Identification of *ANGPT2* as a New Gene for Neovascular Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy in the Chinese and Japanese Populations

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Submitted: August 19, 2016
Accepted: January 8, 2017

Citation: Ma L, Brelen ME, Tsujikawa M, et al. Identification of *ANGPT2* as a new gene for neovascular age-related macular degeneration and polypoidal choroidal vasculopathy in the Chinese and Japanese populations. *Invest Ophthalmol Vis Sci*. 2017;58:1076–1083. DOI:10.1167/iovs.16-20575

Purpose. We determine the *angiopoietin 2* (*ANGPT2*) gene as a new susceptibility gene for neovascular age-related macular degeneration (nAMD) and polypoidal choroidal vasculopathy (PCV).

Methods. A total of 34 haplotype-tagging single-nucleotide polymorphisms (SNPs) were first genotyped in an exploratory Hong Kong Chinese cohort. Suggestive SNPs were replicated in a Shantou Chinese cohort and an Osaka Japanese cohort, with a total of 2343 subjects. The SNP rs800292 in the *complement factor H* (*CFH*) gene was genotyped in all the subjects. Genetic association and gene–gene interaction were analyzed.

Results. In the Hong Kong cohort, four SNPs in *ANGPT2* (rs13255574, rs4455855, rs13269021, and rs11775442) were nominally associated with nAMD and PCV. The four *ANGPT2* SNPs showed the same trends of association in the Shantou and Osaka cohorts. Combining the data from the 3 study cohorts revealed that SNPs rs4455855 and rs13269021 achieved study-wise significance ($P < 0.0016$), conferring an approximately 1.3-fold of increased risk for nAMD and PCV. Interaction analysis revealed the *CFH* SNP rs800292 has a highly significant interaction with the *ANGPT2* SNP rs13269021 in nAMD and PCV in the combined analysis. Subsequent stratification analysis confirmed the interaction.

Conclusions. This study reveals *ANGPT2* as a new susceptibility gene for nAMD and PCV, and it may affect disease susceptibility in association with *CFH*. Thus, this report provides new insights into the genetic architecture of nAMD and PCV.

Keywords: *ANGPT2*, gene association, age-related macular degeneration, polypoidal choroidal vasculopathy, gene–gene interaction

Age-related macular degeneration (AMD) is a leading cause of irreversible central vision loss in the elderly, especially in developed countries. Neovascular AMD (nAMD), a major form of advanced AMD characterized by choroidal neovascularization (CNV), accounts for the majority of severe visual loss in AMD patients. Polypoidal choroidal vasculopathy (PCV) is a macular disease characterized by polyp-like lesions in the choroidal vessels, which are best seen in indocyanine green angiography (ICGA).[1] Polypoidal choroidal vasculopathy and nAMD have similarities in clinical manifestations. For example, they can cause submacular hemorrhage, exudation, and scarring, leading to central vision loss. Interestingly, CNV and PCV can present concurrently in approximately 3.23% of patients,[2] suggesting that they may have a shared mechanism. However, while the prevalence of nAMD (approximately 0.46%) is similar between Caucasian and Asian populations,[3] the prevalence of PCV is approximately 3-fold higher in Asians than in Caucasians,[4] suggesting an ethnic background is related to the occurrence of PCV. Moreover, nAMD and PCV respond differently to anti-VEGF therapy,[5] suggesting differences in their underlying pathophysiology.

Both AMD and PCV are complex diseases resulting from the interaction of multiple genetic and environmental risk factors. Recent genome-wide association studies (GWAS) identified multiple polymorphisms in 34 genetic loci associated with AMD, including the *complement factor H* (*CFH*) and *age-related maculopathy susceptibility 2* (*ARMS2*) genes.[6,7] The majority of these genes also were associated with PCV. However, the effect sizes of some genes, for example *ARMS2*, varied between PCV and AMD.[8] A recent exome sequencing study revealed association of the *FGD6* gene with PCV but not nAMD.[9] Thus, there are similarities and differences in the genetic components of AMD and PCV.[6–10]

The angiopoietin/angiopoietin receptors cascade is an important signaling pathway in regulating angiogenesis.[11] The ligand, angiopoietin 2 (Ang2), activates integrin β1 and leads to endothelial destabilization in vitro and in vivo.[12] In addition, Ang2 is a competitive antagonist for Tie2 (TEK tyrosine kinase, endothelial) and enhances abnormal angiogenesis and destabilizes blood vessels.[13] Moreover, impaired postnatal retinal angiogenesis was detected in the Ang-2–deficient mice, characterized by an incomplete and chaotic vascular plexus.[14] Therefore, *ANGPT2* appears as an excellent candidate gene for nAMD and PCV, which have abnormal angiogenesis in the choroid. Currently, however, the association of *ANGPT2* with AMD and PCV is not known. Therefore, in the present study, we performed a haplotype-tagging SNP association analysis and gene–gene interaction analysis to determine the role of the *ANGPT2* gene, individually and interactively, in the genetic architecture of nAMD and PCV.

Materials and Methods

Study Participants

This study involved 2343 unrelated participants from 3 independent East Asian cohorts, including a Hong Kong Chinese cohort of 214 nAMD and 236 PCV patients, and 433 controls; a Shantou Chinese cohort of 189 nAMD and 187 PCV patients, and 531 controls; and an Osaka Japanese cohort of 192 nAMD and 204 PCV patients, and 157 controls. All Chinese participants are Han Chinese, recruited from the eye clinics of the Prince of Wales Hospital, the Hong Kong Eye Hospital, Hong Kong, and the Joint Shantou International Eye Center, Shantou, China. The Osaka Japanese cohort was recruited from the Department of Ophthalmology, Osaka University Graduate School of Medicine, Osaka, Japan. The study protocol was approved by the institutional Ethics Committee at the respective collaborating institutions. Written informed consent was obtained from each participant. The study procedures were performed in accordance with the tenets of the Declaration of Helsinki.

All patients underwent complete ophthalmic examinations, including best-corrected visual acuity (BCVA) measurement, ocular tonometry, slit-lamp biomicroscopy, fundus photographs, fluorescein angiography (FA) and ICGA. Clinical diagnosis and classification of AMD followed the standardized Age-Related Eye Disease Study criteria.[15] All AMD patients had nAMD in at least one eye. Polypoidal choroidal vasculopathy was diagnosed based on the presence of nodular polypoidal lesions as shown by ICGA.[16–18] Diagnosis of nAMD and PCV was distinguished by FA and ICGA. Patients with other causes of CNV, such as myopic CNV, or with CNV and PCV in the same or fellow eye, were excluded.

Control subjects were recruited from patients seen in the ophthalmology clinics for unrelated eye condition. All control subjects also underwent complete ophthalmic investigations. They had no sign of macular degeneration. Also, they did not have any other major eye diseases, except for mild senile cataract or mild refractive errors.

Single-Nucleotide Polymorphisms (SNPs) Selection and Genotyping

Haplotype-tagging SNPs in the *ANGPT2* gene were selected from the HapMap Beijing Han Chinese (CHB) population, using the HapMap Genome Browser release #27 dataset (available in the public domain at http://hapmap.ncbi.nlm.nih.gov/). The tagger-pairwise method was applied, with an *R* square (r^2) cutoff of 0.8 and a minor allele frequency (MAF) cutoff of 0.15. Totally, 34 SNPs were selected in *ANGPT2*. These tagging SNPs also covered the gene referring to the 1000 genomes reference panel (available in the public domain at http://browser.1000genomes.org/, accessed on December 3, 2016), except rs17553089.

Genomic DNA from peripheral blood was extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In this study, we adopted a two-stage strategy. In the first stage, all of the 34 SNPs were genotyped in the Hong Kong cohort, using *TaqMan* genotyping assays (Applied Biosystems, Foster City, CA, USA) with a Roche LightCycle 480 Real-Time PCR System (Roche Diagnostics; Basel, Switzerland), according to the manufacturer's instructions. In the second stage, six SNPs in *ANGPT2* (rs2515487, rs2922869, rs13255574, rs4455855, rs13269021, and rs11775442) that showed a suggestive disease-association ($P < 0.05$) and a *CFH* SNP rs800292 that showed significant interaction with *ANGPT2* in the Hong Kong cohort (see below) were genotyped in the Shantou and Osaka cohorts, using the same genotyping method.

Statistical Analysis

Hardy-Weinberg equilibrium (HWE) of each SNP in the controls was assessed using the χ^2 test in PLINK (v1.07; available in the public domain at http://pngu.mgh.harvard.edu/purcell/plink/).[19] Allelic and genotypic distributions were compared by the χ^2 test between cases and controls among different study cohorts. The odds ratio (OR) and 95% confidence intervals (CI) for each SNP were calculated. Logistic regression analysis was used to evaluate the genetic effects of the SNPs adjusted for age and sex. In this study, the SNP associations were first assessed in nAMD and PCV separately, and then in combined nAMD and PCV as we found no significant difference in the association profiles between nAMD and PCV.

Pairwise gene–gene interaction analysis was performed using the epistasis option in PLINK between the 6 associated SNPs in *ANGPT2* and our previously-reported AMD and PCV-associated SNPs, including *CFH* rs800292,[20,21] *ARMS2-HTRA1* rs11200638,[22] *SKIV2L* rs429608,[17] *CETP* rs3764261,[18] *ABCG1* rs57137919,[18] *C3* rs17030,[23] and *PGF* rs2268615.[24] A *P* value less than 0.0012 (0.05/42, where 42 is the pairs of interaction) defined a significantly statistical interaction. After identifying a significant interaction between *ANGPT2* rs13269021 and *CFH* rs800292, the study subjects were stratified by using dominant and recessive models of the risk G allele of *ANGPT2* rs13269021. We then performed the association analyses of *CFH* rs800292 with nAMD and PCV in different genotypic strata of *ANGPT2* rs13269021 (i.e., [GG+TG] and TT; GG and [TG+TT]). The homogeneity of the ORs for *CFH* rs800292 in different strata of *ANGPT2* rs13269021 was assessed using the Breslow-Day test.

To combine the data from the 3 study cohorts, we adopted the Mantel-Haenszel χ^2 test to obtain the combined ORs and 95% CIs for the 6 SNPs (rs2515487, rs2922869, rs13255574, rs4455855, rs13269021, and rs11775442) and the gene–gene interaction between *ANGPT2* rs13269021 and *CFH* rs800292, using the fixed-effect ($I^2 \leq 50\%$) or random-effect ($I^2 > 50\%$) model based on heterogeneity test results.[25] The test was performed using Review Manager (RevMan, version 5.2; The Cochrane Collaboration, Copenhagen, Denmark). In this study, we adopted the Bonferroni method to correct the *P* values in multiple testing. A final *P* value of less than 0.0016 (0.05/31, where 31 was the number of SNPs included in data analysis) would be required to conclude a significant disease association.

Results

Individual SNP Association and SNP*SNP Interaction in the Hong Kong Cohort

Supplementary Table S1 shows the characteristics of the participants in the 3 study cohorts. In the Hong Kong exploratory cohort, the genotype call rates of all SNPs were 97.5%. Three SNPs (rs4478599, rs2442604, and rs17077317) in *ANGPT2* showed deviation from HWE in controls and were excluded from further analysis. In the remaining 31 SNPs (Supplementary Table S2), four SNPs, rs13255574 (nAMD: $P = 3.9 \times 10^{-3}$; OR = 1.53; 95% CI, 1.15–2.04; PCV: $P = 4.7 \times 10^{-4}$; OR = 1.69; 95% CI, 1.26–2.28), rs4455855 (nAMD: $P = 0.020$; OR = 1.34; 95% CI, 1.05–1.72; PCV: $P = 4.3 \times 10^{-3}$; OR = 1.43; 95% CI, 1.12–1.82), rs13269021 (nAMD: $P = 0.020$; OR = 1.32; 95% CI, 1.05–1.66; PCV: $P = 2.6 \times 10^{-3}$; OR = 1.43; 95% CI, 1.13–1.81), and rs11775442 (nAMD: $P = 0.016$; OR = 1.43; 95% CI, 1.0–1.92; PCV: $P = 0.044$; OR = 1.34; 95% CI, 1.01–1.79) were associated with nAMD and PCV, while rs2515487 ($P = 4.6 \times 10^{-3}$; OR = 1.45; 95% CI, 1.12–1.88) and rs2922869 ($P = 5.4 \times 10^{-3}$; OR = 1.43; 95% CI, 1.11–1.85) were associated with PCV only (Table 1). Moreover, these 6 SNPs were associated with combined nAMD and PCV (Table 1). Among them, rs13255574 ($P = 1 \times 10^{-4}$; OR = 1.59; 95% CI, 1.26–2.01) and rs13269021 ($P = 1.2 \times 10^{-3}$; OR = 1.37; 95% CI, 1.13–1.66) had the strongest association, which could withstand Bonferroni correction ($P < 0.0016$).

In epistatic analysis, a significant interaction was detected between *ANGPT2* rs13269021 and *CFH* rs800292 in nAMD ($P = 7.3 \times 10^{-4}$). No significant interaction was detected for the remaining SNPs in *ANGPT2*, *ARMS2-HTRA1* rs11200638, *SKIV2L* rs429608, *CETP* rs3764261, *ABCG1* rs57137919, *C3* rs17030, and *PGF* rs2268615.

Replication Study and Combined Analysis

The 6 *ANGPT2* SNPs, rs2515487, rs2922869, rs13255574, rs4455855, rs13269021, and rs11775442, followed HWE in the control groups of the Shantou Chinese and Osaka Japanese cohorts. In the Shantou cohort, the ORs of the 6 SNPs for nAMD, PCV, and combined nAMD and PCV were toward the same trends as in the Hong Kong cohort, although the associations did not reach statistical significance (Table 1). In the Osaka cohort, SNP rs13269021 showed a nominal association with nAMD ($P = 0.025$; OR = 1.59; 95% CI, 1.06–2.37; Table 1).

In the combined analysis of the Hong Kong, Shantou, and Osaka samples by Mantel-Haenszel χ^2 test, two SNPs, rs4455855 (nAMD: $P = 1.15 \times 10^{-3}$; OR = 1.31; 95% CI, 1.11–1.55; $I^2 = 0$; PCV: $P = 1.24 \times 10^{-3}$; OR = 1.30; 95% CI, 1.11–1.53; $I^2 = 0$; combined nAMD and PCV: $P = 1.28 \times 10^{-4}$; OR = 1.30; 95% CI, 1.14–1.49; $I^2 = 0$), and rs13269021 (nAMD: $P = 6.74 \times 10^{-4}$; OR = 1.31; 95% CI, 1.12–1.53; $I^2 = 0$; PCV: $P = 1.15 \times 10^{-3}$; OR = 1.30; 95% CI, 1.11–1.52; $I^2 = 0$; combined nAMD and PCV: $P = 8.49 \times 10^{-5}$; OR = 1.29; 95% CI, 1.14–1.47; $I^2 = 0$), showed a statistically significant association with nAMD, PCV, and combined nAMD and PCV ($P < 0.0016$; Table 2; Supplementary Figs. S1, S2).

Gene–Gene Interaction and Combined Analysis

In the Hong Kong cohort, a significant SNP–SNP interaction was detected between *ANGPT2* rs13269021 and *CFH* rs800292 in nAMD ($P = 7.3 \times 10^{-4}$). However, this interaction was not significant in the Shantou or Osaka cohort alone by using logistic regression analysis. We then performed stratification analyses to elaborate the statistical interaction. We first assessed the associations of *CFH* rs800292 with nAMD and PCV in subjects who carried at least one risk allele (i.e., the GG or TG genotype) of *ANGPT2* rs13269021 versus subjects who carried only the nonrisk TT genotype (i.e., in a dominant model of *ANGPT2* rs13269021). In subjects carrying the rs13269021 GG/TG genotypes, the *CFH* rs800292 conferred a significantly increased risk to nAMD and PCV in the allelic (nAMD: $P = 5.2 \times 10^{-5}$; OR = 1.80; 95% CI, 1.35–2.40; PCV: $P = 2.2 \times 10^{-5}$; OR = 1.81; 95% CI, 1.38–2.39), dominant (nAMD: $P = 1.05 \times 10^{-4}$; OR = 4.14; 95% CI, 1.92–8.91; PCV: $P = 4.5 \times 10^{-5}$, OR = 4.14; 95% CI, 2.00–8.59), and recessive (nAMD: $P = 4 \times 10^{-3}$; OR = 1.73; 95% CI, 1.19–2.50; PCV: $P = 2 \times 10^{-3}$; OR = 1.74; 95% CI, 1.22–2.49; Table 3) models. In contrast, in subjects with the rs13269021 wild-type (TT) genotype, no significant association of *CFH* rs800292 with nAMD or PCV was detected (Table 3). Moreover, the ORs for the *CFH* rs800292 in the allelic (nAMD, $P = 5.0 \times 10^{-4}$; PCV, 1.2×10^{-5}; Breslow-Day test), dominant (nAMD, $P = 0.015$; PCV, $P = 1.1 \times 10^{-4}$), and recessive (nAMD, $P = 2 \times 10^{-3}$; PCV, 5.9×10^{-4}) models were significantly different between the *ANGPT2* rs13269021 GG/TG and TT genotypic strata (Table 3). Thus, results of the stratification analyses confirmed the gene–gene interaction identified by logistic regression.

The *CFH* rs800292 was significantly associated with nAMD and PCV in all 3 study cohorts (Table 1). In stratification analysis of the Shantou and Osaka cohorts, the *CFH* rs800292 G allele was associated with nAMD and PCV in subjects carrying the *ANGPT2* rs13269021 GG/TG genotypes but not in those with the TT genotypes. This was consistent in all 3 study cohorts (Table 3). In the combined analysis, the *CFH* rs800292, in the allelic, dominant, and recessive models, showed significant associations with nAMD and PCV in the *ANGPT2* rs13269021 GG/TG genotypes but not in the TT genotype (Table 4; Supplementary Figs. S3, S4).

When stratified by the dominant model of *CFH* rs800292, the associations of *ANGPT2* rs13269021 with nAMD and PCV appeared inconsistent among different cohorts (Supplementary Table S3), likely due to the small sample sizes in certain strata. However, the combined analysis of Hong Kong, Shantou, and Osaka data revealed that the G allele of *ANGPT2* rs13269021 was significantly associated with nAMD ($P = 4.31 \times 10^{-5}$; OR = 1.38) and PCV ($P = 1 \times 10^{-3}$; OR = 1.29) in subjects carrying the *CFH* rs800292 GG/AG genotypes, but not in those with the AA genotype ($P = 0.25$; OR = 0.53 for nAMD and $P = 0.49$; OR = 0.87 for PCV; Supplementary Table S4).

We then assessed the associations of *CFH* rs800292 with nAMD and PCV in subjects who carried only the risk allele (i.e., the GG genotype) of *ANGPT2* rs13269021 versus subjects who carried the GT or TT genotype (i.e., in a recessive model of *ANGPT2* rs13269021). The ORs were variable across different study cohorts (Supplementary Table S5), likely due to small sample effect. However, in the combined analysis of the 3 cohorts, the ORs for *CFH* rs800292 (in any genetic models) in the stratum of *ANGPT2* rs13269021-GG were consistently higher than the ORs in the *ANGPT2* rs13269021-(TT+TG) stratum (Supplementary Table S6). Similarly, when stratified by *CFH* rs800292 in the recessive model, the association patterns of *ANGPT2* rs13269021 with nAMD and PCV were variable across different cohorts (Supplementary Table S7). However, again, in the combined analysis the ORs for *ANGPT2* rs13269021 (in any genetic models) in the stratum of *CFH* rs800292-GG were consistently higher than the ORs in the *CFH* rs800292-(AA+AG) stratum (Supplementary Table S8).

In addition, we performed stratification analyses in additive effect models in the three cohorts. However, no consistent association pattern was observed in individual cohorts or the combined analysis, likely due to the small sample sizes after stratification (data not shown).

Table 1. Associated SNPs in *ANGPT2* and *CFH* in nAMD and PCV Adjusted for Age and Sex

				MAF				nAMD vs. Control		PCV vs. Control		nAMD+PCV vs. Control	
SNP	Gene	Nucleotide Change	Risk Allele	nAMD	PCV	nAMD+PCV	Ctrl	$P_{adjusted}$	Adjusted OR (95% CI)	$P_{adjusted}$	Adjusted OR (95% CI)	$P_{adjusted}$	Adjusted OR (95% CI)
Hong Kong Chinese													
rs800292	*CFH*	c.184G>A	G	0.713	0.734	0.724	0.614	1.2×10^{-3}	1.52 (1.18–1.96)	2.13×10^{-5}	1.78 (1.37–2.33)	6.61×10^{-6}	1.61 (1.31–1.99)
rs2515487	*ANGPT2*	c.289-10440C>A	A	0.269	0.313	0.292	0.240	0.21	1.18 (0.91–1.53)	4.6×10^{-3}	1.45 (1.12–1.88)	0.012	1.32 (1.06–1.63)
rs2922869	*ANGPT2*	c.289-9785T>C	T	0.703	0.747	0.726	0.677	0.27	1.15 (0.90–1.46)	5.4×10^{-3}	1.43 (1.11–1.85)	0.017	1.28 (1.05–1.57)
rs13255574	*ANGPT2*	c.289-8669C>T	C	0.828	0.839	0.834	0.756	3.9×10^{-3}	1.53 (1.15–2.04)	4.7×10^{-4}	1.69 (1.26–2.28)	1×10^{-4}	1.59 (1.26–2.01)
rs4455855	*ANGPT2*	c.289-7422T>A	A	0.349	0.36	0.355	0.285	0.020	1.34 (1.05–1.72)	4.3×10^{-3}	1.43 (1.12–1.82)	2.2×10^{-3}	1.38 (1.12–1.69)
rs13269021	*ANGPT2*	c.289-6755G>T	G	0.589	0.604	0.597	0.516	0.020	1.32 (1.05–1.66)	2.6×10^{-3}	1.43 (1.13–1.81)	1.2×10^{-3}	1.37 (1.13–1.66)
rs11775442	*ANGPT2*	c.289-6350A>G	A	0.826	0.813	0.819	0.763	0.016	1.43 (1.07–1.92)	0.044	1.34 (1.01–1.79)	7.5×10^{-3}	1.38 (1.09–1.75)
Shantou Chinese													
rs800292	*CFH*	c.184G>A	G	0.681	0.701	0.691	0.569	4.3×10^{-4}	1.59 (1.23–2.05)	3.89×10^{-5}	1.72 (1.33–2.23)	2.15×10^{-6}	1.64 (1.34–2.01)
rs2515487	*ANGPT2*	c.289-10440C>A	A	0.304	0.281	0.293	0.262	0.12	1.23 (0.95–1.59)	0.59	1.07 (0.83–1.39)	0.19	1.15 (0.93–1.42)
rs2922869	*ANGPT2*	c.289-9785T>C	T	0.688	0.722	0.705	0.687	0.63	0.94 (0.73–1.21)	0.43	1.11 (0.86–1.43)	0.77	1.03 (0.84–1.26)
rs13255574	*ANGPT2*	c.289-8669C>T	C	0.783	0.798	0.791	0.773	0.91	1.02 (0.76–1.36)	0.42	1.13 (0.84–1.52)	0.55	1.07 (0.85–1.36)
rs4455855	*ANGPT2*	c.289-7422T>A	A	0.361	0.350	0.356	0.316	0.074	1.26 (0.98–1.61)	0.24	1.16 (0.90–1.49)	0.063	1.21 (0.99–1.48)
rs13269021	*ANGPT2*	c.289-6755G>T	G	0.616	0.612	0.614	0.570	0.13	1.21 (0.95–1.55)	0.17	1.19 (0.93–1.53)	0.072	1.20 (0.98–1.47)
rs11775442	*ANGPT2*	c.289-6350A>G	A	0.811	0.801	0.806	0.784	0.42	1.13 (0.84–1.53)	0.68	1.06 (0.79–1.43)	0.47	1.09 (0.64–1.45)
Osaka Japanese													
rs800292	*CFH*	c.184G>A	G	0.782	0.707	0.743	0.622	2.21×10^{-5}	2.78 (1.73–2.45)	4.6×10^{-3}	1.85 (1.21–2.84)	5.40×10^{-5}	2.22 (1.51–3.27)
rs2515487	*ANGPT2*	c.289-10440C>A	A	0.260	0.260	0.260	0.289	0.70	1.10 (0.69–1.74)	0.88	0.97 (0.64–1.47)	0.95	1.01 (0.69–1.48)
rs2922869	*ANGPT2*	c.289-9785T>C	T	0.747	0.751	0.749	0.723	0.46	0.85 (0.55–1.31)	0.72	0.93 (0.61–1.40)	0.63	0.91 (0.63–1.32)
rs13255574	*ANGPT2*	c.289-8669C>T	C	0.872	0.841	0.856	0.804	0.14	1.48 (0.88–2.48)	0.56	1.15 (0.71–1.87)	0.27	1.27 (0.83–1.95)
rs4455855	*ANGPT2*	c.289-7422T>A	A	0.323	0.326	0.325	0.283	0.13	1.41 (0.90–2.21)	0.13	1.38 (0.91–2.09)	0.10	1.37 (0.94–1.99)
rs13269021	*ANGPT2*	c.289-6755G>T	G	0.622	0.559	0.590	0.560	0.025	1.59 (1.06–2.37)	0.34	1.21 (0.82–1.78)	0.10	1.33 (0.94–1.87)
rs11775442	*ANGPT2*	c.289-6350A>G	A	0.821	0.806	0.813	0.785	0.95	0.98 (0.60–1.61)	0.76	1.08 (0.68–1.70)	0.85	1.04 (0.69–1.57)

ctrl, control.

Table 2. Combined Analysis of Associated *ANGPT2* SNPs in nAMD and PCV Adjusted for Age and Sex

SNP	Risk Allele	nAMD vs. Control			PCV vs. Control			nAMD+PCV vs. Control		
		P	OR (95% CI)	I^2, %	*P*	OR (95% CI)	I^2, %	*P*	OR (95% CI)	I^2, %
rs2515487	A	0.05	1.19 (1.00–1.41)	0	0.03	1.20 (1.01–1.42)	47	0.01	1.20 (1.04–1.38)	0
rs2922869	T	0.89	1.01 (0.86–1.19)	0	0.03	1.20 (1.02–1.42)	46	0.11	1.11 (0.98–1.27)	43
rs13255574	C	0.08	1.29 (0.97–1.73)	53	0.05	1.33 (1.00–1.76)	51	0.06	1.30 (0.99–1.70)	63
rs4455855	A	1.15×10^{-3}	1.31 (1.11–1.55)	0	1.24×10^{-3}	1.30 (1.11–1.53)	0	1.28×10^{-4}	1.30 (1.14–1.49)	0
rs13269021	G	6.74×10^{-4}	1.31 (1.12–1.53)	0	1.15×10^{-3}	1.30 (1.11–1.52)	0	8.49×10^{-5}	1.29 (1.14–1.47)	0
rs11775442	A	0.04	1.23 (1.01–1.49)	6	0.09	1.18 (0.98–1.42)	0	0.02	1.20 (1.03–1.40)	17

Discussion

In this study, we identified 2 haplotype-tagging SNPs, rs4455855 and rs13269021, in the *ANGPT2* gene to be significantly associated with nAMD and PCV in a combined group of subjects from China and Japan. We also confirmed the association of *CFH* rs800292 with nAMD and PCV. In addition, we identified a significant interaction between *ANGPT2* rs13269021 and *CFH* rs800292 in nAMD and PCV. In stratification analysis, *CFH* rs800292 was associated with nAMD and PCV in subjects carrying the *ANGPT2* rs13269021 GG/TG genotypes but not in those with the TT genotypes. These findings have not been reported before to our knowledge. Thus, our study indicates *ANGPT2* to be a new susceptibility gene for nAMD and PCV, and it may affect disease susceptibility in association with *CFH*.

In a previous study, a tagging SNP rs2442598 of the *ANGPT2* gene was associated with psoriasis vulgaris.[26] Another SNP rs3739391 in the *ANGPT2* promoter region had been associated with elevated angiopoietin-2 levels in the blood circulation.[27] In this present study, neither rs2442598 nor rs3739391 was associated with nAMD or PCV. Instead, two tagging SNPs, rs4455855 and rs13269021, located in intron 1 of *ANGPT2*, conferred an increased risk for nAMD and PCV. These two associated SNPs also are located in intronic regions of the *MCPH1* gene. Disorganized and degenerated retinal layers had been found in *MCPH1* knock-out mice,[28] suggesting a role of the *MCPH1* in normal structuring of the retina. These SNPs could be correlated with the expression level of Ang2, having a regulatory role, or in linkage disequilibrium with exonic variants. Whether the SNPs found in our study are correlated with the expression levels of Ang2 remains to be investigated.

The *ANGPT2* gene, spanning a 63.61 kb region on chromosome 8, encodes the angiopoietin 2 (Ang2) protein, which is an antagonist of angiopoietin 1.[13] It is a key regulator in angiogenesis and vascular maturation.[29] Angiopoietin 2 usually is not expressed in healthy adult tissues and its secretion is induced at sites of inflammation and vascular remodeling.[30] Moreover, the mRNA levels of Ang2 are regulated by multiple factors, including VEGF, hypoxia, and TNFα[29] Angiopoietin 2 is immunodetectable in the choroidal neovascular membranes and highly expressed in the vascular enrichment areas where VEGF is colocalized.[31] Angiopoietin 2 also is expressed in surgically excised CNV membranes from neovascular AMD patients.[31,32] Therefore, the Ang2 protein could have a role in the pathogenesis of nAMD and PCV. Recently, in a whole exome sequencing study, by using a gene-based mutational load analysis, we and coworkers found that *ANGPT2* was nominally associated with AMD ($P = 9.85 \times 10^{-3}$) in the East Asian populations.[33] Our current finding that the *ANGPT2* gene is associated with nAMD and PCV provides further evidence to support the pathogenic role of Ang2.

The expression of Ang2 in the vitreous was significantly increased in nAMD patients versus controls.[34] Therefore, an overexpression of *ANGPT2* is likely implicated in the neovascularization of AMD. Anti-VEGF therapy is a current treatment for nAMD and PCV. However, approximately 10% of nAMD patients are resistant to anti-VEGF treatment.[35,36] New and effective therapeutic agents are needed. Angiopoietin 2, which has a role in angiogenesis, appears to be an excellent new treatment target. Recently, AMG386, a selective Ang1/2 neutralizing peptibody, was found to inhibit the neovascular processes in laser-induced CNV in monkeys.[37] A two-in-one VEGF/Ang2 antibody with dual action Fab (DAF) was developed as a potential treatment for nAMD, which enhanced the efficacy compared to monospecific antibody.[38] Therefore, while anti-Ang2 is likely to become a new treatment modality for human nAMD and PCV, the use of combined anti-VEGF and anti-Ang2 therapy would be expected to provide better efficacy. The *ANGPT2* gene could be a biomarker for pharmacogenetics studies on treatment responses in patients with different genotypes.

In this study, we have, for the first time to our knowledge, identified a significant interaction between *ANGPT2* rs13269021 and *CFH* rs800292 in nAMD and PCV. In stratification analysis, the *CFH* rs800292 G allele was associated with nAMD and PCV in subjects carrying the *ANGPT2* rs13269021 GG/TG genotypes but not in those with the TT genotypes. Notably, elevated Ang2 plasma levels have been found to correlate with the activation of the complement pathway in severe trauma patients, particularly in the alternative cascade.[39] An elevated placental C5a level also has been found to be positively associated with Ang2.[40] In addition, TNF-α, an inflammatory mediator, increased the mRNA and protein levels of Ang2 in cultured choroidal endothelial cells from surgically excised CNV membranes.[41] There is a bispecific antibody simultaneously targeting TNF-α and Ang2 available, which reduced the clinical symptoms and histologic scores in a murine inflammatory arthritis model.[42] On the other hand, Ang2 overexpression specifically in endothelial cells promoted inflammation responses, such as leukocyte infiltration in multiple organs, including liver, kidney, lung, and intestine.[43] Thus, our finding of the statistical interaction between *ANGPT2* and *CFH* suggested that genes in the angiopoietin pathway and complement cascade are interactive in the pathogenesis of nAMD and PCV. The exact mechanism of how these two pathways interact at the protein level remains to be elucidated.

In this study, we identified a new putative gene, *ANGPT2*, for nAMD and PCV by a candidate gene approach. In the exploratory stage, there were 6 SNPs in *ANGPT2* showing a suggestive association with *P* values < 0.05 and were subjected to replication. However, only the *P* values for SNP rs13255574 in PCV ($P = 4.7 \times 10^{-4}$), and SNPs rs13255574 ($P = 1 \times 10^{-4}$) and rs13269021 ($P = 1.2 \times 10^{-3}$) in combined nAMD and PCV could withstand the Bonferroni correction ($P < 0.0016$). In the replication study, two SNPs (rs4455855 and rs13269021) showed the same trend of effect in 2 independent replication cohorts from Shantou and Osaka, while in the combined

Table 3. Associations of *CFH* rs800292 With nAMD and PCV Stratified by *ANGPT2* rs13269021 in the Dominant Model

	CFH rs800292 in nAMD			Association in Different Model *P* Value and OR (95% CI)			*CFH* rs800292 in PCV			Association in Different Model *P* Value and OR (95% CI)		
***ANGPT2* rs13269021 in Different Samples**	**GG**	**AG**	**AA**	**Allelic Model, G vs. A**	**Dominant Model, GG+AG vs. AA**	**Recessive Model, GG vs. AG+AA**	**GG**	**AG**	**AA**	**Allelic Model, G vs. A**	**Dominant Model, GG+AG vs. AA**	**Recessive Model, GG vs. AG+AA**
Hong Kong Chinese												
ANGPT2 (GG+TG)												
Case	92	74	8	5.2×10^{-5}	1.05×10^{-4}	4×10^{-3}	104	83	9	2.2×10^{-5}	4.5×10^{-5}	2×10^{-3}
Control	128	143	54	1.80 (1.35–2.40)	4.14 (1.92–8.91)	1.73 (1.19–2.50)	128	143	54	1.81 (1.38–2.39)	4.14 (2.00–8.59)	1.74 (1.22–2.49)
rs13269021 (TT)												
Case	17	13	10	0.66	0.035	0.36	17	17	3	0.26	0.76	0.20
Control	37	59	12	0.89 (0.53–1.50)	0.38 (0.15–0.95)	1.42 (0.68–2.98)	37	59	12	1.38 (0.79–2.43)	1.42 (0.38–5.33)	1.63 (0.76–3.48)
Breslow-Day (*P*)				5.0×10^{-4}	0.015	2×10^{-3}				1.2×10^{-5}	1.1×10^{-4}	5.9×10^{-4}
Shantou Chinese												
ANGPT2 (GG+TG)												
Case	78	62	16	7.4×10^{-5}	0.014	1.63×10^{-4}	90	60	18	5×10^{-5}	0.017	3×10^{-6}
Control	136	200	78	1.75 (1.32–2.31)	2.03 (1.15–3.60)	2.04 (1.41–2.97)	136	200	78	1.89 (1.43–2.48)	1.94 (1.12–3.34)	2.36 (1.64–3.40)
rs13269021 (TT)												
Case	8	14	4	0.86	0.88	0.89	7	8	4	0.86	0.74	0.52
Control	30	55	17	1.06 (0.57–1.95)	1.10 (0.34–3.60)	1.07 (0.42–2.72)	30	55	17	1.06 (0.53–2.15)	0.75 (0.22–2.54)	1.40 (0.50–3.90)
Breslow-Day (*P*)				1.7×10^{-4}	0.02	2.7×10^{-4}				8×10^{-5}	0.037	2×10^{-6}
Osaka Japanese												
ANGPT2 (GG+TG)												
Case	93	54	6	1.4×10^{-5}	0.023	1.0×10^{-5}	79	71	13	0.031	0.39	0.016
Control	44	70	14	2.26 (1.56–3.27)	3.01 (1.12–8.07)	2.96 (1.82–4.82)	44	70	14	1.46 (1.04–2.07)	1.42 (0.64–3.13)	1.80 (1.11–2.89)
rs13269021 (TT)												
Case	23	11	3	0.11	0.72	0.067	20	18	2	0.31	0.37	0.38
Control	11	14	3	1.86 (0.86–4.02)	1.36 (0.25–7.31)	2.54 (0.93–6.96)	11	14	3	1.47 (0.70–3.05)	2.28 (0.36–14.63)	1.55 (0.58–4.12)
Breslow-Day (*P*)				4.0×10^{-6}	0.03	2.0×10^{-6}				0.016	0.25	0.01

Table 4. Combined Analysis of *CFH* rs800292 in nAMD and PCV Stratified by *ANGPT2* rs13269021 in the Dominant Model

	Allelic Model, G vs. A			Dominant Model, GG+AG vs. AA			Recessive Model GG vs. AG+AA		
***CFH* rs800292**	***P***	**OR (95% CI)**	I^2, %	***P***	**OR (95% CI)**	I^2, %	***P***	**OR (95% CI)**	I^2, %
nAMD									
ANGPT2 (GG+TG)	3.40×10^{-12}	1.87 (1.57–2.24)	0	3.16×10^{-6}	2.68 (1.77–4.05)	9	4.97×10^{-10}	2.08 (1.65–2.63)	33
rs13269021 (TT)	0.60	1.10 (0.77–1.57)	18	0.22	0.66 (0.34–1.28)	28	0.11	1.51 (0.92–2.50)	0
PCV									
ANGPT2 (GG+TG)	1.05×10^{-10}	1.74 (1.47–2.06)	0	0.006	2.25 (1.26–4.01)	53	3.64×10^{-9}	1.97 (1.57–2.47)	0
rs13269021 (TT)	0.17	1.30 (0.89–1.89)	0	0.69	1.18 (0.53–2.64)	0	0.10	1.55 (0.92–2.60)	0

analysis their *P* values achieved the study-wise significance level for nAMD, PCV, and combined nAMD and PCV (all $P < 0.0016$), with no intercohort heterogeneities ($I^2 = 0$). Therefore, the results of this study are robust. In this study, we included one Japanese cohort recruited in Osaka. The SNP rs13269021 showed a nominal association with nAMD ($P = 0.025$; OR = 1.59). In PCV, it showed the same trend of effect (OR = 1.21) though the *P* value was not statistically significant. Therefore, despite there was very low intercohort heterogeneity in the combined analysis ($I^2 = 0$), further independent replication in the Japanese population should be warranted. Of note, *ANGPT2* has not been reported in previous GWAS but in a whole exome sequencing study as mentioned above. Therefore, whether the association of *ANGPT2* with AMD and PCV is population-specific should be evaluated in further replication studies in other ethnic groups.

After the identification of the significant interaction between *ANGPT2* rs13269021 and *CFH* rs800292, we performed stratification analyses to evaluate the effects of each of these two SNPs in different genotypic strata (dominant and recessive models) of the other SNP. The ORs and *P* values appeared variable in individual cohorts. This is likely due to the loss of statistical power after stratification. Interestingly, however, when we combined the data from the 3 cohorts using meta-analysis, we found that the ORs for AMD and PCV were consistently higher in the upper strata (i.e., subjects who carry at least one risk allele of either the *ANGPT2* rs13269021 or *CFH* rs800292 SNP; Table 4; Supplementary Tables S4, S6, S8). This indicated that subjects carrying the risk alleles of *ANGPT2* rs13269021 and *CFH* rs800292 could have a higher risk of AMD and PCV development. Further replications in large samples are warranted to confirm the interaction between *ANGPT2* and *CFH*.

In conclusion, we identified 2 haplotype-tagging SNPs rs4455855 and rs13269021 in *ANGPT2* as new susceptibility markers for nAMD and PCV. We also identified an interaction between *ANGPT2* rs13269021 and *CFH* rs800292 in nAMD and PCV, indicating that *ANGPT2* may have a role in the genetic mechanism of nAMD and PCV in association with *CFH*. This report provided new insights into the genetic architecture of nAMD and PCV.

Acknowledgments

Supported in part by the National Natural Science Foundation of China (81500764 [LJC]), a research grant from the General Research Fund, Hong Kong (14120516 [LJC]), and the Direct Grants of the Chinese University of Hong Kong, Hong Kong (4054281 [LJC] and 4054119 [CPP]).

Disclosure: **L. Ma**, None; **M.E. Brelen**, None; **M. Tsujikawa**, None; **H. Chen**, None; **W.K. Chu**, None; **T.Y.Y. Lai**, None; **D.S.C. Ng**, None; **K. Sayanagi**, None; **C. Hara**, None; **N. Hashida**, None; **V.C.K. Chan**, None; **P.O.S. Tam**, None; **A.L. Young**, None; **W. Chen**, None; **K. Nishida**, None; **C.P. Pang**, None; **L.J. Chen**, None

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