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Toyoda, H., Kumada, T., Tada, T., Hayashi, K., Honda, T., Katano, Y., Goto, H., Kawaguchi, T., Murakami, Y. and Matsuda, F.	Antiviral combination therapy with peginterferon and ribavirin does not induce a therapeutically resistant mutation in the HCV core region regardless of genetic polymorphism near the IL28B gene.	*J. Med. Virol.*	83	1559-1564	2011
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Kawaguchi, T. *et al.*	Genetic Polymorphisms of the Human *PNPLA3* Gene are Strongly Associated with Severity of Non-Alcoholic Fatty liver Disease in Japanese.	*PLoS One.* (in the press.)			2012

共著者が10人を超える場合は、筆頭著者名 *et al.* で記載

Ⅶ. 学会発表に関する一覧表

研究代表者：松田　文彦

発表者名	演題名	学会名	会場	日時
Terao, C., Yamada, R., Ohmura, K., Kochi, Y., Okada, Y., Nakamura, Y., Yamamoto, K., Melchers, I. Lathrop, M., Mimori, T. and Matsuda, F.	A haplotype of the human AIRE gene is associated with the risk for rheumatoid arthritis in Japanese population.	EULAR Congress 2011	London, UK	May 27, 2011
松田　文彦	予防医療にとってのバイオマーカー探索の重要性～長浜コホート研究におけるオミックス解析～	第84回日本生化学会バイオインダストリーセミナー	国立京都国際会館（京都）	2011年9月23日
松田　文彦	先制医療の実現を目指した21世紀型ゲノムコホート研究	JST イノベーションサテライト高知研究成果報告会 in 愛媛	愛媛大学南加記念ホール（松山）	2012年1月21日

研究分担者：山田　亮

発表者名	演題名	学会名	会場	日時
Yamada, R., Terao, C., Kawaguchi, T. and Narahara, M.	Evaluation of power for linkage disequilibrium mapping.	International Genetic Epidemiology Society Meeting 2011	Heidelberg Germany	Sep.19, 2011

研究分担者：寺尾　知可史

発表者名	演題名	学会名	会場	日時
Terao, C., Yamada, R., Ohmura, K., Kochi, Y., Okada, Y., Nakamura, Y., Yamamoto, K., Melchers, I. Lathrop, M., Mimori, T. and Matsuda, F.	A haplotype of the human AIRE gene is associated with the risk for rheumatoid arthritis in Japanese population.	EULAR Congress 2011	London, UK	May 27, 2011
寺尾　知可史	関節リウマチの病型と疾患感受性遺伝子、及びその機能解析	日本臨床免疫学会	京王プラザホテル（東京）	2011年9月15日
寺尾　知可史、大村浩一郎、高地 雄太、猪狩 勝則、丸屋 悦子、片山 昌紀、島田浩太、村澤 章、本荘茂、高杉 潔、松尾 恵太郎、田島 和雄、鈴木 亜香里、山本 一彦、桃原 茂樹、山中寿、山田 亮、佐治 博夫、松田　文彦、三森経世	抗CCP抗体陰性関節リウマチ関連HLA-DRB1アレルおよびディプロタイプの同定	日本臨床免疫学会	京王プラザホテル（東京）	2011年9月15日

Terao, C., Ohmura, K., Kochi, Y., Ikari, K., Maruya, E., Katayama, M., Shimada, K., Murasawa, A., Honjo, S., Takasugi, K., Matsuo, K., Tajima, K., Suzuki, A., Yamamoto, K., Momohara, S., Yamanaka, H., Yamada, R., Saji, H., Matsuda, F. and Mimori, T.	A Large-Scale Association Study Identified Multiple HLA-DRB1 Alleles Associated with Anti-Citrullinated Peptide Antibody Negative Rheumatoid Arthritis in Japanese.	Annual Congress of American College of Rheumatology	Chicago, USA	Nov. 6, 2011

Ⅷ．研究成果による特許等の知的財産権の出願・登録状況

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Ⅸ. 研究成果の刊行物・別刷

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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

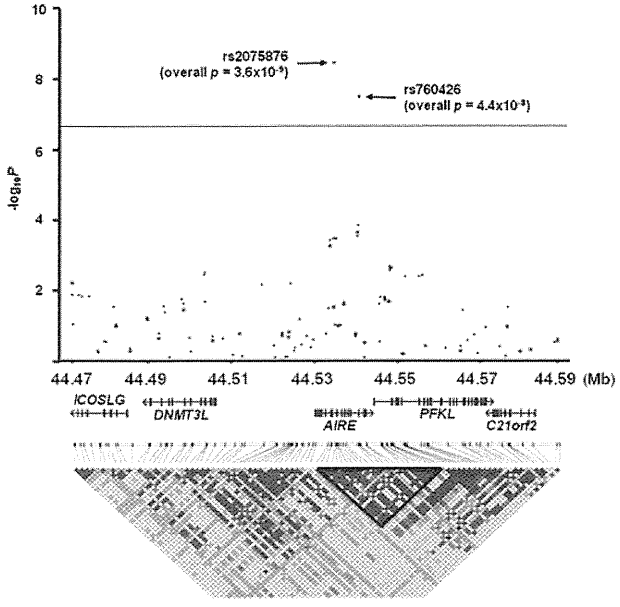

Figure 1. A schematic organization of the human *AIRE* locus at 21q22. *P*-values of the initial genome scan using collection 1 were calculated by the Trend χ^2 test and plotted in red circles. The blue circles indicate *P*-values obtained by imputation using HapMap Japanese results. Overall *P*-values 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.

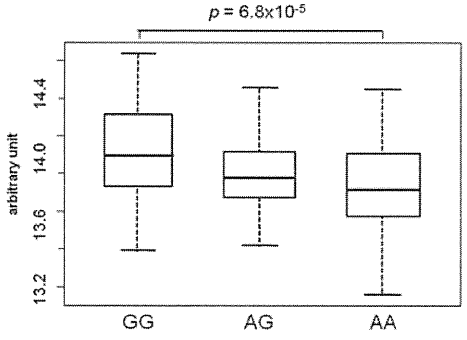

Figure 2. Comparison of the expression levels of *AIRE* among three subgroups of cell lines according to the genotype of rs2075876. 'G' and 'A' correspond, respectively, to the risk and the alternative alleles of rs2075876.

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 Joncheere–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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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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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

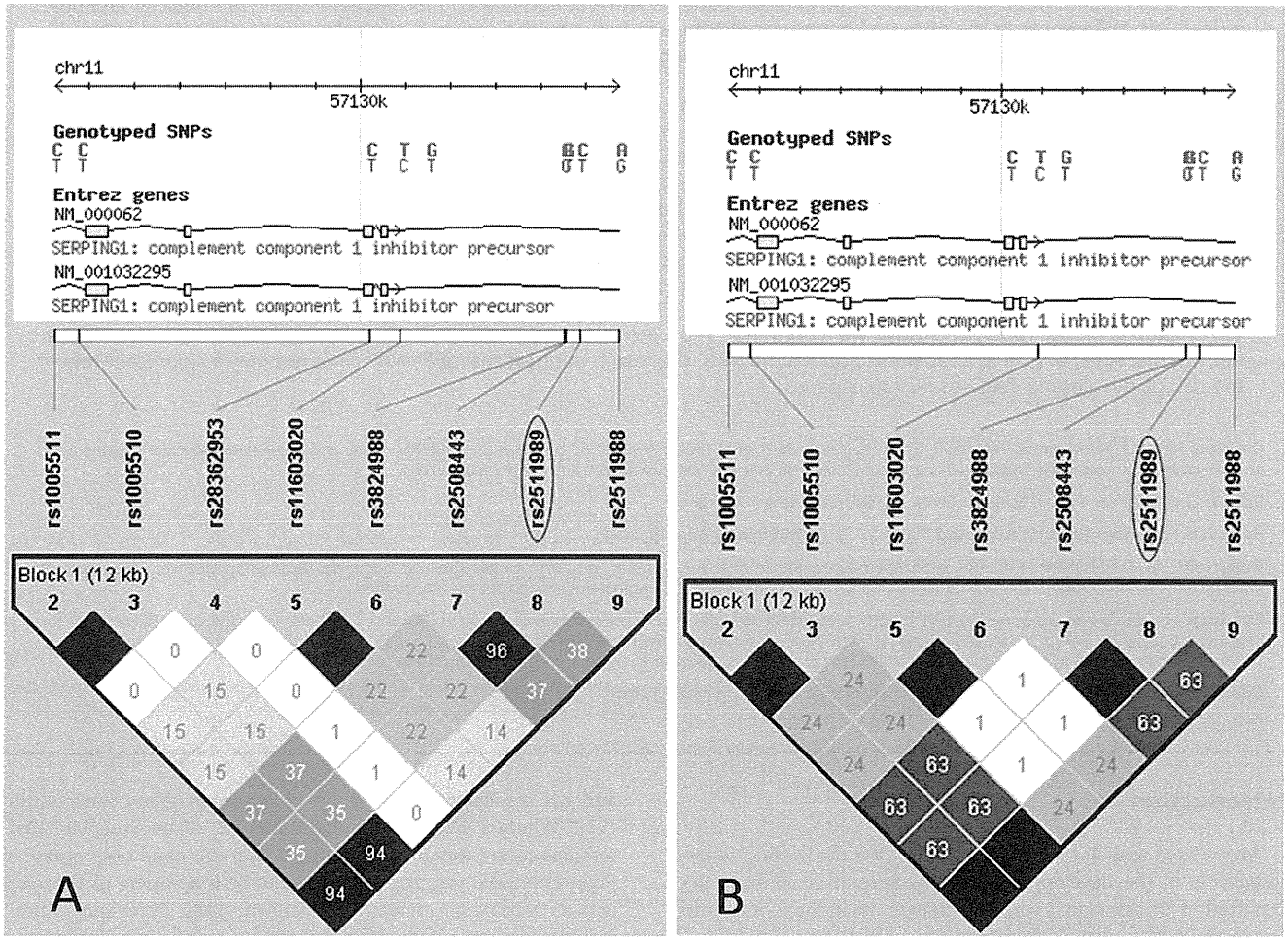

Figure 1. Linkage disequilibrium (LD) structure across the complement component 1 inhibitor (*SERPING1*) gene in Caucasian and Japanese populations. Genotype data were retrieved from HapMap CEU (Utah residents with ancestry from northern and western Europe; A) and JPT (Japanese in Tokyo, Japan; B) data 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 HumanHap 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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