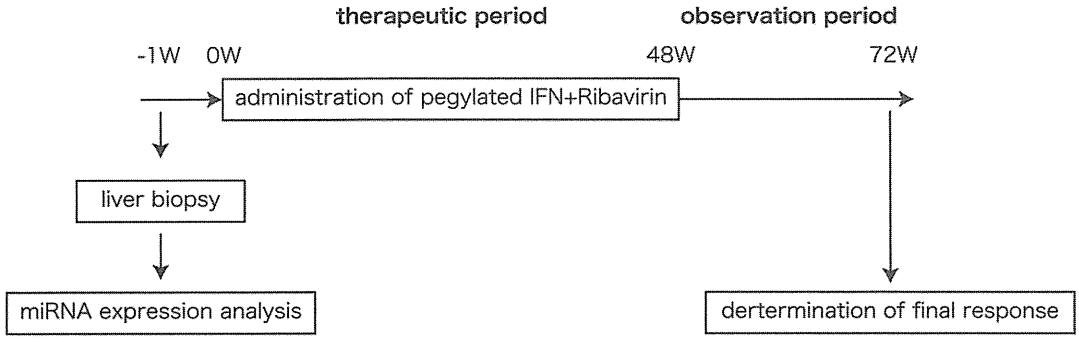

Figure 5. Study design and time line of response to combination therapy. The time frame of liver biopsy, microarray analysis, therapeutic period, observation period after combination therapy, and the judging of clinical outcome is shown.
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cDNA microarray

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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ORIGINAL ARTICLE

A trans-ethnic genetic study of rheumatoid arthritis identified *FCGR2A* as a candidate common risk factor in Japanese and European populations

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Abstract Rheumatoid arthritis (RA) is a common systemic autoimmune disease and its onset and prognosis are controlled by genetic, immunological, and environmental factors. The *HLA* locus, particularly *HLA-DRB1*, is its strongest genetic risk determinant across ethnicities. Several other genes, including *PTPN22* and *PADI4*, show modest association with RA. However, they cover only a part of its genetic components and their relative contribution is different between populations. To identify novel genetic determinants, we took a candidate gene approach in a trans-ethnic manner. After critical selection of 169 genes based on their immunological function, we performed SNP discovery of these genes by the resequencing of exons and surrounding areas using European and Japanese DNAs. We then generated a panel of 1,509 SNPs for case–control association study in both populations. The DerSimonian–Laird test for meta-analysis, using the combined results of the two populations, identified rs7551957 at the 5′-flanking region of the low-affinity Fc-gamma receptor IIa (*FCGR2A*) gene as the strongest candidate for the association ($p = 8.6 \times 10^{-5}$, odds ratio = 1.58 with 95%CI 1.25–1.99). Suggestive signals were also obtained for three SNPs in the dihydropyrimidine dehydrogenase (*DPYD*) gene (rs6685859; $p = 1.3 \times 10^{-4}$, rs7550959; $p = 1.5 \times 10^{-4}$ and rs7531138; $p = 1.7 \times 10^{-4}$) and an intronic SNP, rs2269310, of the erythrocytic spectrin beta (*SPTB*) gene ($p = 7.9 \times 10^{-4}$).

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Introduction

Rheumatoid arthritis (RA [OMIM: 180300]) is a systemic autoimmune disease [1], and is one of the most common forms of inflammatory arthritis, affecting up to 1% of the adult population [2]. Symptoms are chronic, destructive, and debilitating arthritis with a variation in the number of clinical features, such as the presence of autoantibody and joint erosions [3]. Clinical manifestation of RA is related to the development of a variety of autoantibodies, including antibodies to citrullinated peptide antigens and rheumatoid factor, although their pathological role is still unclear [4, 5].

Compelling evidence from genome-wide association (GWA) studies demonstrated that the *HLA* locus is the strongest genetic determinant beyond ethnicity [6]. However, the *HLA* locus contributes to only approximately 37% of the overall genetic susceptibility [7], suggesting the presence of other genes that are genetically associated with pathogenesis, clinical phenotype, and disease heterogeneity. Moreover, the relative contribution of RA-related genes is considered to be different between ethnicities. Indeed, the *PTPN22* gene was shown to be associated with RA in populations of European descent, but not in Asians [8]. Similarly, the *PADI4* gene showed a strong association with RA in Asians [9], but the association was much weaker in Europeans. Intronic SNPs in the *STAT4* gene were identified to be strongly associated with RA in Europeans [10], and their modest statistical association was confirmed in Asians [11], providing the first example of non-*HLA* RA-associated genes in two major ethnicities.

Importantly, however, very few hypothesis-independent GWA studies have succeeded in the identification of non-*HLA* genes associated with RA. A genome scan by the Wellcome Trust study was only able to successfully identify *HLA* and *PTPN22* loci as genome-wide significant [6]. This may be due to the disease heterogeneity with phenocopies, and the insufficient power to detect genes with modest effects [12]. Indeed, SNPs associated with RA in non-*HLA* genes showed only intermediate allelic odds ratios (ORs) of 1.3–2.0. To overcome such shortcomings, studies need to be enlarged, enrolling several thousand patients and a similar number of controls. Alternatively, a candidate gene approach, which focuses on genes critically selected by their biological function, can be an efficient and more cost-effective strategy because SNPs that are associated with RA are mostly within genes that are functionally implicated in the pathogenicity of RA [13]. Above all, comparative genetic analysis across different ethnic groups is ideal.

In this study, we performed a trans-ethnic case–control association study of RA in Japanese and European RA patients by applying an SNP marker panel of 169 genes related to the immune system and drug metabolisms designed for genetic approaches of various immune-related diseases. A total of 1,509 SNPs were exhaustively genotyped in 238 RA cases and 184 controls of Japanese origin, as well as in 182 cases and 273 controls of German and French populations.

Materials and methods

Study populations

Sample collection and genotyping was initiated after approval from the local ethical committees. Written informed consent was obtained from all patients and control individuals after adequate explanation of the study. The disease subjects were recruited in Germany and in Japan. The 420 patients with RA consisted of 182 Germans from the Freiburg area and 238 Japanese from the Kyoto and Kobe areas. All subjects satisfied the American Rheumatism Association's revised criteria (1987) for classification of RA [14]. For population controls, 91 German subjects from the Freiburg area and 184 Japanese subjects were recruited. Additionally, 182 French Caucasian controls from the Epidemiological study on the Genetics and Environment of Asthma (EGEA) were used.

Selection of candidate genes and single nucleotide polymorphisms

A total of 169 genes on autosomes were chosen for case–control association studies of different immune-related diseases, such as autoimmune diseases, immune deficiencies, and allergies, as well as drug response in such diseases. The selection was made according to their biological function in the immune system, such as cytokines and their receptors, cell adhesion molecules, transcriptional factors, and genes involved in signal transduction and cell–cell communication (Table 1). Genes already known for their association with the diseases, in addition to those shown to be related to drug metabolism prior to 2006 when the SNP discovery was performed, were also included in the panel.

The identification of SNPs was performed by an exhaustive resequencing of exons and flanking regions of these genes, using 32 each of French and Japanese control DNAs. Additional SNPs located in the linkage disequilibrium (LD) blocks covering the 169 genes in the International HapMap Project were also included. Among approximately 10,000 SNPs, those having a minor allele frequency (MAF) greater than 0.05 were tested using the

Table 1 Classification of candidate genes by their immunological function

Signal transduction	*ABCB1, ANK1, APOH, CARD4, CARD15, CBLB, CCND2, CCND2P, CCND3, CCNDBP1, CDC37, CDKN1A, CDKN2A2, EIF2AK2, EVI5L, GAB1, GAB2, GRB2, HCK, HSPCA, HSPCB, ITPKB, KIR3DL2, LAT, LCK, LCP2, MBL2, PTPN11, PTPN13, RASGRP1, SOCS3, SOS1, SYK, TANK, TGFB1, VAV1, VAV2, VAV3, XPO1, ZAP70, FTH1, NPM1*
Transcription factors	*AIRE, APOBEC3G, CCNT1, CCNT2, CDK7, CDK9, CD3EAP, NFAT5, NFATC1, NFATC2, NFATC3, NFATC4, STAT1, TBX1, TGFB1I1, TRIM21, TROVE2, TSC22D1, YBX1*
Cell–cell communication	*CD274, CD28, CD36, CD38, CD3Z, CD4, CD58, CD8A, CD8B, COMMD1, CTLA4, FCGR2A, FCGR3A, FER, ICAM2, ICAM3, ITGA1, ITGA2, ITGA4, ITGAL, ITGAV, ITGB1, ITGB4, PECAM1, SELL, TGFB, VCAM1, VIL2, VILL*
Cytokines and receptors	*FAS, FASLG, IFNA2, IFNAR1, IFNG, IFNGR1, IFNGR2, IL10, IL10RA, IL10RB, IL12A, IL12B, IL13, IL15, IL18, IL1A, IL1B, IL1R1, IL1RN, IL2, IL21R, IL2RA, IL2RB, IL4, IL4R, IL6, IL6R, IL6ST, IL7, IL8, L8RA, IL8RB, KIT, TNFRSF11A, TNFRSF13C, TNFRSF18, TNFRSF1A, TNFRSF1B, TNFRSF7, TNFSF15, TNFSF7*
Metabolism	*ALDH2, ANPEP, BCHE, CYP2C19, CYP2D6, CYP2E1, DPP4, DPYD, EPB41, FKBP4, GSTM4, GSTP1, NAT2, NOS2A, NQO1, NQO2, PLCG1, PPIA, SOD1, SOD2, SPTA1, SPTB, TPMT, TRIM5*
Regulation factor of immune response	*GYPA, GYPB, GYPC, OAS1, OAS2, OAS3, THY1*
Genes in the *HLA* locus	*HLA-DOB, PSMB8, PSMB9, TAP2, LTA, CSNK2B*
Other	*C1QBP*: role in complement pathway (binding C1q protein)

GoldenGate technology marker panel selection program by Illumina Inc. (San Diego, CA, USA), and only markers with Illumina Design Scores (IDS) greater than 0.4 were chosen. SNPs in the same LD block with pairwise-r^2 greater than 0.8 were divided into subgroups, and the SNP which showed the highest IDS in each subgroup was selected. When there were multiple SNPs sharing the highest IDS, the one with the highest MAF was chosen. Following these selection steps, a total of 1,509 SNPs were finally chosen as tag SNP markers for the genotyping study.

Genotyping and quality control

Genomic DNAs were extracted from fresh peripheral blood mononuclear cells or from EBV-transformed lymphoblasts in accordance with protocols approved by the appropriate authority.

A panel of 1,509 SNPs was genotyped using a GoldenGate assay on an Illumina BeadArray genotyping platform according to the manufacturer's instructions. DNA samples were tracked using a laboratory information management system (LIMS), and genotypes were called using the Genotyping module of BeadStudio 2 software (Illumina Inc.). The results obtained were filtered on the basis of genotype call rates (success rates of >90% for marker, >95% for DNA sample).

Statistical analysis

Genotype distribution was evaluated by the Hardy–Weinberg equilibrium in the control group (χ^2 test), and the markers with p values less than 0.05 were excluded from the tests [15]. Allele frequency of each SNP was compared for association with RA between cases and controls in each population using the trend χ^2 test and a non-biased exact trend test [16], as well as the DerSimonian–Laird test for meta-analysis, using the combined results of two populations [17].

Expression analyses of the FCGR gene family

A gene-expression dataset in lymphoblastoid cell lines derived from 210 unrelated HapMap populations (GSE6536) was obtained from the Gene Expression Omnibus (GEO) database [18]. The correlation between the expression data of *FCGR1A*, *2A*, *2B*, *2C*, *3A*, and *3B*, and rs7551957 genotypes of 268 individuals (89 European, 44 Japanese, 45 Chinese, and 90 West African Yoruba) available from the HapMap phase 2 data, was examined using the calculation program recommended by the GEO. The association p values were obtained by the Joncheere–Terepstra method using R software or SPSS (version 18).

Results

Association analysis in Japanese and European populations

In order to identify genetic loci associated with susceptibility to RA, 1,509 SNP markers representing 169 candidate genes with an average number of 8.9 SNPs (ranging from 1 to 75) per gene, were genotyped in DNA samples of Japanese and European origins (summarized in Table 1). After quality control of the results, 1,375 and 1,330 SNPs in the Japanese and Europeans, respectively, were tested for association.

In the Japanese case–control analysis, a total of 41 SNPs tagging 26 genes showed a nominal p value <0.005, of which 13 SNPs belonging to ten genes were less than 0.001 (Supplementary Table 1). Of those 41 SNPs, rs17587 in exon4 of the *PSMB9* gene was non-synonymous (arginine to histidine), while the others were either in the gene-flanking regions (11 SNPs), 3′-untranslated region (one SNP) or in introns (28 SNPs). The association analysis using the European sample set identified a putative difference in allele frequency of 20 SNPs in 16 genes with a nominal p value <0.005, of which five SNPs corresponding to three genes showed a p value <0.001 (Supplementary Table 2). One synonymous SNP, rs2302872, was located in exon14 of the *DPP4* gene, while the others were either in the gene-flanking regions (11 SNPs) or in introns (nine SNPs). Markers that are located in the *HLA* locus showed association with RA in both populations. The strongest p value was observed for rs1894408, located adjacent to the *HLA-DOB* gene ($p = 7.5 \times 10^{-5}$) in the Japanese, and for rs1383266 near the *PSMB9* gene ($p = 0.0011$) in the Europeans (Supplementary Tables 1 and 2).

Meta-analysis using combined results of Japanese and European studies

To identify the genes/variants that are associated with RA in both Japanese and Europeans, we performed a meta-analysis combining the genotyping results of the two populations by using the DerSimonian–Laird test. In total, 22 SNPs corresponding to 15 genes showed a p value (*DLp*) less than 0.01 (Table 2). Among them, five SNPs, namely, rs6685859, rs7551957, rs2269304, rs4819522, and rs5746834, showed relatively modest association with RA in both populations (p = <0.05).

The strongest association was obtained for rs7551957 of the *FCGR2A* (low affinity Fc-gamma receptor IIa) gene, with $DLp = 8.6 \times 10^{-5}$ and OR = 1.58 with 95% confidence interval (CI) 1.25–1.99. This SNP showed a strong association with RA in both populations (trend χ^2 p = 0.0035 in the Japanese and $p = 0.0062$ in the Europeans). Three suggestive signals were obtained in the *DPYD* (dihydropyrimidine dehydrogenase) gene by meta-analysis: rs6685859 ($DLp = 1.3 \times 10^{-4}$ and OR = 1.64 with CI 1.27–2.12), rs7550959 ($DLp = 1.5 \times 10^{-4}$ and OR = 1.52 with CI 1.52–1.89), rs7531138 ($DLp = 1.7 \times 10^{-4}$ and OR = 1.51 with CI 1.22–1.88; Table 2). One SNP, rs2269310, in intron1 of the *SPTB* (erythrocytic spectrin beta) gene also showed a modest association ($DLp = 7.9 \times 10^{-4}$ and OR = 1.51 with CI 1.18–1.92) with RA.

Discussion

To our knowledge, this is the first report of a candidate gene-based trans-ethnic association analysis of RA. A panel of 169 genes that play important roles in immune response was extensively studied by SNP genotyping in Japanese and European populations. The *HLA* gene cluster is the major gene locus that contributes to RA susceptibility, and the *HLA-DRB1* gene is reported to be the strongest candidate [6]. In our study, the association between SNPs in the *HLA* region and RA was confirmed in both populations when the results were analyzed separately. However, these SNPs were not found to be significant in the meta-analysis. It is well known that the LD structure in this region is markedly different between ethnic groups. Hence, a larger sample size is required for sufficient statistical power to detect the same SNP as significant in both populations. Moreover, the panel used in this study only contains a single *HLA* gene, *HLA-DOB*, and not the others that are proven to be associated with RA.

The strongest association was obtained in the *FCGR2A* gene, which is located within the cluster of low affinity Fc-gamma receptor genes (*FCGR2A*, *FCGR3A*, *FCGR2C*, *FCGR3B*, and *FCGR2B*) on chromosome 1q22-23 [19]. Rs7551957 showed a strong association with RA in the two ethnicities ($p = 0.0035$ in the Japanese and $p = 0.0062$ in the Europeans), which was further confirmed by meta-analysis ($DLp = 8.6 \times 10^{-5}$;Table 2). In both populations, the variant allele frequency is higher in the RA cases (0.170 in the Japanese and 0.466 in the Europeans) compared with the controls (0.098 in the Japanese and 0.372 in the Europeans). Another SNP in the *FCGR2A* gene, rs1801274, showed a modest association in the meta-analysis ($DLp = 0.0021$). The difference in trend of p value between the two populations ($p = 0.098$ in the Japanese and $p = 0.011$ in the Europeans) may well be explained by the difference in the allele frequencies (0.225 in the Japanese and 0.500 in the Europeans). In line with our results, a recent meta-analysis of European genome-wide association studies confirmed the association of *FCGR2A* with RA risk ($p = 0.0004$) [20]. FCGRs are expressed on the surface of cells involved in the immune

Table 2 Single nucleotide polymorphisms associated with rheumatoid arthritis in meta-analysis using the combined results of Japanese and European populations

dbSNP ID	Chr.	Gene	Location	Nucleotide		Amino acid		Japanese freq. A2		Nominal trend *p*	European freq. A2		Nominal trend *p*	Meta-analysis	
				Ref. (A1)	Var. (A2)	Ref.	Var.	Case	Cont		Case	Cont		*DLp*	OR (95%CI)
rs6685859	1p21.3	*DPYD*	intron16	G	C			0.023	0.050	0.042	0.506	0.619	5.1×10^{-4}	1.3×10^{-4}	1.64 (1.27–2.12)
rs7550959			intron13	G	A			0.166	0.215	0.079	0.387	0.504	6.4×10^{-4}	1.5×10^{-4}	1.52 (1.22–1.89)
rs7531138			intron13	T	A			0.168	0.215	0.090	0.392	0.509	5.9×10^{-4}	1.7×10^{-4}	1.51 (1.22–1.88)
rs7551957	1q23.3	*FCGR2A*	5′-flanking	T	C			0.098	0.170	0.0035	0.372	0.466	0.0062	8.6×10^{-5}	1.58 (1.25–1.99)
rs1801274			exon4	A	G	His	Arg	0.176	0.225	0.098	0.412	0.500	0.011	0.0021	1.40 (1.13–1.74)
rs697846	1q42.12	*ITPKB*	3′-flanking	A	G			0.202	0.283	0.010	0.140	0.181	0.14	0.0042	1.44 (1.12–1.85)
rs1050567	2p15	*XPO1*	3′UTR	C	T			0.236	0.331	0.0053	0.102	0.133	0.17	0.0021	1.50 (1.15–1.94)
rs3770768	2p22.2	*EIF2AK2*	intron13	G	A			0.004	0.014	0.15	0.099	0.155	0.015	0.0082	1.72 (1.15–2.58)
rs926169	2q33.2	*CTLA4*	5′-flanking	G*	T			0.660	0.597	0.091	0.423	0.357	0.047	0.0087	1.31 (1.07–1.61)
rs2686399	3q.26.1	*BCHE*	3′-flanking	C*	G			0.844	0.819	0.36	0.732	0.648	0.0064	0.0077	1.37 (1.08–1.73)
rs6946119	7q21.12	*ABCB1*	3′-flanking	T	C			0.121	0.159	0.12	0.238	0.304	0.028	0.0076	1.39 (1.09–1.78)
rs2269310	14q23.3	*SPTB*	intron26	G	A			0.368	0.475	0.0054	0.108	0.147	0.073	7.9×10^{-4}	1.51 (1.18–1.92)
rs229670			intron1	A*	C			0.413	0.349	0.061	0.268	0.177	0.0011	0.0024	1.48 (1.14–1.91)
rs2269304			intron14	C*	A			0.118	0.075	0.049	0.213	0.153	0.018	0.0025	1.54 (1.16–2.05)
rs4787426	16p12.1	*IL4R*	3′-flanking	T	G			0.104	0.170	0.012	0.122	0.153	0.21	0.0076	1.49 (1.11–2.01)
rs4968681	17q23.3	*ICAM2*	5′-flanking	G	A			0.323	0.409	0.016	0.326	0.371	0.17	0.0082	1.32 (1.07–1.62)
rs7503550	17q23.3	*PECAM1*	intron4	G*	A			0.529	0.467	0.096	0.566	0.474	0.0072	0.0018	1.37 (1.12–1.67)
rs537188	19p13.2	*EVI5L*	intron11	G	A			0.085	0.105	0.34	0.140	0.212	0.0032	0.0045	1.52 (1.13–2.04)
rs347033	19p13.3	*VAV1*	intron4	T	C			0.144	0.227	0.0045	0.163	0.205	0.11	0.0028	1.50 (1.15–1.97)
rs2866370	20q12	*PLCG1*	intron1	G	A			0.039	0.077	0.022	0.050	0.078	0.080	0.0054	1.81 (1.19–2.75)
rs4819522	22q11.21	*TBX1*	exon9	C	T	Thr	Met	0.058	0.097	0.042	0.182	0.254	0.013	0.0014	1.58 (1.19–2.09)
rs5746834			3′-flanking	G	T			0.037	0.069	0.039	0.190	0.248	0.039	0.0049	1.52 (1.13–2.03)

SNPs with a *p* value (*DLp*) less than 0.01 according to the DerSimonian–Laird test are listed with odds ratio (OR) and 95% confidence interval (95%CI).

The risk allele is indicated by an asterisk if it is the reference allele.

Arg arginine, *His* histidine, *Met* methionine, *Thr* threonine, *Ref.* reference allele, *Var.* variant allele

system, and participate in diverse functions such as phagocytosis of immune complexes and modulation of antibody production by B cells. Various genetic polymorphisms of these receptors were reported to be associated with several autoimmune diseases [21, 22]. In particular, *FCGR2A* was shown to be associated to systemic lupus erythematosus [23, 24]. In the mouse model, *Fcgr3*-deficient hosts exhibit resistance to arthritis induced by collagen type II or anti-glucose-6-phosphate isomerase antibody [25]. In contrast, mice deficient for *Fcgr2b* lead to increased susceptibility to collagen-induced arthritis [26]. Theses findings suggest that expression of FCGRs on synovial cells may contribute to the antibody-triggered inflammation in joints [27]. In addition, we examined the association of rs7551957 with the expression level of the other *FCGR* family members according to the four population groups (European, Japanese, Chinese, and West African Yoruba), but linear regression analyses failed to reveal any significant associations (results not shown).

Variation in gene copy number is postulated to influence clinical phenotype. There have been conflicting reports regarding the association of copy number variations (CNV) in the *FCGR* locus with RA in Caucasian studies [28, 29]. In the vicinity of the *FCGR* locus there are at least three reported regions showing CNV, but rs7551957 is located more than 19-kb away from the nearest CNV region, which extends from the 3′-UTR of *FCGR2A* to the 3′-UTR of *FCGR2C* [30]. In addition, a careful examination of the rs7551957 genotype results did not reveal any indications of CNV in the observed cluster signals. Hence the association observed for rs7551957 with *FCGR2A* is unlikely to be caused by CNV of the *FCGR* locus.

Rs6685859 in intron16 of the *DPYD* gene showed $DLp = 1.3 \times 10^{-4}$, although trend p value was at a marginal level ($p = 0.042$) in the Japanese compared with the Europeans ($p = 5.1 \times 10^{-4}$). Again this may be due to the difference in allele frequencies (case versus control; 0.023 versus 0.050 in the Japanese and 0.506 versus 0.619 in the Europeans), because the risk allele is the same in both populations. The two other markers, rs7550959 and rs7531138, showed similar trends as rs6685859. DPYD is a pyrimidine catabolic enzyme, mutation of which leads to dihydropyrimidine dehydrogenase deficiency, putting cancer patients receiving 5-fluorouracil chemotherapy at an increased risk of toxicity [31]. To our knowledge, there have been no published reports on its association with RA or other autoimmune-related diseases to date.

Among the SNPs in the *SPTB* gene examined, three (rs2269310, rs229670, and rs2269304) showed significant *DLp* values. Rs2269310, which showed $DLp = 7.9 \times 10^{-4}$ and a significant trend p value of 0.0054 in the Japanese, did not show a similar level of association in the Europeans ($p = 0.073$). Again this may be due to a lower frequency of the variant allele in Europeans. SPTB acts to stabilize erythrocyte membranes, and mutations in the *SPTB* gene have been associated with spherocytosis type 2 [32], and neonatal hemolytic anemia [33]. Again, its association with RA is not known, although it has been previously reported that there was no significant immunoreactivity observed against spectrin in the sera of 50 RA patients [34].

In the current study, none of the SNPs remained significant after nominal trend p values were corrected for multiple testing using the Bonferroni method (data not shown). Therefore, detection of significant association requires replication analyses using independent sample sets. Nonetheless, we succeeded in the identification of several genetic variants as being associated with RA susceptibility across ethnicities, indicating the usefulness of a trans-ethnic comparison. Additional research is necessary to further confirm their association with the disease, and to elucidate their biological role in the pathophysiology of RA.

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Conflict of interest None.

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Myelin Basic Protein as a Novel Genetic Risk Factor in Rheumatoid Arthritis—A Genome-Wide Study Combined with Immunological Analyses

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Abstract

Rheumatoid arthritis (RA) is a major cause of adult chronic inflammatory arthritis and a typical complex trait. Although several genetic determinants have been identified, they account for only a part of the genetic susceptibility. We conducted a genome-wide association study of RA in Japanese using 225,079 SNPs genotyped in 990 cases and 1,236 controls from two independent collections (658 cases and 934 controls in collection1; 332 cases and 302 controls in collection2), followed by replication studies in two additional collections (874 cases and 855 controls in collection3; 1,264 cases and 948 controls in collection4). SNPs showing $p<0.005$ in the first two collections and $p<10^{-4}$ by meta-analysis were further genotyped in the latter two collections. A novel risk variant, rs2000811, in intron2 of the myelin basic protein (*MBP*) at chromosome 18q23 showed strong association with RA ($p=2.7\times10^{-8}$, OR 1.23, 95% CI: 1.14–1.32). The transcription of *MBP* was significantly elevated with the risk allele compared to the alternative allele ($p<0.001$). We also established by immunohistochemistry that MBP was expressed in the synovial lining layer of RA patients, the main target of inflammation in the disease. Circulating autoantibody against MBP derived from human brain was quantified by ELISA between patients with RA, other connective tissue diseases and healthy controls. As a result, the titer of anti-MBP antibody was markedly higher in plasma of RA patients compared to healthy controls ($p<0.001$) and patients with other connective tissue disorders ($p<0.001$). ELISA experiment using citrullinated recombinant MBP revealed that a large fraction of anti-MBP antibody in RA patients recognized citrullinated MBP. This is the first report of a genetic study in RA implicating MBP as a potential autoantigen and its involvement in pathogenesis of the disease.

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Introduction

Rheumatoid arthritis (RA) is the most common cause of adult inflammatory arthritis, affecting 0.5–1% of the adult population worldwide, and is associated with joint pain, dysfunction and deformity. Both genetic and environmental risk factors have been implicated in RA [1–2]. *HLA-DRB1* is a major genetic component of RA across ethnicities and is estimated to contribute to 30 to 50% of the total genetic risk [3]. However, the other risk loci identified to date show ethnic-specific patterns of disease association. Large-scale genetic analyses including genome-wide association (GWA) studies have shown that more than 20 genes such as *PTPN22*, *TRAF1/C5*, *CD40*, and *TNFAIP3* are associated with RA in populations of European descent [4–12]. A different set of non-*HLA* genes, namely, *PADI4*, *SLC22A4*, *FCRL3*, *CD244* and *CCR6* were first reported for their association with RA using Japanese DNA collections [13–17]. Among them, several genes including *CCR6*, *STAT4* and *TNFAIP3* were later proven their association beyond ethnicity [12,18–19]. On the other hand, some other genes showed strong specificity to a certain ethnic group. The association of the *PTPN22* has been repeatedly reproduced by subsequent genetic studies in Europeans [5,20–21]. However, no evidence of strong disease risk in *PTPN22* was shown in Japanese in part due to a much lower frequency of the risk allele [22]. Similarly the association of *PADI4* with RA, which has been confirmed by multiple genetic studies in Japanese and Koreans [23–24], is found to be much weaker in Europeans [25–26]. Moreover, the size of DNA collections used for GWA studies is much larger in European populations than in Japanese, suggesting the existence of unknown genetic factors in Japanese [12,17]. For these reasons, we decided to conduct a new large-scale GWA study of RA in Japanese. Independent collections of RA patients and controls were enrolled from four clinical centers in our study. The collections from two centers, totaling 990 cases and 1,241 controls, were characterized with genome-wide SNP arrays, and the data were analyzed to identify potential disease-associated loci. For replication, SNPs at these loci were examined in the two remaining collections, totaling 2,138 cases and 1,803 controls.

Results

Genome scan and validation studies

We accumulated data on 3,128 cases and 3,039 controls of four independent RA collections (termed as collections 1,2,3 and 4, Table S1). Collections 1 and 2 (totaling 990 cases and 1,236 controls) were used for GWA analysis and collections 3 and 4 (totaling 2,138 cases and 1,803 controls) were used as replication samples. Quality control of the GWA genotyping results was undertaken separately in cohorts 1 and 2 because of differences in the SNP arrays used (see Methods, Table S2). For 225,079 markers that were common between the arrays and fulfilled our inclusion criteria, we found no evidence of population stratification between cases and controls (genomic control inflation factor $\lambda = 1.03$, Figure S1). We undertook analysis of each collection individually, and a meta-analyses to pool the results in the two collections in the association analysis (see Methods for further details). We report p-values from the meta-analysis unless otherwise stated.

We found a strong association of disease risk with markers in the *HLA* complex [27] ($p = 5.0\times10^{-31}$, Table S3). Although no other chromosomal loci showed genome-wide significance, we detected evidence of association signal in *PADI4* ($p = 2.3\times10^{-5}$), as previously reported in Japanese [13,23,27] (Table S3). The association results of five reported genes in Japanese were shown in Table S4. These results support the quality of our study populations for genetic analysis. In addition, we identified 10 SNPs in five additional chromosomal regions that met our statistical criteria for testing in the replication collections ($p<0.005$ in both collections 1 and 2, and $p<10^{-4}$ in the meta-analysis). None of them showed potential association with p-value being smaller than 10^{-5} in the other Japanese GWA study [17]. From each of these regions, the SNP with the smallest p-value was selected for examination in collections 3 and 4. One of these SNPs, rs2000811 ($p = 0.0036$ in collection1; $p = 5.7\times10^{-4}$ in collection2; $p = 1.2\times10^{-5}$ in the meta-analysis) located on chromosome 18q23, was significantly associated with RA in both replication collections ($p = 0.023$ in collection3; $p = 0.0041$ in collection4, and

Table 1. Association of *MBP* locus with rheumatoid arthritis in the Japanese population.

Chr	dbSNPID	Gene	Allele	DNA Collection		Genotype counts			Success rate	HWE*p*	RAF**	*p*-value	OR	*mhp****
			Ref.(A1)/Var.(A2)			A1A1	A1A2	A2A2					(95%CI)	
18q23	rs2000811	*MBP*	C/T*	1	case	203	303	136	99.8	0.25	0.45	0.0036	1.25	
					control	344	442	148	100	0.76	0.4		(1.08–1.44)	
				2	case	95	152	79	99.7	0.24	0.48	5.7×10^{-4}	1.49	
					control	120	131	46	100	0.31	0.38		(1.19–1.87)	
				3	case	283	392	182	98.1	0.034	0.44	0.023	1.17	
					control	298	404	134	97.8	0.88	0.4		(1.02–1.34)	
				4	case	393	622	233	98.7	0.63	0.44	0.0041	1.19	
					control	341	451	141	98.4	0.68	0.39		(1.06–1.35)	
				3+4	case	676	1014	415	98.5	0.32	0.44	3.0×10^{-4}	1.18	
					control	639	855	275	98.1	0.69	0.4		(1.08–1.30)	
				pooled	case	974	1469	630	98.9	0.078	0.44	4.0×10^{-8}	1.23	2.7×10^{-8}
					control	1103	1428	469	98.9	0.85	0.39		(1.14–1.32)	

*risk allele for the disease,
**risk allele frequency, and
***p-value in meta-analysis using Cochran-Mantel-Haenszel test.
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$p = 3.0 \times 10^{-4}$ in collections 3 and 4). When the four collections were combined, the evidence of association at rs2000811 exceeded genome-wide significance when evaluated either by meta-analysis ($p = 2.7 \times 10^{-8}$; OR = 1.23; 95%CI 1.14–1.32) or by pooling of the genotype counts ($p = 4.0 \times 10^{-8}$; OR = 1.23; 95%CI 1.14–1.32; see Table 1). There was no difference in the effect size among the four collections ($p = 0.28$). To be more conservative, however, we have calculated corrected p-values with Principle Component Analysis (PCA), using subsets of case and control collections for which individual genotypes were available (970 cases and 297 controls, for details, see Materials and Methods). There was no difference in p-values with and without the correction ($p = 6.5 \times 10^{-4}$ and corrected $p = 6.5 \times 10^{-4}$). The four SNPs from the other regions that were tested showed no evidence of association in collection3 for replication study (Table S5).

The disease associated marker rs2000811 is located in the second intron of the *MBP* (myelin basic protein) gene at chromosome 18q23 within a 156-kb region that contains the *MBP* gene (NCBI MapViewer, build 36.3). Linkage disequilibrium (LD) was evaluated using genotyping results obtained in collections 1 and 2; rs2000811 did not show significant LD with other markers from the region ($r^2 < 0.14$; Figure 1), or elsewhere in the genome. An imputation analysis using the Japanese HapMap data identified a SNP, rs9958028, which was 358-bp apart and in strong LD with rs2000811 ($r^2 = 0.96$), as the second strongest association. However, no other marker was in strong LD with these two markers ($r^2 = 0.35$ or smaller) (Figure S2). To determine if unidentified polymorphisms within *MBP* were in LD with rs2000811, we performed a sequencing of the exons and the promoter region of the *MBP* gene in 84 Japanese population control DNAs (Method S1). We identified 66 SNPs, 37 of which were not registered in dbSNP, and three of which were deleterious polymorphisms (Tables S6 and S7). Again, none of these polymorphisms was in strong LD with rs2000811 ($r^2 = 0.35$ or smaller) (Figure S3). An imputation analysis using the genotyping results obtained by sequencing did not discover any other polymorphisms showing stronger association signals ($p > 0.0070$) than that of rs2000811. Taken together, these data suggest that rs2000811 and/or one or more other as yet unidentified non-coding polymorphisms within or near *MBP* are responsible for the genetic association.

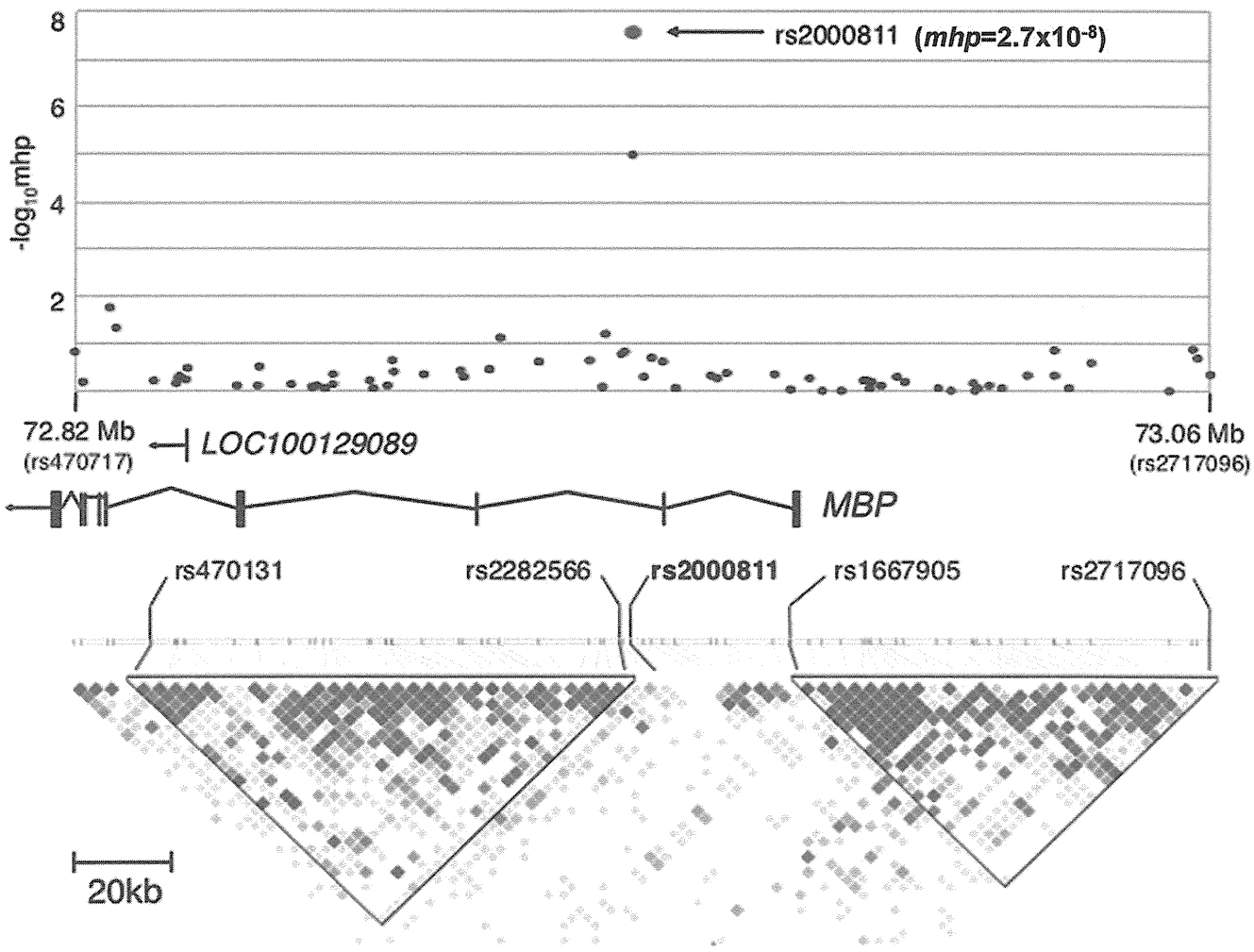

Figure 1. A schematic view of the association results and LD structure of the human *MBP* gene locus at chromosome 18q23. SNPs located between rs470131 and rs2717096 are plotted in $-\log_{10}$ scale according to their chromosomal positions and p-values calculated with Cochran-Mantel-Haenszel test. Red circle indicates *mhp*-value of rs2000811 by meta-analysis using the combined results of collections 1 to 4. Relative locations of the genes in the region are shown with their transcriptional orientations by arrows. LD blocks were generated using the genome scan results.

doi:10.1371/journal.pone.0020457.g001

Evaluation of MBP transcription

Quantitative RT-PCR experiments showed only very low levels of *MBP* expression in RNA from Epstein-Barr virus (EBV)-transformed human B-lymphoblastoid cell lines, and we could detect no discernable correlation of *MBP* transcript levels with different risk genotypes ($p = 0.36$, Figure S4). By a similar reason, *in-silico* expression analysis using GEO database did not return clear association [28]. However, when we performed allele-specific quantitative RT-PCR [16] using genomic DNA and cDNA of these cell lines, we observed elevated allele-specific transcription associated with the risk allele ($p<0.001$, Figure 2, for detailed procedure, see Materials and Methods). This suggests that rs2000811, and/or other variants in linkage disequilibrium with this marker, impact the quantitative pattern of *MBP* transcription. However, bioinformatics analysis identified no known *cis*-acting elements covering rs2000811 that could be inferred to have functional effects (Method S2). In addition, the alignment of the 4-kb region ranging between 2-kb centromeric and 2-kb telomeric to rs2000811 revealed that this segment has very low interspecies conservation among placental mammals.

Expression of the MBP protein in synovial tissues

Next we investigated the expression of the MBP protein in synovial tissue, as this is the main target of inflammation in RA. Microscopic observation revealed that MBP was highly expressed along the lining layer of synovial tissues in 20 out of 23 RA patients tested, while the expression of MBP was observed in only one out of five controls ($p = 0.0017$), and then generally at a weaker level (Figure 3A, B). In synovial tissue from RA patients, the detected MBP expression was weaker in synovial lining layer adjacent to the follicules of infiltrated lymphocytes (Figure 3C). In synoviocytes, the expression of MBP was mainly observed in the plasma membrane (Figure 3D).

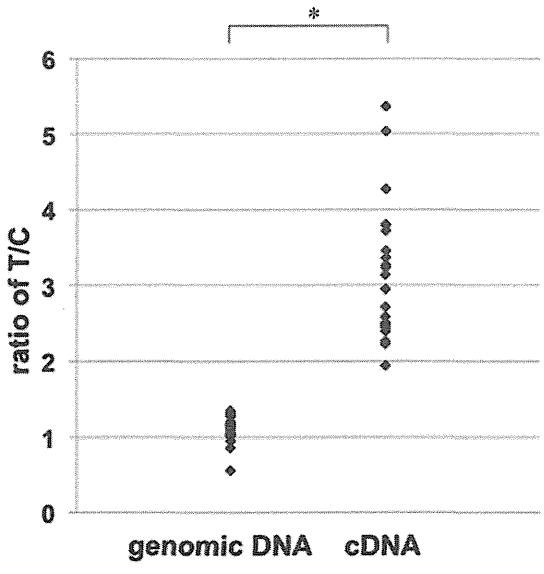

Figure 2. Allelic difference in *MBP* transcription using allele specific quantitative RT-PCR. The amount of *MBP* primary transcripts transcribed from chromosomes carrying rs2000811 risk (T) and alternative (C) alleles was compared in each cell line, and the ratio (T/C) was plotted. Genomic DNA was used as a control for equimoler biallelic representation. Experiments were done twice independently.
doi:10.1371/journal.pone.0020457.g002

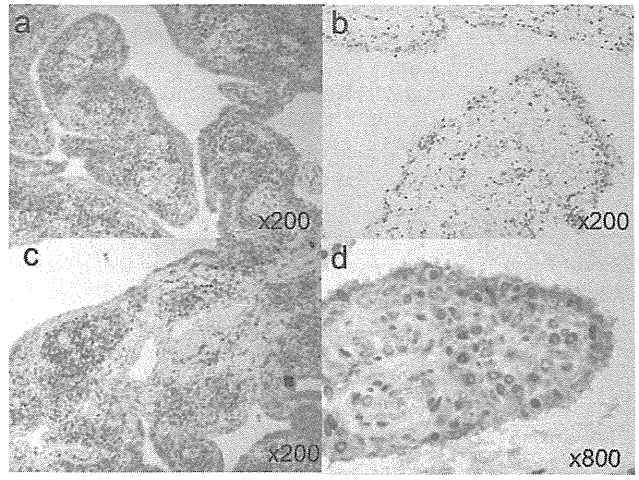

Figure 3. Immunohistochemistry of the MBP protein in human synovial tissues stained by monoclonal anti-MBP antibody. Synovial tissue of RA patients, in particular, along the synovial lining layer strongly expressed MBP (A), whereas that of non-inflammatory osteoarthritis patients was much weaker (B). The expression of MBP in the synovial lining layer was weaker near follicules of infiltrated lymphocytes (C). Localized expression of MBP was observed at the plasma membrane of synoviocytes (D).
doi:10.1371/journal.pone.0020457.g003

Quantification of antibodies to MBP in RA patients

Antibodies to MBP are the major component of autoantibodies in multiple sclerosis, a human autoimmunity with a neurodegenerative phenotype [29]. To assess a possible association of circulating antibodies to MBP with RA, we quantified these in plasma from 323 RA cases, 131 healthy controls and 162 patients with other connective tissue diseases (disease controls) by Enzyme-linked immunosorbent assay (ELISA) with MBP purified from human brain as antigen. The average levels of anti-MBP antibody in plasma of RA patients were much higher than those of healthy controls and patients with seven other connective tissue diseases ($p<0.001$; Figure 4A). Specificity in detection of anti-MBP antibody in ELISA experiments was confirmed by immunoblotting using plasma of a subset of RA patients and controls (Figure S5, Method S3). We also confirmed that the enhancement of ELISA signals by non-specific binding of IgG- and IgM-RF in patients' sera was negligible (for details, see Method S4 and Figure S6).

Amino acid analysis of the MBP protein derived from human brain showed that approximately 21% (citrulline/arginine = 6.0/23.1) of the arginine residues in MBP was citrullinated in human brain under physiological conditions (Method S5). It is possible that anti-MBP antibody recognizes and binds to citrullinated MBP protein. We then performed ELISA using recombinant MBP protein and compared the antibody titers with those of human brain-derived MBP. We could not observe correlation between the two results ($r = -0.19$, Figure 4B). However, when we used recombinant MBP protein artificially citrullinated *in-vitro*, the ELISA results showed strong correlation in titers of the autoantibody ($r = 0.88$, Figure 4C). From these results, we concluded that higher levels of anti-MBP antibody in RA patients than in healthy controls and in patients with other connective tissue diseases was attributed to autoantibodies binding to citrullinated MBP.

Discussion

We conducted a large-scale GWA-based genetic study of RA in the Japanese population. A genome scan of 225,079 SNPs in two

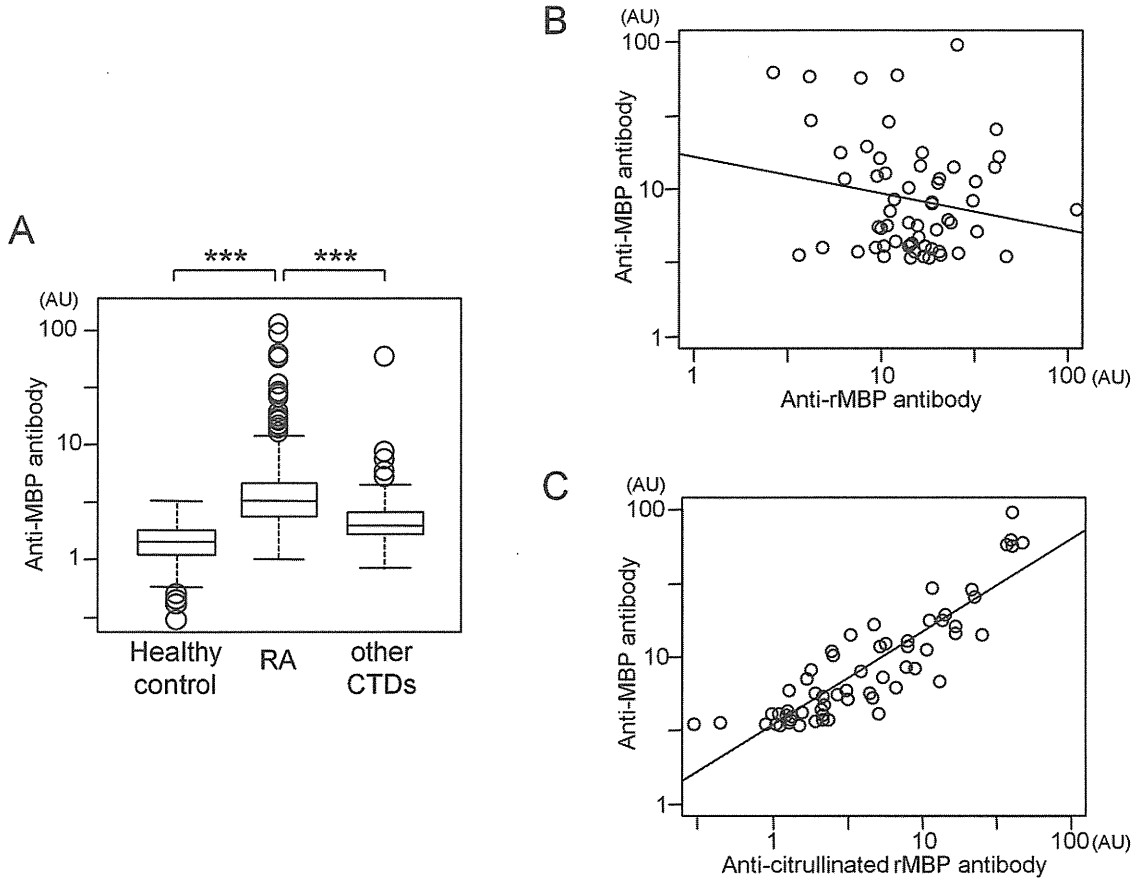

Figure 4. Quantification of circulating antibodies against MBP protein. A. Each boxplot indicates distribution of anti-MBP antibody titers in healthy controls, in RA patients, and in other connective tissue diseases (CTDs) (for detailed composition of the disease patients, see materials and methods). Results were representative of the two independent experiments. *** indicates statistical *p*-value smaller than 0.001. B. Correlation of autoantibody levels between human brain-derived MBP and recombinant MBP (rMBP). C. Correlation of autoantibody levels between human brain-derived MBP and citrullinated rMBP. Sixty RA patients who were positive for anti-MBP antibody were used for the analysis. In each figure, antibody titers were shown as arbitrary unit (AU).
doi:10.1371/journal.pone.0020457.g004

DNA collections of RA patients followed by replication in two additional collections led to identification of a novel risk variant, rs2000811 ($p = 2.7 \times 10^{-8}$, OR 1.23, 95% CI: 1.14–1.32), in the second intron of the *MBP* gene at chromosome 18q23. This is the second largest genome-wide association study to date in the Japanese population, and the first to have identified chromosome 18q23 as a potential susceptibility locus for RA. 18q23 was not reported as a candidate genetic locus in recent GWA study of RA in Japanese [17]. As summarized in Table S4, we confirmed in the current study the association of three out of seven genes reported to date whereas there was no evidence of association for the other four genes. A comprehensive genetic study by a meta-analysis using the results of different genome scans followed by the validation study with a large number of patients and controls will clarify relative contribution of each genetic locus to RA in the Japanese population. There are no GWA studies to date that indicate chromosome 18q23 as a potential genetic locus related to the susceptibility to RA in European populations [5–12]. By taking into account the detection power of the meta-analysis by Stahl *et al.*, it is natural to consider that the association of *MBP* with RA is limited to Japanese (and possibly other Asian populations).

The disease-associated marker rs2000811 is isolated in an 18-kb segment of the intron, and does not show evidence of significant LD with known markers in the region or neighboring genomic regions (Figure 1). The LD structure of the human *MBP* locus was very similar between Europeans and Japanese, and there was no clear LD block in the region encompassing rs2000811 in both populations (Figure S2). Sequencing of the DNAs of 84 healthy controls failed to identify other polymorphisms that were in strong LD with rs2000811 in the exons or the promoter region of the *MBP* gene. While bioinformatics analysis failed to identify any *cis*-acting elements encompassing rs2000811, allele-specific expression analysis demonstrated the elevated transcription level of *MBP* with the risk allele. This raises the possibility that *cis*-acting regulatory elements that encompass unknown genetic variations in strong LD with rs2000811.

A strong expression of the MBP protein was observed in synoviocytes of RA patients while expression was weaker in those of non-inflammatory controls. The fact that the expression of MBP was strongest in the synovial lining layer suggests the role of anti-MBP antibody as a trigger of inflammatory reactions through attacking synoviocytes. Although a study investigating citrullinated proteins in synoviums did not clearly identify MBP [30], MBP is possibly one of the unidentified subset of the citrullinated proteins. MBP expression was weaker in synoviocytes adjacent to the follicules of infiltrated lymphocyte. It would be of interest to examine whether MBP in synovium is

citrullinated and such phenomena are correlated with the disease activity, in particular, at different stages of proliferative synovitis but data for such an investigation are not available in our study. In the absence of a quantitative assay of expression in synoviocytes, it was not possible to discern a correlation between the levels of *MBP* expression with the risk genotype in our experiments.

MBP is a well-known target autoantigen in multiple sclerosis (MS), a human neurodegenerative disease with an active destruction of myelin sheath [29]. The MBP protein has six different isoforms of which isoforms 1, 2, 3 and 4 with shorter open reading frames are expressed preferentially in the central nervous system (CNS). Isoforms 7 and 8 with higher molecular weights (also called Golli-MBP) are known to be expressed relatively ubiquitously including cells of the hematopoietic lineage [31]. The fact that RA patients rarely present CNS symptoms may suggest the involvement of Golli-MBP protein in the generation of anti-MBP antibody and expression in the synovium. An immunological study using relatively small numbers of plasma samples showed that anti-MBP antibody is present in 60% (or six out of ten) of RA patients [32]. To our knowledge, however, there is no study which quantitatively characterized anti-MBP antibody in RA patients as well as in patients of other connective tissue diseases and in healthy controls. On the other hand, ACPA which recognizes citrullinated proteins by peptidylarginine deiminase is considered as a specific and predictive marker for RA [13,33–34]. Indeed, several studies showed the existence of antibodies to citrullinated MBP in RA patients [35–36]. However, the role of MBP and anti-MBP antibody in the pathogenesis of RA is yet to be elucidated. In the current study, we found that a part of brain-derived MBP was citrullinated in physiological conditions and that higher levels of anti-MBP antibody in RA patients can be attributed to MBP citrullination, although anti-MBP antibody is not in complete cross-reactivity with ACPA. We examined whether or not rs2000811 was predominantly associated with patients who were positive for ACPA. However, there was no statistical difference in allele frequency of rs2000811 between patients with and without ACPA ($p = 0.40$, data not shown).

We observed no correlation between the levels of anti-MBP antibody and genotypes at the risk locus. The repertoire of autoantibody is likely to depend on numerous factors such as the immunogenicity and extent of citrullination of the MBP protein. Also, a series of immunological reactions including antigen presentation in thymus and peripheral organs, activation of dendritic cells, T- and B-lymphocytes, and balance of Th1/Th2 and effector/regulatory T-lymphocytes affect antibody production. A possible explanation for the lack of correlation is that the disease-associated genotypes affect these factors in a way that impacts risk without directly influencing the quantitative values of anti-MBP antibody, which we find to be highly variable between RA patients that we have studied. Therefore, it will be of interest to compare the expression levels of *MBP* transcripts and MBP epitopes in synovial tissue, and between different genotypes and disease activity.

Taken together, this is the first genetic study which identified *MBP*, an autoantigen gene, to be associated with RA in Japanese. Transcription of *MBP* was increased with the risk allele of the associated SNP, rs2000811. Strong expression of the MBP protein was observed in the synovial tissues of the patients. Furthermore, significant increase of circulating autoantibodies against MBP protein was demonstrated in RA patients as compared to those with other connective tissue diseases, implicating its role as a disease-associated biomaker.

Materials and Methods

Ethics Statement

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, namely, Kyoto University Graduate School and Faculty of Medicine, Ethics Committee, The Ethic Committee, Sagamihara National Hospital, NHO, Dohgo Spa Hospital Ethical Committee, University of Tokyo Medical Research Center Ethics Committee, Tokyo Women's Medical University Genome Ethics Committee, Ethics Committee of Tokyo Women's Medical University and Aichi Cancer Center Ethical Committee for human genome research.

Study subjects

RA collections 1 to 4 consisted of 658 affected individuals and 934 controls, 332 and 302, 874 and 855, and 1,264 and 948, respectively (summarized in Table S1). The case subjects of collections 1, 2 and 3 were recruited at the rheumatology departments of Kyoto University Hospital, Dohgo Spa Hospital, Sagamihara National Hospital, and Tokyo University Hospital, and those for collection4 were from Tokyo Women's Medical University. All cases fulfilled the revised criteria (1987) of the American College of Rheumatology (ACR) for rheumatoid arthritis [37]. Genotype count data of the Japanese Single Nucleotide Polymorphism (JSNP) database [38] were used as controls for collection1. The control subjects for collection2 were from the Department of Ophthalmology and Visual Science at Kyoto University Hospital [39]. DNA samples of healthy Japanese volunteers in Pharma SNP Consortium [40] and in Aichi Cancer Center Hospital and Research Institute [41] were used for collections 3 and 4, respectively. Plasma of 323 RA patients and 162 patients of other connective tissue diseases (38 of SLE, 25 each of Sjögren's syndrome and systemic sclerosis, 20 each of Behçet's disease and mixed connective tissue disease, 19 of polymyositis/dermatomyositis and 15 of vasculitis) were obtained at Kyoto University Hospital, and those of 131 healthy controls were from Dohgo Spa Hospital.

Genome-wide association analysis

Genome scan for collections 1 and 2 was performed using Infinium Technology (Illumina Inc., San Diego, CA). Case subjects of collection1 were genotyped with Human-Hap300 (version 1.0, 302,627 SNPs) or Human CNV370-Duo (version 1.0, 332,270 SNPs). For collection2, case and control subjects were genotyped on Human610-Quad (version 1.0, 577,348 SNPs), and HumanHap550 (version 3.0, 547,163 SNPs), respectively. Validation studies using collections 3 and 4 were performed using Taqman technology (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's specifications.

Quality control and statistical tests for case-control association

277,420 SNPs that were common among the four arrays described above were chosen for association study. Publicly available genotype counts from the JSNP project were used as control collection1. For this collection, detailed information such as individual genotypes and cluster plots are not disclosed. DNA samples with a call rate smaller than 0.90 (three in collection1 cases), showing high degree of kinship (PI_HAT>0.10 by PLINK [42], eleven in collection1 cases, one in collection2 cases, and four in collection2 controls) and with evidence of possible contamination (one in collection2 controls) were removed from statistical analyses. PCA was performed using the genome scan results of the remaining 644 cases of collection1, in addition to 331 cases and 297 controls of collection2. At this stage, five DNA samples (one in

collection1 cases, and four in collection2 cases) that did not fall into the Japanese cluster were removed. Regarding the SNP markers, a total of 225,079 SNPs with call rate greater than 0.95 for both cases and controls and minor allele frequency greater than 0.05 either in case or in control of each collection were used for analysis. The *p*-value for rs2000811 corrected by PCA was calculated using the remaining 970 cases and 297 controls and compared with the *p*-value without correction.

The case-control association was examined independently for collections 1 and 2 with Cochran-Armitage trend test, followed by meta-analysis with Cochran-Mantel-Haenszel (CMH) test by combining the two collections. Population stratification in collections 1 and 2 was examined and corrected with Genomic Control [43]. The SNPs that showed $p<0.005$ in both collections and meta-analysis *p*-value smaller than 10^{-4} were selected as candidates for further evaluation. Among multiple SNPs in the same region that fulfilled the above criteria, the SNP with the smallest *p*-value in the meta-analysis was chosen for validation with collections 3 and 4. To be more specific, among the four SNPs in the *PLEKHK1* region on chromosome 10q21, namely rs3910172 ($p=4.4\times10^{-5}$), rs6479805 ($p=5.4\times10^{-5}$), rs10733769 ($p=5.8\times10^{-5}$), and rs4147233 ($p=6.6\times10^{-5}$), rs3910172 was selected for the replication study. Likewise, in the region of chromosome 10p14, rs2026628 ($p=1.6\times10^{-5}$) was chosen over rs11253857 ($p=5.3\times10^{-5}$), and rs687848 ($p=5.1\times10^{-5}$) was chosen over rs587404 ($p=7.1\times10^{-5}$) in the *MACF1* region on chromosome 1p31–32. SNPs in the *HLA* and *PADI4* loci were not included in validation studies. Haploview version 4.1 software [44] was used for LD evaluation, and MapViewer (build 36.3) [45] was used to identify the location and structure of the genes in the region.

Quantification of allelic difference in gene expression in MBP transcription

Allele specific gene expression analysis was performed as previously described [16]. Briefly, human B-lymphoblastoid cell lines transformed by EBV were obtained from the Health Science Research Resources Bank of Japan (Osaka). Genomic DNA and total RNA were extracted by standard procedures from 22 cell lines heterozygous for rs2000811 alleles. The ratio of MBP primary transcripts (hnRNA) was quantified between the risk and wild-type alleles by TaqMan assay with SNP genotyping probes. Genomic DNA of a cell line homozygous for the wild-type allele (C) were mixed with DNA homozygous for the risk allele (T) at eight different molar ratios (2:1, 3:2, 1:1, 2:3, 1:2, 1:3, 1:4, 1:6) to draw a standard curve for the evaluation of RNA quantity.

Immunohistochemistry

Synovial tissue specimens of 23 RA patients and five non-inflammatory controls were obtained from Department of Diagnostic Pathology and Department of Orthopaedic Surgery in Kyoto University Hospital. Paraffin-embedded tissues were prepared in a standard method and sectioned at a thickness of 3 μm. The section was mounted on a glass slide coated with 2% 3-aminopropyl triethoxy silane (Tokyo Kasei, Tokyo, Japan). Immunohistochemical staining of MBP was performed by using the standard avidin-biotin-peroxidase complex (ABC) method, as previously described [46]. The sections were incubated overnight at 4°C with an affinity-purified murine anti-human MBP monoclonal antibody (Leica Microsystems, Wetzlar, Germany) diluted at 1:100 in PBS. The sections were then incubated with biotinylated horse anti-mouse IgG antibody (Vector Lab, Burlingame, CA) for 40 minutes, followed by incubation with peroxidase-conjugated streptavidin (Vector Lab, Burlingame, CA) at room temperature for 50 minutes. The coloring reaction was performed with 0.3 mg/ml diaminobenzidine and 0.003% H_2O_2 in 50 mM Tris-HCl (pH 7.6). Each section was counterstained with haematoxylin. Evaluation of MBP expression was performed by a blind test by two rheumatologists as well as a pathologist, and statistical significance in positivity was calculated with Fisher's exact test.

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (Nalge Nunc International K.K., Tokyo, Japan) were coated with 50 μl of MBP protein at 5 μg/ml extracted from human brain (Sigma, St. Louis, MO) or with recombinant human MBP (Genscript, Piscataway, NJ.) in 50 mM bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. Citrullination of recombinant human MBP was done *in-vitro* by rabbit skeletal PAD (Sigma, St. Louis, MO) for 3 hours as previously described [47]. After wells were washed and coated with phosphate-buffered-saline (PBS) containing 2% bovine serum albumin (BSA), 50 μl of plasma samples diluted to 1:150 with 2% BSA in PBS containing 5 U/ml heparin (Mochida Pharmaceutical Co., Ltd. Tokyo, Japan) were added and incubated at room temperature for 2 hours. 50 μl each of purified goat anti-human IgG polyclonal antibody conjugated to alkaline phosphate (Millipore. Billerica, MA) diluted to 1:2000 was added and incubated at room temperature for 1.5 hours. 50 μl of BCIP/NBT substrate (Sigma, St. Louis, MO) in 2 μM $MgCl_2$ was then added and incubated for 45 minutes in the dark. The optical density (OD) value at 405 nm was measured by a SpectraMax Plus^{384} Microplate Reader (Molecular Devices, Sunnyvale, CA). A standard curve was generated by serial dilution (1:50, 1:150, 1:500, 1:1500 and 1:5000) of a plasma sample with a high titer of anti-MBP antibody. The titer of diluted standard sample in 1:50 was set as 100 U for human brain-derived MBP. All samples were examined in duplicate except for negative control in which plasma was replaced by PBS with 2% BSA and was measured in quadruplicate. The specificity of ELISA results was confirmed by standard immunoblotting analysis using sera of 10 each of RA patients and controls.

Statistical analysis of ELISA results

The titer of anti-MBP antibody between RA or its subgroups and controls was compared with Wilcoxon rank-sum test. The correlation between the titers of autoantibody was estimated with Pearson's correlation coefficient in logarithm scale. These statistics were performed in the R statistical system (http://www.R-project.org) and SPSS(ver18).

Supporting Information

Figure S1 QQ plot to show the observed and expected *p*-values of the combined genome scan results. Vertical and horizontal axes indicate observed and expected *p*-values, respectively (A) and in logarithmic scale (B). The analysis using genomic control method showed no significant effect of population stratification ($\lambda_{GC}=1.03$) between the case and control groups.
(TIF)

Figure S2 Imputation analysis and LD structure of the human *MBP* locus. Imputation was performed using the case genotypes of collections 1 and 2 and control genotypes of collection2. Individual genotypes of control population of collection1 (JSNP) were not available. Determination of LD structure was performed by using the GWAS results in this study and the HapMap results of Japanese and Caucasians (Hap JPT and Hap CEU, respectively).
(TIF)

Figure S3 LD structure of the 156-kb region spanning the *MBP* gene. LD plot was generated with Haploview using polymorphisms with reference allele frequencies between 0.05 and 0.95.
(TIF)

Figure S4 Quantification of allelic difference in *MBP* transcription. Human B-lymphoblastoid cell lines transformed by EBV were obtained from the Health Science Research Resources Bank of Japan (Osaka, Japan). Total RNA was extracted by standard procedures from the cell lines that were either homozygous for the wild-type allele (50 cell lines), heterozygous (50 cell lines) or homozygous for the risk allele (49 cell lines) of rs2000811. The amount of *MBP* cDNA in each cell line was measured and normalized to that of β-glucronidase using Taqman Gene Expression Assay (for MBP; Hs00921943-m1, for β-glucronidase; Hs99999908_m1, Applied Biosystems Inc., Foster City, CA) in GeneAmp 7500 Sequence Detection System. The comparative ΔΔCT method and Jonckheere-Terpstra test were used for the analysis.
(TIF)

Figure S5 Immunoblotting of anti-MBP antibody. Immunoblotting analysis was performed to confirm specific binding of circulating anti-MBP antibody. Lane 1, 2 to 6 and 7 were incubated with control plasma, plasma of patients, and rabbit polyclonal anti-human MBP antibody, respectively. The intensity was variable between RA patients whereas no signal was obtained in controls. Similar results were obtained using plasma of the other five RA patients and nine controls.
(TIF)

Figure S6 Comparison of circulating anti-MBP antibody and IgG-RF. We investigated whether the presence of RF in sera enhances the signal in ELISA through binding of IgG-RF to the constant region of anti-MBP antibody or through non-specific binding of IgM-RF to secondary antibody used in the experiments. Initially, correlation between IgG-RF and anti-MBP antibody titers was assessed to examine whether IgG-RF had any influence on anti-MBP antibody titers. 114 patients with RA and 13 other connective tissue disease patients for whom IgG-RF measurement was available were enrolled in this evaluation. As a result, no correlation was observed between the titers of anti-MBP antibody and IgG-RF (Spearman's rank-sum coefficient being 0.145 with *p*-value of 0.103). Subsequently, ELISA experiments using human IgM or IgG as target antigens were undertaken. Non-specific binding of secondary antibodies to human IgM compared to IgG was less than 1%.
(TIF)

Table S1 Summary of the study populations used for the association analysis. Abbreviations were as follows; ACPA: antibodies to citrullinated peptide antigens, RF: rheumatoid factor, SD: standard deviation, N/A, not available.
(DOC)

Table S2 Summary of quality control for genome scan results.
(DOC)

Table S3 Association of *HLA* and *PADI4* loci with rheumatoid arthritis in the Japanese population. *risk allele for the disease, **risk allele frequency, and ***p-value in meta-analysis using Cochran-Mantel-Haenszel test.
(DOC)

Table S4 Association of previously reported non-*HLA* genes in the current study. *p-value in meta-analysis using Cochran-Mantel-Haenszel test.
(DOC)

Table S5 Replication results of the four regions. The order of SNPs is in accordance with *mhp*-value. Chromosome and dbSNPID refer to NCBI build 36.3. *P*-values are calculated using the Cochran-Armitage trend test. *risk allele for the disease, **risk allele frequency, and ***OR, odds ratio with 95% confidence interval. ***p-value in meta-analysis using Cochran-Mantel-Haenszel test.
(DOC)

Table S6 Oligonucleotide primers used for sequencing of the exons and the promoter region of the *MBP* gene.
(DOC)

Table S7 The list of genetic polymorphisms discovered by sequencing the exons and the promoter region of the *MBP* gene. *Positions of polymorphisms are according to NCBI Refseq Build 36.3. Polymorphisms are located between position 1 and position 2.
(DOC)

Method S1 Sequencing of the exons and the promoter region of the MBP gene.
(DOC)

Method S2 Bioinformatics analysis.
(DOC)

Method S3 Immunoblotting of anti-MBP antibody.
(DOC)

Method S4 Evaluation of non-specific binding of secondary antibodies.
(DOC)

Method S5 Amino acid analysis.
(DOC)

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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,