TABLE 3. GENOTYPE COUNTS, ASSOCIATIONS, AND ODDS RATIOS IN PATIENTS WITH HIGH MYOPIA, EXTREME MYOPIA AND POOLED CONTROL PARTICIPANTS.

		Pooled control				High myopia				Extreme myopia			
SNP	**Genotype**	**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.

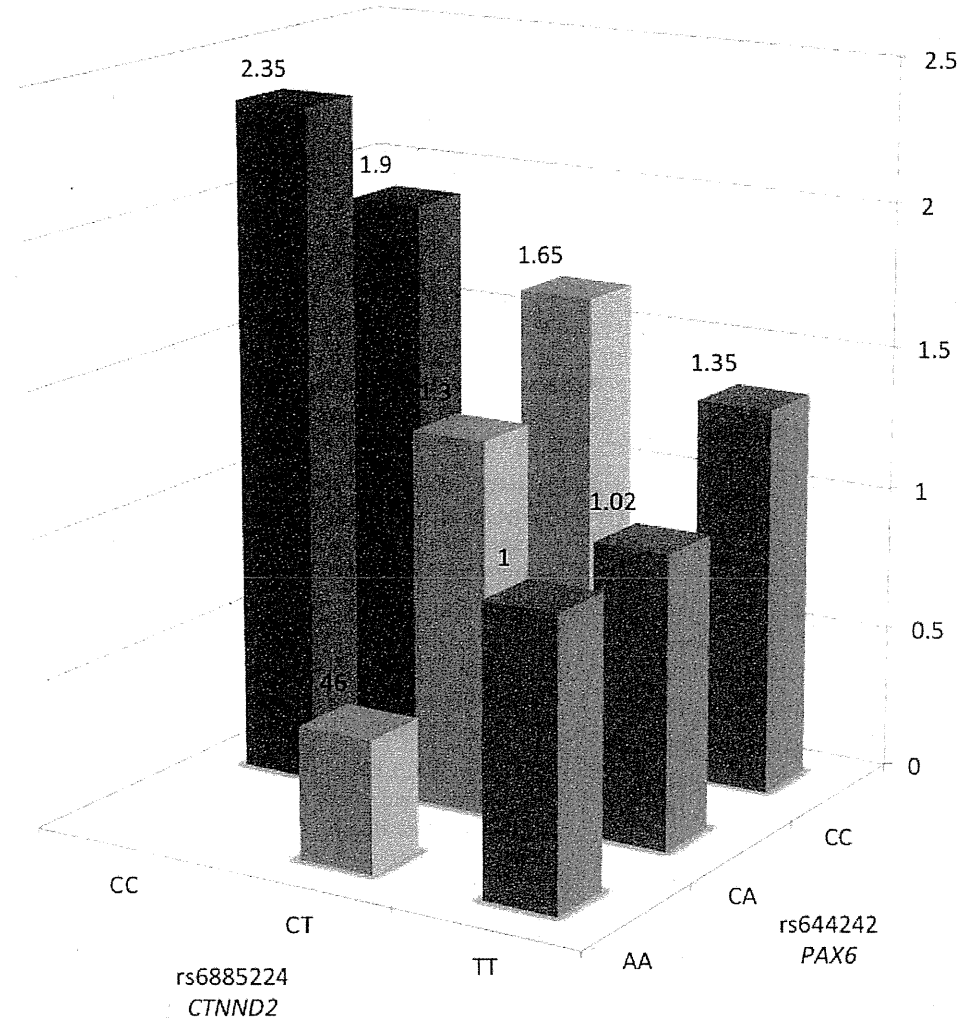

Figure 1. Collaborative effect of *CTNND2* rs6885224 and *PAX6* rs644242 on high myopia. The odds ratio of each genotype-pairs was calculated adjusting for age and sex. Patients with both the rs644242 AA genotype (non-risk homo) and the rs6885224 TT genotype (non-risk homo) are set as the reference (odds 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.

ACKNOWLEDGMENTS

Supported, in part, by grants-in-aid for scientific research (No 21,249,084 and 22,791,653) from the Japan Society for the Promotion of Science, Tokyo, Japan. The funding organization had no role in the design or conduct of this research.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 17 November 2012. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.

Association Between the Cholesteryl Ester Transfer Protein Gene and Polypoidal Choroidal Vasculopathy

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

Submitted: January 4, 2013
Accepted: August 8, 2013

Citation: Nakata I, Yamashiro K, Kawaguchi T, et al; for the Nagahama Study Group. Association between the cholesteryl ester transfer protein gene and polypoidal choroidal vasculopathy. *Invest Ophthalmol Vis Sci.* 2013;54:6068-6073. DOI:10.1167/iovs.13-11605

Purpose. To determine whether genetic variants in the lipid-associated genes are related to the risk of developing polypoidal choroidal vasculopathy (PCV) in a Japanese population.

Methods. Five hundred eighty-one patients with PCV and 793 controls were enrolled in the study. Association analysis of allele and genotype frequencies was performed for the following single-nucleotide polymorphisms (SNPs) that are associated with high-density lipoprotein cholesterol levels in blood: rs493258 at the hepatic lipase gene (*LIPC*), rs3764261 at the cholesteryl ester transfer protein gene (*CETP*), and rs12678919 at the lipoprotein lipase gene (*LPL*). A further model adjusting for age-related maculopathy susceptibility 2 (*ARMS2*) A69S, complement factor H (*CFH*) I62V, age, sex, and smoking status was used to confirm the independent association of these SNPs with other covariates.

Results. *CETP* rs3764261 was significantly associated with the development of PCV; the frequency of the minor allele *A* was higher in the PCV cases (24.0%) than in the control subjects (18.5%) ($P = 0.0025$; odds ratio [OR], 1.41; 95% confidence interval, 1.13-1.75). Furthermore, we found an independent association of *CETP* variants with age, sex, smoking status, and genetic background of *ARMS2* A69S, *CFH* I62V, *LIPC* rs493258, and *LPL* rs12678919 ($P = 0.0013$; OR, 1.50). *LIPC* rs493258 and *LPL* rs12678919 did not show significant associations with the development of PCV ($P > 0.05$).

Conclusion. *CETP* variants are associated a risk of developing PCV among the Japanese population.

Keywords: PCV, lipid, *CETP*, case-control study

Polypoidal choroidal vasculopathy (PCV) is characterized by aneurysmal dilations with interconnecting vessels that are best demonstrated by indocyanine green angiography.[1-3] Clinically, PCV is classified into a specific subtype of age-related macular degeneration (AMD), and the incidence of PCV in Asian populations has been reported to be higher than that in Caucasians.[4-6] Controversies exist about the pathogenesis of PCV; whether this condition represents inner choroidal vascular abnormalities or a particular variety of choroidal neovascularization (CNV) remains undetermined. However, because there are apparent differences in the demographic risk profile, clinical course, and visual prognosis, PCV is thought to be a distinct clinical entity.[7] For example, the response to treatment, particularly in photodynamic therapy for PCV, is completely different from that for typical AMD and CNV.[8,9]

Cholesterol and lipids are reported to accumulate underneath the retinal pigment epithelium (RPE) with age. When sufficient debris, including lipids, accumulates and forms a mound between the RPE cell and its basement membrane, it can be seen clinically as drusen. Because many population-based studies have shown the association between drusen and the progression of AMD, drusen is thought to be one of the determinants of both early and late AMD. In fact, an association between high-density lipoprotein (HDL) cholesterol level and the development of AMD has been reported in several studies.[10-12]

Previous studies[13-15] showed that the prevalence of drusen under RPE was reported to be lower in PCV than in AMD. Therefore, the absence of drusen was thought to be one of the criteria necessary to diagnose PCV.[6,15,16] However, the results of a clinical study[16] suggested that drusen is frequently seen in PCV eyes, and several studies[6,17,18] reported that drusen were observed in 20% to 27% of unaffected, fellow eyes in patients with unilateral PCV. Therefore, whether drusen has a functional role in the development of PCV remains controversial.

While previous investigations showed a lower prevalence of drusen among patients with PCV, lipid deposits that distribute from the inner retina to the outer retina are known to be the paramount features of PCV (Figure). Some recent investiga-

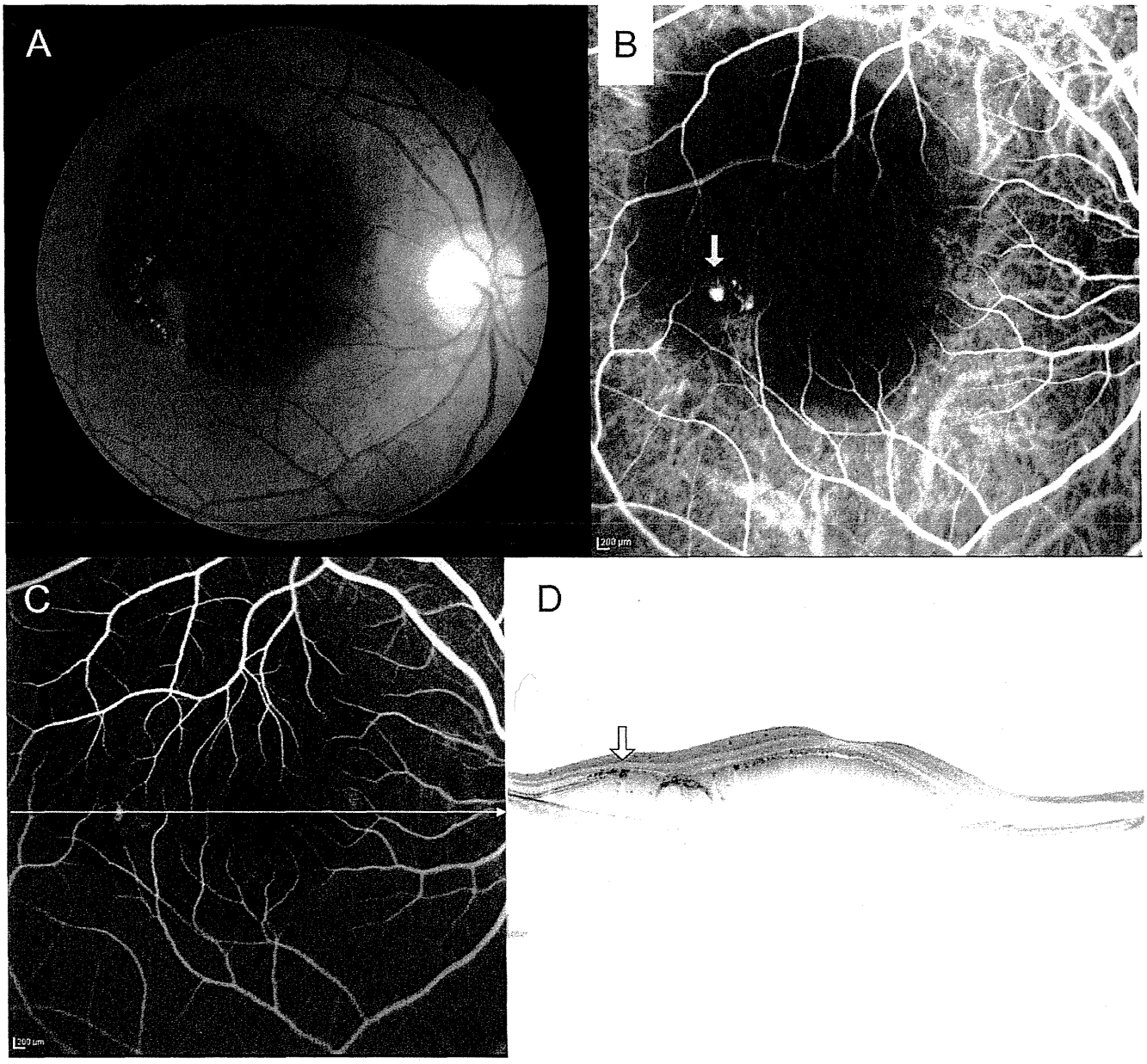

FIGURE. A 64-year-old woman with a typical case of PCV in the right eye. (**A**) Fundoscopic examination shows massive subretinal hemorrhage, lipid deposits, and reddish orange nodules. (**B**) Indocyanine green angiography demonstrates a small branching vascular network terminating in polypoidal lesions (*white arrow*). The speckle noise-reduced spectral-domain optical coherence tomography image of a horizontal section corresponding to the arrow indicated in fluorescein angiography (**C**) shows hyperreflective foci, indicating lipids ([**D**], *arrowhead*), in the outer retina beside the polyp ([**D**], *white arrow*).

tions, including a study[19] in a large cohort of Caucasians, showed significant associations between the lipid-associated genes and the development of AMD. These discoveries of genetic variants in the lipid pathway provided new insight into the pathogenesis of AMD. However, there are limited reports evaluating the association between the lipid-associated genes and the development of PCV. Although several genes are thought to be involved in regulating susceptibility to the development of PCV,[20–23] almost all are identical to those involved in the development of AMD, including the age-related maculopathy susceptibility 2 and high-temperature requirement factor A1 genes (*ARMS2/HTRA1*) locus[24,25] and the complement factor H gene (*CFH*).[26–29] Considering that several studies[13–15] reported a difference in the clinical features of drusen between AMD and PCV, there could be different roles of the lipid-associated genes in these subtypes. Thus, we aimed in this study to determine whether genetic variants in the lipid-associated genes, including variants affecting HDL cholesterol levels, are related to the risk of developing PCV in a Japanese population.

METHODS

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

TABLE 1. Characteristics of the Study Population

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

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

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

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

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

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

* A logistic regression model was used for covariate adjustment.

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

RESULTS

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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Acknowledgments

The authors thank the participants of the Nagahama Study, the Nagahama City Office, and the nonprofit organization Zero-ji Club for Health Promotion.

Supported by grants-in-aid from the following organizations: the Ministry of Education, Culture, Sports, Science, and Technology of Japan (2006–2012); Grants 19390442, 22791706, and 22791653 from the Japan Society for the Promotion of Science; the Japanese National Society for the Prevention of Blindness; and Takeda Science Foundation (2008–2012).

Disclosure: **I. Nakata**, None; **K. Yamashiro**, None; **T. Kawaguchi**, None; **N. Gotoh**, None; **H. Nakanishi**, None; **Y. Akagi-Kurashige**, None; **M. Miyake**, None; **A. Tsujikawa**, None; **A. Oishi**, None; **M. Saito**, None; **T. Iida**, None; **R. Yamada**, None; **F. Matsuda**, None; **N. Yoshimura**, None

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Appendix

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Graefes Arch Clin Exp Ophthalmol (2013) 251:1073–1080
DOI 10.1007/s00417-012-2137-y

RETINAL DISORDERS

Two-year outcome of photodynamic therapy combined with intravitreal injection of bevacizumab and triamcinolone acetonide for polypoidal choroidal vasculopathy

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Received: 16 November 2011 / Revised: 6 June 2012 / Accepted: 6 August 2012 / Published online: 25 August 2012

Abstract
Purpose To compare the 2-year results after photodynamic therapy (PDT) alone and PDT combined with intravitreal injections of bevacizumab and triamcinolone acetonide (triple therapy) for polypoidal choroidal vasculopathy (PCV).
Methods We retrospectively reviewed the medical records of 40 consecutive patients (40 eyes) with subfoveal PCV. Of these 40 eyes, 16 were treated with PDT alone and 24 were treated with triple therapy.
Results The change in visual acuity in the triple therapy group was significantly better than that in the PDT group ($P<0.001$). At 24 months, improvement in visual acuity was seen in only two eyes (12.5 %) of the PDT group, while it was seen in ten eyes (41.7 %) of the triple therapy group. Retreatment was given to 12 eyes (75.0 %) in the PDT group and to nine eyes (37.5 %) in the triple therapy group, although the retreatment-free period was significantly longer in the triple therapy group than in the PDT group ($P<0.001$). Post-treatment vitreous hemorrhage was seen in only two eyes (12.5 %), all of which were in the PDT group.
Conclusion Compared with PDT alone, triple therapy appears to reduce the postoperative hemorrhagic complications and recurrences of PCV and to improve the 2-year visual outcomes of PCV.

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Keywords Age-related macular degeneration · Bevacizumab · Photodynamic therapy · Polypoidal choroidal vasculopathy · Triamcinolone acetonide

Introduction

Previously, photodynamic therapy (PDT) with verteporfin was used primarily for the treatment of classic choroidal neovascularization (CNV) associated with age-related macular degeneration (AMD) [1], while today, intravitreal injections of anti-vascular endothelial growth factor (VEGF) agents (bevacizumab or ranibizumab) have become the principal treatment for exudative AMD [2–4]. Three monthly injections of anti-VEGF agents often lead to visual acuity (VA) improvement, but after these initial injections, repeated injections are usually required in order to maintain initial visual recovery. However, repeated injections increase the risk of ophthalmic and systemic adverse events, such as endophthalmitis and stroke [5]. To achieve better visual outcomes with fewer treatments, several reports have shown promising short-term effects of PDT combined with intravitreal injection of bevacizumab and a steroid (triamcinolone acetonide [TA] or dexamethasone) for exudative AMD [6, 7].

Polypoidal choroidal vasculopathy (PCV) is now recognized as a distinct clinical entity, differing in many ways from exudative AMD [8]. PCV is characterized by a branching vascular network that terminates in polypoidal lesions seen on indocyanine green angiography (IA), and is more common in Asians than in Caucasians [9, 10]. It has been reported that the treatment effects of anti-VEGF agents on the vascular lesions of PCV are limited in short-term follow-up [11, 12]. In contrast, a number of studies have shown encouraging results of PDT for the treatment of PCV. A

small number of sessions of PDT causes regression of the polypoidal lesions, and often results in stable, or even improved VA [13–15]. Subsequent reports, however, have shown extensive hemorrhagic complications and recurrences of the polypoidal lesions after the initially successful treatment with PDT [16, 17].

Recently, PDT combined with anti-VEGF agents has been reported to improve the short-term visual outcome in PCV, compared with PDT alone [18–20]. In addition, it has been suggested that this combination therapy may reduce the risk of postoperative hemorrhagic complications [18]. Furthermore, it has been reported that TA suppresses the early proangiogenic response of retinal pigment epithelium (RPE) cells after PDT treatment [21], and that the intravitreal injection of TA per se has a suppressive effect on CNV [22]. For the treatment of PCV, when PDT is combined with an anti-VEGF agent and also with TA, this triple therapy might reduce postoperative complications and the recurrence rate, and lead to a better visual prognosis. To date, however, no information is available on the effects of this triple therapy for PCV. Accordingly, the study described herein aimed to evaluate the long-term results of this triple therapy on symptomatic subfoveal PCV by comparing it with PDT alone.

Patients and methods

For this retrospective study, we reviewed the medical records of 40 consecutive eyes (40 patients) with treatment-naïve subfoveal PCV, who were treated with PDT alone or with PDT combined with an intravitreal injection of bevacizumab and TA (triple therapy) at Kyoto University Hospital between September 2004 and December 2008. PDT alone was performed on 16 patients (PDT group) between September 2004 and December 2007 and PDT combined with bevacizumab and TA was performed on 24 patients (triple therapy group) between January 2008 and December 2008. Inclusion criteria of the study were: (1) symptomatic PCV in patients older than 50 years, (2) the presence of subfoveal vascular lesions, (3) best-corrected VA of 20/25 or worse, and (4) a minimum follow-up of 24 months after initial treatment. Exclusion criteria were: (1) eyes with other macular abnormalities (i.e., AMD, pathologic myopia, idiopathic CNV, presumed ocular histoplasmosis, angioid streaks, and other secondary CNV), (2) any contraindications for fluorescein angiography (FA), IA, or verteporfin, (3) the presence of an RPE tear, (4) any previous treatment for subfoveal PCV, (5) a history of previous vitrectomy, or (6) any other additional therapy during the study period (i.e., anti-VEGF therapy). This study was approved by the Institutional Review Board at Kyoto University Graduate School of Medicine, and adhered to the tenets of the Declaration of Helsinki.

The diagnosis of PCV was based on IA, which shows a branching vascular network terminating in polypoidal dilation. In the present study, pseudophakic eyes were included. When both eyes with PCV that were treated with PDT or triple therapy met the inclusion criteria, only the eye which was treated initially was included in the current study. Some patients in the PDT group were included in a previous study [17].

At the initial visit, each patient underwent a comprehensive ophthalmologic examination, including measurement of best-corrected VA with a Landolt chart, determination of intraocular pressure, indirect ophthalmoscopy, slit-lamp biomicroscopy with a contact lens, optical coherence tomography examinations, and FA and IA (HRA-2, Heidelberg Engineering, Dossenheim, Germany).

In eyes of the PDT group, standard-fluence PDT was performed using a 689 nm diode laser unit (Visulas PDT system 690S; Carl Zeiss, Dublin, CA, USA) after an injection of verteporfin (Visudyne; Novartis Pharma AG, Basel, Switzerland), according to PDT guidelines for AMD [23]. In eyes that received triple therapy, injection of bevacizumab (1.25 mg) and TA (2 mg) was performed in a sterile manner, and prophylactic topical antibiotics were applied for 1 week after the injection. At 3–4 days after the intravitreal injection, standard-fluence PDT was performed according to PDT guidelines for AMD. The greatest linear dimension was calculated based on FA and IA, as described in detail previously [24]. All polypoidal lesions, the entire branching vascular network, and type 2 CNV detected by FA and IA were included. Serous pigment epithelial detachment was not included in the lesion area when the absence of underlying CNV was confirmed by IA.

After the initial treatment, each patient was scheduled to be seen at 3 months, at which time they again underwent a comprehensive ophthalmologic examination. When IA showed recurrent or residual polypoidal lesions and exudative change was seen on ocular coherence tomography (OCT), retreatment with PDT or the triple therapy was given according to the initial treatment. When residual polypoidal lesions were detected on IA but no exudative change was seen on OCT, no retreatment was given and the patient was reevaluated at the next visit.

All values are presented as mean ± standard deviation. For statistical analysis, best-corrected VA as measured with a Landolt chart was converted to a logarithm of the minimum angle of resolution (logMAR). VA was considered to be improved or deteriorated when the logMAR change was greater than 0.2. On OCT scans, foveal thickness was defined as the distance between the inner surface of the neurosensory retina and the RPE beneath the fovea. In each group, VA or foveal thickness after treatment was studied by one-way repeated measures analysis of variance with the

Table 1 Baseline characteristics of study population

	Photodynamic therapy group	Triple therapy group	*P* value
Number of patients	16	24	
Age (years)	73.3⊠9.9	73.7⊠5.8	0.887*
Gender (female/male)	5/11	6/18	0.665†
Initial visual acuity (logMAR)	0.36⊠0.13	0.50⊠0.31	0.119*
Initial foveal thickness (□m)	264.9⊠87.4	392.1⊠129.5	0.002*
Cystoid macular edema	4 (25.0 %)	11 (45.8 %)	0.182†
Serous retinal detachment	11 (68.8 %)	24 (100 %)	0.003†
Subretinal hemorrhage	8 (50.0 %)	7 (29.2 %)	0.182†
Pigment epithelial detachment	13 (81.3 %)	23 (95.8 %)	0.132†
Greatest linear dimension (□m)	2584⊠998	3193⊠1194	0.101*

logMAR, logarithm of the minimum angle of resolution

*Unpaired *t*-test

†Chi-squared test

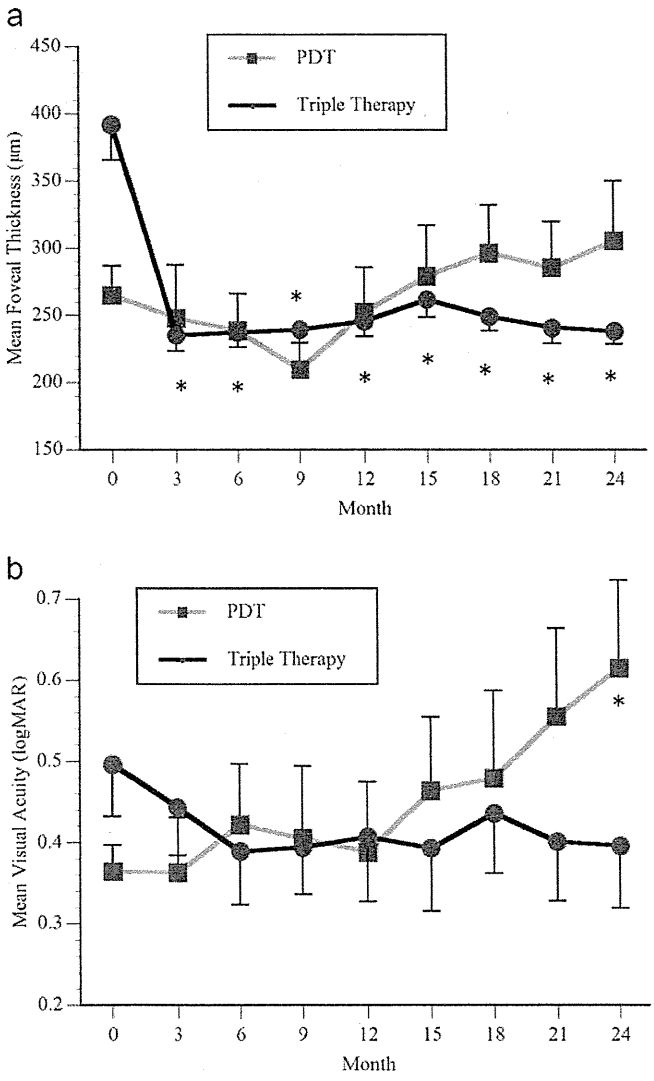

Fig. 1 Mean foveal thickness (**a**) and mean visual acuity (**b**) in eyes with polypoidal choroidal vasculopathy treated with photodynamic therapy (PDT group) or with PDT combined with intravitreal injections of bevacizumab and triamcinolone acetonide (triple therapy group). Visual acuity is shown in logMAR fashion. *$P<0.05$, compared with pretreatment values. *Error bars* represent the standard error

Dunnett test. To compare VA and foveal thickness between the PDT group and the triple therapy group, two-factor repeated measures analysis of variance was used. The retreatment-free period was calculated from the date of the initial therapy to the date when the treating physician determined the necessity of retreatment by PDT or by triple therapy. Survival analysis using Kaplan–Meier methods was used to compare the difference in the retreatment-free period after initial treatment between the PDT and the triple therapy groups. In patients who underwent no retreatment by either PDT or triple therapy, the retreatment-free period was established at 2 years of follow-up. Descriptive statistics for all demographic and clinical variables were calculated, and comparisons made using the unpaired *t*-test for means with continuous data (e.g., age) and the Chi-squared test for categorical data (e.g., gender). Statview version 5.0

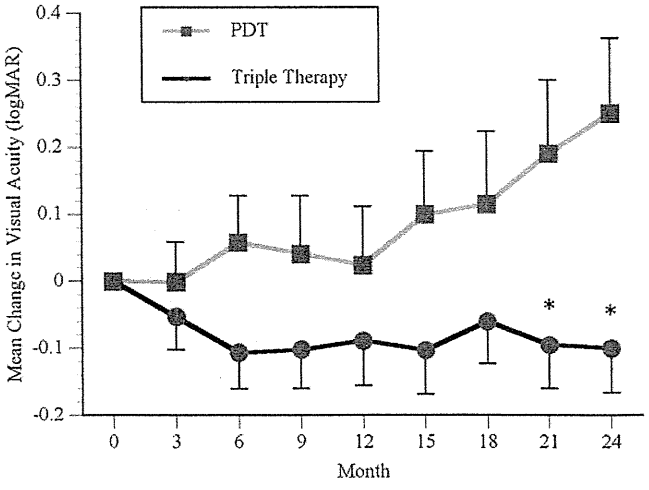

Fig. 2 Change in visual acuity in eyes with polypoidal choroidal vasculopathy treated with photodynamic therapy (PDT group) or with a combination of PDT and intravitreal injection of bevacizumab and triamcinolone acetonide (triple therapy group). Change in visual acuity in eyes treated with triple therapy was significantly better than that in eyes treated with PDT alone ($P<0.001$). Visual acuity is shown in logMAR fashion. *$P<0.05$, compared with values in the PDT group. *Error bars* represent the standard error

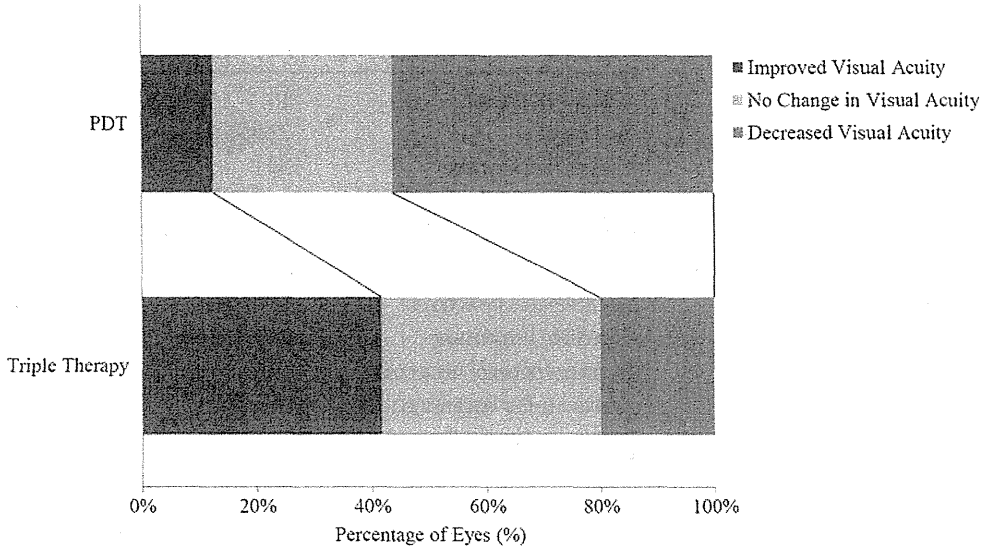

Fig. 3 Percentages of eyes with improved and deteriorated visual acuity at 24 months after treatment. All eyes with polypoidal choroidal vasculopathy were treated with photodynamic therapy (PDT group) or PDT combined with intravitreal injection of bevacizumab and triamcinolone acetonide (triple therapy group). Visual acuity was considered to be improved or deteriorated when the change in logMAR units was greater than 0.2. Improvement of visual acuity was seen more frequently in the triple therapy group (P 0.044)

software (SAS Institute, Inc., Cary, NC, USA) was used for statistical analyses. A P value<0.05 was considered to be statistically significant.

Results

In the study described herein, a total of 40 patients with treatment-naïve PCV were evaluated; 16 eyes which received only treatment with PDT (PDT group) and 24 eyes which received only triple therapy (triple therapy group) during the 24-month study period. Table 1 shows baseline characteristics of each group. There were no significant differences in age, gender, or baseline VA between the two groups. However, baseline foveal thickness of the triple therapy group was significantly larger than that of the PDT group, and the rate of serous retinal detachment was also higher in the triple therapy group.

Figure 1a shows the time-course in mean foveal thickness of each group. In the triple therapy group, the mean foveal thickness decreased immediately after initiation of treatment and remained throughout the 2-year follow-up period. Figure 1b shows the time-course of mean VA in each group. In the PDT group, there was no significant improvement of VA after initiation of treatment; in fact, mean VA was significantly decreased (+0.25±0.45) at 24 months (P 0.041). In contrast, VA somewhat improved after initiation of treatment in the triple therapy group, while the improvement was not statistically significant. Some improvement was maintained throughout the 2-year follow-up period.

Table 2 Final characteristics of study population and complications during study period

	Photodynamic therapy group	Triple therapy group	P value
Number of patients	16	24	
Final visual acuity (logMAR)	0.62 0.43	0.40 0.38	0.099*
Final foveal thickness (m)	283 204	230 66	0.234*
Final conditions			
Cystoid macular edema	5 (31.3 %)	0 (0 %)	0.003
Serous retinal detachment	5 (31.3 %)	0 (0 %)	0.003
Subretinal hemorrhage	2 (12.5 %)	1 (4.2 %)	0.327
Pigment epithelial detachment	11 (68.8 %)	5 (20.8 %)	0.002
Polypoidal lesions	3 (18.8 %)	2 (8.3 %)	0.385
Complications			
Cataract	2 (12.5 %)	1 (4.2 %)	0.327
Suprachoroidal hemorrhage	1 (6.3 %)	0 (0 %)	0.215
Vitreous hemorrhage	2 (12.5 %)	0 (0 %)	0.076
Tear of retinal pigment epithelium	1 (6.3 %)	0 (0 %)	0.215
Number of photodynamic therapy or triple therapy sessions (range)	2.19 0.91 (1 3)	1.50 0.78 (1 3)	0.015*
Retreatment-free period (months)	11.7 8.6	20.6 6.8	<0.001

logMAR, logarithm of the minimum angle of resolution; VEGF, vascular endothelial growth factor

*Unpaired t-test

†Chi-squared test

‡Survival analysis

Figure 2 shows the change in mean VA from baseline in each group. The mean change in VA in the triple therapy group was significantly better than that in the PDT group (P <0.001). At 24 months after initial treatment, mean change in VA in the triple therapy group (□0.10±0.32) was significantly better than that in the PDT group (+0.25±0.45, P0.007). Figure 3 shows the percentage of eyes with improved or decreased VA at 24 months; improvement in VA was seen in two eyes (12.5 %) of the PDT group and in ten eyes (41.7 %) of the triple therapy group, and reduction in VA was seen in nine eyes (56.3 %) of the PDT group and in five eyes (20.1 %) of the triple therapy group. Thus, improvement in VA was seen more frequently in the triple therapy group (P0.044).

Table 2 summarizes final characteristics and complications during the study period. In both groups, the polypoidal lesions disappeared after treatment (Fig. 4). At 24 months, complete disappearance of the polypoidal lesions was confirmed in 13 eyes (81.3 %) in the PDT group and in 22 eyes (91.7 %) of the triple therapy group. Fifteen eyes (62.5 %) of the triple therapy group and four eyes (25.0 %) of PDT group underwent a single session of PDT during the 24-month study period (P0.020). Retreatment by PDT was done in 12 eyes (75.0 %) in that group, and nine eyes (37.5 %) in the triple therapy group received retreatment by triple therapy (Fig. 5). The mean numbers of treatment were 2.19±0.91 in the PDT group and 1.50±0.78 in the triple therapy group (P0.015). Figure 6 shows the overall survival analysis curve for the retreatment-free periods in each group, which was significantly longer in the triple therapy group (20.6±6.8 months) than in the PDT group (11.7±8.6 months, P<0.001).

In the current study, two eyes (12.5 %) in the PDT group developed cataract and underwent surgery during the study period; additionally, one eye (4.2 %) in the triple therapy group (P0.327) underwent similar surgery. In the PDT group, two eyes (12.5 %) developed a vitreous hemorrhage and one eye developed an RPE tear during the study period, while no eye in the triple therapy group developed a vitreous hemorrhage. No eye underwent glaucoma surgery for ocular hypertension after intravitreal injections of TA.

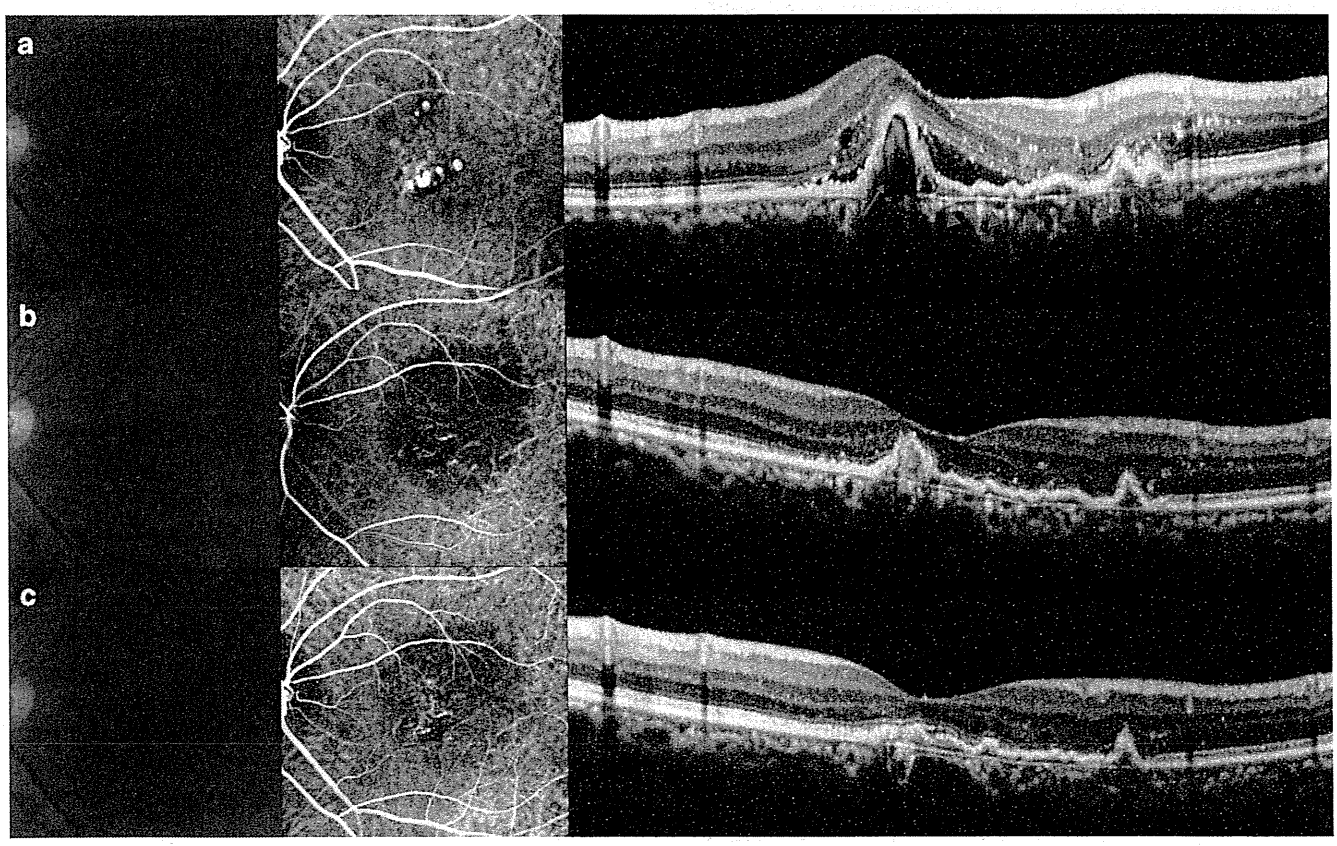

Fig. 4 Polypoidal choroidal vasculopathy successfully treated with photodynamic therapy combined with intravitreal injection of bevacizumab and triamcinolone acetonide (triple therapy). **a** Initial fundus photograph (*left*) shows reddish-orange nodules and fibrin exudate (20/50 OS). Indocyanine green angiography (*middle*) shows a branching vascular network that terminates in polypoidal lesions. A vertical optical coherence tomography section through the fovea (*right*) reveals sharp protrusions of the retinal pigment epithelium due to polypoidal lesions (*arrows*). A branching vascular network is seen as flat protrusions (arrowheads). **b** Three months after triple therapy. No polypoidal lesions are seen on indocyanine green angiogram. A branching vascular network is still seen. Protrusions due to polypoidal lesions have become flattened (20/60 OS). **c** 12 months after treatment, no recurrence is seen (20/50 OS)

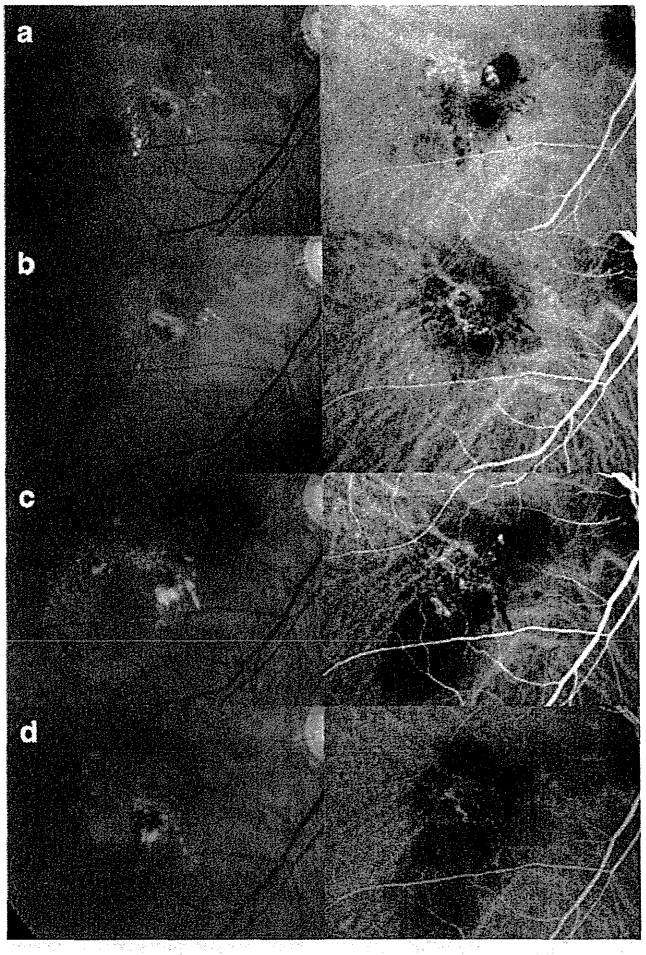

Fig. 5 Recurrence of polypoidal lesions after successful treatment with photodynamic therapy of polypoidal choroidal vasculopathy. **a** Initial fundus photograph (*left*) shows a reddish-orange nodule with a hard exudate and with a subretinal hemorrhage (20/40 OD). Indocyanine green angiography (*right*) shows a branching vascular network that terminates in polypoidal lesions. **b** Three months after photodynamic therapy. Reddish-orange nodules and polypoidal lesions have regressed (20/30 OD). **c** At 12 months after treatment, recurrence has occurred. Fundus photograph shows pigment epithelial detachments with surrounding subretinal hemorrhage and hard exudate (20/100 OD). Polypoidal lesions have recurred at the terminus of the remaining branching vascular network. **d** At 3 months after retreatment with photodynamic therapy, pigment epithelial detachments and polypoidal lesions have completely regressed (20/70 OD)

Discussion

Several investigators have reported the short-term outcomes of anti-VEGF agents for the treatment of PCV, and have shown the limited effect of these agents on the vascular lesions of PCV, even though the anti-VEGF agents reduced the exudative change that was due to PCV. It has been reported that complete resolution of the polypoidal lesions was achieved in only 16.1 % of eyes, with a mean of 3.3 injections of bevacizumab over a 12 month period [25]. In an earlier report, although monthly injections of ranibizumab successfully reduced the exudative manifestations of PCV, a

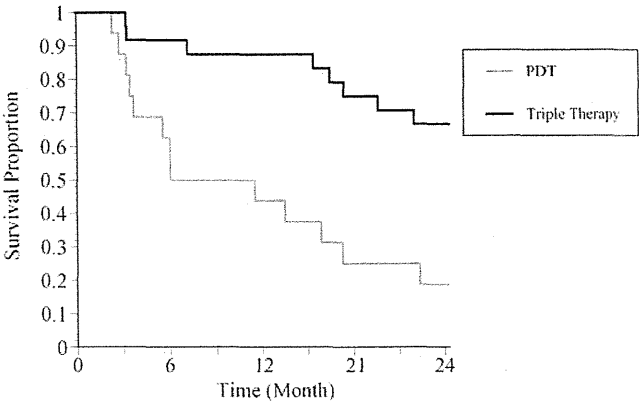

Fig. 6 Overall survival curve for the retreatment-free period in both treatment groups. Eyes with polypoidal choroidal vasculopathy were treated with either photodynamic therapy (PDT group) or PDT combined with intravitreal injection of bevacizumab and triamcinolone acetonide (triple therapy group). The period until the treating physician opted to perform additional treatment was significantly longer in the triple therapy group than in the PDT group ($P<0.001$)

reduction in the polypoidal lesions was seen in only 33 % of eyes [11].

In contrast, a number of studies have shown encouraging results of PDT for the vascular lesions of PCV, with complete regression of the polypoidal lesions achieved in many cases with fewer sessions. In a report by Chan et al. [26], PDT led to complete regression of the polypoidal lesions in 95 % of eyes with PCV, and resulted in either stable or improved VA 1 year after treatment in 95 % of eyes. However, a year or more after successful treatment with PDT, recurrences of the polypoidal lesions sometimes cause a substantial decrease in VA. Using Kaplan–Meier methods, Yamashiro et al. reported that the recurrence of polypoidal lesions after successful PDT treatment was estimated to be 11.5 % at 15 months, 20.4 % at 18 months, and 38.8 % at 21 months [16]. Thus, the recurrence of polypoidal lesions after PDT is a major problem in the treatment of PCV.

In the combination therapy, anti-VEGF agents, which can cause rapid reduction of the exudative change, are thought to contribute to the visual recovery that is associated with regression of the polypoidal lesions induced by PDT. Previous experimental studies have shown increased expression of VEGF shortly after PDT treatment [27, 28], which suggests that an intravitreal injection of bevacizumab before PDT may well exert a protective effect. With regard to the injection of TA, Okubo and colleagues reported a case of PCV successfully treated with trans-Tenons retrobulbar injection of TA [29], and Mukai and colleagues reported the protective effects of TA against occlusion of the choriocapillaris which was induced by PDT [30]. However, Lai et al. reported that the adjunctive use of TA during PDT did not appear to result in additional benefit for the treatment of PCV [31], so the effect of TA on PCV remains controversial.

In the current study, the PDT group showed no significant improvement in VA after initiation of treatment; mean VA was significantly decreased at 24 months. In the triple therapy group, however, VA was improved at 3 months after treatment and mean VA was improved by □0.11 (logMAR) at 6 months and by □0.09 (logMAR) at 12 months. In this triple therapy group, although improvement was not statistically significant, at least some improvement in VA was maintained throughout the 2-year follow-up period. At 24 months, VA improvement was achieved in only 12.5 % of eyes in the PDT group and in 41.7 % in the triple therapy group (P□0.044), so, based on our findings, triple therapy for PCV, compared to PDT alone, results in more rapid visual recovery and improved visual outcome at 24 months.

In our case series, both PDT alone and the triple therapy successfully reduced polypoidal lesions and exudative change, with complete disappearance of the polypoidal lesions confirmed at 24 months in 81.3 % of cases in the PDT group and in 91.7 % of cases in the triple therapy group. There were significant differences in the number of eyes with a recurrence and in the number of PDT treatments between the two groups. Furthermore, the retreatment-free period was significantly longer in the triple therapy group (20.6± 6.8 months) than in the PDT group (11.7±8.6 months).

Following treatment for PCV, one of the most vision-threatening complications of PDT is extensive hemorrhage. A previous report of PCV treated with PDT indicated that postoperative subretinal hemorrhage was seen in 28 of 91 eyes, and that bleeding resulted in a vitreous hemorrhage in six eyes [32]. In the current study, no eye in the triple therapy group developed a vitreous hemorrhage, although two eyes in the PDT group developed a vitreous hemorrhage. Recent reports by Gomi et al. [18] and by Sato et al. [19] suggested a lower incidence of subretinal hemorrhage after PDT when it was combined with bevacizumab, and it has been reported that the vasoconstrictive effect of bevacizumab may contribute to the suppression of postoperative hemorrhages [33].

Major limitations of the current study are its retrospective nature and its relatively small sample size. In addition, there were some statistical differences between the two groups, including baseline foveal thickness and the rate of serous retinal detachment, which may affect the response to treatment. Furthermore, this study was not a randomized, comparative trial. However, selection bias is small as both groups consisted of consecutive eyes that were treated at different time periods. Our findings suggest that intravitreal injection of bevacizumab and TA combined with PDT improves the 2-year visual outcome of PCV and may reduce postoperative hemorrhagic complications and the recurrence rate. However, because our findings are based on an observation period of only 24 months, it remains unclear whether triple therapy has a long-term effect.

Another limitation is that the safety and efficacy of the triple therapy were not compared with PDT combined with anti-VEGF therapy. Recently, the EVEREST study has shown the 6-month effects of PDT in combination with ranibizumab for PCV [34], in which the eyes treated with PDT combined with ranibizumab achieved the highest gains at 6 months. However, it remains unclear whether this combination therapy reduces the recurrence of polypoidal lesions after successful initial treatment. Further prospective, randomized, long-term studies are necessary to determine the efficacy and safety of triple therapy for PCV.

Acknowledgments This study was supported in part by the Japan Society for the Promotion of Science (JSPS), Tokyo, Japan (Grant-in-Aid for Scientific Research, no. 21592256), and by the Japan National Society for the Prevention of Blindness, Tokyo, Japan. We thank the following clinicians at Kyoto University Hospital for their assistance in gathering the treatment histories for our study: Hiroshi Tamura, MD, Hideo Nakanishi, MD, Hisako Hayashi, MD, Satoko Nakagawa, MD, Kohei Takayama, MD, Yumiko Ojima, MD, and Takahiro Horii, MD.

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