4orf14-POLR2B-IGFBP7* on chromosome 4q12, which have been described to have a positive association with development of AMD in a prior study.[27] In the Kyoto cohort, all

Table 1. Characteristics of the Study Population

	Japanese				Chinese			
	Kyoto		Yamanashi		Singapore		Hong Kong	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
n	1364	3057	323	115	240	151	433	275
Age, y	74.3	45.3	74.0	72.5	70.8	65.7	71.6	74.3
SD	8.7	15.4	8.5	9.4	8.7	5.0	9.1	7.6
Range	42 to 96	20 to 79	47 to 93	45 to 91	44 to 93	60 to 84	43 to 94	60 to 94
Male, %	71.6	50.2	74.1	59.8	65.0	53.6	62.8	44.0
PCV, %	52.8	-	65.3	-	49.2	-	53.8	-

TABLE 2. Distribution of Genotypes and the Results of the Association Tests for TNFRSF10-LOC389641 rs13278062

		Genotype								Association Analysis			
		Cases				Controls				Nominal		Adjusted*	
	n	GG	GT	TT	MAF	GG	GT	TT	MAF	P Value	OR (95% CI)	P Value	OR (95% CI)
Japanese													
Kyoto	4421	506	642	205	0.39	1327	1354	348	0.34	5.22×10^{-6}	0.80 (0.73-0.88)	0.040	0.84 (0.71-0.99)
Yamanashi	438	114	163	46	0.39	60	41	14	0.30	0.011	0.66 (0.48-0.91)	0.012	0.65 (0.47-0.91)
Chinese													
Singapore	391	111	103	26	0.32	85	56	10	0.25	0.033	0.71 (0.51-0.97)	0.051	0.72 (0.51-1.00)
Hong Kong	708	206	186	41	0.31	143	113	19	0.27	0.161	0.84 (0.67-1.07)	0.110	0.82 (0.64-1.04)
Meta-analysis													
Total	5958									1.98×10^{-8}	0.79 (0.73-0.86)	1.12×10^{-4}	0.79 (0.70-0.89)

* Adjusted for age and sex.

case samples and PSC samples were genotyped using TaqMan SNP assays with an ABI PRISM 7700 system (Applied Biosystems, Foster City, CA). Controls from the Aichi Cancer Center Research Institute were genotyped using Illumina Human610-Quad BeadChips (Illumina Inc., San Diego, CA). In the Yamanashi cohort, all samples were genotyped using TaqMan genotyping assays with a 7300/7500 Real-Time PCR System (Applied Biosystems). In the Singapore cohort, all samples were genotyped using Illumina Human610-Quad BeadChips (Illumina Inc.). In the Hong Kong cohort, the SNPs were genotyped using TaqMan genotyping assays on an ABI Prism 7000 Sequence Detection System, according to the manufacturer's instructions (Applied Biosystems).

Statistical Analyses

Deviations in genotype distributions from the Hardy-Weinberg equilibrium (HWE) were assessed with the HWE exact test. Statistical analyses for differences in the observed genotypic distributions were performed by the v^2 test for trend; logistic regression analyses were performed for age and sex adjustments. Meta-analyses were conducted using a weighted-inverse variance approach in METAL (http://www.sph.umich.edu/csg/abecasis/metal/; provided in the public domain by Center for Statistical Genetics, The University of Michigan, Ann Arbor, MI).[35] The *P* values of heterogeneity across the population were determined using a Cochran's Q test. The coordinates presented were from NCBI Built 36.1 (http://www.ncbi.nlm.nih.gov/; provided in the public domain by National Center for Biotechnology Information, Bethesda, MD). The statistical power calculation was performed using QUANTO version 1.2.[36] *P* value correction was not performed because the SNPs were analyzed independently. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Demographics of the four Japanese and Chinese cohorts are shown in Table 1. Overall, genotype and allele frequencies of the two reported SNPs (rs13278062 at *TNFRSF10A-LOC389641* and rs1713985 at *REST-C4orf14-POLR2B-IGFBP7*) were analyzed in the 2360 patients with neovascular AMD, and compared with those of the 3598 controls. The genotyping of evaluated SNPs was more than 99.3% successful, and the distributions of the genotypes for all study groups were in HWE ($P > 0.05$). Details of allele frequencies and summary statistics for rs13278062 are shown in Table 2. The minor allele frequency (MAF) of rs13278062 was lower in Chinese populations (0.25-0.32) than in Japanese populations (0.30-0.39). After age and sex adjustments, meta-analyses of the four cohorts revealed a significant association of rs13278062 with developing neovascular AMD ($P = 1.12 \times 10^{-4}$, odds ratio [OR] = 0.79, 95% confidence interval [CI] = 0.70-0.89).

Table 3 shows details of allele frequencies and summary statistics for rs1713985. The MAF of rs1713985 was similar between Japanese and Chinese populations. We found no significant association between rs1713985 and neovascular AMD ($P = 0.785$, OR = 1.02, 95% CI = 0.90-1.15). Had there been a true association between rs1713985 and development of AMD at the level reported in the original study (OR = 1.30),[27] our sample size had more than 99.9% power to detect it (unmatched case-control design, log-additive genotype model, 0.67% for the prevalence of exposure in general population,[37] and 0.22-0.31 for allele frequency). We found no evidence of heterogeneity in these meta-analyses for rs13278062 and rs1713985 (heterogeneity $P > 0.05$).

TABLE 3. Distribution of Genotypes and the Results of the Association Tests for REST-C4orf14-POLR2B-IGFBP7 rs1713985

		Genotype								Association Analysis			
		Cases				Controls				Nominal		Adjusted*	
	n	GG	GT	TT	MAF	GG	GT	TT	MAF	P Value	OR (95% CI)	P Value	OR (95% CI)
Japanese													
Kyoto	4421	109	557	689	0.29	274	1204	1547	0.29	0.730	0.98 (0.89-1.09)	0.359	0.92 (0.77-1.10)
Yamanashi	438	22	147	154	0.30	7	39	69	0.23	0.058	1.40 (0.99-1.99)	0.058	1.39 (0.99-2.05)
Chinese													
Singapore	391	20	102	118	0.30	4	59	88	0.22	0.023	1.47 (1.05-2.06)	0.131	1.32 (0.92-1.89)
Hong Kong	708	39	183	210	0.30	25	121	129	0.31	0.725	0.96 (0.76-1.21)	0.530	0.93 (0.73-1.18)
Meta-analysis													
Total	5958									0.250	1.12 (0.92-1.37)	0.785	1.02 (0.90-1.15)

* Adjusted for age and sex.

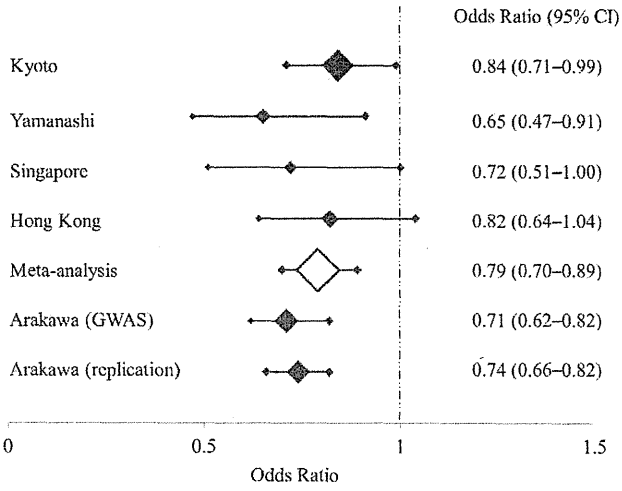

FIGURE 1. Forest plot of the combined analysis of six case-control cohorts for rs13278062 at 8p21. *Horizontal lines* represent the study-specific odds ratio and 95% CI, and various-sized squares correspond to the sample size of cases. Data from all studies suggested that the T allele of rs13278062 conferred risk for AMD. We found no evidence of heterogeneity in this evaluation ($P = 0.53$).

Next, we evaluated whether these two SNPs were associated with developing typical AMD-CNV or PCV. In this subgroup analysis (Table 4), 1013 typical AMD-CNV and 1282 PCV patients were evaluated. In the meta-analysis, *TNFRSF10A-LOC389641* rs13278062 was found to be significantly associated with development of both typical AMD-CNV ($P = 8.21 \times 10^{-3}$, OR = 0.81, 95% CI = 0.70–0.95) and PCV ($P = 3.79 \times 10^{-5}$, OR = 0.74, 95% CI = 0.65–0.86) after age and sex adjustments, although rs1713985 did not show significant association with either typical AMD-CNV or PCV ($P > 0.05$). In the evaluation of rs1713985 in the development of PCV, we found significant evidence of heterogeneity ($P = 0.02$).

DISCUSSION

A recent article by Arakawa et al.[27] suggested that two loci (rs13278062 at *TNFRSF10A-LOC389641* on chromosome 8p21 and rs1713985 at *REST-C4orf14-POLR2B-IGFBP7* on chromosome 4q12) conferred risk for the development of neovascular AMD in case-control studies of a Japanese population. In the present study, we evaluated these variants by using a larger number of cases ($n = 2360$) than in the previous study ($n = 1536$). However, our current study, including four studies of several Asian cohorts analyzed both independently and collectively, shows no significant association between rs1713985 at *REST-C4orf14-POLR2B-IGFBP7* and neovascular AMD ($P = 0.785$, OR = 1.02, 95% CI = 0.90–1.15). Although one of the cohorts (Yamanashi) suggested marginal significance ($P = 0.058$), this result is likely due to the limited sample size of the control group in the Yamanashi cohort, and, thus, is theoretically more prone to sampling error. In fact, the MAF of rs1713985 in the pooled controls (Japanese: 0.287, Chinese: 0.279) were almost the same as in the previous study that used 18,894 individuals with various diseases as controls (0.282–0.286).[27] Considering similarity in the MAF of the controls, the discrepancy of the association for rs1713985 may be due to the population substructure of the cases in the previous study.

On the other hand, the current study confirmed the significance of rs13278062 at *TNFRSF10A-LOC389641* on chromosome 8p21 for development of AMD in Asians ($P =$

TABLE 4. Subgroup Analysis of the Two SNPs for Neovascular AMD

	No. of Cases	rs1713985						rs13278062					
			Nominal		Adjusted*				Nominal		Adjusted*		
		MAF	P	OR (95% CI)	P	OR (95% CI)		MAF	P	OR (95% CI)	P	OR (95% CI)	
tAMD													
Kyoto	579	0.29	0.844	0.99 (0.86–1.13)	0.687	0.95 (0.76–1.20)		0.39	2.89×10^{-4}	0.78 (0.69–0.89)	0.196	0.87 (0.70–1.08)	
Yamanashi	112	0.27	0.303	1.25 (0.82–1.91)	0.340	1.24 (0.81–1.93)		0.42	0.010	0.60 (0.41–0.89)	0.016	0.61 (0.41–0.91)	
Singapore	122	0.31	0.024	1.56 (1.06–2.29)	0.104	1.46 (0.92–2.32)		0.30	0.179	0.77 (0.53–1.13)	0.337	0.80 (0.52–1.25)	
Hong Kong	200	0.30	0.634	0.93 (0.71–1.24)	0.860	0.97 (0.73–1.30)		0.30	0.347	0.87 (0.66–1.16)	0.330	0.86 (0.65–1.16)	
Meta-analysis	1013		0.378	1.09 (0.90–1.34)	0.598	1.04 (0.89–1.22)			7.22×10^{-6}	0.78 (0.70–0.87)	8.21×10^{-3}	0.81 (0.70–0.95)	
PCV													
Kyoto	720	0.29	0.882	0.99 (0.87–1.12)	0.242	0.89 (0.73–1.08)		0.39	1.69×10^{-4}	0.79 (0.71–0.90)	0.044	0.82 (0.68–0.99)	
Yamanashi	211	0.31	0.035	1.49 (1.03–2.15)	0.040	1.95 (1.19–3.19)		0.38	0.032	0.69 (0.49–0.97)	0.029	0.67 (0.48–0.96)	
Singapore	118	0.28	0.099	1.39 (0.94–2.06)	0.256	1.28 (0.84–1.96)		0.34	0.020	0.64 (0.44–0.94)	0.017	0.63 (0.43–0.92)	
Hong Kong	233	0.31	0.890	0.98 (0.75–1.28)	0.830	0.97 (0.72–1.30)		0.32	0.154	0.82 (0.63–1.08)	0.030	0.71 (0.53–0.97)	
Meta-analysis	1282		0.245	1.12 (0.92–1.36)	0.820	1.02 (0.88–1.18)			8.41×10^{-7}	0.78 (0.70–0.86)	3.79×10^{-5}	0.74 (0.65–0.86)	

tAMD, typical age-related macular degeneration with choroidal neovascular membrane.
* Adjusted for age and sex.

1.12×10^{-4}, OR = 0.79, 95% CI = 0.70–0.89). Although the calculated risk for developing neovascular AMD was smaller than that reported in the previous study (OR = 0.73),[27] data from all four studies analyzed here suggested that the T allele of rs13278062 conferred risk for AMD (Fig. 1).

In the subgroup analysis targeting the development of typical AMD-CNV and PCV, an association similar to that of all neovascular AMDs was obtained; rs1713985 did not show a significant association for either typical AMD-CNV or PCV, while rs13278062 showed a significant association for both typical AMD-CNV and PCV. In particular, rs13278062 showed almost the same susceptibility for developing typical AMD-CNV and PCV (OR = 0.82 and 0.75, respectively), which suggests that there is no difference between typical AMD and PCV with regard to the role of rs13278062 in disease development. These results are consistent with previous studies that have investigated the significance of genetic backgrounds for both typical AMD-CNV and PCV.[17–20]

Currently, several genes have been reported to be associated with developing AMD in Asians, including the following major AMD-associated loci in Caucasians: (1) age-related maculopathy susceptibility 2 and high-temperature requirement factor A1 genes (*ARMS2/HTRA1*) locus,[2,3] (2) complement factor H gene (*CFH*),[4–7] and (3) complement component 2 and factor B genes (*C2/CFB*) locus.[8] Considering the reported OR of each major locus was approximately 2.0,[2–8,17–20] the susceptibility of rs13278062 at *TNFRSF10A-LOC389641* for developing AMD would be smaller than those of the other major loci. In fact, the population attributable risk (PAR)[38] for rs13278062 was 5.9% in our cohorts. However, since rs13278062 has been reported to associate with the transcriptional activity of TNF-related apoptosis inducing ligand (TRAIL) receptors (TRAILR1),[39] which are known to be involved in apoptosis and inflammation,[40] rs13278062 might have a functional role in developing AMD.

In the present study, we focused on just two SNPs that were identified as the "peak" associations in the two novel regions reported by Arakawa et al.[27] The failure to replicate the 4q12 association might be due to the limited attempt to replicate only one SNP. However, in the original study,[27] they found that rs1713985 represents a linkage disequilibrium block that spans from 57.421 to 57.611 million bases (Mb) on 4q12. In addition, they showed a successful replication for the observed association in the discovery phase using the additional cohort of 708 AMD cases. Thus, if there were true associations between variants at 4q12 and developing AMD, the association of rs1713985 should be replicated in our cohorts that include the same population.

In conclusion, this study provides an Asian population-wide replication study for associations of rs13278062 at *TNFRSF10A-LOC389641* and rs1713985 at *REST-C4orf14-POLR2B-IGFBP7* with the development of neovascular AMD; we confirmed the significance of rs13278062 for Asian neovascular AMD but found no association for rs1713985. Thus, the results suggest that only one locus (*TNFRSF10A-LOC389641*) of the two suggested loci confers increased risk of developing AMD in Asian populations. Because most of the reported genetic risk factors for developing AMD in Asians are similar to those for developing Caucasian AMD, a replication study using a Caucasian cohort would be needed to confirm the significance of *TNFRSF10A-LOC389641* on chromosome 8p21.

References

1. de Jong PT. Age-related macular degeneration. *N Engl J Med.* 2006;355:1474–1485.
2. Jakobsdottir J, Conley YP, Weeks DE, Mah TS, Ferrell RE, Gorin MB. Susceptibility genes for age-related maculopathy on chromosome 10q26. *Am J Hum Genet.* 2005;77:389–407.
3. Rivera A, Fisher SA, Fritsche LG, et al. Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. *Hum Mol Genet.* 2005; 14:3227–3236.
4. Edwards AO, Ritter R, 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science.* 2005;308:421–424.
5. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A.* 2005;102:7227–7232.
6. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science.* 2005;308:419–421.
7. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science.* 2005;308:385–389.
8. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet.* 2006;38:458–462.
9. Baird PN, Robman LD, Richardson AJ, et al. Gene-environment interaction in progression of AMD: the CFH gene, smoking and exposure to chronic infection. *Hum Mol Genet.* 2008;17: 1299–1305.
10. Seitsonen SP, Onkamo P, Peng G, et al. Multifactor effects and evidence of potential interaction between complement factor H Y402H and LOC387715 A69S in age-related macular degeneration. *PLoS One.* 2008;3:e3833.
11. Anderson DH, Radeke MJ, Gallo NB, et al. The pivotal role of the complement system in aging and age-related macular degeneration: hypothesis re-visited. *Prog Retin Eye Res.* 2010; 29:95–112.
12. Rohrer B, Long Q, Coughlin B, et al. A targeted inhibitor of the alternative complement pathway reduces angiogenesis in a mouse model of age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2009;50:3056–3064.
13. Smailhodzic D, Klaver CC, Klevering BJ, et al. Risk alleles in CFH and ARMS2 are independently associated with systemic complement activation in age-related macular degeneration. *Ophthalmology.* 2012;119:339–346.
14. Kawasaki R, Yasuda M, Song SJ, et al. The prevalence of age-related macular degeneration in Asians: a systematic review and meta-analysis. *Ophthalmology.* 2010;117:921–927.
15. Lavanya R, Jeganathan VS, Zheng Y, et al. Methodology of the Singapore Indian Chinese Cohort (SICC) eye study: quantifying ethnic variations in the epidemiology of eye diseases in Asians. *Ophthalmic Epidemiol.* 2009;16:325–336.
16. Laude A, Cackett PD, Vithana EN, et al. Polypoidal choroidal vasculopathy and neovascular age-related macular degeneration: same or different disease? *Prog Retin Eye Res.* 2010;29: 19–29.
17. Mori K, Horie-Inoue K, Gehlbach PL, et al. Phenotype and genotype characteristics of age-related macular degeneration in a Japanese population. *Ophthalmology.* 2010;117:928–938.
18. Gotoh N, Nakanishi H, Hayashi H, et al. ARMS2 (LOC387715) variants in Japanese patients with exudative age-related macular degeneration and polypoidal choroidal vasculopathy. *Am J Ophthalmol.* 2009;147:1037–1041. 1041 e1031-1032.
19. Nakata I, Yamashiro K, Yamada R, et al. Significance of C2/CFB variants in age-related macular degeneration and polypoidal choroidal vasculopathy in a Japanese population. *Invest Ophthalmol Vis Sci.* 2012;53:794–798.

20. Hayashi H, Yamashiro K, Gotoh N, et al. CFH and ARMS2 variations in age-related macular degeneration, polypoidal choroidal vasculopathy, and retinal angiomatous proliferation. *Invest Ophthalmol Vis Sci.* 2010;51:5914–5919.
21. Spaide RF, Yannuzzi LA, Slakter JS, Sorenson J, Orlach DA. Indocyanine green videoangiography of idiopathic polypoidal choroidal vasculopathy. *Retina.* 1995;15:100–110.
22. Maruko I, Iida T, Saito M, Nagayama D, Saito K. Clinical characteristics of exudative age-related macular degeneration in Japanese patients. *Am J Ophthalmol.* 2007;144:15–22.
23. Ciardella AP, Donsoff IM, Huang SJ, Costa DL, Yannuzzi LA. Polypoidal choroidal vasculopathy. *Surv Ophthalmol.* 2004; 49:25–37.
24. Liu Y, Wen F, Huang S, et al. Subtype lesions of neovascular age-related macular degeneration in Chinese patients. *Graefes Arch Clin Exp Ophthalmol.* 2007;245:1441–1445.
25. Kondo N, Honda S, Kuno S, Negi A. Coding variant I62V in the complement factor H gene is strongly associated with polypoidal choroidal vasculopathy. *Ophthalmology.* 2009; 116:304–310.
26. Terasaki H, Miyake Y, Suzuki T, Nakamura M, Nagasaka T. Polypoidal choroidal vasculopathy treated with macular translocation: clinical pathological correlation. *Br J Ophthalmol.* 2002;86:321–327.
27. Arakawa S, Takahashi A, Ashikawa K, et al. Genome-wide association study identifies two susceptibility loci for exudative age-related macular degeneration in the Japanese population. *Nat Genet.* 2011;43:1001–1004.
28. Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. *Surv Ophthalmol.* 1995;39: 367–374.
29. Japanese Study Group of Polypoidal Choroidal Vasculopathy. Criteria for diagnosis of polypoidal choroidal vasculopathy [in Japanese]. *Nippon Ganka Gakkai Zasshi.* 2005;109:417–427.
30. Miyagishima T, Gushima H, Sumino Y. Research on pharmacokinetics related genetic polymorphism among Japanese population (Pharma SNP Consortium: PCS). *Xenobiotic Metabolism and Disposition.* 2001;16:340–345.
31. Hamajima N, Matsuo K, Saito T, et al. Gene-environment interactions and polymorphism studies of cancer risk in the hospital-based epidemiologic research program at Aichi cancer center II (HERPACC-II). *Asian Pac J Cancer Prev.* 2001;2:99–107.
32. Hirakawa M, Tanaka T, Hashimoto Y, Kuroda M, Takagi T, Nakamura Y. JSNP: a database of common gene variations in the Japanese population. *Nucleic Acids Res.* 2002;30:158–162.
33. Cackett P, Yeo I, Cheung CM, et al. Relationship of smoking and cardiovascular risk factors with polypoidal choroidal vasculopathy and age-related macular degeneration in Chinese persons. *Ophthalmology.* 2011;118:846–852.
34. Cheung CY, Chen D, Wong TY, et al. Determinants of quantitative optic nerve measurements using spectral domain optical coherence tomography in a population-based sample of non-glaucomatous subjects. *Invest Ophthalmol Vis Sci.* 2011;52:9629–9635.
35. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. *Bioinformatics.* 2010;26:2190–2191.
36. Gauderman WJ. Sample size requirements for matched case-control studies of gene-environment interaction. *Statistics in Medicine.* 2002;21:35–50.
37. Oshima Y, Ishibashi T, Murata T, Tahara Y, Kiyohara Y, Kubota T. Prevalence of age related maculopathy in a representative Japanese population: the Hisayama study. *Br J Ophthalmol.* 2001;85:1153–1157.
38. Kawasaki A, Ito I, Hikami K, et al. Role of STAT4 polymorphisms in systemic lupus erythematosus in a Japanese population: a case-control association study of the STAT1-STAT4 region. *Arthritis Res Ther.* 2008;10:R113.
39. Parihar A, Parihar MS, Chen Z. Ghafourifar P. mAtNOS1 induces apoptosis of human mammary adenocarcinoma cells. *Life Sci.* 2008;82:1077–1082.
40. Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nat Rev Cancer.* 2008;8:782–798.

Significance of *C2/CFB* Variants in Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy in a Japanese Population

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Purpose. To determine whether genetic variants in the complement component 2 and factor B gene (*C2/CFB*) locus are associated with the risk for typical age-related macular degeneration (AMD) or polypoidal choroidal vasculopathy (PCV) in a Japanese population.

Methods. Four single nucleotide polymorphisms (SNPs) were genotyped across the *C2/CFB* locus of patients with typical AMD (n = 455) or PCV (n = 581) and of 865 controls. Differences in the observed genotypic distribution between the case and control groups were tested by logistic regression analysis for age and sex adjustments. Significant associations were confirmed using a second control group of 336 cataract patients. A further model adjusting for age-related maculopathy susceptibility 2 (*ARMS2*) A69S, complement factor H (*CFH*) I62V, age, sex and smoking status was performed, to confirm their independent association from other covariates.

Results. *C2* rs547154 and *CFB* rs541862 were significantly associated with typical AMD and PCV in this Japanese sample ($P < 0.05$). These two SNPs were also significantly associated with typical AMD and PCV in evaluation of the second control cohort ($P < 0.05$). Furthermore, an independent association of *C2/CFB* variants was found for both typical AMD and PCV with age, sex, smoking, and genetic background of *ARMS2* A69S and *CFH* I62V (vs. typical AMD: $P = 0.0073$, odds ratio [OR] = 0.47; vs. PCV: $P = 0.0083$, OR = 0.53).

Conclusions. *C2/CFB* variants play a protective role in the risk of developing neovascular AMD and PCV in the Japanese. (*Invest Ophthalmol Vis Sci.* 2012;53:794-798) DOI:10.1167/iovs.11-8468

Age-related macular degeneration (AMD) is the leading cause of visual impairment in the elderly and the most common cause of blindness in developed countries.[1] Recent studies of the genetics of AMD have recognized it as a complex disease caused by the actions and interactions of several genes and environmental factors.[2-10] The three major AMD-associated loci in Caucasians include (1) the age-related maculopathy susceptibility 2 and high-temperature requirement factor A1 (*ARMS2/HTRA1*) gene[2,3] (2) the complement factor H (*CFH*) gene,[4-7] and (3) the complement component 2 and factor B (*C2/CFB*) gene loci.[8]

Some studies have reported that inflammatory processes may play a central role in AMD by contributing to the formation of drusen,[11,12] with *C2* and *CFB* being involved in initiation of the alternative complement cascade and activation of the classic component pathway, respectively. Numerous reports using Caucasian cohorts hold the consensus that genetic variants across *C2/CFB* are involved in protection against AMD.[8,13-15] However, all previous reports that evaluated populations in East Asia have shown an absence of association of *C2/CFB* variants in developing AMD. Thus, it was concluded that these variants are less likely to be associated with the development of AMD in Asians.[16,17]

Polypoidal choroidal vasculopathy (PCV) is clinically classified as a specific type of AMD and is usually diagnosed by indocyanine green angiography.[18] The incidence of PCV in Asian populations with neovascular AMD has been reported to be high, accounting for 54.7% of Japanese AMD patients and 24.5% of Chinese patients, compared with only 8% to 13% of Caucasians.[19-21] Previous studies revealed several genes that are susceptible to the development of PCV.[22-24] However, almost all reported genetic risk factors for developing PCV are identical with those for the development of AMD, which suggests that AMD and PCV share, at least in part, the same genetic background. In fact, PCV has many similarities with neovascular AMD, including demography, pathology, and manifestation.[19,21,25]

There have been studies in which the association between PCV and *C2/CFB* was evaluated in Asian populations, in a relatively smaller cohort size (165 participants by Lee et al.[26] and 313 participants by Kondo et al.[27]), but the results were negative, leading to the conclusion that pathobiological differences between PCV and neovascular AMD were present. However, these studies seemed to be underpowered, and recently, in a Caucasian cohort, Lima et al.[28] showed a positive associ-

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TABLE 1. Characteristics of the Study Population

	Cases		Control
	tAMD (n = 455)	PCV (n = 581)	Healthy Individuals (n = 865)
Age, y			
Mean ± SD	75.59 ± 8.60	72.59 ± 8.13	48.22 ± 16.18
Sex, n (%)			
Men	330 (72.5)	420 (72.3)	431 (49.8)
Women	125 (27.5)	161 (27.7)	434 (50.2)
Smoking, n (%)			
Never	151 (36.7)	200 (38.5)	454 (52.7)
Ever	261 (63.3)	319 (61.5)	408 (47.3)

ation in *C2/CFB* variants of the risk for PCV. Although PCV is more common in Asians than in Caucasians, the association between *C2/CFB* and PCV has not been subjected to detailed evaluation in Asian cohorts. The purpose of this study was to investigate whether the *C2/CFB* variants play a significant role in development of typical AMD or PCV by using almost 2000 participants from a Japanese population.

METHODS

All procedures in this study adhered to the tenets of the Declaration of Helsinki, and the Ethics Committee of each institute involved approved the study protocols. All the patients were fully informed about the purpose and procedures of the study, with each patient providing written consent.

Four hundred and fifty-five patients with typical AMD (tAMD) and 581 patients with PCV were recruited from the Department of Ophthalmology at Kyoto University Hospital, Fukushima Medical University Hospital, and Kobe City Medical Center General Hospital. We used 865 healthy Japanese individuals, recruited from the Aichi Cancer Center Research Institute, as control subjects. They were recruited from first-visit outpatients after it was confirmed that they did not have cancer according to the cancer registry, medical record, and self-report. We recruited them without ophthalmic data and evaluated them as general population controls. When we found a significant association in a studied variant using the general population controls, we confirmed the association using a second control group comprising 336 elderly individuals who had received cataract surgery without age-related maculopathy (ARM), recruited from the Department of Ophthalmology at Kyoto University Hospital, Ozaki Eye Hospital, Japanese Red Cross Otsu Hospital, and Nagahama City Hospital. By funduscopic examination, the cataract control samples were confirmed not to have any drusen or pigment change. The definitions of exudative AMD and ARM were based on those of the International Classification System for ARM and AMD.[29] As proposed by the Japanese Study Group of Polypoidal Choroidal Vasculopathy,[30] the diagnosis of PCV was based on indocyanine green angiography, which showed a branching vascular network terminating in polypoidal swelling. tAMD showed classic CNV, occult CNV, or both. Patients with the following status were excluded from the study subjects: (1) high myopia (spherical equivalent, < -6.00 D), (2) geographic atrophy or drusen only, (3) an eye with both typical choroidal neovascularization and polypoidal lesions, and (4) an old lesion without a clear diagnosis. All diagnoses were made by three retina specialists (KY, AT, and AO); a fourth specialist (NY) was consulted when the subtype classification could not be decided on by the initial three reviewers. All subjects in the present study were unrelated and of Japanese descent.

Information on smoking status was obtained via a self-report questionnaire, with the 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 1 year, and former smokers were those who had quit smoking more than 1 year earlier. As in our previous study,[31] we combined the current smokers and the former smokers into ever smokers; thus, we analyzed the smoking status based on the two groups of never smokers and ever smokers.

We targeted *C2* rs547154 (IVS10) and *CFB* rs2072633 (IVS17), which have been described as having a positive association with the development of AMD in prior studies.[8,13,14,32] We analyzed two additional single-nucleotide polymorphisms (SNPs) on *CFB* (rs541862 and rs4151672) because they had relatively higher allele frequencies on the *C2/CFB* locus. Genomic DNAs were prepared from peripheral blood by using a DNA extraction kit (QuickGene-610L; Fujifilm, Minato, Tokyo, Japan). All case samples and cataract samples were then genotyped (Taqman SNP assay with the PRISM 7700 system; Applied Biosystems, Inc. [ABI], Foster City, CA). Individuals recruited from the Aichi Cancer Center Research Institute were genotyped with another system (Human-Hap610 chips; Illumina Inc., San Diego, CA).

Deviations in genotype distributions from the Hardy-Weinberg equilibrium (HWE) were assessed with the HWE exact test. Statistical analyses for differences in the observed genotypic distribution were performed by logistic regression analysis for age and sex adjustments. Haploview software was used to perform haplotype analysis and to infer the linkage disequilibrium (LD) among the evaluated SNPs.[33] P value correction was performed with the Bonferroni method, using the ratio of the number of selected SNPs across a gene. $P < 0.05$ was considered statistically significant.

RESULTS

Demographics of the study population are shown in Table 1. Genotype and allele frequencies of the four SNPs were analyzed in the 455 patients with tAMD and 581 patients with PCV and compared with those of 865 healthy Japanese individuals. Details of allele frequencies and summary statistics are shown in Table 2. The genotyping of all evaluated SNPs was more than 98.2% successful, and the distributions of the genotypes for all study groups were in HWE ($P > 0.05$). In logistic regression analyses adjusted for age and sex, *C2* rs547154 and *CFB* rs541862 were significantly associated with both tAMD

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

			Minor Allele Frequency			Association Test Results					
			tAMD	PCV	Control	Control vs. tAMD		Control vs. PCV		PCV vs. tAMD	
		Minor Allele	n = 455	n = 581	n = 865	P*	OR (95% CI)*	P*	OR (95% CI)*	P*	OR (95% CI)*
C2	rs547154	T	0.045	0.052	0.076	0.018	0.57 (0.35–0.91)	0.0062	0.54 (0.35–0.84)	0.385	0.83 (0.56–1.25)
CFB	rs541862	C	0.044	0.053	0.076	0.016	0.56 (0.35–0.90)	0.0061	0.54 (0.35–0.84)	0.317	0.81 (0.54–1.22)
CFB	rs2072633	A	0.436	0.413	0.454	0.875	0.98 (0.78–1.24)	0.240	0.88 (0.71–1.09)	0.425	1.08 (0.90–1.29)
CFB	rs4151672	T	0.018	0.022	0.024	0.845	0.92 (0.38–2.22)	0.842	0.93 (0.44–1.95)	0.584	0.84 (0.44–1.59)

* Adjusted for age and sex.

TABLE 3. Replication Study Using Cataract Patients

		Cataract Patients (n = 336)		vs. tAMD (n = 455)		vs. PCV (n = 579)	
		Minor Allele	MAF	P*	OR (95% CI)*	P*	OR (95% CI)*
C2	rs547154	T	0.092	0.0028	0.51 (0.33-0.79)	0.0038	0.56 (0.38-0.83)
CFB	rs541862	C	0.091	0.0028	0.51 (0.33-0.79)	0.0046	0.57 (0.38-0.84)

MAF, minor allele frequency in the cataract patients.
* Adjusted for age and sex.

(rs547154: P = 0.018, odds ratio [OR] = 0.57, 95% confidence interval [CI] = 0.35-0.91; rs541862: P = 0.016, OR = 0.56, 95% CI = 0.35-0.90) and PCV (rs547154: P = 0.0062, OR = 0.54, 95% CI = 0.35-0.84; rs541862: P = 0.0061, OR = 0.54, 95% CI = 0.35-0.84). These associations remained significant, even after a permutation procedure for multiple test correction (corrected $P < 0.05$). There was no SNP in the four tested SNPs across the *C2/CFB* locus, which showed a significant difference between tAMD and PCV.

Because the age of the controls was definitely younger than that of the cases, to adjust for a birth cohort effect, differential survival, or survivorship, we also performed a stratification analysis using 420 controls aged 50 years or older. This stratified cohort included 212 (50.5%) men, 208 (49.5%) women, 223 (53.3%) never smokers, and 195 (46.7%) ever smokers. The mean age ± SD of the group was 62.68 ± 7.67 years. We found that the associations of *C2* rs547154 and *CFB* rs541862 remained statistically significant in both tAMD (rs547154, P = 0.0048; rs541862, P = 0.0042) and PCV (rs547154, P = 0.0076; rs541862, P = 0.0075) in this stratification analysis, as well.

Next, we confirmed the positive associations using a second control cohort of 336 elderly cataract patients. The mean age ± SD of the cataract patients was 74.16 ± 8.42 years (range, 43- 94), and 142 (42.3%) male and 194 (57.7%) female patients were included. Table 3 shows the result of this replication analysis. Significant associations for developing both tAMD and PCV with *C2* rs547154 and *CFB* rs541862 were also shown in this evaluation ($P < 0.05$). In addition, we performed haplotype analysis using the cataract controls (Table 4). Haplotype analysis revealed that a common haplotype across the *C2/CFB* locus conferred a significant risk for both tAMD and PCV (P = 0.0030 and 0.0001, respectively) and a rare haplotype protectively associated with both tAMD and PCV (P = 0.0001 and 0.0016, respectively).

Finally, we conducted a logistic regression analysis that included the effects of the most robust Japanese AMD/PCV-associated variants, *ARMS2* A69S (rs10490924) and *CFH* I62V (rs800292), as well as age, sex, and smoking status. Because *C2* rs547154 and *CFB* rs541862 were in strong LD (pair-wise D′ = 1.0 and r^2 = 1.0), we analyzed rs547154 as the representative SNP of the *C2/CFB* locus. Table 5 shows the result of this logistic regression analysis. *C2/CFB* rs547154 remained significant both in tAMD and PCV, even after including the effects of these covariates (vs. tAMD: P = 0.0073, OR = 0.47, 95% CI = 0.27-0.82; vs. PCV: P = 0.0083, OR = 0.53, 95% CI = 0.33-0.85). After considering the effects of three major AMD-associated loci, we found that the effect of smoking was diminished in the risk for PCV (P = 0.292), and just a marginal association was found for tAMD (P = 0.0693).

DISCUSSION

The present study shows the significance of polymorphisms in *C2* and *CFB* for development of tAMD and PCV in a relatively large sample of Japanese patients. As a result of comparing the genotypic distributions of *C2/CFB* variants in a sample of Japanese patients with tAMD (n = 455) or PCV (n = 581) and in two independent control groups (865 healthy Japanese individuals and 336 cataract patients), we found that *C2* rs547154 (IVS10) and *CFB* rs541862 showed significant associations with the risk for both tAMD and PCV, with protective effects against the risk of the diseases.

Replication is the gold standard for assessing statistical results from genetic studies. However, a real result may fail to be replicated for numerous reasons, including inadequate sample size or variability in phenotype definitions across independent samples.[34] Although numerous reports have shown a significant association between *C2/CFB* variants and AMD in Caucasians, all studies in Asians have been unable to replicate these results.[16,17] However, in the present study, we clearly showed a significant association of *C2/CFB* variants for developing AMD in a Japanese cohort. Considering that minor allele frequency is similar between our cohort and previous reports, this discrepancy would be due to the small sample size used in the previous reports on Asian cohorts. Our study indicates that previous studies on the same subject did not reach statistical significance, and that large cohorts are needed to have enough statistical power to detect the association of the *C2/CFB* locus.

To date, all reports on Asian cohorts have shown a lack of association between *C2/CFB* polymorphism and PCV.[26,27] However, recently, Lima et al.[28] showed a positive association with PCV in Caucasians, even though incidence of PCV is lower in Caucasians than in Asians. In the present study,

TABLE 4. Distribution of Haplotypes and the Results of the Association Tests

Haplotype				Frequencies		P		
rs547154	rs541862	rs2072633	rs4151672	tAMD	PCV	Cataract Controls	Cataract vs. tAMD	Cataract vs. PCV
G	T	G	C	0.55	0.57	0.47	0.0030	0.0001
G	T	A	C	0.39	0.36	0.41	0.527	0.0467
T	C	A	C	0.044	0.053	0.092	0.0001	0.0016
G	T	G	T	0.018	0.022	0.029	0.109	0.264

tAMD, typical age-related macular degeneration.

TABLE 5. Comparison of the Significance of Major AMD-Associated Factors

	tAMD		PCV	
	P*	OR (95% CI)	P*	OR (95% CI)
Age, y	<0.0001	1.21 (1.18–1.24)	<0.0001	1.18 (1.15–1.20)
Sex, women/men	0.0127	1.87 (1.14–3.07)	<0.0001	2.64 (1.72–4.04)
ARMS2/HTRA1 rs10490924 (G/T)	<0.0001	2.43 (1.85–3.20)	<0.0001	2.02 (1.60–2.56)
CFH rs800292 (A/G)	<0.0001	1.89 (1.41–2.55)	<0.0001	2.02 (1.57–2.60)
C2/CFB rs547154 (G/T)	0.0073	0.47 (0.27–0.82)	0.0083	0.53 (0.33–0.85)
Smoking, never/ever	0.0693	1.56 (0.97–2.52)	0.292	1.25 (0.83–1.87)

* A logistic regression model was used to analyze the association between covariates.

C2/CFB variants were clearly associated with PCV. Our result is therefore in agreement with that of the Caucasian cohort used by Lima et al. Hence, our study also supports that there is no difference between tAMD and PCV in the role of *C2/CFB* for development of the disease.

In addition, we found the association of *C2/CFB* variants was unchanged, even when we adjusted for the effects of other established risk factors for AMD (age, sex, smoking, and a genetic background including *ARMS2* A69S and *CFH I62V*). In this study, common genetic variations at all three loci were associated with PCV, similar to that already documented in AMD—that is, SNPs that conferred a higher risk or protection from the disease in AMD were associated with the same in PCV. Furthermore, logistic regression analysis revealed that the role of environmental factors (smoking) diminishes when the effects of the three major AMD-associated loci (*ARMS2/HTRA1*, *CFH*, and *C2/CFB*) are taken into consideration. This result indicates that genetic factors have an enormous influence on whether people develop AMD and/or PCV. Among all covariates, *ARMS2/HTRA1* variants had the largest effect on the risk for tAMD (OR = 2.43), whereas sex had the largest effect on development of PCV (OR = 2.64). In a previous meta-analysis study,[35] the prevalence of late AMD in Asian women was reported to be much lower than in Asian men; on the other hand it is said that those with PCV are predominately male.[20] Considering the high prevalence of PCV in Asian populations, these results suggest that men would be more likely to develop PCV. In our study, genetic factors had important roles in the development of both tAMD and PCV. Thus, our results indicate that differences in sex would affect phenotypic differences in AMD.

In the present study, we evaluated different SNPs from those examined in the original study,[8] because minor allele frequencies of the SNPs evaluated in Caucasians were extremely low in the Japanese. To confirm the association reported in Caucasians, we also genotyped *C2* rs9332739, reported to be positive in the original study in cataract controls. However, there was no significant association in *C2* rs9332739 for development of tAMD and PCV in our cohort, because of its low allele frequency (data not shown), and *C2* rs9332739 did not have an impact on the result of haplotype analysis

We also grouped the current smokers and the former smokers into ever smokers, because this group had the highest tendency to develop PCV.[31] However, smoking was not found to have a significant independent association with the development of either tAMD or PCV in this study. Considering that smoking status was obviously different between the cases and the controls, this association should reach statistical significance if the number of participants were increased. Another reason for the lack of association could be because of the heterogeneity of smoking status. As with the general trend, the former smokers were older than others, and more men than women had smoked in our cohort (data not shown). In addition, we could not exclude the possibility that there were interactions between genetic and environmental factors or between genes; several studies have reported the presence of interactions between *ARMS2/HTRA1*, *CFH*, and smoking in AMD[9,10,36] and PCV.[31] Hence, further studies should be performed to ascertain the effects of interaction of different risk factors in the development of disease, including AMD-associated genes and smoking.

Another limitation of the present study is the difference between the case and control samples. The control samples were definitely younger than those in the case group, which means that some of these young controls may develop AMD or PCV in the future. To exclude a potential confounder of genetic background in age, we confirmed that our results were unchanged, even after a stratification analysis adjusting for the difference in age. In addition, to avoid a sampling error, we performed a replication study using another control group of a much closer age to the cases (cataract patients without ARM) and found a significant association between *C2/CFB* variants and development of AMD/PCV. However, because the prevalence of late AMD in the Japanese population is reported to be 0.5%,[37] the magnitude of the statistical bias of an association analysis should be negligible. In addition, considering that the case-control association analyses using such subjects are less apt to be statistically significant, our positive results should be acceptable.

Recently, subretinal drusenoid deposits, called reticular pseudodrusen, were differentiated from soft drusen with spectral domain optical coherence tomography (SD-OCT)[38] and were reported to be associated with late AMD.[39,40] We also evaluated whether *C2/CFB* variants are associated with developing reticular pseudodrusen in a small number of participants (n = 91) who had SD-OCT and autofluorescence imaging. However, we could not find a significant association between *C2/CFB* variants and the incidence of reticular pseudodrusen (data not shown). Further studies are needed to ascertain the association between *C2/CFB* variants and developing reticular pseudodrusen.

In conclusion, this study provides the first evidence that *C2/CFB* variants play a role in the risk of both neovascular AMD and PCV in Asians. Inflammation plays a central role in the pathobiology of AMD, with *C2* and *CFB* both encoding regulatory proteins that activate the complement pathway. As the inhibition of CFB with a specific chemical binding entity has been suggested to be a viable approach for the treatment of neovascular AMD,[41] our findings may suggest the potential effectiveness of such treatments by using anti-inflammatory agents, not only for AMD but also for PCV.

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References

1. de Jong PT. Age-related macular degeneration. *N Engl J Med.* 2006;355:1474-1485.
2. Jakobsdottir J, Conley YP, Weeks DE, et al. Susceptibility genes for age-related maculopathy on chromosome 10q26. *Am J Hum Genet.* 2005;77:389-407.
3. Rivera A, Fisher SA, Fritsche LG, et al. Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. *Hum Mol Genet.* 2005;14:3227-3236.
4. Edwards AO, Ritter R 3rd, Abel KJ, et al. Complement factor H polymorphism and age-related macular degeneration. *Science.* 2005;308:421-424.
5. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A.* 2005;102:7227-7232.
6. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science.* 2005;308:419-421.
7. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science.* 2005;308:385-389.
8. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet.* 2006;38:458-462.
9. Baird PN, Robman LD, Richardson AJ, et al. Gene-environment interaction in progression of AMD: the CFH gene, smoking and exposure to chronic infection. *Hum Mol Genet.* 2008;17:1299-1305.
10. Seitsonen SP, Onkamo P, Peng G, et al. Multifactor effects and evidence of potential interaction between complement factor H Y402H and LOC387715 A69S in age-related macular degeneration. *PLoS One.* 2008;3:e3833.
11. Anderson DH, Mullins RF, Hageman GS, Johnson LV. A role for local inflammation in the formation of drusen in the aging eye. *Am J Ophthalmol.* 2002;134:411-431.
12. Bok D. Evidence for an inflammatory process in age-related macular degeneration gains new support. *Proc Natl Acad Sci U S A.* 2005;102:7053-7054.
13. Spencer KL, Hauser MA, Olson LM, et al. Protective effect of complement factor B and complement component 2 variants in age-related macular degeneration. *Hum Mol Genet.* 2007;16:1986-1992.
14. Richardson AJ, Islam FM, Guymer RH, Baird PN. Analysis of rare variants in the complement component 2 (C2) and factor B (BF) genes refine association for age-related macular degeneration (AMD). *Invest Ophthalmol Vis Sci.* 2009;50:540-543.
15. Chen W, Stambolian D, Edwards AO, et al. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. *Proc Natl Acad Sci U S A.* 2010;107:7401-7406.
16. Liu X, Zhao P, Tang S, et al. Association study of complement factor H, C2, CFB, and C3 and age-related macular degeneration in a Han Chinese population. *Retina.* 2010;30:1177-1184.
17. Chu J, Zhou CC, Lu N, Zhang X, Dong FT. Genetic variants in three genes and smoking show strong associations with susceptibility to exudative age-related macular degeneration in a Chinese population. *Chin Med J (Engl).* 2008;121:2525-2533.
18. Spaide RF, Yannuzzi LA, Slakter JS, Sorenson J, Orlach DA. Indocyanine green videoangiography of idiopathic polypoidal choroidal vasculopathy. *Retina.* 1995;15:100-110.
19. Maruko I, Iida T, Saito M, Nagayama D, Saito K. Clinical characteristics of exudative age-related macular degeneration in Japanese patients. *Am J Ophthalmol.* 2007;144:15-22.
20. Ciardella AP, Donsoff IM, Huang SJ, Costa DL, Yannuzzi LA. Polypoidal choroidal vasculopathy. *Surv Ophthalmol.* 2004;49:25-37.
21. Liu Y, Wen F, Huang S, et al. Subtype lesions of neovascular age-related macular degeneration in Chinese patients. *Graefes Arch Clin Exp Ophthalmol.* 2007;245:1441-1445.
22. Gotoh N, Nakanishi H, Hayashi H, et al. ARMS2 (LOC387715) variants in Japanese patients with exudative age-related macular degeneration and polypoidal choroidal vasculopathy. *Am J Ophthalmol* 2009;147:1037-1041, 1041 e1031-e1032.
23. Kondo N, Honda S, Kuno S, Negi A. Coding variant I62V in the complement factor H gene is strongly associated with polypoidal choroidal vasculopathy. *Ophthalmology* 2009;116:304-310.
24. Hayashi H, Yamashiro K, Gotoh N, et al. CFH and ARMS2 variations in age-related macular degeneration, polypoidal choroidal vasculopathy, and retinal angiomatous proliferation. *Invest Ophthalmol Vis Sci.* 2010;51:5914-5919.
25. Terasaki H, Miyake Y, Suzuki T, Nakamura M, Nagasaka T. Polypoidal choroidal vasculopathy treated with macular translocation: clinical pathological correlation. *Br J Ophthalmol.* 2002;86:321-327.
26. Lee KY, Vithana EN, Mathur R, et al. Association analysis of CFH, C2, BF, and HTRA1 gene polymorphisms in Chinese patients with polypoidal choroidal vasculopathy. *Invest Ophthalmol Vis Sci.* 2008;49:2613-2619.
27. Kondo N, Honda S, Kuno S, Negi A. Role of RDBP and SKIV2L variants in the major histocompatibility complex class III region in polypoidal choroidal vasculopathy etiology. *Ophthalmology.* 2009;116:1502-1509.
28. Lima LH, Schubert C, Ferrara DC, et al. Three major loci involved in age-related macular degeneration are also associated with polypoidal choroidal vasculopathy. *Ophthalmology.* 2010;117:1567-1570.
29. Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. *Surv Ophthalmol.* 1995;39:367-374.
30. Japanese Study Group of Polypoidal Choroidal Vasculopathy. Criteria for diagnosis of polypoidal choroidal vasculopathy. *Nippon Ganka Gakkai Zasshi* 2005;109:417-427.
31. Nakanishi H, Yamashiro K, Yamada R, et al. Joint effect of cigarette smoking and CFH and LOC387715/HTRA1 polymorphisms on polypoidal choroidal vasculopathy. *Invest Ophthalmol Vis Sci.* 2010;51:6183-6187.
32. Kaur I, Katta S, Reddy RK, et al. The involvement of complement factor B and complement component C2 in an Indian cohort with age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2010;51:59-63.
33. Barrett JC, Fry B, Maller J, Daly MJ Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics.* 2005;21:263-265.
34. Chanock SJ, Manolio T, Boehnke M, et al. Replicating genotype-phenotype associations. *Nature.* 2007;447:655-660.
35. Kawasaki R, Yasuda M, Song SJ, et al. The prevalence of age-related macular degeneration in Asians: a systematic review and meta-analysis. *Ophthalmology.* 2010;117:921-927.
36. Kabasawa S, Mori K, Horie-Inoue K, et al. Associations of cigarette smoking but not serum fatty acids with age-related macular degeneration in a Japanese population. *Ophthalmology.* 2011;118:1082-1088.
37. Kawasaki R, Wang JJ, Ji GJ, et al. Prevalence and risk factors for age-related macular degeneration in an adult Japanese population: the Funagata study. *Ophthalmology.* 2008;115:1376-1381, 1381 e1371-e1372.
38. Zweifel SA, Spaide RF, Curcio CA, Malek G, Imamura Y. Reticular pseudodrusen are subretinal drusenoid deposits. *Ophthalmology.* 2010;117:303-312 e301.
39. Zweifel SA, Imamura Y, Spaide TC, Fujiwara T, Spaide RF. Prevalence and significance of subretinal drusenoid deposits (reticular pseudodrusen) in age-related macular degeneration. *Ophthalmology.* 2010;117:1775-1781.
40. Smith RT, Sohrab MA, Busuioc M, Barile G. Reticular macular disease. *Am J Ophthalmol.* 2009;148:733-743 e732.
41. Zarbin MA, Rosenfeld PJ. Pathway-based therapies for age-related macular degeneration: an integrated survey of emerging treatment alternatives. *Retina.* 2010;30:1350-1367.

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Association of *paired box 6* with high myopia in Japanese

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Purpose: The objective of this study was to investigate whether genetic variations in the *paired box 6* (*PAX6*) gene are associated with high myopia in Japanese subjects.

Methods: A total of 1,307 unrelated Japanese patients with high myopia (axial length ≥26 mm in both eyes) and two independent control groups were evaluated (333 cataract patients without high myopia and 923 age-matched healthy Japanese individuals). We genotyped three tag single-nucleotide polymorphisms (SNPs) in *PAX6*: rs2071754, rs644242, and rs3026354. These SNPs provided 100% coverage of all phase II HapMap SNPs within the *PAX6* region (minor allele frequency ≥0.10; r^2 threshold: 0.90). Chi-square tests for trend and multivariable logistic regression were conducted.

Results: Genotype distributions in the three SNPs were in accordance with the Hardy–Weinberg equilibrium. After adjusting for age and sex, evaluation of cataract control showed a marginal association with high myopia in rs644242 (odds ratio [95% confidence interval]=0.69 [0.49–0.96], p=0.026), and a significant association was observed in healthy Japanese controls (0.79 [0.66–0.96], p=0.015). We pooled two control cohorts to evaluate the association. This analysis revealed a strong association between rs644242 and high myopia (0.78 [0.65–0.92], p=0.0045). The rs644242 A allele was a protective allele for development of high myopia. Subanalysis also revealed that rs644242 was significantly associated with extreme high myopia (0.78 [0.64–0.95], p=0.0165). The other two SNPs did not show a significant association with this condition.

Conclusions: The current study showed a significant association of *PAX6* with high and extreme myopia in Japanese participants. The A allele of rs644242 is a protective allele.

Myopia is the most common visual disorder in the world and presents major public health concerns, especially in East Asian populations. Eyes with long axial lengths (≥26 mm) or a high degree of myopic refractive error (≤−6 diopter [D]) were diagnosed with high myopia [1]. High myopia is associated with various ocular complications [2], and pathological myopia is one of the leading causes of legal blindness in developed countries [3-5]. Therefore, clarifying the pathological pathway that leads to high myopia and developing methods for preventing or delaying its onset are important.

Myopia is a complex disease caused by environmental and genetic factors. Although linkage analysis studies have revealed more than 20 myopia-susceptibility loci and various candidate genes have been evaluated, most of these genes were not consistently responsible for high myopia. Recently, several groups performed genome-wide association studies (GWAS); we determined a susceptible locus at 11q14.1 [6] and 5p15 [7], while studies of Caucasians revealed myopia-susceptibility loci on chromosome 15 [8,9]. We demonstrated the association of these susceptibility loci on chromosome 15 with high myopia in Japanese [10], and a Chinese study successfully replicated the association between high myopia and the *catenin δ2* (*CTNND2*) gene polymorphism in the susceptibility loci 5p15 we determined [11]. However, although the C allele of *CTNND2* single nucleotide polymorphism (SNP) rs6885224 was a risk allele for high myopia in our study, the replication study showed this allele was protective against high myopia. Since the expression of the catenin δ2 protein is regulated by transcription factor Pax6 [12] and *PAX6* is another myopia-susceptibility gene, *PAX6* and *CTNND2* might cooperatively affect myopia development. Although several studies have examined the association between *PAX6* and myopia, whether *PAX6* is a susceptibility gene for myopia remains controversial [13-20]. To determine whether *PAX6* is associated with high myopia, we conducted a large-cohort case–control study of Japanese participants.

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METHODS

All procedures adhered to the tenets of the Declaration of Helsinki. The Institutional Review Board and the Ethics Committee of each participating institute approved the protocols. All the patients were fully informed of the purpose and procedures of the study, and written consent was obtained from each patient.

Patients and control subjects: In total, 1,307 unrelated Japanese patients with high myopia from the Kyoto University Hospital, Tokyo Medical and Dental University Hospital, Fukushima Medical University Hospital, Kobe City Medical Center General Hospital, and Ozaki Eye Hospital were included in the study. Comprehensive ophthalmic examinations were conducted on all the patients, which included dilated indirect and contact lens slit-lamp biomicroscopies, automatic objective refractions, and measurements of axial length using applanation A-scan ultrasonography or partial coherence interferometry (IOLMaster; Carl Zeiss Meditec, Dublin, CA). An axial length of at least 26 mm in both eyes confirmed the patient had high myopia.

Two control cohorts were recruited for this study. The first cohort was categorized as the selected control group, and comprised 333 cataract patients with axial lengths of less than 25.0 mm in both eyes (control 1). These patients were recruited from the Department of Ophthalmology at Kyoto University Hospital, the Ozaki Eye Hospital, the Japanese Red Cross Otsu Hospital, and the Nagahama City Hospital. In this group, the mean age ± standard deviation (SD) was 75.2±7.9 years; 37.2% were men, and 59.5% were women. Axial length was measured with applanation A-scan ultrasonography or partial coherence interferometry before cataract surgery, and post-surgery, a dilated fundus examination was performed. If the fundus examination revealed that myopic changes had occurred, such as lacquer cracks/peripapillary atrophy, staphyloma, or choroidal neovascularization, the subject was eliminated from the group.

The second cohort was recruited as a general-population control. In total, 923 healthy unrelated Japanese individuals were recruited from the Aichi Cancer Center Research Institute (control 2). Only individuals at least 35 years of age were selected to participate in this group, meaning that the controls were age-matched with the high-myopia cohort. The mean age ± SD of this cohort was 56.9±11.4 years (p=0.855 compared with the high-myopia cohort); 39.3% were men, and 60.7% were women.

Genotyping and statistical analyses: Genomic DNAs were prepared from peripheral blood using a DNA extraction kit (QuickGene-610L; Fujifilm, Minato, Tokyo, Japan) according to the manufacturer's protocol, and the A260/A280 optical density was measured. Extracted DNA was stored at -80 °C until used. Three tag SNPs (rs2071754, rs644242, and rs3026354) were selected using Tagger software, and provided 100% coverage for all common phase II HapMap SNPs (minor allele frequency: >10%; Build: 36.1) within a 22.4-kb region that covered the *PAX6* gene on chromosome 11 (r^2 threshold: 0.90). The samples from patients with high myopia and the cataract controls were genotyped using a commercially available assay (TaqMan SNP assay with the ABI PRISM 7700 system; Applied Biosystems, Foster City, CA). The individuals recruited from the Aichi Cancer Center Research Institute were genotyped using Illumina HumanHap 610 Chips (Illumina Inc., San Diego, CA). The genotype for rs3026354 was obtained from imputed data using MACH software because it was not included in the Illumina BeadChip. Phase II HapMap (Build: 36.1) was referred to for reference sequences.

Deviations from the Hardy–Weinberg equilibrium (HWE) in genotype distributions were assessed for each group using the HWE exact test. The chi-square test for trend or its exact counterpart was used to compare the genotype distributions of the two groups. Multiple regression and logistic regression analysis were performed to adjust for age and sex. These statistical analyses were conducted using Software R (R Foundation for Statistical Computing, Vienna, Austria). A p value of less than or equal to 0.05 was considered statistically significant. Bonferroni correction was used for multiple comparisons.

RESULTS

The demographics of the study population are shown in Table 1. The mean axial length of the 2,614 eyes with high myopia was 29.17±1.84 mm. Of the eyes in this group, 1,878 (71.8%) were phakic, with a mean refraction of −12.71±4.57 D. In the control 1 group, the mean axial length of the 666 eyes was 22.87±0.80 mm, and the mean refraction of the phakic eyes in this group was −0.355±2.96 D.

The genotype counts, associations, and odds ratios (ORs) for the three SNPs in the high-myopia and control groups are shown in Table 2. The genotype distributions of the three SNPs were in HWE (p>0.05). After corrections for age and sex differences had been made, based on a logistic regression model, rs644242 showed a marginal association (p=0.026) with high myopia when evaluated with control 1 (n=333), and a significant association (p=0.015) when evaluated with control 2 (n=923); further analysis demonstrated that this association was still significant after Bonferroni correction. For the high-myopia group, the odds ratios were 0.69 (95%

TABLE 1. CHARACTERISTICS OF THE STUDY POPULATION.

Population characteristics	High myopia*	Case		Control	
		Control 1†	P value	Control 2	P value
Patients (n)	1307	333		923	
Age (mean±SD; years)	57.1±15.0	75.2±7.9	<0.0001‡	56.9±11.4	0.8549‡
Sex (n)					
Male	427 (32.7%)	124 (37.2%)	0.05626§	363 (39.3%)	0.0015§
Female	879 (67.3%)	198 (59.5%)		560 (60.7%)	
Axial length (mm±SD)					
Right eyes	29.23±1.85	22.84±0.81	NA		
Left eyes	29.10±1.82	22.88±0.78	NA		
Refraction of the phakic eyes (D)					
Right eyes	−12.86±4.44	−0.411±3.15	NA		
Left eyes	−12.57±4.71	−0.296±2.77	NA		

* Axial length >26.00 mm in both eyes. † Individuals who underwent cataract surgery and who had an axial length of <25.00 mm in both eyes.‡ Unpaired t test. Compared with the high-myopia group. § Chi-square test. Compared with the high-myopia group. SD: standard deviation, D: diopter, NA: Not applicable.

confidence interval [CI]: 0.49–0.96) for the rs644242 A allele when evaluated with control 1, and 0.79 (95% CI: 0.66–0.96) when evaluated with control 2. Chi-square tests for the trend also showed that rs644242 was significantly associated with high myopia when this group was evaluated with control 2 (p=0.015). The two other SNPs did not have any significant associations with the condition.

Since the allele frequency and the genotype frequency of the three SNPs were not significantly different (p>0.20) between control 1 and control 2, we pooled the controls for further analysis (Table 3). The genotype distributions in the pooled control were still within HWE. This analysis revealed that the rs644242 polymorphism was strongly associated with high myopia. The p value of a chi-square test for the trend was 0.011, and was 0.0045 after adjusting for age and sex with a logistic regression model. Since previous studies have reported on SNP associations with extreme myopia, the genotype distributions of the three SNPs between the extreme myopia cases were compared (axial length ≥28 mm in both eyes) as a pooled control. After age and sex adjustment and Bonferroni correction, this analysis also showed a significant association between rs644242 and extreme myopia (p=0.0165). The OR of this analysis was similar to the OR for the high-myopia analysis (0.78 [95% CI:0.64–0.95]). To investigate whether there are more appropriate genetic association models, we applied other possible ones: dominant, recessive, and codominant. However, we did not find a more significant association than the additive model.

Comparisons between the results of the current study and those of previous studies are summarized in Table 4. The current study is the first study to prove significant associations between a *PAX6* SNP and high myopia and extreme myopia.

DISCUSSION

In the present study, using a relatively large cohort of 2,563 individuals, we showed that *PAX6* is associated with high and extreme myopia in Japanese. The minor A allele of rs644242 was a protective allele for high and extreme myopia.

The association of *PAX6* with common myopia was first evaluated in a Caucasian cohort. Although genome-wide linkage scans in a twins study suggested the *PAX6* region was strongly linked to common myopia, further case–control studies using tag SNPs rejected the hypothesis of an association between *PAX6* and common myopia [13-15]. Regarding high myopia, although Han et al., in a Chinese nuclear family study, reported that two SNPs in *PAX6* were associated with the condition [17], the subsequent case–control study did not replicate these associations, while haplotype analyses using 16 SNPs revealed the association [19]. Two Chinese reports also denied an association of *PAX6* with high myopia, while the subgroup analysis showed *PAX6* was associated with extreme myopia [16,20]. However, only 67 and 55 cases were used in these subgroup analyses, respectively, and therefore, caution should be applied when interpreting the findings, as pointed out by Zayats et al. [21].

Table 2. Genotype counts, associations, and odds ratios in participants with high myopia and control participants.

Single nucleotide polymorphisms	Genotype	High myopia	Control 1				Control 2			
		n	n	Nominal p value*	Adjusted p value†	Adjusted OR (95% CI)	n	Nominal p value*	Adjusted P value†	Adjusted OR (95% CI)
rs2071754 (C/T)	CC	326	90	0.61	0.26	1.12 (0.92-1.38)	232	0.485	0.497	1.04 (0.93-1.17)
	CT	632	156				466			
	TT	344	87				225			
rs644242 (C/A)	CC	1052	258	0.12	0.026	0.69 (0.49-0.96)	710	0.0153	0.0152	0.79 (0.66-0.96)
	CA	237	68				195			
	AA	14	7				18			
rs3026354 (A/G)	AA	544	142	0.33	0.78	1.03 (0.83-1.29)	376	0.611	0.638	0.97 (0.86-1.10)
	AG	590	155				421			
	GG	171	34				126			

* Differences in the observed genotypic distribution were examined by a chi-square test for trend. † Age and sex adjustment were performed based on a logistic regression model. CI: Confidence interval, OR: Odds ratio.

Table 3. Genotype counts, associations, and odds ratios in patients with high myopia, extreme myopia and pooled control participants.

SNP	Genotype	Pooled control				High myopia				Extreme myopia			
		n	Genotype frequency p value*	Allele frequency p value*	HWE P value	n	Nominal p value†	Adjusted p value‡	Adjusted odds ratio (95% confidence interval)	n	Nominal p value†	Adjusted p value‡	Adjusted odds ratio (95% confidence interval)
rs2071754	CC	322	0.53	0.99	0.74	326	0.44	0.343	1.06	196	0.327	0.317	1.07
(C/T)	CT	622				632			(0.94–1.18)	397			(0.94–1.21)
	TT	312				344				215			
rs644242	CC	968	0.95	0.95	0.15	1052	0.0105	0.00445	0.78	651	0.0294	0.0165	0.78
(C/A)	CA	263				237			(0.65–0.92)	149			(0.64–0.95)
	AA	25				14				8			
rs3026354	AA	518	0.28	0.22	1	544	0.99	0.834	0.99	346	0.656	0.585	0.96
(A/G)	AG	576				590			(0.88–1.11)	359			(0.84–1.10)
	GG	160				171				104			

* The difference in genotype and allele frequency between control 1 and control 2 were analyzed using a chi-square test. † Differences in the observed genotypic distribution were examined by chi-square test for trend. ‡ Age and sex adjustment were performed based on a logistic regression model. SNP: Single-nucleotide polymorphism, HWE: Hardy–Weinberg equilibrium

Table 4. Summary of previous reports that evaluated an association between PAX6 and high myopia.

Author (year)	Definition of cases		Cases	Controls	Reported single-nucleotide polymorphisms*			Remarks
	Criteria of high myopia	Affected eye			rs667773‡ rs644242 rs662702	rs3026390 rs3026393 rs2071754	rs3026354 rs628224	
Tsai et al., 2008	SE<6 D	Both	255	87	n.s.	-	-	Significant association of AC- and AG-repeat lengths in the P1 promoter
Ng et al., 2009	SE≤6 D	-	379	349	n.s.	-	-	
Han et al., 2009	SE<6 D	Both	FBAT† with 164 nuclear family	n.s.	p=0.0011	n.s.		Significant association in haplotype analysis
Liang et al., 2011	SE≤6 D	At least one eye	1083	1096	n.s.	n.s.	n.s.	
Jiang et al., 2011	SE≤8 D	Both	300	300	n.s.	n.s.	n.s.	
	SE≤8 D	Both	299	299	-	n.s.	-	
Current Study	AL≥26 mm	Both	1307	1256	p=0.0045	n.s.	n.s.	
Tsai et al., 2008	SE<10 D	Both	67	87	p<0.001	-	-	
Liang et al., 2011	SEM≤11 D	At least one eye	55	619	p=0.0074	n.s.	n.s.	
Current Study	AL≥28 mm	Both	810	1256	p=0.0165	n.s.	n.s.	

* Single-nucleotide polymorphism pairs with r2 0.90 in HapMap Phase II are in the same column. † Family-based association test ‡ Although this single-nucleotide polymorphism is not included in HapMap SNP, it is reported to be in strong linkage disequilibrium (r2=0.92) with rs644242. D: diopter, n.s.: Not significant, SE: Spherical equivalent, AL: Axial length.

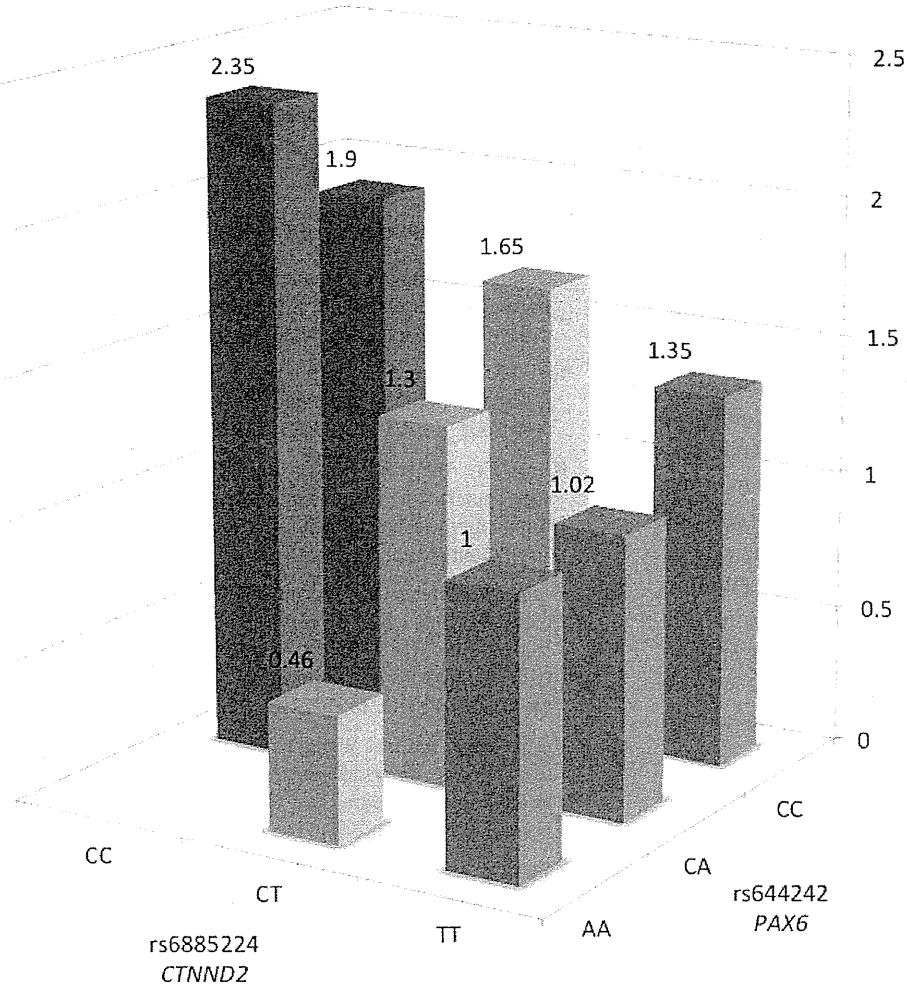

Figure 1. Collaborative effect of *CTNND2* rs6885224 and *PAX6* rs644242 on high myopia. The odds ratio of each genotype-pairs was calculated adjusting for age and sex. Patients with both the rs644242 AA genotype (non-risk homo) and the rs6885224 TT genotype (non-risk homo) are set as the reference (odds ratio=1.0). The number of subjects with rs6885224 CC and rs644242 CC were 55 in the case group and 42 in the control group, 286 in the case group and 252 in the control group with rs6885224 CT and rs644242 CC, 392 in the case group and 416 in the control group with rs6885224 TT and rs644242 CC, 15 in the case group and nine in the control group with rs6885224 CC and rs644242 CA, 70 in the case group and 78 in the control group with rs6885224 CT and rs644242 CA, 76 in the case group and 108 in the control group with rs6885224 TT and rs644242 CA, three in the case group and nine in the control group with rs6885224 CT and rs644242 AA, and six in the case group and nine in the control group with rs6885224 TT and rs644242 AA.

Table 4 summarizes the SNPs that have been evaluated previously to discover if *PAX6* is associated with high/extreme myopia. Rs667773 and rs662702 are reportedly in strong linkage disequilibrium with rs644242 [20], which showed significant association with high/extreme myopia in the present study. The association of these SNPs with extreme myopia was reported by Tsai et al. and Liang et al. [16,20], as well as in the current study, and the direction was the same in these three studies.

There are three possible reasons previous studies did not identify the association of rs644242 (or SNPs in strong linkage disequilibrium with rs644242) with high myopia. First, the parameter used to define high myopia was axial length, while all of the previous studies used standard error of the mean (SEM). Currently, *PAX6* is considered the "master gene" in eye development, owing to the gene's pivotal role during the induction of lens and retina differentiation [22]. At an early stage of eye development, *PAX6* expression alone forms the eyeball and, with *SOX2*, affects the crystalline lens [23]. Hence, using SEM to define high myopia, which is affected by lens and eye shape, does not convey the direct effects of *PAX6*. However, high myopia defined by axial length, which is determined by changes to the shape of the eye only, demonstrates the direct effects of *PAX6*. This is why previous studies showed a significant association only in extreme myopia; almost all cases of extreme myopia present an abnormal eye shape. The second reason for the discrepancy between the studies is the number of cases. All the previous studies (except the study by Liang et al. [20]) had fewer than 600 participants [16,17,19], which is less than half the number of cases we included in our study. The variance in the inclusion criteria for patients with high myopia is the last possible reason previous studies failed to identify the association. Although Liang et al. included more than 1,000 cases, the researchers defined high myopia as SEM no greater

than −6 D in at least one eye [20]. Considering the effect of the *PAX6* gene, the current inclusion criterion, which is to enroll patients who have two highly myopic eyes, is more suitable for selecting genetic-dependent high myopia. Indeed, the inclusion criteria of other studies are the same as in the current study [16,17,19].

Recently, our GWAS showed that *CTNND2* is a susceptibility gene for high myopia [7]. *CTNND2* encodes catenin δ2, also known as δ-catenin. Catenin δ2/δ-catenin belongs to the catenin δ1/p120-catenin protein family, which regulates cell adhesion and intracellular signaling pathways [24-26]. P120-catenin and β-catenin bind to the cytoplasmic tail of cadherin, which stabilizes the adherence junctions composed of cadherin, p120-catenin, β-catenin, α-catenin, and the actin cytoskeleton. δ-catenin competes with p120-catenin for interaction with cadherin and destabilizes the adherens junction [26,27]. In addition, δ-catenin can also affect the gene expression of other molecules associated with the wingless (Wnt)/β-catenin signaling pathway [28]. Since *CTNND2* expression is regulated by Pax6 [12], and that the distribution of Pax6 and δ-catenin/catenin δ2 is remarkably similar [29,30], the collaboration of *PAX6* and *CTNND2* might be associated with myopia. In genetic studies on age-related macular degeneration (AMD), its association with the *CFH* gene led to the discovery that other molecules in the complement pathway were also associated with the condition, such as *C2/CFB*, *C3*, and *CFI* [31-34]. Similar to these collaborative associations of several complement factors to AMD, molecules associated with the adherence junction and Wnt/β-catenin signaling might contribute to the development of myopia. When we calculate the odds ratio of each genotype-pairs of *PAX6* and *CTNND2* using samples shared between the present study and our previous study [7], the C allele of *CTNND2* rs6885224 seems to be a risk allele for high myopia in populations with the CC/CA genotype in *PAX6* rs644242, while the T allele of CTNND2 rs6885224 seems to be a risk allele in populations with the AA genotype in *PAX6* rs644242 (Figure 1). However, since the number of patients with the *PAX6* AA genotype are small, replication studies are needed.

In conclusion, we proved the significant association of rs644242 in *PAX6* with high and extreme myopia. The A allele for rs644242 is protective for high and extreme myopia, and the collaboration of *PAX6* and *CTNND2* might be associated with the development of this condition. The adherens junction and Wnt/β-catenin signaling are possible attractive targets for further study of myopia development.

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REFERENCES

1. Jacobi FK, Zrenner E, Broghammer M, Pusch CM. A genetic perspective on myopia. Cellular and molecular life sciences Cell Mol Life Sci 2005; 62:800-8. [PMID: 15868405].
2. Saw SM, Gazzard G, Shih-Yen EC, Chua WH. Myopia and associated pathological complications. Ophthalmic Physiol Opt 2005; 25:381-91. [PMID: 16101943].
3. Klaver CC, Wolfs RC, Vingerling JR, Hofman A, de Jong PT. Age-specific prevalence and causes of blindness and visual impairment in an older population: the Rotterdam Study. Arch Ophthalmol 1998; 116:653-8. [PMID: 9596502].
4. Evans JR, Fletcher AE, Wormald RP. Causes of visual impairment in people aged 75 years and older in Britain: an add-on study to the MRC Trial of Assessment and Management of Older People in the Community. Br J Ophthalmol 2004; 88:365-70. [PMID: 14977771].
5. Xu L, Wang Y, Li Y, Wang Y, Cui T, Li J, Jonas JB. Causes of blindness and visual impairment in urban and rural areas in Beijing: the Beijing Eye Study. Ophthalmology 2006; 113:1134-[PMID: 16647133].
6. Nakanishi H, Yamada R, Gotoh N, Hayashi H, Yamashiro K, Shimada N, Ohno-Matsui K, Mochizuki M, Saito M, Iida T, Matsuo K, Tajima K, Yoshimura N, Matsuda F. A genome-wide association analysis identified a novel susceptible locus for pathological myopia at 11q24.1. PLoS Genet 2009; 5:e1000660-[PMID: 19779542].
7. Li YJ, Goh L, Khor CC, Fan Q, Yu M, Han S, Sim X, Ong RT, Wong TY, Vithana EN, Yap E, Nakanishi H, Matsuda F, Ohno-Matsui K, Yoshimura N, Seielstad M, Tai ES, Young TL, Saw SM. Genome-wide association studies reveal genetic variants in CTNND2 for high myopia in Singapore Chinese. Ophthalmology 2011; 118:368-75. [PMID: 21095009].
8. Hysi PG, Young TL, Mackey DA, Andrew T, Fernandez-Medarde A, Solouki AM, Hewitt AW, Macgregor S, Vingerling JR, Li YJ, Ikram MK, Fai LY, Sham PC, Manyes L, Porteros A, Lopes MC, Carbonaro F, Fahy SJ, Martin NG, van Duijn CM, Spector TD, Rahi JS, Santos E, Klaver CC, Hammond CJ. A genome-wide association study for myopia and refractive error identifies a susceptibility locus at 15q25. Nat Genet 2010; 42:902-5. [PMID: 20835236].
9. Solouki AM, Verhoeven VJ, van Duijn CM, Verkerk AJ, Ikram MK, Hysi PG, Despriet DD, van Koolwijk LM, Ho L, Ramdas WD, Czudowska M, Kuijpers RW, Amin N, Struchalin M, Aulchenko YS, van Rij G, Riemslag FC, Young TL, Mackey DA, Spector TD, Gorgels TG, Willemse-Assink JJ, Isaacs A, Kramer R, Swagemakers SM, Bergen AA, van Oosterhout AA, Oostra BA, Rivadeneira F, Uitterlinden AG, Hofman

A, de Jong PT, Hammond CJ, Vingerling JR, Klaver CC. A genome-wide association study identifies a susceptibility locus for refractive errors and myopia at 15q14. Nat Genet 2010; 42:897-901. [PMID: 20835239].

10. Hayashi H, Yamashiro K, Nakanishi H, Nakata I, Kurashige Y, Tsujikawa A, Moriyama M, Ohno-Matsui K, Mochizuki M, Ozaki M, Yamada R, Matsuda F, Yoshimura N. Association of 15q14 and 15q25 with high myopia in Japanese. Invest Ophthalmol Vis Sci 2011; 52:4853-8. [PMID: 21436269].
11. Lu B, Jiang D, Wang P, Gao Y, Sun W, Xiao X, Li S, Jia X, Guo X, Zhang Q. Replication study supports CTNND2 as a susceptibility gene for high myopia. Invest Ophthalmol Vis Sci 2011; 52:8258-61. [PMID: 21911587].
12. Duparc RH, Boutemmine D, Champagne MP, Tetreault N, Bernier G. Pax6 is required for delta-catenin/neurojugin expression during retinal, cerebellar and cortical development in mice. Dev Biol 2006; 300:647-55. [PMID: 16973151].
13. Hammond CJ, Andrew T, Mak YT, Spector TD. A susceptibility locus for myopia in the normal population is linked to the PAX6 gene region on chromosome 11: a genomewide scan of dizygotic twins. Am J Hum Genet 2004; 75:294-304. [PMID: 15307048].
14. Mutti DO, Cooper ME, O'Brien S, Jones LA, Marazita ML, Murray JC, Zadnik K. Candidate gene and locus analysis of myopia. Mol Vis 2007; 13:1012-9. [PMID: 17653045].
15. Simpson CL, Hysi P, Bhattacharya SS, Hammond CJ, Webster A, Peckham CS, Sham PC, Rahi JS. The Roles of PAX6 and SOX2 in Myopia: lessons from the 1958 British Birth Cohort. Invest Ophthalmol Vis Sci 2007; 48:4421-5. [PMID: 17898260].
16. Tsai YY, Chiang CC, Lin HJ, Lin JM, Wan L, Tsai FJA. PAX6 gene polymorphism is associated with genetic predisposition to extreme myopia. Eye (Lond) 2008; 22:576-81. [PMID: 17948041].
17. Han W, Leung KH, Fung WY, Mak JY, Li YM, Yap MK, Yip SP. Association of PAX6 polymorphisms with high myopia in Han Chinese nuclear families. Invest Ophthalmol Vis Sci 2009; 50:47-56. [PMID: 19124844].
18. Ng TK, Lam CY, Lam DS, Chiang SW, Tam PO, Wang DY, Fan BJ, Yam GH, Fan DS, Pang CP. . AC and AG dinucleotide repeats in the PAX6 P1 promoter are associated with high myopia Mol Vis 2009; 15:2239-48. [PMID: 19907666].
19. Jiang B, Yap MK, Leung KH, Ng PW, Fung WY, Lam WW, Gu YS, Yip SP. PAX6 haplotypes are associated with high myopia in Han chinese. PLoS ONE 2011; 6:e19587-[PMID: 21589860].
20. Liang CL, Hsi E, Chen KC, Pan YR, Wang YS, Juo SH. A functional polymorphism at 3'UTR of the PAX6 gene may confer risk for extreme myopia in the Chinese. Invest Ophthalmol Vis Sci 2011; 52:3500-5. [PMID: 21421876].
21. Zayats T, Guggenheim JA, Hammond CJ, Young TL. Comment on 'A PAX6 gene polymorphism is associated with genetic predisposition to extreme myopia'. Eye (Lond) 2008; 22:598-9. , author reply 9. [PMID: 18219338].
22. Tsonis PA, Fuentes EJ. Focus on molecules: Pax-6, the eye master. Exp Eye Res 2006; 83:233-4. [PMID: 16563385].
23. Kondoh H, Uchikawa M, Kamachi Y. Interplay of Pax6 and SOX2 in lens development as a paradigm of genetic switch mechanisms for cell differentiation. Int J Dev Biol 2004; 48:819-27. [PMID: 15558474].
24. Davis MA, Ireton RC, Reynolds AB. A core function for p120-catenin in cadherin turnover. J Cell Biol 2003; 163:525-34. [PMID: 14610055].
25. Casagolda D, Del Valle-Perez B, Valls G, Lugilde E, Vinyoles M, Casado-Vela J, Solanas G, Batlle E, Reynolds AB, Casal JI, de Herreros AG, Dunach M. A p120-catenin-CK1epsilon complex regulates Wnt signaling. J Cell Sci 2010; 123:2621-31. [PMID: 20940130].
26. Yang I, Chang O, Lu Q, Kim K. Delta-catenin affects the localization and stability of p120-catenin by competitively interacting with E-cadherin. Mol Cells 2010; 29:233-7. [PMID: 20108168].
27. Ireton RC, Davis MA, van Hengel J, Mariner DJ, Barnes K, Thoreson MA, Anastasiadis PZ, Matrisian L, Bundy LM, Sealy L, Gilbert B, van Roy F, Reynolds AB. A novel role for p120 catenin in E-cadherin function. J Cell Biol 2002; 159:465-76. [PMID: 12427869].
28. Kim H, He Y, Yang I, Zeng Y, Kim Y, Seo YW, Murnane MJ, Jung C, Lee JH, Min JJ, Kwon DD, Kim KK, Lu Q, Kim K. delta-Catenin promotes E-cadherin processing and activates beta-catenin-mediated signaling: Implications on human prostate cancer progression. Biochim Biophys Acta 2012; 1822:509-21. [PMID: 22261283].
29. Zhou J, Liyanage U, Medina M, Ho C, Simmons AD, Lovett M, Kosik KS. Presenilin 1 interaction in the brain with a novel member of the Armadillo family. Neuroreport 1997; 8:2085-90. [PMID: 9223106].
30. Davis J, Duncan MK, Robison WG Jr, Piatigorsky J. Requirement for Pax6 in corneal morphogenesis: a role in adhesion. J Cell Sci 2003; 116:2157-67. [PMID: 12692153].
31. Nakanishi H, Gotoh N, Yamada R, Yamashiro K, Otani A, Hayashi H, Tsujikawa A, Shimada N, Ohno-Matsui K, Mochizuki M, Saito M, Saito K, Iida T, Matsuda F, Yoshimura N. ARMS2/HTRA1 and CFH polymorphisms are not associated with choroidal neovascularization in highly myopic eyes of the elderly Japanese population. Eye (Lond) 2010; 24:1078-84. [PMID: 19680273].
32. Gold B, Merriam JE, Zernant J, Hancox LS, Taiber AJ, Gehrs K, Cramer K, Neel J, Bergeron J, Barile GR, Smith RT, Hageman GS, Dean M, Allikmets R. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. Nat Genet 2006; 38:458-62. [PMID: 16518403].
33. Yates JR, Sepp T, Matharu BK, Khan JC, Thurlby DA, Shahid H, Clayton DG, Hayward C, Morgan J, Wright AF, Armbrecht AM, Dhillon B, Deary IJ, Redmond E, Bird AC, Moore AT. Complement C3 variant and the risk of age-related macular degeneration. N Engl J Med 2007; 357:553-61. [PMID: 17634448].

34. Ennis S, Jomary C, Mullins R, Cree A, Chen X, Macleod A, Jones S, Collins A, Stone E, Lotery A. Association between the SERPING1 gene and age-related macular degeneration: a two-stage case-control study. Lancet 2008; 372:1828-34. [PMID: 18842294].

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