Table 4. Characteristics of the training and validation set.

	non NR (SVR+R) group	non NR (SVR+R) group		NR group	NR group	
	average (training set)	average (validation set)	p-value	average (training set)	average (validation set)	p-value
No.	32	32		12	11	
Age	59.3	57.1	0.38	60.6	61.7	0.74
HCVRNA ($\times 10^6$ IU/ml)	1.77	2.08	0.48	1.51	1.52	0.97
AST (IU/L)	44.6	65.3	0.06	55.3	56.9	0.89
ALT (IU/L)	50	87.3	0.05	67.7	66.8	0.96
WBC($\times 10^3$/mm^3)	5220	5440	0.57	4610	4860	0.6
Platelet ($\times 10^4$/mm^3)	15.8	17.6	0.15	15	15.2	0.95
Total bilirubin (mg/dl)	0.71	0.69	0.78	0.68	0.68	0.92
weight	58.1	59.2	0.67	57	53.8	0.28
ALP (IU/L)	251	249	0.92	298	326	0.64
gGTP (IU/L)	48	57.4	0.54	73.3	73.8	0.98
Hemoglobin (g/dl)	13.9	14.1	0.53	13.7	13.5	0.78
Albumin (g/dl)	4.15	4.21	0.41	4.11	3.98	0.52

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SVR, clinicians could potentially reduce the side effects and costs associated with these regimens and provide a more personalized approach to treating CH patients.

Materials and Methods

Patients and sample preparation

Eighty seven CH patients with HCV genotype 1b in the Department of Gastroenterology at the Ogaki Municipal Hospital were enrolled between 2004 and 2006 (Table 1). Patients with autoimmune hepatitis, alcohol-induced liver injury, and patients positive for hepatitis B virus associated antigen/antibody or anti-human immunodeficiency virus antibody were excluded. None of the patients had received IFN therapy or immunomodulatory therapy prior to enrollment. Five normal liver specimens were obtained by surgical resection. Three of these were obtained from Osaka City University Hospital and were taken from gall bladder cancer, cholangiocarcinoma, and hemangioma patients whose liver tissue were normal based on histological, virological and blood examination of their liver function. The remaining two normal liver samples were obtained from the Liver Transplantation Unit of Kyoto University Hospital.

Patients' serum HCV RNA was quantified before IFN treatment using Amplicor-HCV Monitor Assay (Roche Molecular Diagnostics Co., Tokyo, Japan). Histological grading and staging of liver biopsy specimens from the CH patients were performed according to the Metavir classification system. Pretreatment blood samples were analyzed to determine the level of aspartate aminotransferase, alanine aminotransferase (ALT), total bilirubin, alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (γGTP), white blood cell (WBC), platelets, and hemoglobin. Written informed consent was obtained from all patients or their guardians and provided to the Ethics Committee of the Graduate School of Kyoto University, Osaka City University and Ogaki Municipal Hospital, who approved this study in accordance with the Helsinki Declaration.

Treatment protocol

For all enrolled patients, treatment with PegIFN- α-2b (Schering-Plough Corporation, Kenilworth, NJ, USA) and ribavirin (Schering-Plough) was initiated at the beginning of the 1st week and lasted for 48 weeks. PegIFN was administrated at a dose of 1.5 μg kg/week and ribavirin was administrated at the dose recommended by the manufacturer.

Definition of drug response to therapy

The patients were classified into the following three groups at the completion of follow-up period (24 weeks): (1) sustained virological responder (SVR): a patient who was negative for serum HCV RNA during the 24 weeks following the completion of the

Table 5. Quality of NR-prediction by DLDA.

		Predicted		
		NR	nonNR(SVR+R)	Total
Diagnosed	NR	9	2	11
	nonNR(SVR+R)	4	28	32
	Total	13	30	43

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Table 6. Result of the IL28B polymorphism (rs8099917).

		rs8099917		
		TT	TG	GG
outcome	NR	7	12	1
	Relapse	18	3	0
	SVR	30	1	0
	Total	55	16	1

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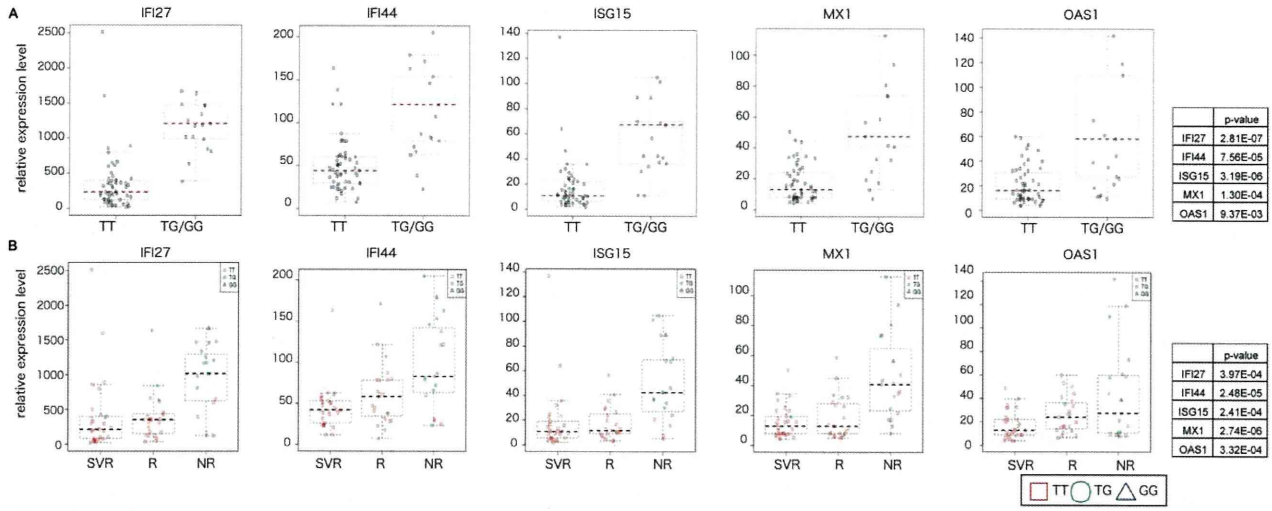

Figure 2. The relationship among the expression of IFN-related genes, IL28B polymorphism and clinical outcome. (A) The relationship between expression of ISG and five related genes (MX1, OAS1, ISG15, IFI27, and IFI44) in the liver of CH patients and IL28B with the major (TT) or minor (TG or GG) genotype (rs8099917) is shown. The p-value of the relationship between gene expression level and IL28B genotype is also depicted. (B) The relationship among the expression level of the above five genes, clinical outcome, and IL28 genotype in individual cases. Red square, green circle, and blue rectangle represent TT, TG, and GG in IL28B genotype, respectively. The p value was calculated from a linear regression employing outcome as an explanatory variable (in which SVR, R and NR are encoded to 0, 1 and 2 respectively) and expression level as the response variable. We tested the null hypothesis that the coefficient of the outcome is 0. Summary table of the p-value is also shown. NS shows no significant difference.
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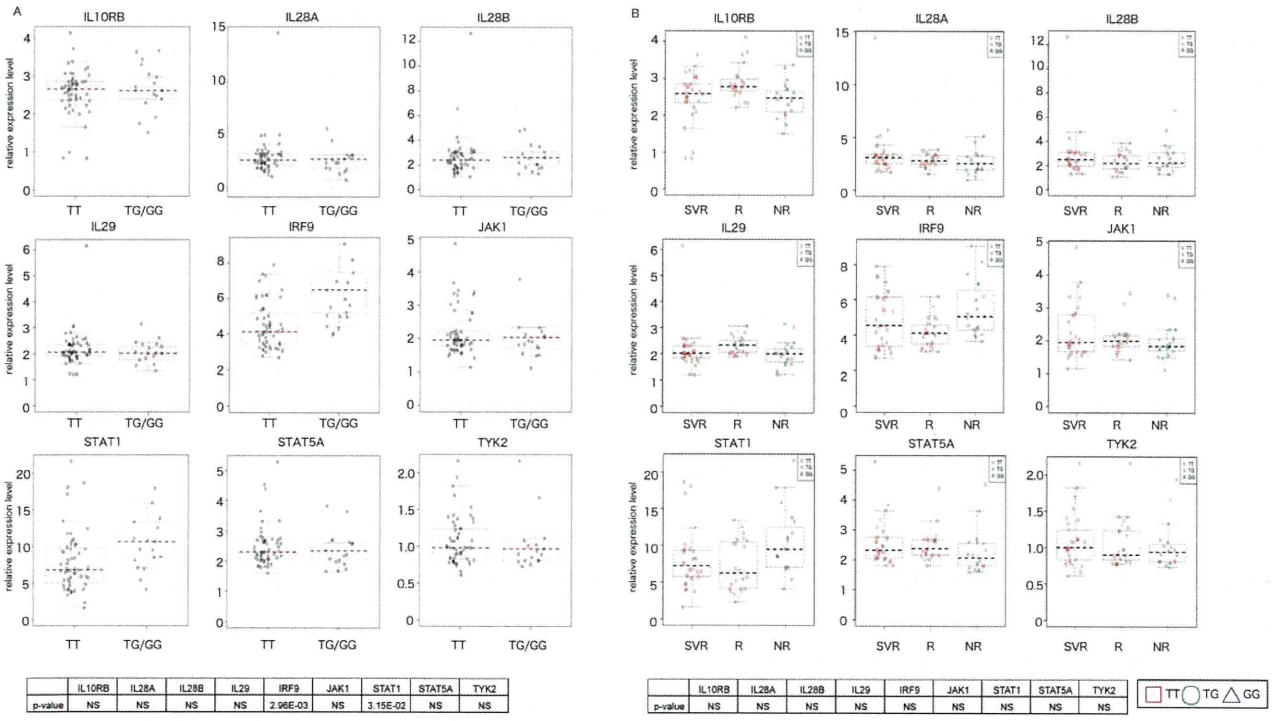

Figure 3. The relationship among the expression of IFN lambda-related genes, IL28B polymorphism and clinical outcome. (A) The relationship between the expression level of IFN lambda related genes (TYK2, STAT5A, STAT1, IL10RB, IL29, IL28A, IL28B, JAK1, and IRF9) in the liver of CH patients and IL28B with genotype. The p-value of the relationship between gene expression level and IL28B genotype is also presented. (B) The relationship among IFN lambda related genes, clinical outcome, and IL28 genotype in individual cases. Summary table of the p-value is also shown. NS was not significantly different.
doi:10.1371/journal.pone.0019799.g003

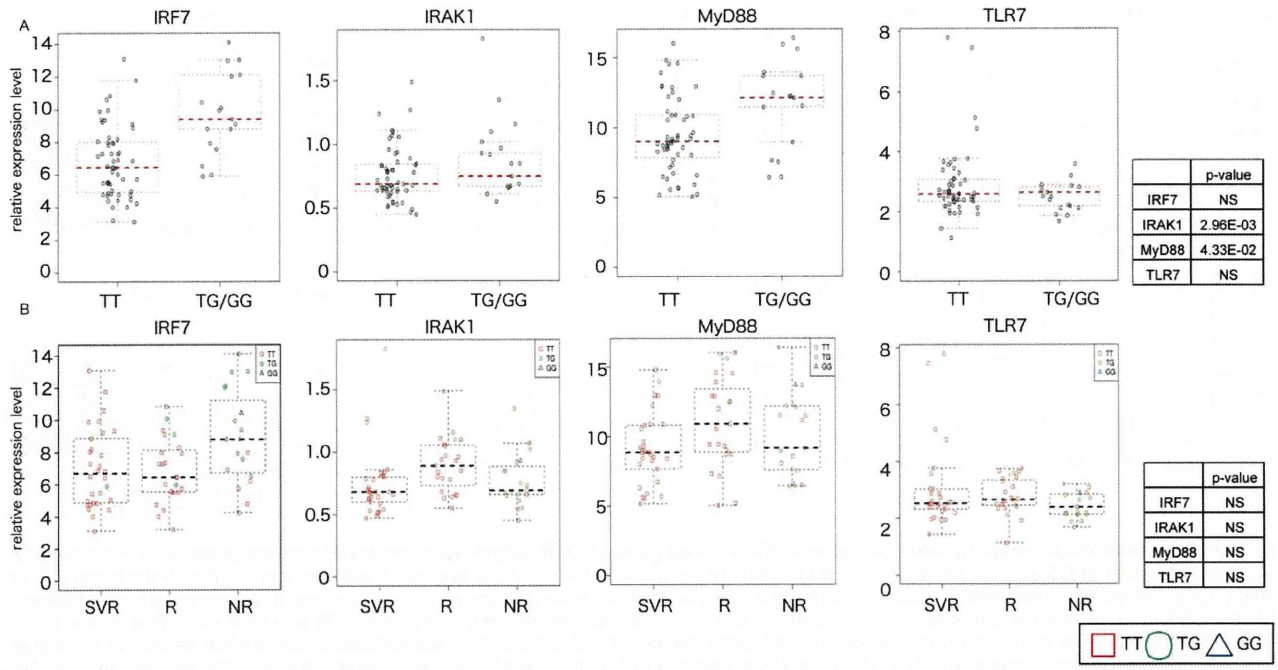

Figure 4. The relationship between the expression level of genes which participate in IFN production (TLR7, MyD88, IRAK1, and IRF7) in the liver of CH patients and IL28B genotype. (A) The relationship between IFN early response genes and clinical outcome is shown. A summary table of the p-value is also presented. NS shows no significant difference. (B) The relationship between IFN early response genes and IL28 genotype is shown. The p-value is also presented.
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combination therapy; (2) relapse (R): a patient whose serum HCV RNA was negative by the end of the combination therapy but reappeared during the 24 week observation period; and (3) non responder (NR): a patient who was positive for serum HCV RNA during the entire course of the combination therapy (Figure 5). No patients were withdrawn from the study due to side effects or any other reason.

RNA preparation and real-time qPCR

Total RNA from tissue samples was prepared using a mirVana miRNA extraction Kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction. cDNA was synthesized by Transcriptor High Fidelity cDNA synthesis Kit (Roche, Basel, Switzerland). Total RNA (2 μg) in 11 μl of nuclease free water was added to 1 μl of 50 μM random hexamer and denatured for 10 min at 65°C. The denatured RNA mixture was added to 4 μl of 5x reverse transcriptase buffer, 2 μl of 10 mM dNTP, 0.5 μl of 40 U/ml RNase inhibitor, and 0.5 μl of reverse transcriptase (FastStart Universal SYBR Green Master (Roche) in a total volume of 20 μl. cDNA synthesis was performed for 30 min at 50°C, and enzyme denaturation for 5 min at 85°C. Chromo 4 detector (Bio-Rad, Hercules, CA, USA) was used to detect mRNA expression. Assays were performed in triplicate, and the expression levels of target genes were normalized to that of the ß-actin gene, as quantified using real-time qPCR as internal controls. Nucleotide sequences of primers were as follows: IFI27 (sense) 5′-ctaggccacggaattaaccc-3′, IFI27 (anti-sense) 5′-gactgcagagtagc-cacaag-3′, IFI44 (sense) 5′-gcatgtaacgcatcaggctt-3′, IFI44 (anti-sense) 5′-ccacaccagcgtttaccaac-3′, ISG15 (sense) 5′-ctttgccagta-caggagctt-3′, ISG15 (anti-sense) 5′-gcccttgttattcctcacca-3′, MX1 (sense) 5′-aatcagcctgctgacattgg-3′, MX1 (anti-sense) 5′-gtgatgagctcgctggtaag-3′, OAS1 (sense) 5′-gtgcgctcagcttcgtactg-3′, OAS1 (anti-sense) 5′-actaggcggatgaggctctt-3′, and β-actin (sense) 5′-ccactggcatcgtgatggac-3′, β-actin (anti-sense) 5′-tcattgccaatggtgatgacct-3′.

Table 7. Quality of NR-prediction by DLDA with IFN related gene and IL28B polymorphism A.IFN+IL28B.

		Predicted		
		NR	nonNR	Total
Diagnosed	NR	7	2	9
	nonNR	4	23	27
	Total	11	25	36

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Table 8. Quality of NR-prediction by DLDA with IFN related gene only.

		Predicted		
		NR	nonNR	Total
Diagnosed	NR	8	1	9
	nonNR	5	22	27
	Total	13	23	36

doi:10.1371/journal.pone.0019799.t008

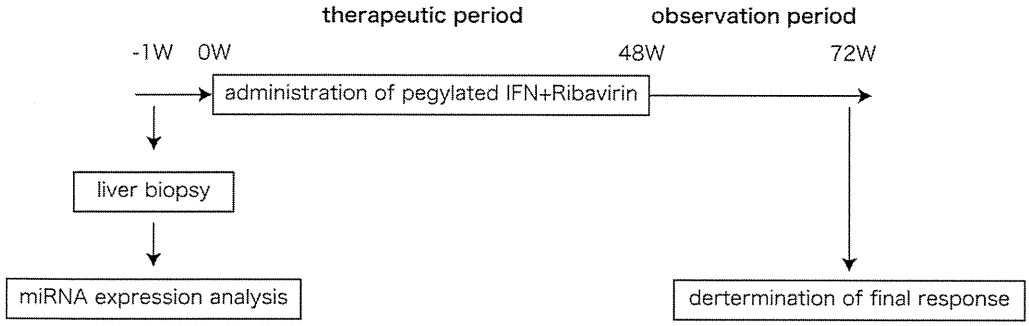

Figure 5. Study design and time line of response to combination therapy. The time frame of liver biopsy, microarray analysis, therapeutic period, observation period after combination therapy, and the judging of clinical outcome is shown.
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cDNA microarray

RNA was amplified and biotinylated using the MessageAmp-Biotin Enhanced Kit (Ambion). DNA oligonucleotide probes were synthesized onto a DNA microarray chip called Genopal (Mitsubishi Rayon) in order to detect the 237 genes (200 genes on Chip1 and 37 genes on Chip2) related to the innate immune response. Hybridization was carried out overnight at 65°C using Genopal in an hybridization buffer[0.12 M Tris-HCl/0.12 M NaCl/0.05% Tween-20]. After hybridization, Genopal was washed with hybridization buffer twice at 65°C for 20 min followed by washing in 0.12 M Tris-HCl/0.12 M NaCl at 65°C for 10 min. Genopal was then labeled with streptavidin-Cy5 (GE Healthcare Bioscience, Tokyo, Japan). The fluorescent labeled-Genopal was washed for 5 min four times with hybridization buffer at RT and scanned at multiple exposure times ranging from 0 to 40s by DNA microarray reader (Yokogawa Electric Co, Tokyo, Japan). Intensity values with the best exposure condition for each spot were selected. The data presented here have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE20119: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=xlmbxyyumcwkeba&acc=GSE20119. All data are MIAME compliant, and are also registered with GEO.

Statistical analysis

To identify the genes that varied significantly among NR, R, SVR and NL groups, one-way ANOVA and Turkey's post hoc tests were used to assess each of the 237 IFN related-genes on the arrays. Benjamini-Hochberg correction for multiple hypotheses testing was applied to all tests. P values <0.05 were considered statistically significant.

Method of predicting prognosis

The patients were randomly divided into two groups: one was used as a TS and the other VS to calculate the prediction discriminant. A prognosis signature (PS) was defined in terms of the expression levels of the six genes that differed significantly between NR and non-NR groups using post hoc analysis (IFI27, IFI44, interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), ISG15, MX1, OAS1). A prognosis predictor (PP) was computed by applying a diagonal linear DLDA to the TS [33] and then using it to predict the prognoses of the VS. The predicted and actual prognoses of VS patients were compared to obtain the following five measures of prognosis prediction performance: accuracy (proportion of correctly predicted prognoses), sensitivity (proportion of correctly predicted non-NR), specificity (proportion of correctly predicted NR), PPV (proportion of actual non-NR versus predicted non-NR) and NPV (proportion of actual NR versus predicted NR).

Genetic Variation of IL28B Polymorphism

Genotypes rs8099917 was determined in 72 out of 87 patients by Taqman SNP assays (Applied Biosystems) using a pre-designed and functionally tested probe (ABI assay ID (C_11710096_10). The experiment was carried out according to the manufacturer's instruction.

Supporting Information

Figure S1 Real-time qPCR validation of the five IFN related genes. Each column represents the relative amount of mRNAs normalized to expression level of β-actin. The data shown are means+SD of three independent experiments. Asterisk was indicated to the significant difference at $p<0.05$.
(TIF)

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Author Contributions

Conceived and designed the experiments: KS YM. Performed the experiments: KO SM T. Kawaguchi YM. Analyzed the data: T. Kawaguchi MT MK. Contributed reagents/materials/analysis tools: HT T. Kumada. Wrote the paper: HT KU T. Kawaguchi FM TF YM.

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Association between the SERPING1 Gene and Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy in Japanese

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Abstract

Purpose: Recently, a complement component 1 inhibitor (*SERPING1*) gene polymorphism was identified as a novel risk factor for age-related macular degeneration (AMD) in Caucasians. We aimed to investigate whether variations in *SERPING1* are associated with typical AMD or with polypoidal choroidal vasculopathy (PCV) in a Japanese population.

Methods: We performed a case-control study in a group of Japanese patients with typical AMD (n = 401) or PCV (n = 510) and in 2 independent control groups—336 cataract patients without age-related maculopathy and 1,194 healthy Japanese individuals. Differences in the observed genotypic distribution between the case and control groups were tested using chi-square test for trend. Age and gender were adjusted using logistic regression analysis.

Results: We targeted rs2511989 as the haplotype-tagging single nucleotide polymorphism (SNP) for the *SERPING1* gene, which was reported to be associated with the risk of AMD in Caucasians. Although we compared the genotypic distributions of rs2511989 in typical AMD and PCV patients against 2 independent control groups (cataract patients and healthy Japanese individuals), *SERPING1* rs2511989 was not significantly associated with typical AMD (P = 0.932 and 0.513, respectively) or PCV (P = 0.505 and 0.141, respectively). After correction for age and gender differences based on a logistic regression model, the difference in genotypic distributions remained insignificant (P>0.05). Our sample size had a statistical power of more than 90% to detect an association of a risk allele with an odds ratio reported in the original studies for rs2511989 for developing AMD.

Conclusions: In the present study, we could not replicate the reported association between *SERPING1* and either neovascular AMD or PCV in a Japanese population; thus, the results suggest that *SERPING1* does not play a significant role in the risk of developing AMD or PCV in Japanese.

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Age-related macular degeneration (AMD) is the leading cause of visual loss in the developed world [1]. Several genes have been reported to be associated with this disease, including complement factor H [2–4] and the age-related maculopathy susceptibility 2/HtrA serine peptidase 1 (ARMS2/HTRA1) region [5,6], and subsequent studies have replicated the association between susceptibility genes and the development of AMD using a different ethnic cohort [7–10].

Inner choroidal vascular networks that terminate in polypoidal lesions are defined as polypoidal choroidal vasculopathy (PCV), and are typically visualized by indocyanine green angiography [11]. Whether PCV represents a subtype of neovascular AMD remains controversial; moreover, whether this condition represents inner choroidal vascular abnormalities or is a variety of choroidal neovascularization remains unknown [12]. Previous studies identified several genes that contribute to the development of PCV; however, almost all reported genetic risk factors for PCV are the same as for AMD [13–15], and this suggests that AMD and PCV share, at least in part, the same genetic background.

Studies in cohorts from both the United Kingdom and the United States have shown that the complement component 1 inhibitor (*SERPING1*) gene is positively associated with AMD [16]. However,

Table 1. Characteristics of the Study Population.

		Cases		Controls	
		tAMD	PCV	Control 1*	Control 2†
No. of participants		401	510	336	1194
Age	Mean ± SD	77.38±8.39	74.98±7.77	74.16±8.42	50.34±15.9
Gender	Men	287	372	142	493
	Women	114	138	194	701

tAMD, typical age-related macular degeneration; PCV, polypoidal choroidal vasculopathy; SD, standard deviation.
*Cataract patients without age-related maculopathy.
†Healthy Japanese individuals.
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another study in a larger cohort (n = 7723 and 2327) which involved the same population could not replicate the finding of the previous study [17,18]. Recently, Lee et al. have shown that *SERPING1* is positively associated with AMD in Caucasians [19], but whether this gene is truly associated with AMD remains controversial.

Furthermore, the association of *SERPING1* with AMD has been evaluated also in Asians. Lu et al. examined the association in 194 AMD patients and 285 controls and reported that *SERPING1* is not associated with AMD in the Chinese population [20]. The association between PCV and *SERPING1* has also been evaluated in a smaller Chinese cohort (118 patients and 115 controls), also with negative findings [21]. So far, all Asian studies for *SERPING1* did use smaller cohorts than those of original studies and not consider their statistical power. For evaluating the true gene-disease association, it would be helpful to replicate the positive association reported in previous studies using a different ethnic cohort. The aim of this study, which involved a relatively large number of participants, was to investigate whether the *SERPING1* gene variants are associated with typical AMD or PCV in a Japanese population.

Materials and Methods

All procedures in this study adhered to the tenets of the Declaration of Helsinki. This study was approved by the Ethics Committee of each institute involved (Kyoto University Graduate School and Faculty of Medicine, Ethics Committee, the Ethical

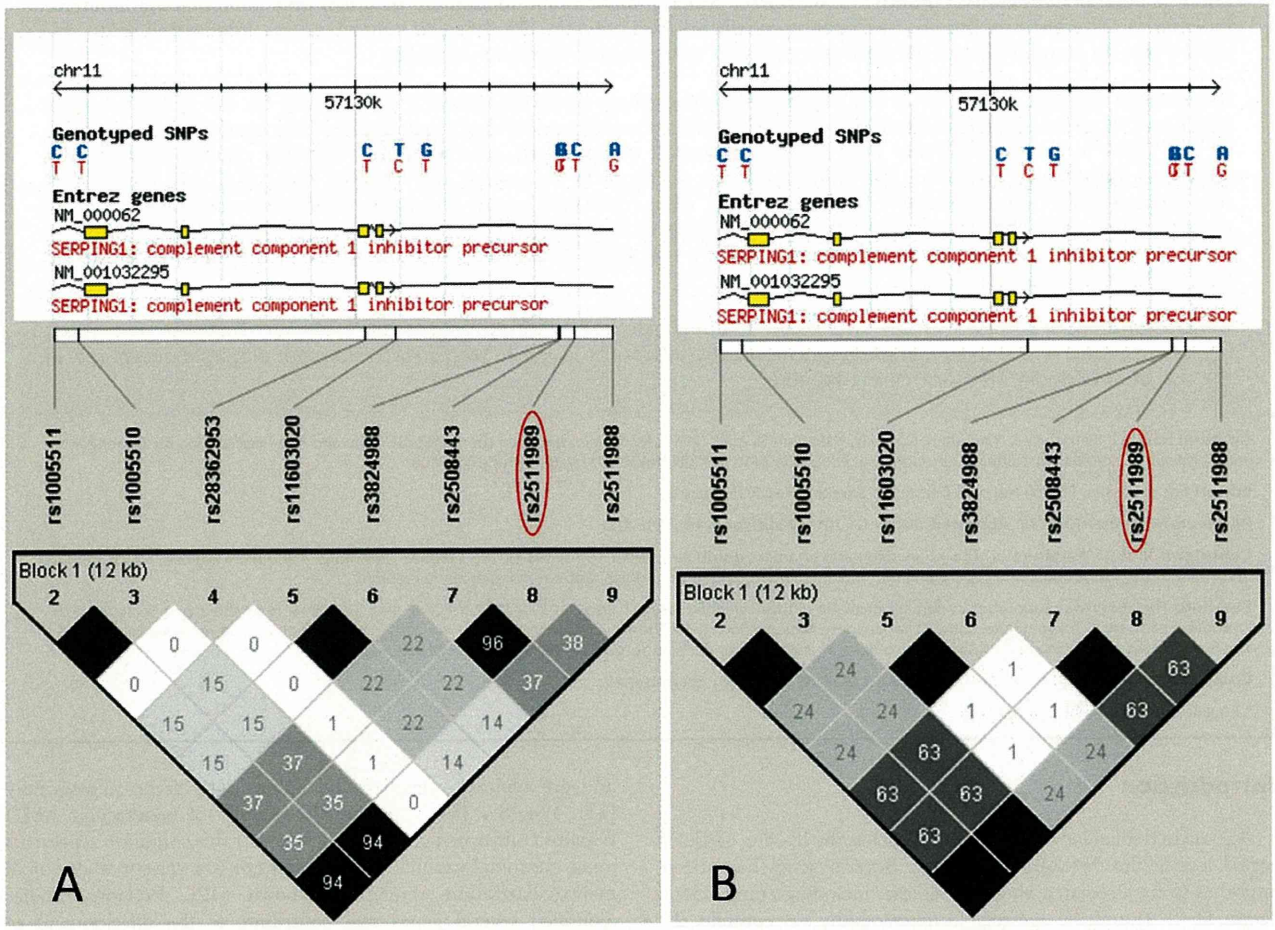

Figure 1. Linkage disequilibrium (LD) structure across the complement component 1 inhibitor (*SERPING1*) gene in Caucasian and Japanese populations. Genotype data were retrieved from HapMap CEU (Utah residents with ancestry from northern and western Europe; A) and JPT (Japanese in Tokyo, Japan; B) data sets. Haplotype blocks were determined using the "four-gamete rule" option in Haploview; all HapMap single nucleotide polymorphisms on *SERPING1* gene are in the same block in both populations. Each box provides estimated statistics of the coefficient of determination (r^2), with darker shades representing stronger LD.
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Committee of Fukushima Medical University, the Ethical Committee of Kobe City Medical Center General Hospital, the Ethical Committee of Ozaki Eye Hospital, the Ethical Committee of the Otsu Red Cross Hospital, the Ethical Committee of Nagahama City Hospital, and the Ethical Committee at Aichi Cancer Center). All of the patients were fully informed about the purpose and procedures of this study, and written consent was obtained from each.

In this study, 401 patients with typical AMD and 510 patients with PCV were recruited from the Department of Ophthalmology at Kyoto University Hospital, Fukushima Medical University Hospital, and Kobe City Medical Center General Hospital. The control group included 2 populations: (1) 336 individuals who underwent cataract surgery and had no age-related maculopathy (ARM) (Control 1) were recruited from the Department of Ophthalmology, Kyoto University Hospital, Ozaki Eye Hospital, Japanese Red Cross Otsu Hospital, and Nagahama City Hospital; and (2) 1194 healthy individuals who were recruited from the Aichi Cancer Center Research Institute as the general population control (Control 2). AMD and ARM were defined according to the International Classification System for ARM and AMD [22]. The diagnosis of PCV was based on indocyanine green angiography, which showed a branching vascular network that terminated in polypoidal swelling. Typical AMD were late AMD which showed classic choroidal neovascularization (CNV), occult CNV, or both. All diagnoses were made by 3 retina specialists (K.Y., A.T., and A.O.); a fourth specialist (N.Y.) was consulted when the subtype classification could not be decided on by the initial 3 reviewers. All of the subjects were unrelated and were of the Japanese descent.

Genomic DNAs were isolated from the peripheral blood of the subjects by using a DNA extraction kit (QuickGene-610L, Fujifilm, Minato, Tokyo, Japan). The samples of all the patients with typical AMD and PCV and of cataract patients were genotyped using a Taqman single nucleotide polymorphism (SNP) assay with the ABI PRISM 7700 system (Applied Biosystems, Foster City, CA). The individuals recruited from the Aichi Cancer Center Research Institute were genotyped using Illumina Human-Hap 610 chips (Illumina Inc., San Diego, CA).

Linkage disequilibrium (LD) structures across the *SERPING1* gene were compared between the Caucasian and Japanese populations, using genotype data retrieved from the HapMap CEU and JPT data sets [23]. The retrieved data were loaded into Haploview to estimate LD parameters and to identify haplotype blocks [24]. Deviations in genotype distributions from the Hardy–Weinberg equilibrium (HWE) were assessed using the HWE exact test. Statistical analyses for differences in the observed genotypic distribution were performed by the chi square test for trend; logistic regression analysis was performed for age and gender adjustments. The statistical power calculation was performed using QUANTO version 1.2 [25]. P values less than 0.05 were considered statistically significant.

Results

The demographic details of the study population are presented in Table 1. Because all SNPs of the *SERPING1* gene are in the same haplotype block, rs2511989 was selected as the haplotype-tagging SNP; rs2511989 was reported to be associated with the risk of AMD in previous studies [16,19] (Fig. 1). Details of allele and genotype counts and summary statistics for rs2511989 are shown in Table 2. The success rate of genotyping of rs2511989 was 98.1%, and the distributions of the genotypes for all study groups were in the Hardy–Weinberg equilibrium ($P>0.05$). Although we compared the genotype distributions of rs2511989 in typical AMD and PCV patients against 2 independent control groups (cataract patients without ARM and healthy Japanese individuals), *SERPING1* rs2511989 was not significantly associated with typical AMD ($P = 0.932$ and 0.513, respectively); furthermore, it was not significantly associated with PCV ($P = 0.505$ and 0.141, respectively). After correction for age and gender differences based on a logistic regression model, the difference in the genotype distributions remained insignificant ($P>0.05$). Table 3 shows the odds ratios adjusted for age and gender under various genetic models. We could not find a significant association in any genetic model.

Next, we calculated our statistical power to detect an association of a risk allele with the odds ratio reported in the previous study that investigated the association of rs2511989 with developing AMD. When we targeted the original study reported by Ennis (odds ratio 0.63) [16], our sample size had more than 90% power to detect the association (Table 2). In addition, the statistical power calculation revealed that our sample size could detect the gene-disease association for an odds ratio of 0.797 by more than 80%.

Discussion

In the present study, we investigated whether *SERPING1* gene variants are associated with typical AMD or with PCV in a Japanese population. We selected rs2511989 as the haplotype-tagging SNP, because this has been reported to be positively associated with the risk of AMD in Caucasians. The results of this study showed that *SERPING1* rs2511989 was not associated with the risk for typical AMD in a Japanese population; thus, the results did not support the hypothesis that an association between the *SERPING1* gene and AMD exists. Our sample size had more than 90% power to detect the association determined in the previous

Table 2. *SERPING1* rs2511989 Genotypic Distributions and Results of Association Tests and Power Analysis.

						vs Control 1			vs Control 2		
		GG	GA	AA	MAF	P Value	Adjusted P*	Power†	P Value	Adjusted P*	Power†
Cases	tAMD	293	102	6	0.142	0.932	0.687	93.6%	0.513	0.860	99.3%
	PCV	380	125	5	0.132	0.505	0.855	95.7%	0.141	0.678	99.2%
Controls	Control 1	248	76	10	0.144	-	-	-	-	-	-
	Control 2	859	308	27	0.152	-	-	-	-	-	-

tAMD, typical age-related macular degeneration; PCV, polypoidal choroidal vasculopathy; MAF, minor allele frequency.
*Adjusted for age and gender.
†Statistical power for detecting the association reported in the previous study (odds ratio 0.63).
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Table 3. Odds Ratios in Various Genetic Models.

		Adjusted Odds Ratio (95% Confidence Interval)*	
Group	**Model**	**vs tAMD**	**vs PCV**
Control 1	Additive	0.938 (0.687–1.281)	0.972 (0.72–1.312)
	Dominant	1.283 (0.746–2.204)	0.598 (0.338–1.056)
	Recessive	0.934 (0.783–1.114)	1.283 (0.746–2.204)
Control 2	Additive	1.034 (0.716–1.491)	0.933 (0.673–1.294)
	Dominant	0.940 (0.470–1.879)	0.709 (0.349–1.440)
	Recessive	1.025 (0.839–1.254)	0.983 (0.823–1.174)

*Adjusted for age and gender.
tAMD, typical age-related macular degeneration; PCV, polypoidal choroidal vasculopathy.
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study in a Caucasian cohort (odds ratio 0.63) [16]. Furthermore, we found no evidence to support the role played by *SERPING1* rs2511989 in the susceptibility to PCV, and this finding is in agreement with that of the previous study in a Chinese population [21].

The reported association between AMD and *SERPING1* rs2511989 is shown in Table 4. The size of our Japanese cohort was similar to that of the original study [16]. Furthermore, the statistical power calculation revealed that our sample size could detect the gene-disease association for an odds ratio of 0.797 by more than 80%. Had there been a true protective effect of *SERPING1* gene variants for developing AMD at the same level as was reported in previous studies [16,19], the statistical power of our study would have detected such an association. Differences in the ethnicities of subjects might be 1 reason for the difference observed between the results of this study in a Japanese cohort and those of the previous study in a Caucasian cohort. Frequency of the minor allele of rs2511989 was reportedly greater in the earlier study in a Caucasian population than that of the present study in a Japanese population. In fact, in reference to the allele frequency data from the HapMap, all genetic variants across the *SERPING1* gene showed smaller minor allele frequency in Japanese than in Caucasians.

Another possible explanation for the differences between our findings and those of other studies in different ethnic cohorts may include a difference in the phenotypes of AMD. Numerous studies have reported that distinguishing features of Asian AMD include male predominance, unilateral presentation, comparatively low incidence of soft drusen, and greater prevalence of neovascular AMD and PCV [26–29]. To address these concerns, we classified AMD patients into those with typical AMD and those with PCV, but the possible hidden differences in the phenotypes cannot be excluded. Alternatively, considering the fact that genetic variants that are associated with a particular disease in 1 population may not necessarily be associated in another population [30–32]; moreover, it is possible that gene-disease association of *SERPING1* in populations from East Asia is very weak or absent as compared with Caucasian populations.

In this study, we used general population-based controls (Control 2). The possibility exists that some of the eyes in the control 2 group might have or develop AMD or PCV, and this might be a possible explanation for the negative results in this study. However, because the prevalence of AMD in the general population is estimated to be 0.5% in the Japanese population [33], the loss of the statistical power of association analysis must be negligible. In addition, we also performed a subset analysis on

Table 4. Comparison of Association Observed between AMD and *SERPING1* rs2511989.

Subject Group		**Current Study (JP)**			**Mayo Subjects (US)**		**AREDS Subjects (US)**		**Ennis et al. (UK)**		**Ennis et al. (US)**		**Lee et al. (US)**		**Lu et al. (CH)**	
Subjects		**Case**	**Control 1**	**Control 2**	**Case**	**Control**	**Case**	**Control**	**Case**	**Control**	**Case**	**Control**	**Case**	**Control**	**Case**	**Control**
No. of participants		401	336	1194	470	310	1221	295	479	479	248	252	556	256	194	285
Allele count	G	688	572	2026	569	363	1435	357	597	500	322	282	669	283	336	493
	A	114	96	362	371	257	1007	233	355	454	174	222	413	229	52	69
Genotype count	GG	293	248	859	179	103	436	115	191	132	100	79	213	74	147	215
	GA	102	76	308	211	157	563	127	215	236	122	124	273	135	42	63
	AA	6	10	27	80	50	222	53	70	109	26	49	70	47	5	3
MAF		0.142	0.144	0.152	0.395	0.415	0.412	0.395	0.373	0.475	0.351	0.441	0.382	0.447	0.134	0.123
P. values		-	0.932	0.513	-	0.46	-	0.41	-	5.4×10^{-6}	-	0.0037	-	0.011	-	0.61

MAF, minor allele frequency.
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controls 2 with 55 years of age or older to minimize the possibility that some of the eyes in the control group might develop AMD or PCV. However, no new significant differences in the genotypic distributions were found in the current study (data not shown). Thus, we concluded that the result of the analysis using control 2 is valuable as reference data which supports a lack of association between *SERPING1* and both typical AMD and PCV in a Japanese population. Another limitation is about geographical difference of Control 1, which may influence genetic background of the participants. However, because the Japanese population has been reported to have a rather small genetic diversity, according to data from the SNP discovery project in Japan [34], geographical difference should not be affect our statistical results.

In conclusion, this study showed a lack of association between *SERPING1* and both typical AMD and PCV in a Japanese population; thus, the results suggest that *SERPING1* does not play a significant role in the risk of developing AMD or PCV in Japanese.

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Author Contributions

Conceived and designed the experiments: IN KY HN NY. Performed the experiments: IN NG HN HH. Analyzed the data: IN RY. Contributed reagents/materials/analysis tools: IN KY RY NG HN HH AT AO MS TI AO KM KT FM NY. Wrote the paper: IN KY RY.

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The human *AIRE* gene at chromosome 21q22 is a genetic determinant for the predisposition to rheumatoid arthritis in Japanese population

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Rheumatoid arthritis (RA) is a typical complex trait and the major cause of chronic inflammation worldwide. Although multiple genetic loci have been shown for their association with the onset of RA, they cover only a part of its genetic components and are largely ethnicity-specific. To identify novel genetic factors related to the predisposition and prognosis of RA in Japanese, we conducted a large-scale genome-wide association (GWA) study. We performed a GWA analysis by scanning the genome of 1247 RA cases and 1486 controls for 277 420 single nucleotide polymorphisms (SNPs), followed by replication analysis using two independent sample sets consisting of 1865 cases and 1623 controls, and 2303 cases and 3380 controls. We identified two SNPs, rs2075876 and rs760426, in intron of the autoimmune regulator *AIRE* gene at chromosome 21q22 that showed strong associations with the disease ($P = 3.6 \times 10^{-9}$ and $P = 4.4 \times 10^{-8}$, respectively). Rs1800250, in exon7 of *AIRE*, was in strong linkage disequilibrium ($r^2 = 0.94$) with rs2075876 and introduced an amino acid alteration (S278R) in the SAND domain of the AIRE protein. *In silico* analysis showed the decreased transcription of *AIRE* by the risk allele of rs2075876 compared with the alternative allele ($P = 6.8 \times 10^{-5}$). No correlation was observed between the rs2075876 genotype and quantitative traits reflecting the progression of RA. As AIRE is a key molecule which regulates the expression and presentation of self-antigens in thymic negative selection, its downregulation by genetic polymorphisms may result in the survival of auto-reactive T cells to trigger auto-inflammation in RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a major cause of chronic arthritis worldwide and results in severe functional impairment and joint destruction. The impairment of joints and disability for social activity bring strong social and economic impact (1). Both environmental and genetic factors are considered to be associated with its onset and progression (2). Twin studies of

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the European populations showed that ~60% of RA onset could be attributed to genetic factors (3). In them, *HLA-DRB1* is the strongest genetic component of the disease beyond ethnicity, and is estimated to correspond to 30–50% of the genetic components in Europeans (4). Although extensive genetic analyses including hypothesis-independent genome-wide association (GWA) studies identified >20 genes in Europeans (5–13) and 7 genes in East Asians (14–19) as genetic risk loci for RA, they account for only a part of its genetic components. Moreover, trans-ethnic comparison demonstrated that their association with RA is mostly specific to a particular ethnicity and as little as three genes, namely, *CCR6*, *STAT4* and *TNFAIP3*, have shown their association in both populations. These results strongly suggest the existence of additional susceptibility loci to RA in East Asian populations (14–19). By these reasons, we have conducted a GWA study using large DNA collections of Japanese RA patients.

RESULTS

GWA analysis

We performed a large-scale genome scan using a Japanese DNA collection (collection 1) consisting of 1247 RA cases and 1486 general population controls with Illumina Infinium arrays (Supplementary Material, Table S1). After a standard procedure of quality control (see Materials and Methods), 241 523 single nucleotide polymorphisms (SNPs) were examined for their association with RA. Quantile-quantile plot to estimate population stratification resulted in a small inflation factor ($\lambda = 1.05$). The strongest association was detected for markers in the *HLA* locus with the strongest *P*-value of 2.4×10^{-38} for rs9296015. Another known genetic determinant, *PADI4*, also showed strong association (strongest $P = 1.8 \times 10^{-8}$ for rs2240335). Also, a modest association was found in the *CCR6* gene (strongest $P = 9.7 \times 10^{-4}$ for rs1556413) (16). However, there was no evidence of association for *STAT4* and the disease in our study ($P > 0.070$). There were no other loci that showed significant association ($P < 2.1 \times 10^{-7}$) after Bonferroni's correction for multiple testing.

We then took a strategy to select candidate genes/markers for further genotyping analysis based on their functional relevance in the immune system. For this purpose, we generated a list of SNP markers showing potential association with the disease (nominal $P < 0.001$), and investigated their chromosomal locations and corresponding genes in the order of association strength. Among the top 471 SNPs with *P*-value smaller than 0.001, we found two SNPs located in intron of the *AIRE* gene at chromosome 21q22, which is known as an autoimmune regulator. They were rs2075876 and rs760426 with *P*-value of 5.1×10^{-4} and 2.0×10^{-4}, respectively, and were ~6.7 kb apart from each other and in moderate linkage disequilibrium (LD) ($r^2 = 0.63$, Fig. 1). We performed genotyping of these two markers using an additional DNA collection (termed as collection 2) consisting of 1865 cases and 1623 controls. All the RA cases and 855 controls were newly genotyped with the Taqman method, and the genotypes of the other 768 controls were extracted from genome scan results of other population-based genetic studies. We successfully confirmed the association of rs2075876 ($P = 5.1 \times 10^{-4}$) in collection 2. The other marker, rs760426, showed a moderate association ($P = 0.011$) (Table 1).

We further examined whether or not the results of our study were reproducible in another Japanese RA GWA study of Biobank Japan Project recruiting 2303 cases and 3380 controls (termed as collection 3) (16). The statistical test again returned significant associations for these markers ($P = 3.6 \times 10^{-4}$ for rs2075876 and $P = 8.2 \times 10^{-4}$ for rs760426, Table 1). When the genotyping results of the three collections were pooled, the association *P*-value reached $P = 3.6 \times 10^{-9}$ for rs2075876 and $P = 4.4 \times 10^{-8}$ for rs760426 (Table 1).

We then investigated whether or not the association of the *AIRE* gene with RA was observed in Europeans. Our own genome scan results of German RA samples (I.M., M.L. and F.M., unpublished data) showed no associations for the SNP markers in the *AIRE* locus. Two large-scale GWA studies of European descents, namely, Wellcome Trust Case Control Consortium (9) and a meta-analysis of multiple GWA studies (12), did not identify *AIRE* as a risk locus, strongly suggesting its limited contribution to RA in East Asian populations.

Structure and organization of the human *AIRE* locus

LD structure of the chromosomal region containing rs2075876 and rs760426 was generated using Japanese HapMap results. As shown in Figure 1, rs2075876 and rs760426 are located in an LD block encompassing the 32 kb region between intron 5 of the *AIRE* gene and intron 12 of the liver phosphofructokinase *PFKL* gene. As the SNPs around the *PFKL* gene showed weaker association with RA ($P > 0.002$) than the two SNPs, we considered that the observed association with RA was most likely with the *AIRE* polymorphisms. However, both of these SNPs were located in intron and no other SNP markers in the genotyping arrays were mapped in this LD block and showed similar degree of association with RA. Hence, we searched for SNPs in dbSNP that were located in exons of *AIRE* and introduce functional alterations of the AIRE protein. There were five non-synonymous SNPs in the coding region of *AIRE* out of which rs1800520 in exon7 showed an allele frequency similar to that of rs2075876 (0.420). Rs1800520 introduced an amino acid alteration from serine to arginine at amino acid residue 278 (S278R). We genotyped rs1800520 in the DNA samples of all the cases ($n = 1865$) and a part of controls ($n = 855$) of collection 2 and found that rs1800520 was in strong LD with rs2075876 ($r^2 = 0.94$) and was also associated with RA ($P = 0.0071$).

AIRE polymorphism and expression

Although both rs2075876 and rs760426 are located in intron, they may have functional roles such as regulation of *AIRE* transcription. The correlation between these SNPs and transcription levels of *AIRE* was examined by using the expression profiles of 210 lymphoblastoid cells in Gene Expression Omnibus (GEO) database (20). As the result, the transcription of *AIRE* was decreased by the risk allele (A) of rs2075876 ($P = 6.8 \times 10^{-5}$, Fig. 2) but not by that of rs760426 ($P = 0.24$). Although we hypothesized the presence of a transcription factor-binding site around rs2075876, *in silico* study

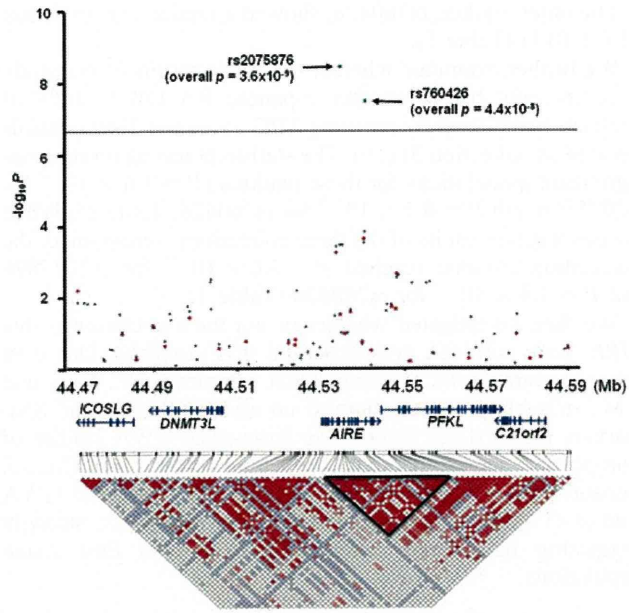

Figure 1. A schematic organization of the human *AIRE* locus at 21q22. *P*-values of the initial genome scan using collection 1 were calculated by the Trend χ^2 test and plotted in red circles. The blue circles indicate *P*-values obtained by imputation using HapMap Japanese results. Overall *P*-values of rs2075876 and rs760426 using the combined results of collections 1, 2 and 3 were also shown in green circles. A horizontal line indicates Bonferroni-adjusted $P = 2.5 \times 10^{-7}$. The structure and orientation of four genes were shown below the plots with their transcriptional orientations according to the NCBI Reference Sequence Build 36.3. LD blocks were generated according to the pairwise LD estimates of the SNPs in HapMap Japanese results.

did not predict a motif of transcription factor-binding site spanning rs2075876. Multiple nucleotide sequence alignment around rs2075876 showed a high degree of conservation among seven mammalian species (human, chimpanzee, rhesus macaque, bushbaby, horse, cow and dog). The corresponding region of rodents (mouse and rat) showed much weaker conservation (Supplementary Material, Fig. S1).

AIRE polymorphism and difference in clinical phenotypes and disease activity

RA is often subdivided into two groups based on the presence of circulating antibodies to citrullinated peptide antigen (ACPA), a specific predictive biomarker for destructive RA (21–22). In our patient collections (collection 1 and collection 2), there were 803 patients with ACPA quantification of which 176 patients were negative for ACPA. We compared the allele frequency of rs2075876 between APCA(+) and ACPA(−) groups and found no significant difference [0.39 for ACPA(+) and 0.40 for ACPA(−), $P = 0.66$]. We next tested whether rs2075876 was associated with the disease activity and prognosis. For this purpose, 212 RA patients for whom the quantitative DAS28 score was available were chosen to evaluate the correlation of RA activity and rs2075876 genotypes. Statistical analysis did not return correlations between rs2075876 genotypes and DAS28 (Supplementary Material, Fig. S2).

DISCUSSION

AIRE is a transcriptional regulator primarily expressed in medullary thymic epithelial cells (mTEC), and plays a functional role in thymocyte education and negative selection by controlling the expression of peripheral antigens in thymus (23). The expression of *AIRE* in non-thymic tissues is still controversial; some studies detected *AIRE* transcripts at a lower level in secondary lymphoid organs and in periphery while others did not (24–25), and the expression of the AIRE protein in such tissues is yet to be established. In human, dysfunction of *AIRE* caused a rare systemic multi-organ autoimmune disease known as autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) (26). However, the patients rarely show joint destruction as observed in RA (27). In mice deficient for *aire* which develop APECED-like multi-organ autoimmune features and do not manifest with arthritis, a dramatic decrease in the expression of type II collagen was observed in mTEC and the incidence and severity of collagen-induced arthritis were augmented when compared with the wild-type (28). Such observations indicate the possible involvement of *AIRE* in immunopathology both in the human and in the mouse. However, the involvement of *AIRE* in human multigenetic autoimmune diseases still remains to be elucidated. Our study is the first successful case which clearly showed the involvement of *AIRE* in systemic autoimmunity. The function of the AIRE protein in the secondary lymphoid organs is not fully understood. Elucidation of the functions of AIRE in peripheral organs may provide hints to the involvement of *AIRE* in the predisposition or progression in RA.

In silico analysis using the GEO database showed that the risk allele of rs2075876 decreased the transcription level of *AIRE*. This may cause lower expression of various peripheral tissue antigens (PTAs), resulting in the failure of negative selection in the thymus resulting in the survival of autoreactive T cells. Although low amount of *AIRE* transcripts in B-lymphocytes was detected in most of the reported experiments, the conclusive answer for the functional impact of rs2075876 to the immune regulation needs further studies using the tissues in which *AIRE* is strongly expressed. The S278R replacement by rs1800520 is located in the SAND domain, a conserved sequence motif in nuclear proteins including Sp100 family and plays a key role in transcription regulation. However, the SAND domain of AIRE lacks the canonical KDWF motif for the interaction with DNA. Also amino acid sequence alignment of the SAND domains in different nuclear proteins revealed that S278R was located at the poorly conserved carboxyl terminal (29). Moreover, the interaction of AIRE with histone H3 through a plant homeodomain finger was suggested to be important to up-regulation of PTA genes (30). On the other hand, an assessment of mRNA stability by a computerized modeling showed lower stability of *AIRE* mRNA with the risk allele of rs1800520 than the alternative allele, suggesting the possibility of shorter half-life of the transcripts and thus lower amount of the AIRE protein. As such, we cannot conclude whether or not these SNPs have functional impact to the regulation of *AIRE* expression. The existence of unidentified SNPs that are in strong LD with them and play important functional roles is also conceivable. Extensive analyses of the *AIRE* locus by fine mapping and

Table 1. Association analysis of two SNPs in the *AIRE* gene with RA in Japanese

rs2075876		Genotype counts			Frequency A	OR (95% CI)	*P*-value
		GG	GA	AA			
Collection 1	Case	480	554	201	0.39	1.22 (1.09–1.36)	5.1×10^{-4}
	Control	639	680	167	0.34		
Collection 2	Case	706	887	243	0.37	1.18 (1.07–1.31)	9.4×10^{-4}
	Control	710	671	192	0.34		
Collection 3	Case	905	1061	330	0.37	1.15 (1.07–1.25)	3.6×10^{-4}
	Control	1462	1506	398	0.34		
Combined study	Case	2091	2502	774	0.38	1.18 (1.11–1.24)	3.6×10^{-9}
	Control	2811	2857	757	0.34		
rs760426		Genotype counts			Frequency G	OR (95% CI)	*P*-value
		AA	AG	GG			
Collection 1	Case	464	559	219	0.40	1.23 (1.10–1.38)	2.0×10^{-4}
	Control	608	709	169	0.35		
Collection 2	Case	684	897	265	0.39	1.13 (1.03–1.25)	0.011
	Control	666	741	205	0.36		
Collection 3	Case	866	1078	357	0.39	1.14 (1.06–1.23)	8.2×10^{-4}
	Control	1408	1520	450	0.36		
Combined study	Case	2014	2534	841	0.39	1.16 (1.10–1.22)	4.4×10^{-8}
	Control	2682	2970	824	0.36		

OR, odds ratio; 95% CI, 95% confidence interval.

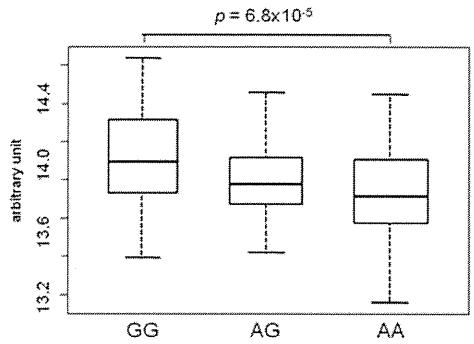

Figure 2. Comparison of the expression levels of *AIRE* among three subgroups of cell lines according to the genotype of rs2075876. 'G' and 'A' correspond, respectively, to the risk and the alternative alleles of rs2075876.

extensive sequencing in combination with examination of promoter activity will answer this question.

There was no association between *AIRE* and RA in Europeans even in the large-scale meta-analysis of GWA studies with a strong detection power (12). Although the frequency of the risk allele of rs2075876 is much lower in Caucasians (0.15 in Caucasian HapMap results and 0.097 in our own genome scan results) compared with that of the current study (0.34), this does not fully explain the lack of association in Europeans. This suggests that the association of *AIRE* with RA is, like that of *PADI4*, specific to East Asian populations including Japanese. The future validation study using other Asian population will address this issue.

MATERIALS AND METHODS

Study subjects

RA collections 1–3 consisted of 1247 affected individuals and 1486 controls, 1865 cases and 1623 controls, and 2303 cases and 3380 controls, respectively (summarized in Supplementary Material, Table S1). The case subjects of collections 1 and 2 were recruited at the rheumatology departments of Kyoto University Hospital, Dohgo Spa Hospital, Sagamihara National Hospital, Tokyo University Hospital and Tokyo Women's Medical University. The control subjects for collection 1 were from Aichi Cancer Center Hospital and Research Institute and the Department of Ophthalmology and Visual Science at Kyoto University Hospital. DNA samples of healthy Japanese volunteers in collection 2 were from Pharma SNP Consortium (31) and the Center for Genomic Medicine, Graduate School of Medicine, Kyoto University. The case and control subjects in collection 3 were recruited in the Biobank Japan Project at the Institute of Medical Science, the University of Tokyo; the Department of Allergy and Rheumatology, Graduate School of Medicine, the University of Tokyo (32). All cases fulfilled the revised criteria (1987) of the American College of Rheumatology for RA. Among the RA cases, DAS28 score for RA activity in 212 RA patients was obtained at each institution. Written informed consent was obtained from all the participants at the institute of sample collection after being approved for genetic studies by the local ethical committee.

GWA analysis

Genome scan for collection 1 was performed using Infinium Technology (Illumina Inc., San Diego, CA, USA). Case subjects were genotyped with Human-Hap300 (version 1.0, 302 627 SNPs), Human CNV370-Duo (version 1.0, 332 270 SNPs) or Human610-Quad (version 1.0, 577 348 SNPs). For control subjects, they were genotyped with Human610-Quad (version 1.0, 577 348 SNPs) and HumanHap550 (version 3.0, 547 163 SNPs). For validation analysis, Taqman

technology (Life Technologies Corp., Foster City, CA, USA) was employed.

Quality control and statistical tests for case–control association

A total of 277 420 SNPs that were common among the four types of arrays described above were selected for the association study. One thousand two hundred and forty-six cases and 1486 controls with call rate being >0.90 and not showing high degree of kinship (PI_HAT < 0.10 by PLINK) were examined for the association analysis. A total of 241 523 SNPs with call rate >0.95 for both cases and controls and minor allele frequency >0.05 either in the case or in the control were used for the analysis. The case–control association was examined with the Cochran–Armitage trend for each collection as well as for the combined pooled study. Population stratification was examined and corrected with Genomic Control. SNPs that showed *P*-value $<10^{-3}$ were selected as candidates for further evaluation. SNPs in the *HLA*, *PADI4* and *CCR6* loci were not selected for validation studies. Haploview version 4.1 software (33) was used for LD evaluation, and MapViewer (build 36.3) (34) was used to identify the location and structure of the genes in the region.

Analysis of *AIRE* expression

A gene-expression data set in lymphoblastoid cell lines derived from 210 unrelated HapMap populations was obtained from GEO database (20). The correlation between the expression of *AIRE* and genotypes of SNPs in the region was examined using the calculation program recommended by GEO. The association *P*-values were obtained by the Jonchheere–Terepstra method using R software or SPSS (version 18).

Bioinformatics analysis

Genome sequence alignment of 14 placental mammals was obtained from the UCSC genome browser (http://genome.ucsc.edu). Motif search was carried out by the Jaspar database (35) (http://jaspar.cgb.ki.se) using 'Jasper Core Subset' which contains 138 matrices for known *cis*-acting elements. The matrices were converted into bit scores and used to search against the genomic sequences around the SNP of interest. Identification of orthologs of the *AIRE* gene in different mammals and multiple nucleotide sequence alignment was performed using KEGG SSDB Database (www.genome.jp/kegg/ssdb).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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APPENDIX

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Genetic Variants in Pigment Epithelium-Derived Factor Influence Response of Polypoidal Choroidal Vasculopathy to Photodynamic Therapy

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Purpose: To investigate whether photodynamic therapy (PDT) outcomes of polypoidal choroidal vasculopathy (PCV) are related to baseline clinical characteristics, smoking history, or genetic factors by analyzing the retreatment-free period after the first PDT.

Design: Retrospective cohort study.

Participants: The study consisted of 167 patients with PCV who underwent PDT as their first treatment.

Methods: We targeted 638 single nucleotide polymorphisms (SNPs) in 42 possible susceptible genes for age-related macular degeneration to evaluate their relation to the effectiveness of PDT for PCV. For this evaluation, we used 2 methods: (1) survival analysis, with the retreatment-free period as the target; and (2) logistic regression test between the need for additional therapy within 3 months after the first PDT and the genotypes, with age, gender, smoking status, and greatest linear dimension (GLD) at baseline as covariates. The contributions of smoking status and GLD at baseline for the retreatment-free period also were evaluated. Contributions of these factors to visual prognosis were evaluated for 1 year after PDT.

Main Outcome Measures: Retreatment-free period after the first PDT for PCV. Secondary outcome measures included correlation of the susceptible factor to the retreatment requirement within the 3-month follow-up and the mean visual acuity change.

Results: In survival analyses, SERPINF1 rs12603825 showed a significant association with the retreatment-free period after the first PDT; those patients homozygous for the minor allele A of rs12603825 received additional treatment after PDT within significantly shorter times than those with other genotypes ($P = 0.0038$). There was no significant difference in the retreatment-free period between baseline GLD and smoking status. Retreatment within 3 months was required significantly more in patients with the AA genotype, even after taking into consideration the effect of clinical characteristics (age, gender), baseline PCV lesion size, and smoking status ($P = 0.0027$). Furthermore, patients with the AA genotype showed significantly worse visual prognosis after PDT ($P = 0.013$).

Conclusions: Pigment epithelium-derived factor (SERPINF1 or PEDF) polymorphisms may influence the initial response to and visual prognosis after PDT for PCV. Our findings may lead to understanding the pathogenesis of PCV and modification of the effects of PDT.

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Polypoidal choroidal vasculopathy (PCV) is observed frequently in Asian patients diagnosed with exudative age-related macular degeneration (AMD),[1,2] and PCV recently has been considered to be a separate clinical entity differing from neovascular AMD and other diseases associated with subretinal neovascularization.[3] Recent studies on the genetics of AMD and PCV have recognized them as complex diseases caused by the actions and interactions of numerous genes and environmental factors.[4–8]

Photodynamic therapy (PDT) with verteporfin was previously one of the main therapeutic options for neovascular AMD, and several studies have shown that the treatment effects of PDT for AMD vary according to the baseline composition, including lesion size of choroidal neovascularization, visual acuity, and genotype.[9–12] Many studies have reported that PDT is more effective in treating PCV than neovascular AMD,[13–15] although PDT for PCV often has to be repeated, either because of persistent disease or

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recurrence.[16,17] There are limited reports of the association between clinical or pathologic features and the response of PCV to PDT. When evaluating the effect of PDT for PCV, it is essential to consider both genetic and environmental factors, which has been done in the evaluation of AMD. As shown in the AMD study, studies have shown that smoking is associated with the development of PCV.[18–20]

The objectives of the current study were to discern whether the response of PCV to PDT was related to baseline clinical characteristics, smoking history, and genetic background by analyzing multiple single nucleotide polymorphisms (SNPs) and focusing primarily on the clinical retreatment-free period.

Materials and Methods

All procedures in this study adhered to the tenets of the Declaration of Helsinki. The institutional review board and ethics committee of each institute involved approved the protocols of this study. All patients were fully informed of the purpose and procedures of this study, and written consent was obtained from each patient.

Patients and Methods

The study consisted of 167 Japanese patients with PCV who underwent PDT at Kyoto University Hospital, Fukushima Medical University Hospital, or Kobe City Medical Center General Hospital between August 2004 and February 2009. All patients enrolled in the study met the criteria of PCV as proposed by the Japanese Study Group of Polypoidal Choroidal Vasculopathy.[21] Each subject underwent a complete ophthalmic examination, including measurement of best-corrected visual acuity, indirect ophthalmoscopy and slit-lamp biomicroscopy with a contact lens by a retina specialist, fluorescein angiography and indocyanine green angiography (ICGA), and optical coherence tomography. Best-corrected visual acuity was measured with a Landolt chart and converted to a logarithm of the minimal angle of resolution for statistical analysis. The inclusion criteria for this study were (1) diagnosis of PCV, (2) treatment with PDT as the first therapy, (3) age $\geq$ 50 years, (4) presence of a subfoveal lesion, and (5) best-corrected Snellen visual acuity equivalent of 20/200 to 20/40 at baseline. Exclusion criteria were (1) choroidal neovascularization caused by other diseases (e.g., pathologic myopia, uveitis) and (2) combined treatment (e.g., PDT in combination with antivascular endothelial growth factor drugs). If a patient had bilateral PCV treated with PDT, the eye treated earlier that fulfilled the criteria of this study was selected as the study eye for analysis. The greatest linear dimension (GLD) used for PDT was based on the ICGA findings and covered the entire PCV vascular lesion, including polypoidal lesions and branching vascular network vessels.[22] All patients received PDT with verteporfin following the standard protocol of treatment[23] except for determination of the GLD. At 3 months after the first PDT for PCV, all patients underwent a repeat ophthalmologic examination, including optical coherence tomography or fluorescein angiography and ICGA, on which the need for additional treatment was based. This sequence was followed during the follow-up time at intervals of patient visits to the outpatient clinic for up to 3 months. The retreatment-free period was calculated as the date of the first PDT to the date that the treating physician opted for additional treatment for a persistent or new lesion.

To evaluate the effect of GLD size, patients were divided into 3 groups according to the guidelines for PDT in Japan.[24] The GLD was $\leq$1800 μm in the first group, 1800 to 5400 μm in the second group, and $\geq$5400 μm in the third group. Information on smoking status (never smoked, ex-smoker, or current smoker) was obtained by self-reported questionnaire.

Two methods were used for the current PDT study: (1) survival analysis, with the retreatment-free period after the first PDT being the target; and (2) logistic regression test between 2 subgroups to evaluate the initial response to PDT. Because additional treatment with PDT is usually considered at 3 months after the first PDT,[14,25] the patients were classified into 1 of 2 groups by whether additional treatment was required within the first 3-month follow-up. Those patients who required additional therapy within 3 months after the first PDT (i.e., they continued to show an exudative lesion or had a worsened exudative lesion) were regarded as having a retreatment-free period of less than 3 months (Fig 1).

Multiplexing Single Nucleotide Polymorphism Analysis

To identify susceptible SNPs for the retreatment-free period after the first PDT, we used 31 of 160 PCV samples that were genotyped with the Illumina GoldenGate assay across 638 SNPs of 42 genes on a BeadStation 500G Genotyping System (Illumina, Inc., San Diego, CA); this was customized to evaluate possible AMD/PCV susceptible genes (listed in Table 1, available at http://aaojournal.org.). Haploview[26] software was used to infer the linkage disequilibrium (LD) in the targeted regions; among the candidate SNPs, LD indices (D' and r^2) were calculated with Haploview. To detect an association between the gene and the response to PDT, 1 representative SNP was chosen from each region. To confirm the positive association seen in the screening samples, 136 additional patients were genotyped for the SNPs with the Taqman SNP assay, which used the ABI PRISM 7700 system (Applied Biosystems, Foster City, CA). The 31 PCV samples used in the initial screening were also genotyped to validate concordance between the GoldenGate assay and the Taqman assay. Samples with a low successful call rate (<95%) were excluded from the study.

Statistical Analyses

Survival analysis was conducted using Kaplan–Meier methods to estimate differences among genotypes in the retreatment-free period after the first PDT. The retreatment-free period of the patients with no additional treatment was censored at the time of last contact. To detect differences in survival, Breslow–Gehan–Wilcoxon tests were used. When a significant association was found, the best fitting model (additive, dominant, or recessive) was then investigated. The Hardy–Weinberg equilibrium for genotypic distribution was evaluated using the Hardy–Weinberg equilibrium exact test. Descriptive statistics for all demographic and clinical variables were calculated and comparisons were made using the unpaired t test for means with continuous data (e.g., age) and the chi-square test for categoric data (e.g., gender). Logistic regression analysis was used to evaluate the association for adjusting age, gender, smoking status, GLD, and genotype considering the best fitting model. Visual prognosis after treatment was compared by a repeated-measures analysis of variance. P value correction was performed with the Bonferroni method using the ratio of the number of all genotyped SNPs in the screening procedure. For overall survival analysis, P value correction was performed with the Bonferroni method using the ratio of the number of

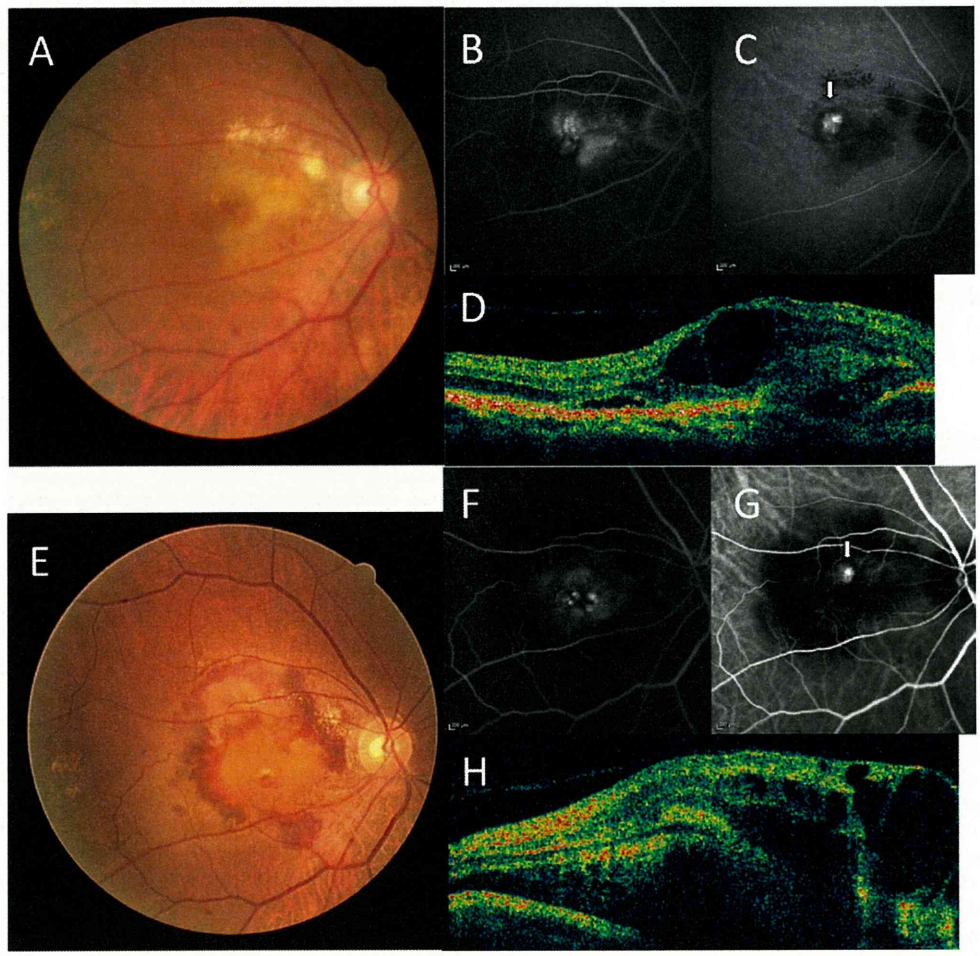

Figure 1. Fundus photographs **(A, E)**, fluorescein angiographs **(B, F)**, indocyanine green angiographs **(C, G)**, and optical coherence tomographs **(D, H)** of a patient who received additional treatment within 3 months after the first PDT. This 72-year-old man with PCV in his right eye underwent PDT as his first therapy. Before the treatment **(A–D)**, his best-corrected visual acuity was 20/200 and ICGA revealed an active polyp **(C,** *white arrow*). Seventy-six days after PDT **(E–H)**, the treating physician opted to perform additional treatment because his best-corrected visual acuity decreased to 20/400, a new macular hemorrhage appeared, and the polyp **(G,** *white arrow*) and exudative lesion **(F, H)** remained active.

selected SNPs from the screening. Significance was defined at the 5% level.

Results

A total of 167 patients with PCV who underwent PDT as their first therapy at 1 of 3 institutes were enrolled in the current study. Demographic and clinical characteristics of each patient by institute involved are shown in Table 2.

Survival Analysis for the Retreatment-free Period

Of the 160 patients with PCV who were genotyped by the Illumina GoldenGate assay, which launches 638 SNPs across 42 genes in our previous study, 31 met the inclusion criteria of the current PDT study and were used for the screening of genotype data. Because 57 SNPs with no call or scattered or overlapping clusters were excluded from the analysis, 581 SNPs were evaluated by survival analysis with the retreatment-free period. We identified 6 SNPs in 4 genes (FBLN5, CX3CR1, SERPINF1, and TLR4), with the P value adjusted for multiple testing <0.05 (Table 3). At SERPINF1 gene, rs12103559 and rs1894286 were in strong LD (pair-wise D' = 1.0 and r^2 = 1.0). By considering the LD and minor allele frequency of 3 SNPs of this region, we selected rs12603825 as the representative SNP of the SERPINF1 gene and tested a total of 4 SNPs in other patients. A total of 136 additional patients from the 3 institutes were genotyped by the Taqman method. Genotyping success rates of the 4 SNP markers in the additional 136 samples were greater than 98.8%. In overall survival analyses, SERPINF1 rs12603825 showed a significant association with the retreatment-free period (P = 0.0117). Patients homozygous for the minor allele of rs12603825 (i.e., a recessive model) were given an additional treatment after the first PDT in significantly shorter time periods than were the other genotypes (P = 0.0038), and this association remained significant after a permutation procedure for multiple test correction (corrected P = 0.015) (Table 3, Fig 2).

There was no significant difference in the retreatment-free period among the 3 GLD groups and the smoking status groups

Table 2. Baseline Characteristics of the Study Population

	Kyoto	Kobe City	Fukushima	Total
No. of patients	79	51	37	167
Mean age (yrs)	73.01	70.92	70.64	71.86
Gender				
Women	21 (26.6)	18 (35.3)	8 (21.6)	47 (28.1)
Men	58 (73.4)	33 (64.7)	29 (78.4)	120 (71.9)
Mean visual acuity (logMAR)	0.552	0.605	0.573	0.573
Smoking history				
Never	26 (36.1)	22 (44.9)	15 (40.5)	63 (39.9)
Previous	27 (37.5)	21 (42.9)	12 (32.5)	60 (38.0)
Current	19 (26.4)	6 (12.2)	10 (27.0)	35 (22.1)
Mean follow-up (days)	1156.4	1084.6	1198.8	1143.8
GLD				
≤1800 μm	13 (16.9)	3 (6.3)	7 (18.9)	23 (14.2)
1800–5400 μm	60 (77.9)	41 (85.4)	28 (75.7)	129 (79.6)
>5400 μm	4 (5.2)	4 (8.3)	2 (6.4)	10 (6.2)
Mean (μm)	2817.5	3476.7	3150.5	3209.0

GLD = greatest linear dimension; logMAR = logarithm of the minimal angle of resolution.

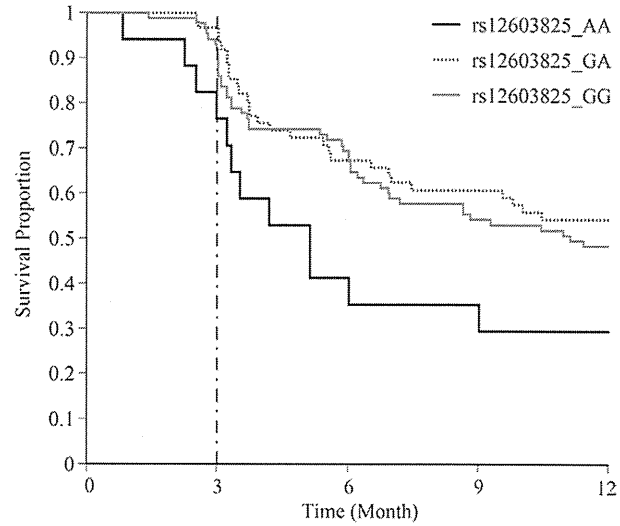

Figure 2. Overall survival analysis curve for the retreatment-free period among patients with the genotype of rs12603825. Patients with AA genotype were administered additional treatment after the first PDT within a significantly shorter period of time than those with other genotypes (P = 0.0038).

based on overall survival analysis (P = 0.214 and 0.166, respectively), although borderline evidence of an association was observed between never smoked and ex-smokers plus current smokers (P = 0.060) (Fig 3).

Effect of Photodynamic Therapy

We investigated the association between the susceptible SNP for the retreatment-free period and initial clinical response to PDT. Of the 167 eyes eligible for this analysis, 13 required additional treatment within 3 months after their first PDT, and 150 did not (Table 4); 4 patients with a follow-up of less than 3 months were excluded. Logistic regression analysis revealed an independent association between SERPINF1 rs12603825 and these subgroups for age, gender, smoking status, and GLD (P = 0.0027). We next conducted a survival analysis of the retreatment-free period in 150 PCV eyes that had been inactivated with a single PDT to evaluate whether this SNP was associated with recurrence of PCV, but there was no significant difference in the retreatment-free period among genotypes of rs12603825 (P = 0.36), even after adjusting the recessive model (P = 0.16) (Fig 4, available at http://aaojournal.org.).

Visual Outcomes

The visual outcomes after PDT were examined. Seventy-five patients from Kyoto University Hospital were followed up for more than 1 year after their first treatment. Although no significant difference in visual outcomes was observed in lesion size or smoking status (P = 0.523 and 0.468, respectively) (Fig 5, available at http://aaojournal.org.), visual outcomes of patients with the AA genotype of SERPINF1 rs12603825 were significantly worse than those with other genotypes (P = 0.013) (Fig 6).

Table 3. Association Results of Survival Analysis from Screening and Overall Genotyping

						Screening Sample (n = 31)	All Sample (n = 167)		
SNP	Chr*	Position*	Ref.†	Var.†	Gene*	*Corrected P*§	*MAF*	*HWE P*‡	*Nominal P*
rs17732513	14	91456132	C	T	FBLN5	0.000129	0.33	0.39	0.834
rs17793056	3	39284219	C	T	CX3CR1	0.00482	0.31	0.54	0.198
rs12603825	17	1620155	G	A	SERPINF1	0.000195	0.28	0.65	0.0117
rs12103559	17	1622128	G	A	SERPINF1	0.000107	–	–	–
rs1894286	17	1623659	C	T	SERPINF1	0.000162	–	–	–
rs11536889	9	119517952	G	C	TLR4	0.00021	0.23	0.24	0.733
Best-fitting model for significant results									
rs12603825	Recessive model								0.0038

HWE = Hardy–Weinberg equilibrium; MAF = minor allele frequency; SNP = single nucleotide polymorphism.
*Chromosome and position of markers refer to NCBI Build 36.1.
†Ref. and Var. are the reference and variant nucleotides, respectively, that are defined on the reference sequence of NCBI Build 36.1.
‡Hardy–Weinberg equilibrium for genotypic distribution was examined by the Hardy–Weinberg equilibrium exact test.
§P value corrected for multiple testing using the Bonferroni method.