Fallin, D., Cohen, A., Essioux, L., et al. 2001. Genetic analysis of case/control data using estimated haplotype frequencies: application to APOE locus variation and Alzheimer's disease. *Genome Res.* 11, 143–151.

Falush, D., Stephens, M., and Pritchard, J.K. 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* 164, 1567–1587.

Gao, X., and Martin, E.R. 2009. Using allele sharing distance for detecting human population stratification. *Hum. Hered.* 68, 3.

Gao, X., and Starmer, J. 2007. Human population structure detection via multilocus genotype clustering. *BMC Genet.* 8, 34.

Gaudieri, S., Leelayuwat, C., Tay, G.K., et al. 1997. The Major Histocompatability Complex (MHC) contains conserved polymorphic genomic sequences that are shuffled by recombination to form ethnic-specific haplotypes. *J. Mol. Evol.* 45, 17–23.

Gonzalez, E., Bamshad, M., Sato, N., et al. 1999. Race-specific HIV-1 disease-modifying effects associated with CCR5 haplotypes. *Proc. Natl. Acad. Sci. USA* 96, 12004–12009.

Haiman, C.A., Stram, D.O., Pike, M.C., et al. 2003. A comprehensive haplotype analysis of CYP19 and breast cancer risk: The Multiethnic Cohort. *Hum. Mol. Genet.* 12, 2679–2692.

International HapMap Consortium. 2005. A haplotype map of the human genome. *Nature* 437, 1299–1320. Available at www.hapmap.org. Accessed November 1, 2011.

Isaev, A. 2004. *Introduction to mathematical methods to bioinformatics*. Springer, New York.

Jakobsson, M., Scolz, S.W., Scheet, P., et al. 2008. Genotype, haplotype and copy-number variation in worldwide human populations. *Nature* 451, 998–1003.

Jin, L., Zhu, W., and Guo, J. 2010. Genome-wide association studies using haplotype clustering with a new haplotype similarity. *Genet. Epidemiol.* 34, 633–641.

Johnson, G.C.L., Esposito, L., Barratt, B.J., et al. 2001. Haplotype tagging for the identification of common disease genes. *Nat. Genet.* 29, 233–237.

Kaufman, L, and Rousseeuw, P. 1990. *Finding Groups in Data: An Introduction to Cluster Analysis*. John Wiley and Sons, New York.

Ke, X., and Cardon, L.R. 2003. Efficient selective screening of haplotype tag SNPs. *Bioinformatics* 19, 287–288.

Kim, S., and Misra, A. 2007. SNP genotyping: technologies and biomedical applications. *Annu. Rev. Biomed. Eng.* 9, 289–320.

Lesk, A.M. 2005. Introduction to Bioinformatics, 2nd ed. *Oxford*, New York.

Li, J., and Jiang, T. 2005. Haplotype-based linkage disequilibrium mapping via direct data mining. *Bioinformatics* 21, 4384–4393.

Li, J., Zhou, Y., and Elston, R.C. 2006. Haplotype-based quantitative trait mapping using a clustering algorithm. *BMC Bioinform.* 7, 258.

Mao, X., Bigham, A.W., Mei, R., et al. 2007. A genomewide admixture mapping panel for Hispanic/Latino populations. *Am. J. Hum. Genet.* 80, 1171–1178.

Meng, Z., Zaykin, D.V., Xu, C., et al. 2003. Selection of genetic markers for association analyses, using linkage disequilibrium and haplotypes. *Am. J. Hum. Genet.* 73, 115–130.

Pritchard, J.K., Stephens, M., and Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155, 945–959.

Rabiner, L.R., and Juang, B.H. 1986.An introduction to hidden Markov models. *IEEE ASSP Mag.* 3, 4–16.

Rastas, P., Koivisto, P.M., Mannila, H., et al. 2005. A hidden Markov technique for haplotype reconstruction. *Lect. Notes Bioinform.* 3692, 140–151.

Rinaldo, A., Bacanu, S., Devlin, B., et al. 2005. Characterization of multilocus linkage disequilibrium. *Genet. Epidemiol.* 28, 193–206.

Saitou, N., and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Small, K.M., Mialet-Perez, J., Seman, C.A., et al. 2004. Polymorphisms of cardiac presynaptic α_{2C} adrenergic receptors: diverse intragenic variability with haplotype-specific functional effects. *Proc. Natl. Acad. Sci. USA* 101, 13020–13025.

Team RDC. 2007. *R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing.*

Tzeng, J.Y., Devlin, B., Wasserman, L., et al. 2003. On the identification of disease mutations by the analysis of haplotype similarity and goodness of fit. *Am. J. Hum. Genet.* 72, 891–902.

Ward, J.H. 1963. Hierarchical grouping procedure to optimize an objective function. *J. Am. Stat. Assoc.* 58, 236–244.

Ward, J.H., and Hook, M.E. 1963. Application of an hierarchical grouping procedure to a problem of grouping profiles. *Educ. Psychol. Measure.* 23, 69–81.

Wiencke, J.K. 2004. Impact of race/ethnicity on molecular pathways in human cancer. *Nat. Rev. Cancer* 4, 79–84.

Wilson, F.W., Weale, M.E., Smith, A.C., et al. 2001. Population genetic structure of variable drug response. *Nat. Genet.* 29, 265–269.

Witherspoon, D.J., Wooding, S., Rogers, A.R., et al. 2007. Genetic similarities within and between human populations. *Genetics* 176, 351–359.

Yang, Y., and Tabus, I. 2007. Haplotype block partitioning using a normalized maximum likelihood model. *Proc. IEEE Genomic Signal Process. Stat.* 1–4.

Address correspondence to:
Ms. Ritsuko Onuki
Bioinformatics Center
Institute for Chemical Research
Kyoto University
Gokasho, Uji
Kyoto 611-0011, Japan

E-mail: onuki@hgc.jp

OPEN ACCESS Freely available online

PLOS | ONE

PADI4 and HLA-DRB1 Are Genetic Risks for Radiographic Progression in RA Patients, Independent of ACPA Status: Results from the IORRA Cohort Study

Taku Suzuki[1,2☯], Katsunori Ikari[1*☯], Koichiro Yano[1☯], Eisuke Inoue[1], Yoshiaki Toyama[2], Atsuo Taniguchi[1], Hisashi Yamanaka[1], Shigeki Momohara[1]

1 Institute of Rheumatology, Tokyo Women's Medical University, Shinjuku, Tokyo, Japan, **2** Department of Orthopaedic Surgery, Keio University School of Medicine, Shinjuku, Tokyo, Japan

Abstract

Introduction: Rheumatoid arthritis (RA) is a systemic, chronic inflammatory disease influenced by both genetic and environmental factors, leading to joint destruction and functional impairment. Recently, a large-scaled GWAS meta-analysis using more than 37,000 Japanese samples were conducted and 13 RA susceptibility loci were identified. However, it is not clear whether these loci have significant impact on joint destruction or not. This is the first study focused on the 13 loci to investigate independent genetic risk factors for radiographic progression in the first five years from onset of RA.

Methods: Sharp/van der Heijde score of hands at 5-year disease duration, which represents joint damage, were measured retrospectively and used as an outcome variable in 865 Japanese RA patients. Genetic factors regarded as putative risk factors were RA-susceptible polymorphisms identified by the Japanese GWAS meta-analysis, including HLA-DRB1 (shared epitope, SE), rs2240340 (*PADI4*), rs2230926 (*TNFAIP3*), rs3093024 (*CCR6*), rs11900673 (*B3GNT2*), rs2867461 (*ANXA3*), rs657075 (*CSF2*), rs12529514 (*CD83*), rs2233434 (*NFKBIE*), rs10821944 (*ARID5B*), rs3781913 (*PDE2A-ARAP1*), rs2841277 (*PLD4*) and rs2847297 (*PTPN2*). These putative genetic risk factors were assessed by a stepwise multiple regression analysis adjusted for possible non-genetic risk factors: autoantibody positivity (anti-citrullinated peptide antibody [ACPA] and rheumatoid factor), history of smoking, gender and age at disease onset.

Results: The number of SE alleles ($P = 0.002$) and risk alleles of peptidyl arginine deiminase type IV gene (*PADI4*, $P = 0.04$) had significant impact on progressive joint destruction, as well as following non-genetic factors: ACPA positive ($P = 0.0006$), female sex ($P = 0.006$) and younger age of onset ($P = 0.02$).

Conclusions: In the present study, we found that *PADI4* risk allele and HLA-DRB1 shared epitope are independent genetic risks for radiographic progression in Japanese rheumatoid arthritis patients. The results of this study give important knowledge of the risks on progressive joint damage in RA patients.

Citation: Suzuki T, Ikari K, Yano K, Inoue E, Toyama Y, et al. (2013) *PADI4* and HLA-DRB1 Are Genetic Risks for Radiographic Progression in RA Patients, Independent of ACPA Status: Results from the IORRA Cohort Study. PLoS ONE 8(4): e61045. doi:10.1371/journal.pone.0061045

Editor: Sunil K. Ahuja, South Texas Veterans Health Care System and University Health Science Center San Antonio, United States of America

Received October 9, 2012; **Accepted** March 5, 2013; **Published** April 8, 2013

Funding: This work was supported by grants provided by the Japanese Ministry of Education, Culture, Sports, Science and Technology Grant-in-Aid for Scientific Research (KI), the Japanese Ministry of Health, Labour and Welfare (SM, HY) and the Japan Science and Technology Agency (HY). The IORRA cohort was supported by non-restricted research grants from commercial companies (Asahikasei Kuraray Medical Co., Ltd., Abbott Japan Co., Ltd., Asahikasei Pharma Corporation, Astellas Pharma Inc., AstraZeneca K. K., Bristol-Myers Squibb, Chugai Pharmaceutical Co., Ltd., Daiichi Fine Chemical Co., Ltd., Daiichi Sankyo Co., Ltd., Dainippon Sumitomo Pharma Co., Ltd., Dentsu, Sudler & Hennessey Inc., Eisai Co., Ltd., GlaxoSmithKline K. K., Hisamitsu Pharmaceutical Co.,Inc., Janssen Pharmaceutical K. K., Japan Tobacco Inc., Kaken Pharmaceutical Co., Ltd., Kissei Pharmaceutical Co., Ltd., Kowa Pharmaceutical Co., Ltd., Mitsubishi Chemical Medience Corporation, Mitsubishi Tanabe Pharma Corporation, Mochida Pharmaceutical Co., Ltd., MSD K. K., Mundipharma K.K., Nippon Chemiphar Co., Ltd., Nippon Shinyaku Co., Ltd., Novartis Pharma K. K., Otsuka Pharmaceutical Co., Ltd., Pfizer Japan Inc., Sanofi-Aventis K. K., Santen Pharmaceutical Co., Ltd., Sanwa Kagaku Kenkyusho Co., Ltd., Sekisui Medical Co., Ltd., Taishotoyama Pharmaceutical Co., Ltd., Takeda Pharmaceutical Company Limited, Teijin Pharma Limited, Torii Pharmaceutical Co., Ltd., UCB Japan Co., Ltd., ZERIA Pharmaceutical Co., Ltd.). However, these funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

Competing Interests: This work was supported by grants provided by the Japanese Ministry of Education, Culture, Sports, Science and Technology Grant-in-Aid for Scientific Research (KI), the Japanese Ministry of Health, Labour and Welfare (SM, HY) and the Japan Science and Technology Agency (HY). The IORRA cohort was supported by non-restricted research grants from commercial companies (Asahikasei Kuraray Medical Co., Ltd. Abbott Japan Co., Ltd. Asahikasei Pharma Corporation Astellas Pharma Inc. AstraZeneca K.K. Bristol-Myers Squibb Chugai Pharmaceutical Co., Ltd. Daiichi Fine Chemical Co., Ltd. Daiichi Sankyo Co., Ltd. Dainippon Sumitomo Pharma Co., Ltd. Dentsu, Sudler & Hennessey Inc. Eisai Co., Ltd. GlaxoSmithKline K.K. Hisamitsu Pharmaceutical Co.,Inc. Janssen Pharmaceutical K.K. Japan Tobacco Inc. Kaken Pharmaceutical Co., Ltd. Kissei Pharmaceutical Co., Ltd. Kowa Pharmaceutical Co., Ltd. Mitsubishi Chemical Medience Corporation Mitsubishi Tanabe Pharma Corporation Mochida Pharmaceutical Co., Ltd. MSD K.K., Mundipharma K.K. Nippon Chemiphar Co., Ltd. Nippon Shinyaku Co., Ltd. Novartis Pharma K.K. Otsuka Pharmaceutical Co., Ltd. Pfizer Japan Inc. Sanofi-Aventis K.K. Santen Pharmaceutical Co., Ltd. Sanwa Kagaku Kenkyusho Co., Ltd. Sekisui Medical Co., Ltd. Taishotoyama Pharmaceutical Co., Ltd. Takeda Pharmaceutical Company Limited Teijin Pharma Limited Torii Pharmaceutical Co., Ltd. UCB Japan Co., Ltd. ZERIA Pharmaceutical Co., Ltd). However, these funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: kikari@ior.twmu.ac.jp

☯ These authors contributed equally to this work.

Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by the chronic synovitis and the localized destruction of cartilage and bone resulting in deteriorated physical function and reduced quality of life. It has been recognized that early therapeutic intervention can prevent progress of joint damage and provide long-term benefits to the patients of RA. The therapeutic recommendations for the management of RA indicate patients may use non-biologic and/or biologic disease-modifying anti-rheumatic drugs (DMARDs) in consideration of the presence of poor prognostic factors.[1–3].

To date, prognostic markers of joint damage have been studied extensively and reported; anti-cyclic citrullinated peptides antibody (ACPA) positive,[4–7] rheumatoid factor (RF) positive, [6,7] the history of smoking, [8,9] the high level of disease activity measured using composite measures,[10–12] gender [4,13] and the age of disease onset.[13–15].

Since RA is a complex disease influenced by both genetic and environmental factors, susceptibility genes to the disease have been widely investigated and identified, especially in the era of genome-wide association studies (GWAS) and GWAS meta-analyses.[16–18] Recently, a large-scaled GWAS meta-analysis was conducted using samples from more than 9,000 Japanese RA patients and 38,000 controls. As a result, nine novel RA susceptibility loci were identified; *B3GNT2*, *ANXA3*, *CSF2*, *CD83*, *NFKBIE*, *ARID5B*, *PDE2A-ARAP1*, *PLD4* and *PTPN2*. [16] The study also showed that some previously reported RA susceptibility genes satisfied the genome-wide significance threshold ($P<5.0 \times 10^{-8}$); HLA-DRB1, *PADI4*, *TNFAIP3* and *CCR6*. [16] Of these 13 RA-susceptible loci, HLA-DRB1 shared epitope (SE) have been reported to have impact on disease severity.[19–21] However, the question remains whether if the other RA-susceptible genes have significant impact on joint destruction.

The purpose of this study is to explore genetic risk factors associated with radiographic progression in RA patients.

Methods

Patients and Evaluation of Radiographic Joint Damage

Tokyo Women's Medical University Genome Ethics Committee approved the present study and each individual signed an informed consent form after receiving a verbal explanation of the study. All the patients satisfied the American College of Rheumatology 1987 revised criteria for RA. [22] DNA samples from RA patients were obtained from the IORRA (Institute of Rheumatology Rheumatoid Arthritis cohort study) DNA collection. [16] IORRA is a project of observational RA cohort with an enrollment of over 5,000 Japanese RA patients, and DNA samples were collected from 2,068 patients. [23,24] All these DNA samples were included in the Japanese GWAS meta-analysis. [16].

Radiographic data at 5-year disease duration were collected retrospectively from the medical records of the patients. Of the patients who donated DNA samples, Sharp/van der Heijde score (SHS) of the hands representing radiographic joint damage (a higher score indicating more damage) was available in 865 patients who have not received biologic agents. [25] Proper anteroposterior radiographs of the hands were scored by a single experienced reader as described elsewhere. [26] Since it has been well known that the rate of radiologic progression develops rapidly in early disease course of RA, joint damage scores of the same disease duration, 5 years, were used. Interobserver and intraobserver agreements (0.85 and 0.95, respectively) indicated good reliability.

The reasons of the exclusion for the patients who treated with biologic agents were as follows: the apparent reported dissociation between clinical and radiologic outcomes in patients with RA who are treated with biologic agents, which could be a confounding factor for the study; [27] the year of RA onset for most patients in this study was before 2000 (70.2%), while the first biologic agent was not launched in the Japanese market until 2003, and the number of the patients who have ever used biologic agents in the first 5-year of disease duration was not sufficient for the sub-analysis targeted on biologic agents.

Assessment Measures, Non-genetic Factors

From the IORRA database and medical records of the patients, demographic, clinical, biological and therapeutic data during the first 5-year after onset of RA were collected, including ACPA status (ACPA titers were measured with second [MESACUP CCP test, Medical and biological laboratories] or third generation [QUANTA Lite CCP3 IgG ELISA, Inova Diagnostics] kit), [28] RF status (determined by a latex agglutination turbidimetric immunoassay method), history of smoking, gender and the age at onset. The age at onset was defined as the age at the onset of first symptoms, according to the patient's self-report, and it did not mean the age that satisfied the 1987 ACR criteria.

ACPA, RF, history of smoking and gender were categorized into two dichotomous variables: ACPA (positive [> = 4.5 IU/ml] = 1, negative = 0), RF (positive [> = 15.0 IU/ml] = 1, negative = 0; maximum value in the first 5 years was used), history of smoking (ever smoked = 1, never = 0) and gender (female = 1, male = 0). Data of age at onset was used as continuous variables.

Assessment Measures, Genetic Factors

HLA-DRB1 SE and twelve single nucleotide polymorphisms that have been reported as RA susceptibility polymorphisms using a large-scaled GWAS meta-analysis of Japanese were chosen for the study. [16] There were rs2240340 (*PADI4*, peptidyl arginine deiminase type IV), rs2230926 (*TNFAIP3*, tumor necrosis factor, alpha-induced protein 3), rs3093024 (*CCR6*, C-C chemokine receptor type 6), rs11900673 (*B3GNT2*, UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2), rs2867461 (*ANXA3*, annexin A3), rs657075 (*CSF2*, colony stimulating factor 2), rs12529514 (*CD83*, CD83 molecule), rs2233434 (*NFKBIE*, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon), rs10821944 (*ARID5B*, AT rich interactive domain 5B [MRF1-like]), rs3781913 (*PDE2A-ARAP1*, *PDE2A*; phosphodiesterase 2A, cGMP-stimulated, *ARAP1*; ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1), rs2841277 (*PLD4*, phospholipase D family, member 4) and rs2847297 (*PTPN2*, protein tyrosine phosphatase, non-receptor type 2). The risk alleles were defined as the allele that increases the risk of RA based on a prior report. [16].

Genotyping

Duplicate samples and negative controls were included to ensure accuracy of genotyping. High-resolution polymerase chain reaction (PCR) based DNA typing of HLA-DRB1 locus was performed using the sequence-based typing method with the AlleleSEQR DRB1 typing kit (Abbott Japan), according to the manufacturer's instructions. Assignment of HLA-DRB1 alleles was performed using Assign software. HLA-DRB1 SE were defined as alleles encoding amino acid sequences of QKRAA/QRRAA/RRRAA in positions 70–74 of HLA-DRB1. Genotyping of non-HLA RA susceptibility single-nucleotide polymorphisms (SNPs) were performed using the TaqMan fluorogenic 5′ nuclease assay according to the manufacturer's instructions (Applied Biosystems,

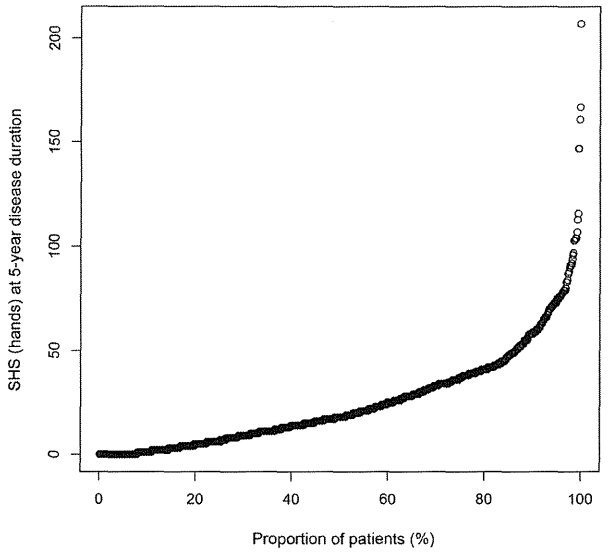

Figure 1. Probability plot of Sharp/van der Heijde score of the hands at the 5-year disease duration. Each point on the plot represents the Sharp/van der Heijde score (SHS) of the hands at the 5-year disease duration, which representing approximate value of the radiographic progression in the first 5 years after onset of RA, in an individual patient. A zero value represents a patient without any radiographic progression, and the right-side tail represents patients with the most progression.
doi:10.1371/journal.pone.0061045.g001

Tokyo, Japan) as described elsewhere. [16] All PCRs were performed using GeneAmp PCR System 9700 (Applied Biosystems), DNA sequencing for HLA typing on 3130×l Genetic Analyzer (Applied Biosystems) and endpoint fluorescent readings for TaqMan assays on ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems).

Statistical Analysis

First, the putative risk factors including non-genetic factors on joint damage were assessed using univariate linear regression analyses (univariate-based feature selection process). Any variable showing a significance level (alpha = 0.05) was selected as a candidate for a stepwise multiple regression analysis (backward elimination) to evaluate the putative risk factor as an independent risk of radiographic damage in RA patients. Number of reported risk alleles on disease susceptibility (0, 1 and 2) was used for the RA susceptible polymorphisms to test the additive effect of the alleles. [16] The dependent variable was the radiographic progression in the first 5 years after onset of RA, calculated as SHS of hands at the 5-year disease duration. Since some RA patients may show more rapid radiographic progression than others[29–31], the SHS (hands) were log-transformed to obtain a normal distribution for all statistical analyses. [32,33].

All valuables were standardized using "scale" command in R software to calculate standardized regression coefficients (β) in the stepwise multiple regression analysis. Statistical analyses were performed using the R software package (http://www.r-project.org/).

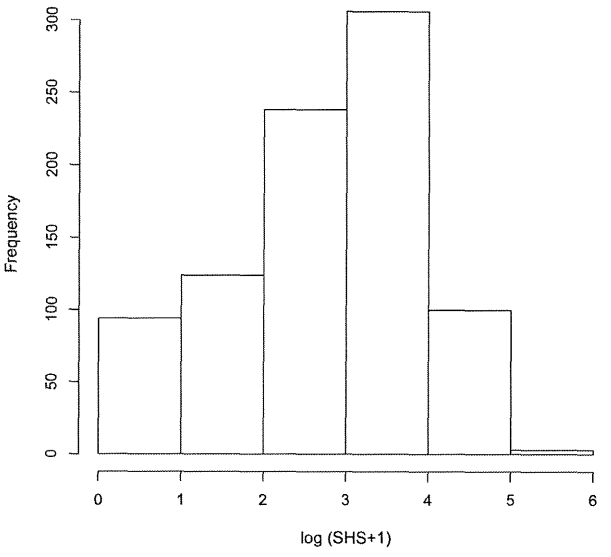

Figure 2. Histogram of distribution of the log-transformed SHS (hands).
doi:10.1371/journal.pone.0061045.g002

Results

Demographic, Clinical and Biological Characteristics of the Patients

Demographic, clinical, biological and therapeutic characteristics of the patients are shown in Table 1. Median age of the patients at

Table 1. Demographic and clinical characteristics of patients at 5 years from onset.

Age at 5-year disease duration, years	54 (46–62)
Sex, female	738 (85.3)
Year of disease onset	
<1990	141 (16.3)
1990<1995	195 (22.5)
1995<2000	271 (31.3)
2000<	258 (29.8)
SHS (hands)	18 (6–37)
ACPA, positive*	739 (87.8)
RF, positive†	781 (90.3)
History of smoking, ever	301 (35.2)
Medication in the first 5-year from the onset	
DMARDs use, ever	735 (92.3)
Methotrexate use, ever	399 (50.1)
Biologic agents use, ever	0 (0)
Corticosteroid use, ever	375 (47.4)

Data are presented as median (interquartile range) or n (%).
*Cut-off = 4.5 IU/ml.
†Maximum value in the first 5-year period of the disease was used, cut-off = 15.0 IU/ml.
SHS, Sharp/van der Heijde score; ACPA, anti-citrullinated peptide antibody; RF, rheumatoid factor; DMARDs, disease-modifying anti-rheumatic drugs.
doi:10.1371/journal.pone.0061045.t001

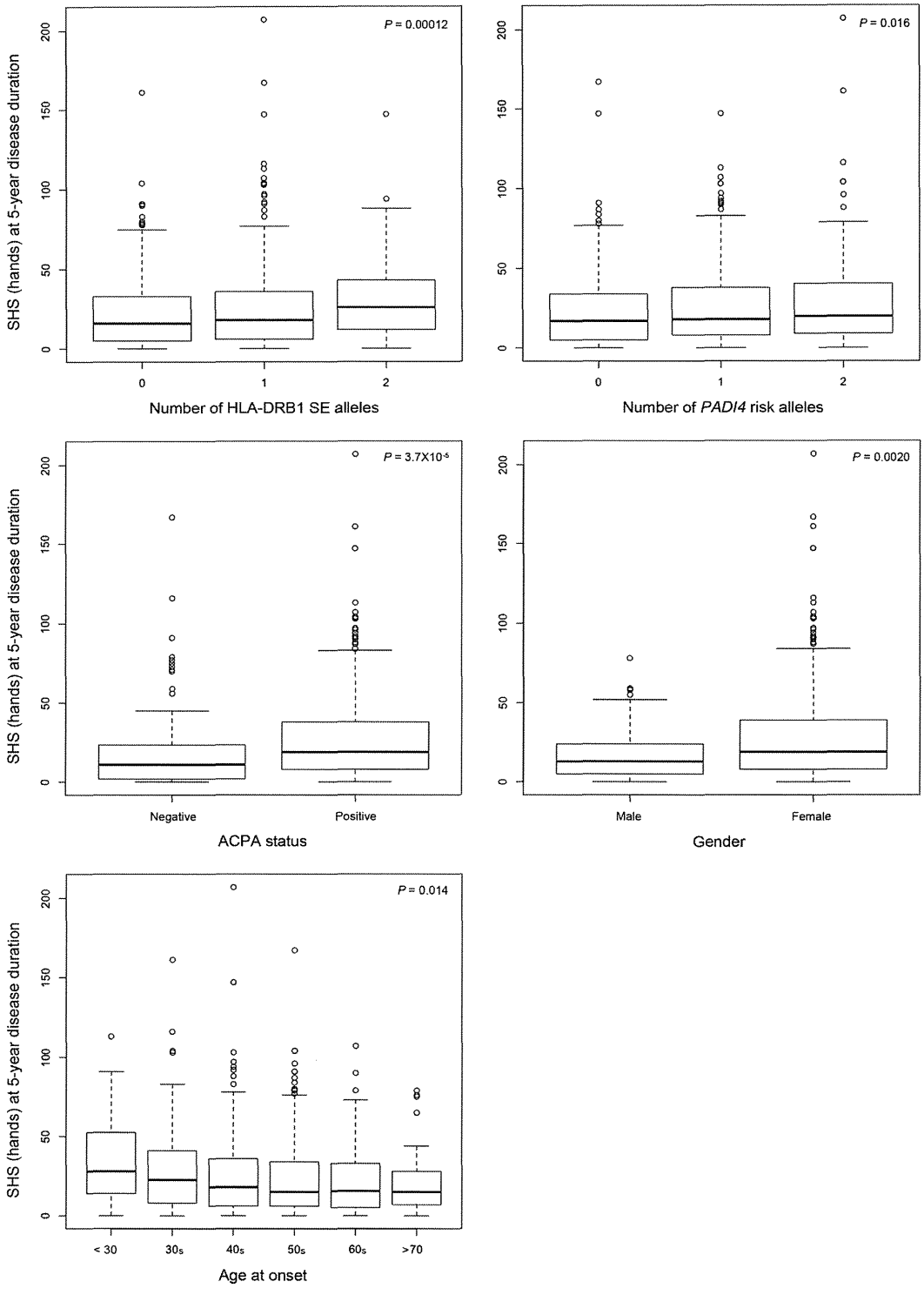

Figure 3. Boxplots representing the distribution of Sharp/van der Heijde score (SHS) of the hands in each category of independent risk factors for joint destruction. Risk factors; the number of HLA-DRB1 shared epitope, the number of PADI4 risk alleles, ACPA status (negative [<4.5 IU/ml] and positive), gender (female and male) and age at onset (categorized as "age under 30", "30 s", "40 s", "50 s", "60 s" and "age over 70"). Each box represents the interquartile range of values, with the bold line showing the median value. The vertical lines show maximum and minimum value that fall within 1.5 box lengths, the open circles show extreme values >1.5 box plot lengths. The P values were given by the univariate linear regression analyses (a log-transformed SHS was used as the dependent variable). PADI4, peptidyl arginine deiminase type IV ACPA, anti-citrullinated peptide antibody.
doi:10.1371/journal.pone.0061045.g003

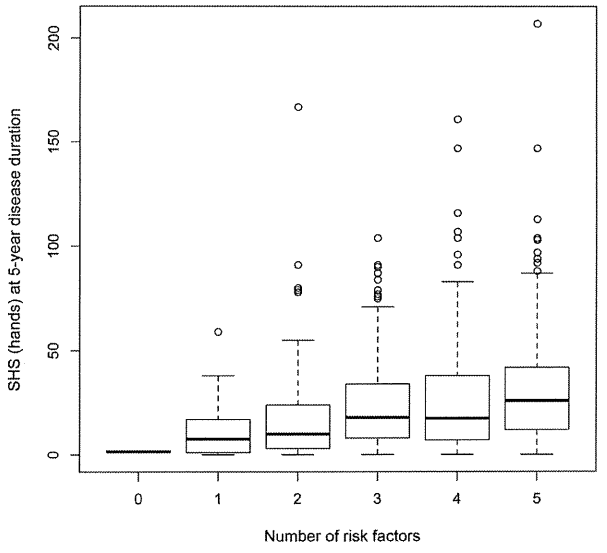

Figure 4. Boxplots representing the distribution of Sharp/van der Heijde score (SHS) of the hands according to the number of the risk factors. Risk factors; SE allele carrier, PADI4 risk allele carrier, ACPA positive, female and age at onset under 50. Each box represents the interquartile range of values, with the bold line showing the median value. The vertical lines show maximum and minimum value that fall within 1.5 box lengths, the open circles show extreme values >1.5 box plot lengths. PADI4, peptidyl arginine deiminase type IV ACPA, anti-citrullinated peptide antibody.
doi:10.1371/journal.pone.0061045.g004

5-year disease duration was 54 years, 85.3% of the patients were female, 87.8% were ACPA positive and 90.3% were RF positive. Median SHS (hands) at 5-year disease duration was 18 (interquartile range 6–37) and yearly progression rate (SHS/disease duration) was 3.6 (Figure 1 and 2). The distribution of SHS (hands) was similar to those in recent clinical studies in which some patients had extreme progressive joint destruction compared to others.[29–31] Half of the patients had prior use of MTX (50.1%) for their treatment of RA in the first 5 years of the disease. The patients who had used biologic agents in the first 5-year disease duration were excluded from the study. Since ACPA measurements started only in the early 2000 s in Japan, data of ACPA in the first 5-years from the onset could not be collected in most patients in this study, and they were substituted by recent data.

SNPs and HLA-DRB1 Genotyping

The overall genotyping success rate was 98.1% and the genotype concordance rate was 100% as assessed by duplicate samples. After the application of quality control criteria for genotyping (remove samples that consistently fail for ≥20% [3/13] SNPs, SNP call rate >95% overall after removing samples that consistently fail), 857 of 865 samples and all polymorphisms passed for the analyses. The following HLA-DRB1 alleles were classified as belonging to SE: DRB1*0101, DRB1*0401, DRB1*0404, DRB1*0405, DRB1*0410, DRB1*1001, DRB1*1402 and DRB1*1406. Frequency of SE carrier was 70.4% (n = 605) and 130 patients were homozygous for SE (15.1%).

Risk Factors for Radiographic Joint Damage

The univariate analysis identified 6 covariates initially as potential candidates; ACPA positive, RF positive, female sex, younger age at onset, HLA-DRB1 SE and *PADI4* risk allele (Table 2). The stepwise multiple regression analysis revealed all tested candidates except RF as independent risks for radiographic joint destruction (Table 3 and Figure 3). Patients with higher number of risk factors had more joint damage (Figure 4). Patients with extremely high joint damage score (SHS [hands] at 5-year disease duration more than 100, n = 13) were all females and had either SE or *PADI4* risk allele.

In the power calculation with a sample size of 830 (the number of samples used in the stepwise multivariate analysis), a 22% change of SHS of the hands with and without a risk by power 0.69 and an 11% change by power 0.23 could be detected.

Discussion

To date, a lot of studies focused on disease severity of RA have been conducted using various endpoints: radiographic progression, disease activity, functional impairment, presence of extra-articular features, complication or death.[34–36] Since a major symptom of RA is the chronic synovitis of multiple joints, which leads to highly damaged joints, restriction of activities of daily living and deterioration of quality of life, SHS that represent radiographic damage in joints has been thought to be a reliable index to assess the disease severity.

One of the difficulties in a study using joint damage score to evaluate RA severity is that the radiographic change is highly influenced by the disease duration. The patients with longer disease duration tend to have more accumulated damage; furthermore, rates of progression in joint damage are nonlinear, it is significantly faster in the early stage than the late phase of the disease. [37] Though the problem can be solved by using the radiographic joint damage score of the same disease duration, such data must be collected from a large number of patients. One of the strong points of this study was that we could obtain hundreds of SHS data from the same disease duration of 5 years, from a large RA cohort project, IORRA. As a result, we were able to perform powerful statistical analyses on joint destruction.

RA is caused by a combination of genetic and environmental factors, and to date, plenty of RA-susceptible polymorphisms have been identified, especially in the era of GWAS. However, genetic factors associated with joint destruction in RA patients have not been extensively studied. Although we had tested the association between joint destruction and some susceptible polymorphisms, no significant association was found thus far. [26,38,39] One of the reasons for the negative association may be due to the small sample size. By utilizing a larger size of DNA samples, we could find that HLA-DRB1 SE and *PADI4* risk allele were genetic risk factors for joint destruction in RA patients.

Hence, the genetic background of disease severity of RA is not yet fully known, although one thing may be for sure; there is little doubt that HLA-DRB1 SE, the strongest genetic factor to RA susceptibility, has impact on the disease severity, as was confirmed in this study.[19–21,40] HLA-DRB1 SE may play a central role for genetic component of RA, and the association between HLA-DRB1 SE and RA susceptibility or severity has been repeatedly reported across the different ethnic populations.

However, RA susceptible genes outside the HLA region have not been fully replicable across racial or ethnic groups. A representative example is *PADI4*, which was first reported in 2003 as RA susceptible gene in a Japanese population. [41] Since then, several reports using Caucasian samples showed negative association between RA susceptibility and *PADI4* polymorphisms, while the association was repeatedly confirmed in Asian populations.[42–47] Currently, based on amassing of research evidence,

Table 2. Univariate linear regression analysis on putative risk factors for radiographic progression: non-genetic and genetic factors.

Putative risk/gene(s)	Polymorphism	alleles *	MAF	risk allele	n	ß	*P* value
ACPA (positive)					834	0.14	3.7×10^{-5}†
RF (positive)					857	0.12	0.00043†
Smoking status (ever)					848	−0.056	0.10
Gender (female)					857	0.11	0.0020†
Age of onset					857	−0.084	0.014†
HLA-DRB1	SE	+/−	0.428	SE	853	0.13	0.00012†
PADI4	rs2240340	G/A	0.442	A	856	0.082	0.016†
TNFAIP3	rs2230926	T/C	0.089	C	847	−0.027	0.43
CCR6	rs3093024	C/T	0.487	T	852	−0.011	0.74
B3GNT2	rs11900673	C/T	0.320	T	852	0.015	0.66
ANXA3	rs2867461	A/G	0.454	G	822	−0.020	0.56
CSF2	rs657075	G/A	0.391	A	832	0.019	0.59
CD83	rs12529514	T/C	0.163	C	843	−0.030	0.39
NFKBIE	rs2233434	T/C	0.239	C	828	0.028	0.42
ARID5B	rs10821944	T/G	0.398	G	842	−0.032	0.35
PDE2A-ARAP1	rs3781913	A/C	0.278	A	848	0.062	0.073
PLD4	rs2841277	T/C	0.287	T	853	−0.013	0.70
PTPN2	rs2847297	A/G	0.360	G	854	−0.032	0.36

*Alleles shown as major allele/minor allele.
†$P<0.05$.
ACPA, anti-citrullinated peptide antibody; RF, rheumatoid factor. MAF; Minor allele frequency in the tested population, SE, shared epitope; *PADI4*, peptidyl arginine deiminase type IV; *TNFAIP3*, tumor necrosis factor, alpha-induced protein 3; *CCR6*, C-C chemokine receptor type 6; *B3GNT2*, UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2; *ANXA3*, annexin A3; *CSF2*, colony stimulating factor 2; *CD83*, CD83 molecule; *NFKBIE*, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon; *ARID5B*, AT rich interactive domain 5B [MRF1-like]; *PDE2A*, phosphodiesterase 2A, cGMP-stimulated; *ARAP1*, ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1; *PLD4*, phospholipase D family, member 4; *PTPN2*, protein tyrosine phosphatase, non-receptor type 2.
doi:10.1371/journal.pone.0061045.t002

PADI4 is considered as RA susceptibility gene even in Caucasian populations though its impact on disease susceptibility is lower than in Asian populations. [16].

It is interesting that *PADI4* risk allele had impact on joint damage independent of ACPA status, which is the most significant finding of this study. *PADI4* gene encodes one of PADI enzymes that catalyse the post-translational modification reaction generating citrulline residues from arginine, [41] and the serum titer of antibodies against citrullinated peptides, ACPA, which is an established prognostic marker for joint destruction in RA patients, is significantly correlated to *PADI4* risk alleles.[48–50] Thus, to date, the relationship between *PADI4* gene and disease severity of RA have been reported mainly in the context of association of *PADI4* haplotypes (or alleles) with serum titer (or positivity) of ACPA.[41,48–50] Recently, Bang et al. [51] indicated that *PADI4* gene contributed to the development of RA, regardless of ACPA status. Combined with our results, the *PADI4* gene is likely to play an additional role in the development and disease progression of RA along with its role in ACPA formation. Subsequent studies should elucidate the unidentified role of *PADI4* in the pathogenesis of RA.

Table 3. Stepwise multiple regression analysis on risk factors for radiographic progression (n = 830).

Risk factors	ß	95% CI for ß	*P* value
ACPA (positive)	0.12	0.05–0.17	0.00056
Gender (female)	0.09	0.03–0.16	0.0059
Age of onset	−0.07	−0.14– −0.01	0.024
HLA-DRB1 SE	0.11	0.04–0.17	0.0021
PADI4 risk allele	0.07	0.004–0.14	0.037

Multiple R squared value = 0.055.
95% CI, 95% confidence interval; ACPA, anti-citrullinated peptide antibody; SE, shared epitope; *PADI4*, peptidyl arginine deiminase type IV.
doi:10.1371/journal.pone.0061045.t003

Numerous clinical studies have indicated that severe, tight control with aggressive treatment in RA patients with remission as a target would help to lower the risk of progression of joint damage, which is especially critical in patients with uncontrollable risk factors. Although prediction of progressive joint damage in RA patients is still far from perfect, the use of identified risk factors (HLA-DRB1-SE positive, *PADI4* risk allele positive, ACPA positive, younger age of onset and female sex) should make it easier for rheumatologists to make their treatment decisions in the future.

Our cohort study has strong points, but also still has some limitations. Since the study was a retrospective cohort study, we were able to collect radiographic data from only 865 of 2,068 patients with DNA sample. Loss of patients could affect the results, although the baseline characteristics of the patients with radiographic data were similar to the whole DNA cohort of IORRA. As a result of the limited sample size, the study was underpowered to detect minor effect on joint destruction. Though we used the data of SHS (hands) at the same disease duration, because radiographs at baseline (onset of the disease) were not available in most

patients, they are only approximate values of delta-SHS in the first 5 years of the disease.

Conclusions

In conclusion, we have identified HLA-DRB1 SE and *PADI4* risk alleles as independent risk factors for progressive joint destruction in the first five years from onset of RA, as well as several non-genetic factors; ACPA positive, younger age of onset and female sex. Results of this study may help patients with these risk factors receive early aggressive intervention to change their natural disease course of RA.

Acknowledgments

We thank all DNA donors for making this study possible. We appreciate the members of Institute of Rheumatology, Tokyo Women's Medical University for their effort on the IORRA cohort. We are also grateful to the members of the Genetics and Allied research in Rheumatic diseases Networking (GARNET) consortium for their helpful advices in conducting the study. We also thank Ms Kaori Arai for her technical assistance, and Dr. Noriko Iikuni for her assistance in preparing the manuscript.

Author Contributions

Conceived and designed the experiments: KI. Performed the experiments: TS KI EI. Analyzed the data: TS KI KY EI. Contributed reagents/materials/analysis tools: KI KY YT AT HY SM. Wrote the paper: TS KI.

References

1. Smolen JS, Aletaha D, Bijlsma JW, Breedveld FC, Boumpas D, et al. (2010) Treating rheumatoid arthritis to target: recommendations of an international task force. Ann Rheum Dis 69: 631–637.
2. Smolen JS, Landewe R, Breedveld FC, Dougados M, Emery P, et al. (2010) EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs. Ann Rheum Dis 69: 964–975.
3. Singh JA, Furst DE, Bharat A, Curtis JR, Kavanaugh AF, et al. (2012) 2012 update of the 2008 american college of rheumatology recommendations for the use of disease-modifying antirheumatic drugs and biologic agents in the treatment of rheumatoid arthritis. Arthritis Care Res (Hoboken) 64: 625–639.
4. Syversen SW, Gaarder PI, Goll GL, Odegard S, Haavardsholm EA, et al. (2008) High anti-cyclic citrullinated peptide levels and an algorithm of four variables predict radiographic progression in patients with rheumatoid arthritis: results from a 10-year longitudinal study. Ann Rheum Dis 67: 212–217.
5. Forslind K, Ahlmen M, Eberhardt K, Hafstrom I, Svensson B (2004) Prediction of radiological outcome in early rheumatoid arthritis in clinical practice: role of antibodies to citrullinated peptides (anti-CCP). Ann Rheum Dis 63: 1090–1095.
6. Berglin E, Johansson T, Sundin U, Jidell E, Wadell G, et al. (2006) Radiological outcome in rheumatoid arthritis is predicted by presence of by IgA-RF at disease onset. Ann Rheum Dis 65: 453–458.
7. Lindqvist E, Eberhardt K, Bendtzen K, Heinegard D, Saxne T (2005) Prognostic laboratory markers of joint damage in rheumatoid arthritis. Ann Rheum Dis 64: 196–201.
8. Manfredsdottir VF, Vikingsdottir T, Jonsson T, Geirsson AJ, Kjartansson O, et al. (2006) The effects of tobacco smoking and rheumatoid factor seropositivity on disease activity and joint damage in early rheumatoid arthritis. Rheumatology (Oxford) 45: 734–740.
9. Wolfe F (2000) The effect of smoking on clinical, laboratory, and radiographic status in rheumatoid arthritis. J Rheumatol 27: 630–637.
10. van der Heijde DM, van Riel PL, van Leeuwen MA, van 't Hof MA, van Rijswijk MH, et al. (1992) Prognostic factors for radiographic damage and physical disability in early rheumatoid arthritis. A prospective follow-up study of 147 patients. Br J Rheumatol 31: 519–525.
11. Welsing PM, Landewe RB, van Riel PL, Boers M, van Gestel AM, et al. (2004) The relationship between disease activity and radiologic progression in patients with rheumatoid arthritis: a longitudinal analysis. Arthritis Rheum 50: 2082–2093.
12. Aletaha D, Funovits J, Breedveld FC, Sharp J, Segurado O, et al. (2009) Rheumatoid arthritis joint progression in sustained remission is determined by disease activity levels preceding the period of radiographic assessment. Arthritis Rheum 60: 1242–1249.
13. Kuiper S, van Gestel AM, Swinkels HL, de Boo TM, da Silva JA, et al. (2001) Influence of sex, age, and menopausal state on the course of early rheumatoid arthritis. J Rheumatol 28: 1809–1816.
14. van der Heijde DM, van Riel PL, van Leeuwen MA, van 't Hof MA, van Rijswijk MH, et al. (1991) Older versus younger onset rheumatoid arthritis: results at onset and after 2 years of a prospective followup study of early rheumatoid arthritis. J Rheumatol 18: 1285–1289.
15. Pease CT, Bhakta BB, Devlin J, Emery P (1999) Does the age of onset of rheumatoid arthritis influence phenotype?: a prospective study of outcome and prognostic factors. Rheumatology (Oxford) 38: 228–234.
16. Okada Y, Terao C, Ikari K, Kochi Y, Ohmura K, et al. (2012) Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population. Nat Genet 44: 511–516.
17. Zhernakova A, Stahl EA, Trynka G, Raychaudhuri S, Festen EA, et al. (2011) Meta-analysis of genome-wide association studies in celiac disease and rheumatoid arthritis identifies fourteen non-HLA shared loci. PLoS Genet 7: e1002004.
18. Stahl EA, Raychaudhuri S, Remmers EF, Xie G, Eyre S, et al. (2010) Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nat Genet 42: 508–514.
19. Marotte H, Farge P, Gaudin P, Alexandre C, Mougin B, et al. (2006) The association between periodontal disease and joint destruction in rheumatoid arthritis extends the link between the HLA-DR shared epitope and severity of bone destruction. Ann Rheum Dis 65: 905–909.
20. Wagner U, Kaltenhauser S, Pierer M, Seidel W, Troltzsch M, et al. (2003) Prospective analysis of the impact of HLA-DR and -DQ on joint destruction in recent-onset rheumatoid arthritis. Rheumatology (Oxford) 42: 553–562.
21. Gorman JD, Lum RF, Chen JJ, Suarez-Almazor ME, Thomson G, et al. (2004) Impact of shared epitope genotype and ethnicity on erosive disease: a meta-analysis of 3,240 rheumatoid arthritis patients. Arthritis Rheum 50: 400–412.
22. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, et al. (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31: 315–324.
23. Yamanaka H, Inoue E, Singh G, Tanaka E, Nakajima A, et al. (2007) Improvement of disease activity of rheumatoid arthritis patients from 2000 to 2006 in a large observational cohort study IORRA in Japan. Mod Rheumatol 17: 283–289.
24. Yamanaka H, Tohma S (2006) Potential impact of observational cohort studies in Japan on rheumatoid arthritis research and practice. Mod Rheumatol 16: 75–76.
25. van der Heijde DM (2000) How to read radiographs according to the Sharp/van der Heijde method. J Rheumatol 27: 261–263.
26. Nishimoto K, Kochi Y, Ikari K, Yamamoto K, Suzuki A, et al. (2010) Association study of TRAF1-C5 polymorphisms with susceptibility to rheumatoid arthritis and systemic lupus erythematosus in Japanese. Ann Rheum Dis 69: 368–373.
27. Keystone EC (2009) Clinical implications of understanding radiographic findings in relation to clinical outcomes in rheumatoid arthritis. J Rheumatol Suppl 82: 11–16.
28. Shidara K, Inoue E, Tanaka E, Hoshi D, Seto Y, et al. (2011) Comparison of the second and third generation anti-cyclic citrullinated peptide antibody assays in the diagnosis of Japanese patients with rheumatoid arthritis. Rheumatol Int 31: 617–622.
29. Ideguchi H, Ohno S, Hattori H, Senuma A, Ishigatsubo Y (2006) Bone erosions in rheumatoid arthritis can be repaired through reduction in disease activity with conventional disease-modifying antirheumatic drugs. Arthritis Res Ther 8: R76.
30. Landewe R, van der Heijde D (2005) Radiographic progression in rheumatoid arthritis. Clin Exp Rheumatol 23: S63–68.
31. de Vries-Bouwstra JK, Goekoop-Ruiterman YP, Verpoort KN, Schreuder GM, Ewals JA, et al. (2008) Progression of joint damage in early rheumatoid arthritis: association with HLA-DRB1, rheumatoid factor, and anti-citrullinated protein antibodies in relation to different treatment strategies. Arthritis Rheum 58: 1293–1298.
32. Keene ON (1995) The log transformation is special. Stat Med 14: 811–819.
33. McDonald JH (2009) Handbook of Biological Statistics. Baltimore: Sparky House Publishing. 160–164 p.
34. Tanaka E, Mannalithara A, Inoue E, Hara M, Tomatsu T, et al. (2008) Efficient management of rheumatoid arthritis significantly reduces long-term functional disability. Ann Rheum Dis 67: 1153–1158.
35. Nakajima A, Inoue E, Tanaka E, Singh G, Sato E, et al. (2010) Mortality and cause of death in Japanese patients with rheumatoid arthritis based on a large observational cohort, IORRA. Scand J Rheumatol 39: 360–367.
36. Nakajima A, Inoue E, Shidara K, Hoshi D, Sato E, et al. (2011) Standard treatment in daily clinical practice for early rheumatoid arthritis improved disease activity from 2001 to 2006. Mod Rheumatol 21: 594–597.
37. van der Heijde DM (1995) Joint erosions and patients with early rheumatoid arthritis. Br J Rheumatol 34 Suppl 2: 74–78.
38. Tsukahara S, Shinozaki M, Ikari K, Mochizuki T, Inoue E, et al. (2008) Effect of matrix metalloproteinase-3 functional SNP on serum matrix metalloproteinase-3 level and outcome measures in Japanese RA patients. Rheumatology (Oxford) 47: 41–44.
39. Kobayashi S, Ikari K, Kaneko H, Kochi Y, Yamamoto K, et al. (2008) Association of STAT4 with susceptibility to rheumatoid arthritis and systemic

lupus erythematosus in the Japanese population. Arthritis Rheum 58: 1940–1946.
40. Rojas-Villarraga A, Diaz FJ, Calvo-Paramo E, Salazar JC, Iglesias-Gamarra A, et al. (2009) Familial disease, the HLA-DRB1 shared epitope and anti-CCP antibodies influence time at appearance of substantial joint damage in rheumatoid arthritis. J Autoimmun 32: 64–69.
41. Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, et al. (2003) Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat Genet 34: 395–402.
42. Kang CP, Lee HS, Ju H, Cho H, Kang C, et al. (2006) A functional haplotype of the PADI4 gene associated with increased rheumatoid arthritis susceptibility in Koreans. Arthritis Rheum 54: 90–96.
43. Iwamoto T, Ikari K, Nakamura T, Kuwahara M, Toyama Y, et al. (2006) Association between PADI4 and rheumatoid arthritis: a meta-analysis. Rheumatology (Oxford) 45: 804–807.
44. Barton A, Bowes J, Eyre S, Spreckley K, Hinks A, et al. (2004) A functional haplotype of the PADI4 gene associated with rheumatoid arthritis in a Japanese population is not associated in a United Kingdom population. Arthritis Rheum 50: 1117–1121.
45. Burr ML, Naseem H, Hinks A, Eyre S, Gibbons LJ, et al. (2010) PADI4 genotype is not associated with rheumatoid arthritis in a large UK Caucasian population. Ann Rheum Dis 69: 666–670.
46. Martinez A, Valdivia A, Pascual-Salcedo D, Lamas JR, Fernandez-Arquero M, et al. (2005) PADI4 polymorphisms are not associated with rheumatoid arthritis in the Spanish population. Rheumatology (Oxford) 44: 1263–1266.
47. Too CL, Murad S, Dhaliwal JS, Larsson PT, Jiang X, et al. (2012) Polymorphisms in peptidylarginine deiminase (PADI) associate with rheumatoid arthritis in diverse Asian populations: evidence from MyEIRA study and meta-analysis. Arthritis Res Ther 14: R250.
48. Hoppe B, Haupl T, Egerer K, Gruber R, Kiesewetter H, et al. (2009) Influence of peptidylarginine deiminase type 4 genotype and shared epitope on clinical characteristics and autoantibody profile of rheumatoid arthritis. Ann Rheum Dis 68: 898–903.
49. Harris ML, Darrah E, Lam GK, Bartlett SJ, Giles JT, et al. (2008) Association of autoimmunity to peptidyl arginine deiminase type 4 with genotype and disease severity in rheumatoid arthritis. Arthritis Rheum 58: 1958–1967.
50. Cha S, Choi CB, Han TU, Kang CP, Kang C, et al. (2007) Association of anti-cyclic citrullinated peptide antibody levels with PADI4 haplotypes in early rheumatoid arthritis and with shared epitope alleles in very late rheumatoid arthritis. Arthritis Rheum 56: 1454–1463.
51. Bang SY, Han TU, Choi CB, Sung YK, Bae SC, et al. (2010) Peptidyl arginine deiminase type IV (PADI4) haplotypes interact with shared epitope regardless of anti-cyclic citrullinated peptide antibody or erosive joint status in rheumatoid arthritis: a case control study. Arthritis Res Ther 12: R115.

OPEN ACCESS Freely available online

PLoS one

ACPA-Negative RA Consists of Two Genetically Distinct Subsets Based on RF Positivity in Japanese

Chikashi Terao[1,2]*, Koichiro Ohmura[1]*, Katsunori Ikari[3], Yuta Kochi[4], Etsuko Maruya[5], Masaki Katayama[1], Kimiko Yurugi[6], Kota Shimada[7], Akira Murasawa[8], Shigeru Honjo[9], Kiyoshi Takasugi[10], Keitaro Matsuo[11], Kazuo Tajima[11], Akari Suzuki[4], Kazuhiko Yamamoto[12], Shigeki Momohara[3], Hisashi Yamanaka[3], Ryo Yamada[2], Hiroo Saji[5], Fumihiko Matsuda[2,13,14], Tsuneyo Mimori[1]

1 Department of Rheumatology and Clinical Immunology, Kyoto University Graduate School of Medicine, Kyoto, Japan, **2** Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan, **3** Institute of Rheumatology, Tokyo Women's Medical University, Tokyo, Japan, **4** Laboratory for Autoimmune Diseases, Center for Genomic Medicine, RIKEN, Yokohama, Japan, **5** HLA Laboratory, Kyoto, Japan, **6** Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, Kyoto, Japan, **7** Department of Rheumatology, Sagamihara National Hospital, National Hospital Organization, Sagamihara, Japan, **8** Department of Rheumatology, Niigata Rheumatic Center, Niigata, Japan, **9** Rheumatoid Arthritis Center, Saiseikai Takaoka Hospital, Toyama, Japan, **10** Department of Internal Medicine, Center for Rheumatic Diseases, Dohgo Spa Hospital, Matsuyama, Japan, **11** Aichi Cancer Center Hospital and Research Institute, Nagoya, Japan, **12** Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan, **13** CREST program, Japan Science and Technology Agency, Kawaguchi, Saitama, Japan, **14** Institut National de la Sante et de la Recherche Medicale (INSERM) Unite U852, Kyoto University Graduate School of Medicine, Kyoto, Japan

Abstract

HLA-DRB1, especially the shared epitope (SE), is strongly associated with rheumatoid arthritis (RA). However, recent studies have shown that SE is at most weakly associated with RA without anti-citrullinated peptide/protein antibody (ACPA). We have recently reported that ACPA-negative RA is associated with specific HLA-DRB1 alleles and diplotypes. Here, we attempted to detect genetically different subsets of ACPA-negative RA by classifying ACPA-negative RA patients into two groups based on their positivity for rheumatoid factor (RF). HLA-DRB1 genotyping data for totally 954 ACPA-negative RA patients and 2,008 healthy individuals in two independent sets were used. HLA-DRB1 allele and diplotype frequencies were compared among the ACPA-negative RF-positive RA patients, ACPA-negative RF-negative RA patients, and controls in each set. Combined results were also analyzed. A similar analysis was performed in 685 ACPA-positive RA patients classified according to their RF positivity. As a result, HLA-DRB1*04:05 and *09:01 showed strong associations with ACPA-negative RF-positive RA in the combined analysis ($p = 8.8 \times 10^{-6}$ and 0.0011, OR: 1.57 (1.28–1.91) and 1.37 (1.13–1.65), respectively). We also found that HLA-DR14 and the HLA-DR8 homozygote were associated with ACPA-negative RF-negative RA ($p = 0.00022$ and 0.00013, OR: 1.52 (1.21–1.89) and 3.08 (1.68–5.64), respectively). These association tendencies were found in each set. On the contrary, we could not detect any significant differences between ACPA-positive RA subsets. As a conclusion, ACPA-negative RA includes two genetically distinct subsets according to RF positivity in Japan, which display different associations with HLA-DRB1. ACPA-negative RF-positive RA is strongly associated with HLA-DRB1*04:05 and *09:01. ACPA-negative RF-negative RA is associated with DR14 and the HLA-DR8 homozygote.

Citation: Terao C, Ohmura K, Ikari K, Kochi Y, Maruya E, et al. (2012) ACPA-Negative RA Consists of Two Genetically Distinct Subsets Based on RF Positivity in Japanese. PLoS ONE 7(7): e40067. doi:10.1371/journal.pone.0040067

Editor: Pierre Bobé, Institut Jacques Monod, France

Received March 10, 2012; **Accepted** May 31, 2012; **Published** July 6, 2012

Funding: This study was supported by Grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, as well as by research grants from the Japan Rheumatism Foundation, the Waksman Foundation, and the Mitsubishi Pharma Research Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding received for this study.

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

* E-mail: a0001101@kuhp.kyoto-u.ac.jp (CT); ohmurako@kuhp.kyoto-u.ac.jp (KO)

Introduction

Rheumatoid arthritis (RA) is the most common cause of chronic arthritis worldwide and results in severe joint destruction [1]. Genetic and environmental factors have been shown to be associated with its onset [2–3]. Among the susceptibility genes to RA, HLA-DRB1 has been shown to be the strongest genetic determinant of RA susceptibility, and its association with RA susceptibility has been repeatedly shown to be independent of ethnicity [4–5]. A common amino acid sequence extending from the 70th to 74th in the HLA-DRβ chain, which is known as the "shared epitope (SE)", is considered to be the reason for the association between HLA-DRB1 and RA, and the association between the SE and RA has been reported to be ethnicity-independent [6–8]. However, recent studies have shown that the SE is strongly associated with RA patients who have anti-citrullinated peptide/protein antibodies (ACPA), which is a highly specific marker of RA [9], but that it is not or only weakly associated with RA without ACPA [7,10–11]. Among the various HLA-DRB1 alleles, HLA-DR3 [12] and HLA-DR13 [13] were reported to be associated with ACPA-negative RA in populations of European descent, but these results were not confirmed in a

meta-analysis of a large Caucasian cohort [8]. In Asian populations, we recently reported that DRB1*12:01 is a HLA-DRB1 susceptibility allele for ACPA-negative RA in Japanese populations and that DRB1*04:05, the most common SE allele in Japanese, and *14:03 showed moderate associations with ACPA-negative RA susceptibility [14]. We also reported that DRB1*15:02 and *13:02 displayed protective associations with ACPA-negative RA and that being homozygous for HLA-DR8 was associated with ACPA-negative RA susceptibility. While a very small Japanese study suggested that HLA-DRB1*09:01 is associated with ACPA-negative RA [15], our study did not detect a significant association between them. These findings suggest that ACPA-negative RA is genetically different from ACPA-positive RA in terms of its associations with HLA-DRB1 alleles. While some specific alleles and diplotypes seem to be associated with ACPA-negative RA, the genetic characteristics of ACPA-negative RA have not been fully elucidated. Recently, UK group reported that SE is associated with ACPA-negative RF-positive RA in UK population [16]. However, whether this is true to other population is uncertain. Moreover, the associations of other alleles than SE with subgroups of ACPA-negative RA have never been reported. Here, we show that when we classified ACPA-negative RA into two subsets based on rheumatoid factor (RF) positivity, we were able to clearly distinguish them from each other according to their associations with HLA-DRB1 alleles, not only with SE, but with other alleles. We also compared ACPA-positive RA patients based on their RF positivity to examine whether we can apply this classification to ACPA-positive RA.

Results

HLA-DRB1 Alleles Associated with ACPA-negative RF-positive RA

We compared 179 ACPA-negative RF-positive RA with 1508 controls in collection 1 for their frequency of HLA-DRB1 alleles, followed by comparison of 267 ACPA-negative RF-positive RA with 500 controls in collection 2. Significant association was evaluated in the combined analysis. Regarding HLA-DRB1 alleles that were previously shown to be associated with ACPA-negative RA, we found that all of the alleles, namely, HLA-DRB1*12:01, *04:05, *13:02, *14:03, and *15:02 showed association tendency with ACPA-negative RF-positive RA in the combined study (Table 1). Interestingly, HLA-DRB1*04:05 ($p = 8.8 \times 10^{-6}$, odds ratio (OR): 1.57) showed the strongest association, while its association with entire ACPA-negative RA was moderate in the previous study. When we analyzed the associations of the SE, we found that it displayed a significant association ($p = 0.00013$, OR: 1.37). HLA-DRB1*04:05 was responsible for most of the association of SE because none of the other SE alleles showed significant associations with ACPA-negative RF-positive RA. We also found that HLA-DRB1*09:01, which was not associated with ACPA-negative RA as a single allele, was found to be significantly associated with ACPA-negative RF-positive RA ($p = 0.0011$, OR: 1.37). Importantly, these association tendencies written above were observed in both collections (Table 1). Logistic regression analysis was carried out to examine whether the susceptibility associations were dependent on a lack of protective alleles or vice versa. As a result, it was demonstrated that HLA-DRB1*04:05, *09:01, and *12:01 showed significant associations ($p<0.0005$), while the associations of HLA-DRB1*14:03, *13:02, and *15:02 were moderate to suggestive (Table S1). Next, we analyzed the dosage effects of the alleles and found that the association between HLA-DRB1*09:01 and ACPA-negative RF-positive RA showed a clear dosage effect (Figure S1). HLA-DRB1*12:01 also showed a dosage effect (data not shown due to small number). HLA-DRB1*04:05 did not show a dosage effect, suggesting that the effect of HLA-DRB1*04:05 on the predisposition to ACPA-negative RF-positive RA is a dominant effect.

HLA-DRB1 Alleles Associated with ACPA-negative RF-negative RA

Next we compared 274 ACPA-negative RF-negative RA with 1,508 controls, followed by comparison between 234 ACPA-negative RF-negative RA and 500 controls. Interestingly, we did not observe association of HLA-DRB1*04:05 and *09:01 with ACPA-negative RF-negative RA, while HLA-DRB1*12:01, *13:02, *14:03, and *15:02 were moderately associated with ACPA-negative RF-negative RA (Table 2). The SE was not associated with ACPA-negative RF-negative RA. DR14 was found to be significantly associated with ACPA-negative RF-negative RA and HLA-DRB1*14:03 and *14:06 comprised the association of HLA-DR14 (Table S2). These association tendencies in ACPA-negative RF-negative RA were observed in both sets (Table 2). Logistic regression analysis confirmed that none of the associations were mutually dependent and that the association of DR14 remained significant ($p = 0.00069$, Table S3). DR14 could not be evaluated the dosage effect because neither the cases nor controls included DRB1*14:03 or *14:06 homozygotes or the DRB1*14:03 and *14:06 diplotype.

HLA Diplotype Analysis: DR8 Homozygote and *12:01/*09:01 Diplotype

As we previously showed that the DR8 homozygote was significantly associated with susceptibility to ACPA-negative RA, we analyzed its associations with ACPA-negative RF-positive RA and RF-negative RA. As a result, we found that the HLA-DR8 homozygote is exclusively associated with ACPA-negative RF-negative RA in the combined study ($p = 0.00013$, OR: 3.08 for ACPA-negative RF-negative RA, Table 2; $p = 0.86$, OR: 1.08 for ACPA-negative RF-positive RA, Table 1). The effect of DR8 on the susceptibility to ACPA-negative RF-negative RA was not dose-dependent (OR: 1.04 for HLA-DR8 heterozygote).

We also found that the combination of HLA-DRB1*12:01 and *09:01, the diplotype that was most strongly associated with susceptibility to ACPA-negative RA in the previous study, was especially strongly associated with ACPA-negative RF-positive RA ($p = 5.0 \times 10^{-6}$, OR: 4.97 for ACPA-negative RF-positive RA; $p = 0.040$, OR: 2.46 for ACPA-negative RF-negative RA).

We found that the similar associations were seen between the alleles/diplotypes and ACPA-negative RF-positive erosive RA and ACPA-negative RF-negative erosive RA (except for that between HLA-DRB1*12:01 and the ACPA-negative RF-negative subset), even though the number of patients was limited (Table S4).

Comparison between ACPA-negative RF-positive RA and ACPA-negative RF-negative RA

To compare the usage of HLA-DRB1 allele between ACPA-negative RF-positive RA and ACPA-negative RF-negative RA, we directly compared the allele and diplotype frequencies between the two groups (Table 3). As expected, HLA-DRB1*09:01 and *04:05 showed significant differences in their frequencies between the two subsets ($p = 0.0018$ and 0.0034, respectively). The SE was more common in the ACPA-negative RF-positive RA patients ($p = 0.0047$), whereas DR14 was more prevalent in the ACPA-negative RF-negative RA patients ($p = 0.028$). The DR8 homozygote was more frequently seen in the ACPA-negative RF-negative RA patients than in the ACPA-negative RF-positive RA patients

Table 1. Association of HLA-DRB1 alleles with ACPA-negative RF-positive RA.

	1st set				2nd set				combined analysis			
HLA-DRB1 allele	§ACPA (-)RF(+)RA	§control	*p*	OR	§ACPA (-)RF(+)RA	§control	*p*	OR	§ACPA (-)RF(+)RA	§control	*p*	OR
*04:05	65 (18.2%)	340 (11.3%)	0.00015	1.75 (1.30–2.34)	88 (16.5%)	129 (12.9%)	0.055	1.33 (0.99–1.79)	153 (17.2%)	469 (11.7%)	8.8×10^{-6}	1.57 (1.28–1.91)
*09:01	70 (19.6%)	432 (14.3%)	0.0086	1.45 (1.10–1.92)	99 (18.5%)	154 (15.4%)	0.11	1.25 (0.95–1.65)	169 (18.9%)	586 (14.6%)	0.0011	1.37 (1.13–1.65)
*12:01	13 (3.6%)	91 (3%)	0.53	1.21 (0.67–2.19)	35 (6.6%)	37 (3.7%)	0.012	1.83 (1.14–2.93)	48 (5.4%)	128 (3.2%)	0.0014	1.73 (1.23–2.43)
*13:02	21 (5.9%)	273 (9.1%)	0.043	0.63 (0.40–0.99)	18 (3.4%)	52 (5.2%)	0.10	0.64 (0.37–1.1)	39 (4.4%)	325 (8.1%)	0.00013	0.52 (0.37–0.73)
*14:03	7 (2.0%)	39 (1.3%)	0.31	1.52 (0.68–3.43)	13 (2.4%)	14 (1.4%)	0.14	1.76 (0.82–3.77)	20 (2.2%)	53 (1.3%)	0.040	1.71 (1.02–2.88)
*15:02	43 (12.0%)	369 (12.2%)	0.90	0.98 (0.70–1.37)	37 (6.9%)	113 (11.3%)	0.0060	0.58 (0.4–0.86)	80 (9.0%)	482 (12.0%)	0.010	0.72 (0.56–0.93)
SE	106 (29.6%)	677 (22.4%)	0.0024	1.45 (1.14–1.85)	150 (28.1%)	233 (23.3%)	0.039	1.29 (1.01–1.63)	256 (28.7%)	910 (22.7%)	0.00013	1.37 (1.17–1.62)
DR14	29 (8.1%)	253 (8.4%)	0.85	0.96 (0.64–1.44)	48 (9.0%)	73 (7.3%)	0.24	1.25 (0.86–1.83)	78 (8.7%)	326 (8.1%)	0.55	1.08 (0.83–1.40)
Diplotype												
DR8/DR8	3 (1.7%)	17 (1.1%)	0.46	1.49 (0.28–5.24)	3 (1.1%)	8 (1.6%)	0.76	0.70 (0.12–2.94)	6 (1.3%)	25 (1.2%)	0.86	1.08 (0.44–2.65)
*12:01/*09:01	5 (2.8%)	10 (0.66%)	0.0041	4.30 (1.45–12.74)	9 (3.3%)	3 (0.60%)	0.0051	5.76 (1.42–33.42)	14 (3.1%)	13 (0.6%)	5.0×10^{-6}	4.97 (2.32–10.66)

OR: odds ratio.

SE: shared epitope: HLA-DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *04:10, *04:13, *04:16, *10:01, *14:02, and *14:06.

doi:10.1371/journal.pone.0040067.t001

(p = 0.021). When we applied logistic regression analysis to the HLA-DRB1*09:01, *04:05, and HLA-DR14, their associations were revealed to be significant and do not depend on each other (p = 0.00067 and 0.00072, respectively, Table S5), except for that of DR14 (p = 0.30).

Comparison between ACPA-positive RF-positive RA and ACPA-positive RF-negative RA

Next, we analyzed whether these allele usage differences are also seen in ACPA-positive RA. We collected data about the HLA-DRB1 genotypes of 154 ACPA-positive RF-negative RA patients and 531 ACPA-positive RF-positive RA patients. As the SE and HLA-DRB1*09:01 were found to be associated with ACPA-positive RA, we analyzed the differences in the frequencies of these alleles [17]. In comparison with the healthy controls, SE and HLA-DRB1*09:01 were associated with a predisposition to ACPA-positive RF-positive RA as well as ACPA-positive RF-negative RA and displayed comparable odds ratios in logistic regression analysis (Table 4). No HLA-DRB1 alleles showed a strong specific association with a particular subset. When we directly compared the two subsets of ACPA-positive RA, no alleles displayed significant associations (Figure 1, Table S6). However, whether the two subsets of ACPA-positive RA share most of HLA-DRB1 susceptibility associations is inconclusive due to the small number of RF-negative subset.

Discussion

In this study, we demonstrated that classifying Japanese ACPA-negative RA patients based on their RF positivity successfully divided them into two genetically different subsets, which displayed different associations with HLA-DRB1. We showed that HLA-DRB1*09:01 and *04:05, strong susceptibility alleles to ACPA-positive RA, were also associated with ACPA-negative RF-positive subset, and that DR14 and the DR8 homozygote were associated only with the ACPA-negative RF-negative subset (Figure 1). Since the titer of RF fluctuates along with disease activity much more than that of ACPA, we were very careful to take the maximum RF titer when multiple titers were available for a particular patient, in order to prevent the RF positive subset from being contaminated with RF negative RA patients. The Recent UK population study reported the association of SE with ACPA-negative RF-positive RA [16]. Our study not only confirmed this association in Japanese RA, but also showed that the association of SE with ACPA-negative RF-positive RA is mainly due to the effect of HLA-DRB1*04:05 and that HLA-DRB1*09:01, HLA-DR14, and homozygote of HLA-DR8 are specifically associated with subsets of ACPA-negative RA.

These above-mentioned association tendencies were observed in the first set and successfully replicated in the second set, indicating that we can avoid population stratification or sampling bias. The effect sizes (odds ratio) of the alleles were comparable in each cohort (Tables 1 and 2) and the associations in the combined analysis reached significant level, although the p-values in each set did not reach the significance level due to the limited number of samples they contained. These data indicate that our results are reliable, at least in Japanese populations, although further replication studies including other populations are favorable. In the current study, we used logistic regression analysis to confirm independency of associated alleles in each comparison. When we used relative predispositional effects (RPE) method [18] to stratify associated alleles, we obtained the similar results to those we obtained by logistic regression analysis (data not shown).

Table 2. Association of HLA-DRB1 alleles with ACPA-negative RF-negative RA.

	1st set				2nd set				combined analysis			
HLA-DRB1 allele	§ACPA(-)RF (-)RA	§control	*p*	OR	§ACPA(-)RF (-)RA	§control	*p*	OR	§ACPA(-)RF (-)RA	§control	*p*	OR
*04:05	69 (12.6%)	340 (11.3%)	0.37	1.13 (0.86–1.49)	57 (12.2%)	129 (12.9%)	0.70	0.94 (0.67–1.31)	126 (12.4%)	469 (11.7%)	0.52	1.07 (0.87–1.32)
*09:01	74 (13.5%)	432 (14.3%)	0.61	0.93 (0.72–1.22)	65 (13.9%)	154 (15.4%)	0.45	0.89 (0.65–1.21)	139 (13.7%)	586 (14.6%)	0.46	0.93 (0.76–1.13)
*12:01	28 (5.1%)	91 (3.0%)	0.012	1.73 (1.12–2.67)	27 (5.8%)	37 (3.7%)	0.070	1.59 (0.96–2.65)	55 (5.4%)	128 (3.2%)	0.00071	1.74 (1.26–2.40)
*13:02	28 (5.1%)	273 (9.1%)	0.0023	0.54 (0.36–0.81)	34 (7.3%)	52 (5.2%)	0.070	1.59 (0.96–2.65)	62 (6.1%)	325 (8.1%)	0.033	0.74 (0.56–0.98)
*14:03	12 (2.2%)	39 (1.3%)	0.10	1.71 (0.89–3.29)	10 (2.1%)	14 (1.4%)	0.30	1.54 (0.68–3.49)	22 (2.2%)	53 (1.3%)	0.047	1.65 (1.00–2.73)
*15:02	51 (9.3%)	369 (12.2%)	0.051	0.74 (0.54–1.00)	36 (7.7%)	113 (11.3%)	0.033	0.65 (0.44–0.97)	87 (8.6%)	482 (12.0%)	0.0020	0.69 (0.54–0.87)
SE	131 (23.9%)	677 (22.4%)	0.45	1.09 (0.88–1.34)	103 (22%)	233 (23.3%)	0.58	0.93 (0.71–1.21)	234 (23.0%)	910 (22.7%)	0.80	1.02 (0.87–1.2)
DR14	69 (12.6%)	253 (8.4%)	0.0016	1.57 (1.19–2.09)	51 (10.9%)	73 (7.3%)	0.021	1.55 (1.07–2.26)	120 (11.8%)	326 (8.1%)	0.00022	1.52 (1.21–1.89)
Diplotype												
DR8/DR8	12 (4.4%)	17 (1.1%)	9.1×10^{-5}	4.02 (1.90–8.51)	7 (3.0%)	8 (1.6%)	0.21	1.90 (0.68–5.29)	19 (3.7%)	25 (1.2%)	0.00013	3.08 (1.68–5.64)
*12:01/*09:01	4 (1.5%)	10 (0.66%)	0.25	2.22 (0.50–7.76)	4 (1.7%)	3 (0.60%)	0.22	2.88 (0.48–19.80)	8 (1.6%)	13 (0.6%)	0.040	2.46 (1.01–5.96)

doi:10.1371/journal.pone.0040067.t002

In our previous study [14], HLA-DRB1*09:01 was not significantly associated with ACPA-negative RA, in spite of the association it displayed in combination with HLA-DRB1*12:01. In the current study, we showed that HLA-DRB1*09:01 displayed a strong dose-dependent association with ACPA-negative RF-positive RA, but not with ACPA-negative RF-negative RA. These findings were confirmed by a direct comparison between the two subsets. A small study in Japan suggested that HLA-DRB1*09:01 is associated with ACPA-negative RA [15]. Our results suggest that their study mainly included ACPA-negative RF-positive RA patients. HLA-DRB1*09:01 was shown to reduce the ACPA titer in Japanese ACPA-positive RA patients [19–20]. Therefore, HLA-DRB1*09:01 might increase the titer of RF and decrease that of ACPA, although our study also showed that HLA-DRB1*09:01 is associated with ACPA-positive RF-negative RA.

HLA-DRB1*04:05, which is a major component of the SE in Asians [17], was shown to be significantly associated with ACPA-negative RA in our previous study. The current study showed that it is only associated with ACPA-negative RF-positive RA. This predisposition was also confirmed by direct comparison of the two subsets. As we could not detect a dosage effect of HLA-DRB1*04:05, its susceptibility effect might occur in a dominant manner. It is interesting that of the many SE alleles only HLA-DRB1*04:05 is associated with ACPA-negative RF-positive RA. This does not seem to be due to the relatively low frequencies of the other SE alleles (Table 1). Therefore, the common amino acid sequence that extends from the 70^{th} to the 74^{th} amino acid of the HLA-DRβ chain might not be important for the development of ACPA-negative RF-positive RA. As immunization of citrullinated peptide induced arthritis in HLA-DR4 transgenic mice [21] and citrullinated peptides were shown to have higher affinity to HLA-DR4 [22], high affinity of SE to citrullinated antigen is hypothesized to be the link between SE and RA development. Our findings may raise possibility of another mechanism of SE in developing arthritis.

It is quite interesting that HLA-DRB1*04:05 and *09:01, strongly associated alleles with ACPA-positive RA, are associated with ACPA-negative RF-positive RA. Although there are genetic similarities between ACPA-negative RF-positive RA and ACPA-positive RA, they should be considered to be different subsets as SE alleles other than HLA-DRB1*04:05 are not associated with ACPA-negative RF-positive RA and the HLA-DRB1*09:01 and *12:01 diplotype is strongly associated with ACPA-negative RF-positive RA.

When we analyzed the HLA-DR14 serotype, it showed a strong association with ACPA-negative RF-negative RA, largely due to HLA-DRB1*14:03 and *14:06. When we compared the frequency of DR14 in each ACPA-negative subset after stratifying the data according to the presence of HLA-DRB1*09:01 and *04:05, DR14 did not display a significant effect. In this sense, the specific association of DR14 with ACPA-negative RF-negative RA needs to be confirmed.

The HLA-DR8 homozygote displayed an association with ACPA-negative RA in our previous study [14]. The current study demonstrated that its association is specific to ACPA-negative RF-negative RA. As the number of HLA-DR8 homozygote is limited, further replication is necessary for this association. No association between the HLA-DR8 and 14 diplotype and susceptibility to ACPA-negative RF-negative RA was found (data not shown).

It is interesting that HLA-DR14 and HLA-DR8, associated serotype with ACPA-negative RF-negative RA, were reported association with psoriatic arthritis [23]. HLA-DR14 is often linked with HLA-Cw*06, susceptibility serotype to psoriasis arthritis in European [24]. HLA-Cw*06 is rare in Japanese (<1%) and the

Table 3. Direct comparison of HLA-DRB1 allele frequency between ACPA-negative RF-positive RA and ACPA-negative RF-negative RA.

HLA-DRB1	ACPA(−)RF(+)RA Number of allele (%)	ACPA(−)RF(−)RA Number of allele (%)	*p*	OR (95%CI)
*09:01	169 (18.9%)	139 (13.7%)	0.0018	1.47 (1.15–1.88)
*04:05	153 (17.2%)	126 (12.4%)	0.0034	1.46 (1.13–1.89)
*08:02	24 (2.7%)	52 (5.1%)	0.0068	0.51 (0.31–0.84)
*14:06	8 (0.9%)	21 (2.1%)	0.037	0.43 (0.19–0.97)
SE	256 (28.7%)	234 (23.0%)	0.0047	1.35 (1.09–1.65)
DR14	78 (8.7%)	120 (11.8%)	0.028	0.72 (0.53–0.97)
DR8/DR8	6 (1.3%)	19 (3.7%)	0.021	0.35 (0.14–0.89)

doi:10.1371/journal.pone.0040067.t003

strong association between HLA-Cw*06 and HLA-DR14 is not observed in Japan (<10%). While psoriatic arthritis is not reported to be associated with these serotypes in Japan, association between these serotypes and arthritis is interesting.

It could be argued that ACPA-negative RA includes some non-RA arthritic diseases such as psoriasis, seronegative spondyloarthropathy and other collagen vascular diseases. Thus, we analyzed the associations between the above-mentioned alleles and diplotypes with ACPA-negative RA displaying bone erosion to examine whether the same association patterns were present in this strictly defined cohort. The typical bone erosions of RA are rarely seen in other arthritic disorders. As a result, we found the same associations. Therefore, we are convinced that our findings were not caused by the contamination of our study population by patients with other diseases. Since RF sometimes normalizes after treatment, the RF-negative RA patients whose RF titers were not measured at multiple points might not have been RF-negative. So, we re-analyzed our data by excluding the RA patients for whom consecutive RF titers were not available. As a result, we found the same tendency of associations for each allele and diplotype in each subset (data not shown), indicating that these subsets are stable.

Analysis using ACPA-positive RF-positive RA and ACPA-positive RF-negative RA patients compared with healthy controls did not result in distinct differences in HLA-DRB1 association. The SE is associated with both ACPA-positive RF-positive and RF-negative RA. HLA-DRB1*09:01 was found to be associated with both subsets after stratifying the patients according to their SE alleles. We also did not detect an association between HLA-DR14 or the HLA-DR8 homozygote and either subset. While 154 ACPA-positive RF-negative RA patients in our study are too small in number to detect the difference in HLA-DRB1 alleles with weak effect size between the two ACPA-positive subsets, these results suggest that there are no big differences in the HLA usage of the two subsets in ACPA-positive RA. To confirm our results and to detect possible different frequency of other HLA-DRB1 alleles in the two ACPA-positive subsets, replication study is necessary.

Table 4. Logistic regression analysis of HLA-DRB1 alleles with ACPA-positive RF-positive RA and ACPA-positive RF-negative RA.

	ACPA(+)RF(+)RA		ACPA(+)RF(−)RA	
HLA-DRB1	*p**	OR (95%CI)*	*p**	OR (95%CI)*
SE	$<2\times10^{-16}$	3.21 (2.72–3.78)	$<2\times10^{-16}$	3.03 (2.33–3.94)
*09:01	2.4×10^{-9}	1.83 (1.5–2.25)	0.0035	1.67 (1.17–2.37)

*p-values and odds ratios in logistic regression analysis using SE and HLA-DRB1*09:01.

doi:10.1371/journal.pone.0040067.t004

In the current study, we performed multiple comparisons in each subset and between subsets. The associations should be evaluated in the combined analysis with significant level corrected by Bonferronii's method and independency of each association should be evaluated by logistic regression analysis or RPE method. In this sense, p-values around cut-off level in the combined analysis should be taken with caution and the associations should be confirmed by independent study.

We have shown that ACPA-negative RA includes two genetically distinct subsets in Japanese population: RF-positive and RF-negative RA. This is the first report in Asians to show that these subsets are genetically distinct. We have to clarify the clinical difference between these two subsets. We also have to clarify whether non-HLA genes display different associations with each subset. So far, many genome wide association studies (GWAS) of RA and ACPA-positive RA have been performed, and more than twenty genes or loci have been shown to be susceptibility loci [25–38]. However, no GWAS studies have detected susceptibility genes for ACPA-negative RA with genome-wide significance [39]. This is probably due to the relatively small number of patients studied, but it might be overcome by stratifying ACPA-negative RA patients into RF-positive and RF-negative subsets. Since RA susceptibility genes usually cross ethnic boundaries [40], global collaboration might result in a fruitful dissection of these minor subsets.

Materials and Methods

Ethics Statement

This study was approved by the local ethical committees at each institution, namely, Kyoto University Graduate School and Faculty of Medicine, Ethics Committee, Tokyo Women's Medical University Genome Ethics Committee, and the ethics committee of RIKEN, and written informed consent was obtained from all patients.

Study Subjects

DNA samples were collected from ACPA-negative RA patients at Kyoto University Hospital, Tokyo Women's Medical University [41], and RIKEN with the support of BioBank Japan. All patients were Japanese and had been diagnosed by rheumatologists

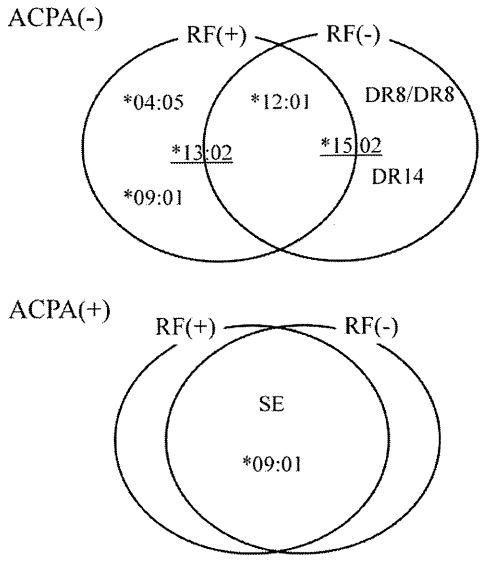

Figure 1. Summary of the HLA-DRB1 alleles associated with ACPA-negative RA and ACPA-positive RA. The relationships between the RF-positive and RF-negative subsets of ACPA-negative and ACPA-positive RA in terms of their associations with HLA-DRB1 alleles are illustrated. While the two subsets of ACPA-positive RA seem to share most associations with HLA-DRB1, the two ACPA-negative RA subsets possess specific alleles and HLA-DRB1 diplotypes. The underlined alleles are protective alleles.
doi:10.1371/journal.pone.0040067.g001

according to the 1987 American College of Rheumatology revised criteria for RA [42]. The control DNA samples were collected at Aichi Cancer Center Hospital, the DNA banks of the Pharma SNP Consortium [43], and HLA laboratory. A more detailed description of the collection procedure was given in a previous study [14]. We performed association studies using similar study design of the two collections to our previous study; namely, collection 1 for 456 ACPA-negative RA and 1508 healthy subjects, and collection 2 for 501 ACPA-negative RA and 500 healthy people. RF data were available for 453 out of 456 cases in collection 1 and all of 501 cases in collection 2. 179 patients were RF-positive in collection 1 and 267 patients were RF-positive in collection 2. We also collected DNA samples from 531 ACPA-positive RF-positive RA patients at Kyoto University Hospital and 154 ACPA-positive RF-negative RA patients at Kyoto University and Tokyo Women's Medical University.

ACPA Detection

The MESACUP CCP ELISA kit (Medical and Biological Laboratories Co., Ltd, Nagoya, Japan) was used to detect 2nd generation ACPA in each RA patient, according to the manufacturer's instructions. A cut-off value of 4.5 U/ml was used to define ACPA positivity.

RF Detection

The serum RF concentrations of samples in collection 1 were quantified using a latex agglutination turbidimetric immunoassay. An ELISA assay was used to determine the RF levels of samples in collection 2. When multiple values for RF had been obtained at different visits, we used the maximum RF value for each patient. The cut off values of each detection kit in each hospital were employed.

HLA-DRB1 Genotyping

The HLA-DRB1 typing methods were previously described [14]. Briefly, the WAKFlow system or the AlleleSEQR HLA-DRB1 typing kit (Abbott, Tokyo, Japan) was used for the HLA-DRB1 typing. The following HLA-DRB1 alleles were classified as belonging to the SE: DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *04:10, *04:13, *04:16, *10:01, *14:02, and *14:06.

Statistical Analysis

The frequency of each allele or diplotype was compared among the ACPA-negative RF-positive RA, ACPA-negative RF-negative RA patients, and the healthy controls in each set and combined set using the chi-square test or Fisher's exact test. The same analyses were performed in ACPA-positive RA patients classified according to their RF possession. Ninety-five percent confidence intervals (CI) for the OR were also calculated. Logistic regression analysis was used to evaluate the effects of each allele by adjusting for the influence of strongly-associated alleles. Single alleles were regarded as significant when they showed p-values of less than 0.0026 in a combined study, which is obtained by Bonferroni's correction. For diplotype analyses, we regarded 0.025 as the cut off level for significance because we performed just two tests. All statistical analyses were performed using the R statistic system (http://www.R-project.org) or SPSS (version 18).

Supporting Information

Figure S1 Dosage effects of HLA-DRB1*04:05 and *09:01 alleles on ACPA-negative RF-positive RA susceptibility. Each column represents the odds ratio for developing ACPA-negative RF-positive RA associated with possessing one (red column) or two (green column) alleles of HLA-DRB1*04:05 or *09:01.
(TIF)

Table S1 Logistic regression analysis of associated alleles with ACPA-negative RF-positive RA. *p-values and odds ratios in logistic regression analysis using the six alleles listed above.
(DOC)

Table S2 Association between HLA-DR14 and ACPA-negative RF-negative RA.
(DOC)

Table S3 Logistic regression analysis of associated alleles with ACPA-negative RF-negative RA. *p-values and odds ratios in logistic regression analysis using HLA-DR14 and three HLA-DRB1 alleles listed above.
(DOC)

Table S4 Association of HLA-DRB1 with ACPA-negative RA erosive subsets. [a]Total allele number is 268. [b]Total allele number is 212.
(DOC)

Table S5 Logistic regression analysis of assoicated alleles with ACPA-negative RF-positive RA, compared with ACPA-negative RF-negative RA. *p-values and odds ratios in logistic regression analysis using HLA-DRB1*09:01, *04:05, and HLA-DR14. [a]HLA-DRB1 alleles which showed $p<0.05$ in Table 3 were used for analysis.
(DOC)

Table S6 Comparison between ACPA-positive RF-positive RA and ACPA-positive RF-negative RA. [a] Alleles with frequency more than 1% in any groups are shown.
(DOC)

Acknowledgments

We would like to thank Dr. Naoichiro Yukawa, Dr. Hajime Yoshifuji, Dr. Daisuke Kawabata, Dr. Takaki Nojima, Dr. Takashi Usui, and Dr. Takao Fujii of Kyoto University for collecting the DNA samples. We would also like to thank Mr. Taishi Shigeki for developing the clinical database software used at Dohgo Spa hospital. We would like to thank Dr. Yasuo Miura and Dr. Taira Maekawa of Kyoto University for their support of HLA-DRB1 genotyping. Moreover, we wish to thank all of the doctors and medical staff who collected the samples. This study was performed with the support of the Genetics and Allied research in Rheumatic diseases Networking (GARNET) consortium.

Author Contributions

Conceived and designed the experiments: CT KO KI YK RY FM TM. Performed the experiments: CT KI YK EM K. Yurugi MK AS HS. Analyzed the data: CT. Contributed reagents/materials/analysis tools: KI EM KS AM SH K. Takasugi KM K. Tajima SM HY K. Yamamoto HS TM. Wrote the paper: CT KO.

References

1. Firestein GS (2003) Evolving concepts of rheumatoid arthritis. Nature 423: 356–361.
2. MacGregor AJ, Snieder H, Rigby AS, Koskenvuo M, Kaprio J, et al. (2000) Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. Arthritis Rheum 43: 30–37.
3. Kallberg H, Ding B, Padyukov L, Bengtsson C, Ronnelid J, et al. (2011) Smoking is a major preventable risk factor for rheumatoid arthritis: estimations of risks after various exposures to cigarette smoke. Ann Rheum Dis 70: 508–511.
4. Deighton CM, Walker DJ, Griffiths ID, Roberts DF (1989) The contribution of HLA to rheumatoid arthritis. Clin Genet 36: 178–182.
5. Gorman JD, Lum RF, Chen JJ, Suarez-Almazor ME, Thomson G, et al. (2004) Impact of shared epitope genotype and ethnicity on erosive disease: a meta-analysis of 3,240 rheumatoid arthritis patients. Arthritis Rheum 50: 400–412.
6. Gregersen PK, Silver J, Winchester RJ (1987) The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 30: 1205–1213.
7. Ohmura K, Terao C, Maruya E, Katayama M, Matoba K, et al. (2010) Anti-citrullinated peptide antibody-negative RA is a genetically distinct subset: a definitive study using only bone-erosive ACPA-negative rheumatoid arthritis. Rheumatology (Oxford) 49: 2298–2304.
8. van der Woude D, Lie BA, Lundstrom E, Balsa A, Feitsma AL, et al. (2010) Protection against anti-citrullinated protein antibody-positive rheumatoid arthritis is predominantly associated with HLA-DRB1*1301: a meta-analysis of HLA-DRB1 associations with anti-citrullinated protein antibody-positive and anti-citrullinated protein antibody-negative rheumatoid arthritis in four European populations. Arthritis Rheum 62: 1236–1245.
9. Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, et al. (2000) The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. Arthritis Rheum 43: 155–163.
10. Morgan AW, Thomson W, Martin SG, Carter AM, Erlich HA, et al. (2009) Reevaluation of the interaction between HLA-DRB1 shared epitope alleles, PTPN22, and smoking in determining susceptibility to autoantibody-positive and autoantibody-negative rheumatoid arthritis in a large UK Caucasian population. Arthritis Rheum 60: 2565–2576.
11. Huizinga TW, Amos CI, van der Helm-van Mil AH, Chen W, van Gaalen FA, et al. (2005) Refining the complex rheumatoid arthritis phenotype based on specificity of the HLA-DRB1 shared epitope for antibodies to citrullinated proteins. Arthritis Rheum 52: 3433–3438.
12. Verpoort KN, van Gaalen FA, van der Helm-van Mil AH, Schreuder GM, Breedveld FC, et al. (2005) Association of HLA-DR3 with anti-cyclic citrullinated peptide antibody-negative rheumatoid arthritis. Arthritis Rheum 52: 3058–3062.
13. Lundstrom E, Kallberg H, Smolnikova M, Ding B, Ronnelid J, et al. (2009) Opposing effects of HLA-DRB1*13 alleles on the risk of developing anti-citrullinated protein antibody-positive and anti-citrullinated protein antibody-negative rheumatoid arthritis. Arthritis Rheum 60: 924–930.
14. Terao C, Ohmura K, Kochi Y, Ikari K, Maruya E, et al. (2011) A large-scale association study identified multiple HLA-DRB1 alleles associated with ACPA-negative rheumatoid arthritis in Japanese subjects. Ann Rheum Dis 70(12): 2134–2139.
15. Furuya T, Hakoda M, Ichikawa N, Higami K, Nanke Y, et al. (2007) Differential association of HLA-DRB1 alleles in Japanese patients with early rheumatoid arthritis in relationship to autoantibodies to cyclic citrullinated peptide. Clin Exp Rheumatol 25: 219–224.
16. Mackie SL, Taylor JC, Martin SG, Wordsworth P, Steer S, et al. (2012) A spectrum of susceptibility to rheumatoid arthritis within HLA-DRB1: stratification by autoantibody status in a large UK population. Genes Immun 13: 120–128.
17. Lee HS, Lee KW, Song GG, Kim HA, Kim SY, et al. (2004) Increased susceptibility to rheumatoid arthritis in Koreans heterozygous for HLA-DRB1*0405 and *0901. Arthritis Rheum 50: 3468–3475.
18. Payami H, Joe S, Farid NR, Stenszky V, Chan SH, et al. (1989) Relative predispositional effects (RPEs) of marker alleles with disease: HLA-DR alleles and Graves disease. Am J Hum Genet 45: 541–546.
19. Okada Y, Suzuki A, Yamada R, Kochi Y, Shimane K, et al. (2010) HLA-DRB1*0901 lowers anti-cyclic citrullinated peptide antibody levels in Japanese patients with rheumatoid arthritis. Annals of the Rheumatic Diseases 69: 1569–1570.
20. Terao C, Ikari K, Ohmura K, Suzuki T, Iwamoto T, et al. (2012) Quantitative effect of HLA-DRB1 alleles to ACPA levels in Japanese rheumatoid arthritis: no strong genetic impact of shared epitope to ACPA levels after stratification of HLA-DRB1*09:01. Annals of the Rheumatic Diseases 71: 1095–1097.
21. Hill JA, Bell DA, Brintnell W, Yue D, Wehrli B, et al. (2008) Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice. Journal of Experimental Medicine 205: 967–979.
22. Hill JA, Southwood S, Sette A, Jevnikar AM, Bell DA, et al. (2003) Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. Journal of Immunology 171: 538–541.
23. Queiro-Silva R, Torre-Alonso JC, Tinture-Eguren T, Lopez-Lagunas I (2004) The effect of HLA-DR antigens on the susceptibility to, and clinical expression of psoriatic arthritis. Scand J Rheumatol 33: 318–322.
24. Ho PY, Barton A, Worthington J, Plant D, Griffiths CE, et al. (2008) Investigating the role of the HLA-Cw*06 and HLA-DRB1 genes in susceptibility to psoriatic arthritis: comparison with psoriasis and undifferentiated inflammatory arthritis. Ann Rheum Dis 67: 677–682.
25. Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, et al. (2003) Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat Genet 34: 395–402.
26. Kochi Y, Yamada R, Suzuki A, Harley JB, Shirasawa S, et al. (2005) A functional variant in FCRL3, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities. Nat Genet 37: 478–485.
27. Suzuki A, Yamada R, Kochi Y, Sawada T, Okada Y, et al. (2008) Functional SNPs in CD244 increase the risk of rheumatoid arthritis in a Japanese population. Nat Genet 40: 1224–1229.
28. Kochi Y, Okada Y, Suzuki A, Ikari K, Terao C, et al. (2010) A regulatory variant in CCR6 is associated with rheumatoid arthritis susceptibility. Nat Genet 42: 515–519.
29. Terao C, Ohmura K, Katayama M, Takahashi M, Kokubo M, et al. (2011) Myelin basic protein as a novel genetic risk factor in rheumatoid arthritis-a genome-wide study combined with immunological analyses. PLoS One 6: e20457.
30. Terao C, Yamada R, Ohmura K, Takahashi M, Kawaguchi T, et al. (2011) The human AIRE gene at chromosome 21q22 is a genetic determinant for the predisposition to rheumatoid arthritis in Japanese population. Hum Mol Genet 20: 2680–2685.
31. Plenge RM, Seielstad M, Padyukov L, Lee AT, Remmers EF, et al. (2007) TRAF1-C5 as a risk locus for rheumatoid arthritis–a genomewide study. N Engl J Med 357: 1199–1209.
32. Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G, et al. (2007) STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. N Engl J Med 357: 977–986.
33. Plenge RM, Cotsapas C, Davies L, Price AL, de Bakker PI, et al. (2007) Two independent alleles at 6q23 associated with risk of rheumatoid arthritis. Nat Genet 39: 1477–1482.
34. Wellcome Trust Case Control Consortium (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447: 661–678.
35. Thomson W, Barton A, Ke X, Eyre S, Hinks A, et al. (2007) Rheumatoid arthritis association at 6q23. Nat Genet 39: 1431–1433.
36. Raychaudhuri S, Remmers EF, Lee AT, Hackett R, Guiducci C, et al. (2008) Common variants at CD40 and other loci confer risk of rheumatoid arthritis. Nat Genet 40: 1216–1223.
37. Gregersen PK, Amos CI, Lee AT, Lu Y, Remmers EF, et al. (2009) REL, encoding a member of the NF-kappaB family of transcription factors, is a newly defined risk locus for rheumatoid arthritis. Nat Genet 41: 820–823.
38. Raychaudhuri S, Thomson BP, Remmers EF, Eyre S, Hinks A, et al. (2009) Genetic variants at CD28, PRDM1 and CD2/CD58 are associated with rheumatoid arthritis risk. Nat Genet 41: 1313–1318.
39. Padyukov L, Seielstad M, Ong RT, Ding B, Ronnelid J, et al. (2011) A genome-wide association study suggests contrasting associations in ACPA-positive versus ACPA-negative rheumatoid arthritis. Ann Rheum Dis 70: 259–265.
40. Okada Y, Terao C, Ikari K, Kochi Y, Ohmura K, et al. (2012) Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population. Nature Genetics 44: 511–516.

41. Matsuda Y, Singh G, Yamanaka H, Tanaka E, Urano W, et al. (2003) Validation of a Japanese version of the Stanford Health Assessment Questionnaire in 3,763 patients with rheumatoid arthritis. Arthritis Rheum 49: 784–788.
42. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, et al. (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31: 315–324.
43. Kamatani N, Sekine A, Kitamoto T, Iida A, Saito S, et al. (2004) Large-scale single-nucleotide polymorphism (SNP) and haplotype analyses, using dense SNP Maps, of 199 drug-related genes in 752 subjects: the analysis of the association between uncommon SNPs within haplotype blocks and the haplotypes constructed with haplotype-tagging SNPs. Am J Hum Genet 75: 190–203.

ARTHRITIS & RHEUMATISM
Vol. 65, No. 2, February 2013, pp 472–480
DOI 10.1002/art.37777

PLD4 as a Novel Susceptibility Gene for Systemic Sclerosis in a Japanese Population

Chikashi Terao,[1] Koichiro Ohmura,[1] Yasushi Kawaguchi,[2] Tetsuya Nishimoto,[3] Aya Kawasaki,[4] Kazuhiko Takehara,[5] Hiroshi Furukawa,[6] Yuta Kochi,[7] Yuko Ota,[2] Katsunori Ikari,[2] Shinichi Sato,[8] Shigeto Tohma,[6] Ryo Yamada,[1] Kazuhiko Yamamoto,[8] Michiaki Kubo,[7] Hisashi Yamanaka,[2] Masataka Kuwana,[3] Naoyuki Tsuchiya,[4] Fumihiko Matsuda,[1] and Tsuneyo Mimori[1]

Objective. **Systemic sclerosis (SSc) is an autoimmune disease for which multiple susceptibility genes have been reported. Genome-wide association studies have shown that large numbers of susceptibility genes are shared among autoimmune diseases. Recently, our group identified 9 novel susceptibility genes associated with rheumatoid arthritis (RA) in a Japanese population. The aim of this study was to elucidate whether the 18 genes that displayed associations or suggestive associations for RA in our previous study are associated with SSc in Japanese.**

Methods. **We performed an association study that included 415 patients with SSc and 16,891 control subjects, followed by a replication study that included 315 patients and 21,054 control subjects. The 18 markers reported to display association with RA were analyzed for their associations with SSc in the first study, and 5 markers were further analyzed in the replication study. The inverse variance method was used to evaluate the associations of these markers with SSc in a combined study.**

Results. **In the phospholipase D4 gene (*PLD4*), rs2841277 displayed a significant association with SSc in Japanese patients ($P = 0.00017$). We observed that rs2841280 in exon 2 of *PLD4* was in strong linkage disequilibrium with rs2841277 and introduced an amino acid alteration. We also observed associations between SSc and rs6932056 in *TNFAIP3* and rs2280381 in *IRF8* ($P = 0.0000095$ and $P = 0.0030$, respectively), both of which displayed associations with SSc in a European population.**

Conclusion. **We determined that *PLD4* is a novel susceptibility gene for SSc in Japanese, thus confirming the involvement of *PLD4* in autoimmunity. Associations between SSc and *TNFAIP3* or *IRF8* were also detected in our Japanese population. SSc and RA appear to share relatively large proportions of their genetic backgrounds.**

Systemic sclerosis (SSc) is a connective tissue disease that affects 7–489 individuals per million worldwide and is characterized by the excess production of extracellular matrix molecules and fibrosis (1). Patients with SSc display skin sclerosis, obliterative microvasculopathy such as Raynaud's phenomenon, and multiorgan involvement. Severe complications of SSc sometimes develop, including interstitial lung disease, pulmonary hypertension, and renal crisis. These severe symptoms

Supported by grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, by research grants from the Japan Rheumatism Foundation, the Waksman Foundation, and the Mitsubishi Pharma Research Foundation, and by the Genetics and Allied Research in Rheumatic Diseases Networking consortium.

[1]Chikashi Terao, MD, PhD, Koichiro Ohmura, MD, PhD, Ryo Yamada, MD, PhD, Fumihiko Matsuda, PhD, Tsuneyo Mimori, MD, PhD: Kyoto University, Kyoto, Japan; [2]Yasushi Kawaguchi, MD, PhD, Yuko Ota, MD, Katsunori Ikari, MD, PhD, Hisashi Yamanaka, MD, PhD: Tokyo Women's Medical University, Tokyo, Japan; [3]Tetsuya Nishimoto, PhD, Masataka Kuwana, MD, PhD: Keio University, Tokyo, Japan; [4]Aya Kawasaki, PhD, Naoyuki Tsuchiya, MD, PhD: University of Tsukuba, Tsukuba, Japan; [5]Kazuhiko Takehara, MD, PhD: Kanazawa University, Kanazawa, Japan; [6]Hiroshi Furukawa, MD, PhD, Shigeto Tohma, MD: Sagamihara National Hospital, National Hospital Organization, Sagamihara, Japan; [7]Yuta Kochi, MD, PhD, Michiaki Kubo, MD, PhD: RIKEN, Yokohama, Japan; [8]Shinichi Sato, MD, PhD, Kazuhiko Yamamoto, MD, PhD: University of Tokyo, Tokyo, Japan.

Address correspondence to Chikashi Terao, MD, PhD, or Koichiro Ohmura, MD, PhD, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan. E-mail: a0001101@kuhp.kyoto-u.ac.jp or ohmurako@kuhp.kyoto-u.ac.jp.

Submitted for publication June 4, 2012; accepted in revised form October 23, 2012.

and complications of SSc result in a poor prognosis and a shortened lifespan (2,3). No effective method for preventing or curing SSc has been established (4).

It is well known that SSc has genetic components (5); for example, a US study revealed that the incidence of SSc was much higher among the families of patients with SSc compared with the general population (6). Recent technologic developments enabled the use of genome-wide association studies (GWAS) to identify novel susceptibility loci for autoimmune diseases (7). GWAS of European patients with SSc revealed that *CD247* (8), HLA (8), *TNIP1*, *PSORS1C1*, and *RHOB* (9) are susceptibility loci for SSc. In addition, another GWAS identified associations between *IRF8*, *GRB10*, and *SOX5* and limited cutaneous SSc (lcSSc) in a European population (10). Furthermore, studies adopting a candidate gene approach based on subjecting genes to functional inference analysis led to the identification of *STAT4* (11), *IRF5* (12), *TBX21* (13), *NLRP1* (14), *TNFSF4* (15), *CD226* (16), *BLK* (17), and *TNFAIP3* (18) as novel susceptibility genes for SSc in Europeans. SSc association studies in Japanese populations confirmed that *STAT4* (19), *IRF5* (20), and *BLK* (21) are associated with SSc and identified *UBE2L3* as a susceptibility gene for diffuse cutaneous SSc (dcSSc) (22). An association between HLA and SSc was also detected in Asians (23). These findings suggest a clear overlap in the genetic background of SSc between different populations.

It is well known that susceptibility genes are shared by various autoimmune diseases (24). In fact, HLA (25), *STAT4* (26), and *TNFAIP3* (27,28), which are susceptibility genes for SSc, have also been reported to be associated with rheumatoid arthritis (RA). In addition, *PTPN22*, which was shown to be strongly associated with RA in a European population (29), showed a suggestive association with SSc in Europeans (30). The sharing of these susceptibility genes between RA and SSc raises the possibility that newly identified susceptibility genes for RA could also be susceptibility genes for SSc. Recently, a large Japanese consortium, the Genetic and Allied research in Rheumatic diseases Networking consortium, identified 9 novel susceptibility genes and 6 candidate susceptibility genes for RA using a meta-analysis of GWAS and replication studies (31). Four other genes, namely, HLA, *PADI4*, *CCR6*, and *TNFAIP3*, were also confirmed to display associations with RA. Here, we performed a 2-stage association study of Japanese patients with SSc, in which we genotyped these genes as candidate susceptibility loci.

PATIENTS AND METHODS

Study subjects. DNA samples were obtained from 415 patients with SSc at Kyoto University Hospital and Tokyo Women's Medical University; these samples comprised the first set. Independent DNA samples were obtained from 315 patients with SSc at Keio University Hospital, Sagamihara National Hospital, and Kanazawa University Hospital; these samples were used as the replication set. All patients were Japanese, all had a diagnosis of SSc as determined by a rheumatologist, and all fulfilled the 1980 American College of Rheumatology classification criteria for SSc (32). The patients with SSc for whom clinical information was available were classified as having lcSSc or dcSSc, according to the definitions developed by LeRoy et al (33). The control samples were described in detail in our previous study (31). The current study was approved by the local ethics committees at each institution, and written informed consent was obtained from all subjects. The basic characteristics of the study subjects are shown in Table 1.

Genotyping. The 9 novel susceptibility markers, 6 potentially associated markers, and 4 confirmed markers of RA that were identified in our previous study in a Japanese population (31) were chosen as candidate susceptibility markers for SSc in Japanese. Eighteen of the 19 markers (HLA was excluded; see Results), none of which had previously been reported to be associated with SSc in Japanese individuals, were genotyped in the current study. The 5 candidate markers in the first set that showed associations with P values less than 0.1 were further genotyped in the replication study. Single-nucleotide polymorphisms (SNPs) rs2841280 and rs894037 were chosen as candidate causative variants in the phospholipase D4 gene (*PLD4*) region. Because rs894037 was shown to be monomorphic in Japanese, rs2841280 was genotyped in 334 control subjects, in addition to all patients, for imputation reference. The patients in the first and replication studies were genotyped at Kyoto University or Tokyo Women's Medical University and at Keio University or University of Tsukuba, respectively, using TaqMan assays (Applied Biosystems). The genotyping methods in control subjects were described in detail in our previous study (31).

Briefly, control genotypes in the first set were imputed based on the genome-scanning data, using mach2dat software with HapMap Phase II East Asian Populations as reference. The control genotypes for the replication study were extracted from genome-scanning data for the markers included on Illumina HumanHap610 Quad BeadChips. The genotypes for rs6932056 (which is not included in the array) were imputed based on the genome-scanning data, using mach2dat software with HapMap Phase II East Asian Populations as reference, and were used as control data for the replication set. The genotypes for rs2841280 (which is not included in the HapMap data or the array) were also imputed in control subjects, based on the genome-scanning data, using mach2dat software. Genotyping data for the 334 control subjects as determined by TaqMan assay in combination with genome-scanning data were used as reference.

Statistical analysis. The associations between the genotyped markers and SSc were analyzed using a Cochran-Armitage trend test in both the first and replication studies. Subanalyses were performed by comparing the genotypes of